MOLECULAR SWITCHES OF THE IMMUNE SYSTEM: THE E PROTEIN/ID AXIS IN HEMATOPOIETIC DEVELOPMENT AND FUNCTION

EDITED BY: Michele Kay Anderson, Juan Carlos Zuniga-Pflucker, Mikael Sigvardsson and Barbara L. Kee

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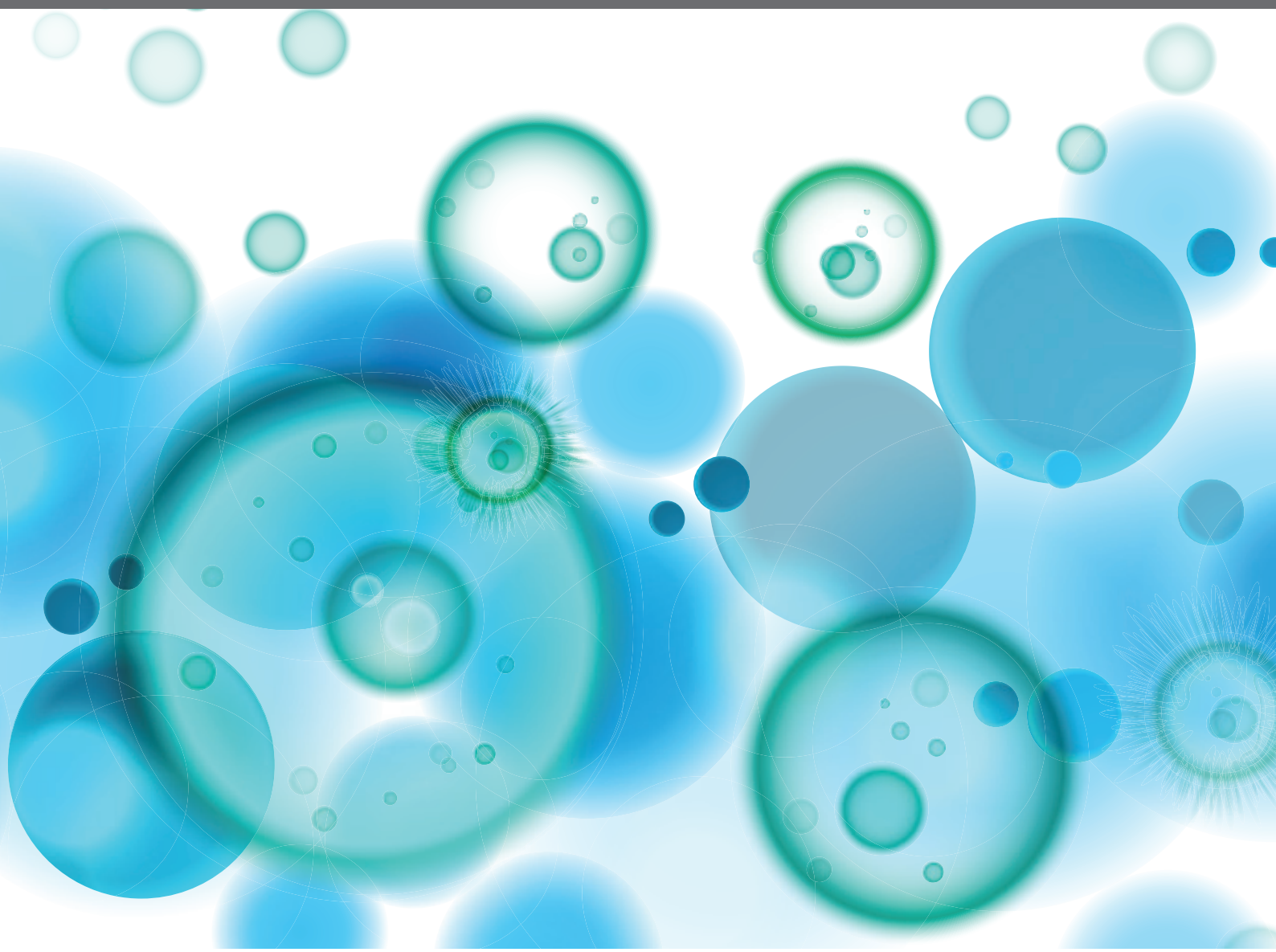

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MOLECULAR SWITCHES OF THE IMMUNE SYSTEM: THE E PROTEIN/ID AXIS IN HEMATOPOIETIC DEVELOPMENT AND FUNCTION

Topic Editors:
Michele Kay Anderson, University of Toronto, Canada
Juan Carlos Zuniga-Pflucker, University of Toronto, Canada
Mikael Sigvardsson, Linköping University, Sweden
Barbara L. Kee, The University of Chicago, United States

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Andrew D. Wells,
Children's Hospital of Philadelphia,
United States

*CORRESPONDENCE
Mikael Sigvardsson
mikael.sigvardsson@ibk.liu.se

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Editorial: Molecular switches of the immune system: The E-protein/Id axis in hematopoietic development and function

Mikael Sigvardsson[1,2]*, Barbara L. Kee[3],
Juan Carlos Zúñiga-Pflücker[4,5] and Michele K. Anderson[4,5]

[1]BKV, Linköping University, Linköping, Sweden, [2]Division of Molecular Hemathology, Lund University, Lund, Sweden, [3]Department of Pathology, The University of Chicago, Chicago, IL, United States, [4]Department of Immunology, University of Toronto, Toronto, ON, Canada, [5]Biological Sciences, Sunnybrook Research Institute (SRI), Toronto, ON, Canada

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Editorial on the Research Topic

Molecular switches of the immune system: The E protein/Id axis in hematopoietic development and function

This Frontiers in Immunology Research Topic is composed by a set of review and original articles, all highlighting the roles of E-proteins in hematopoiesis.

The first evidence for a role of E-box binding (E) proteins in the immune system came from the identification of protein bound "E-boxes" in regulatory elements at the immunoglobulin locus (1, 2). The subsequent identification of these DNA binding proteins (3) revealed amino-acid homology to MyoD as well as Myc (3, 4), and created the foundation for a new research area in molecular immunology. The complexity of the regulatory networks involving these "basic-helix-loop-helix" (bHLH) transcription factors became obvious, as both broadly expressed and tissue specific family members became identified (4). These proteins are able to form homo- as well as hetero-dimeric complexes targeting E-boxes in transcription regulatory elements of both ubiquitously expressed and tissue restricted genes (4–6). Among the broadly expressed prot*ins are E12, E47 [both encoded by the E2A gene (*TCF3*) (3)], HEB (*TCF12*) (7) and E2-2 (*TCF4*) (8), that due to their overlapping activities and dimerization patterns have been denoted as E proteins [Reviewed in (9)]. In aggregate, E proteins display a high degree of redundancy, and it has been proposed that functional dose, rather than expression of any specific protein, controls developmental trajectories in hematopoiesis (10–13).

The complexity of the E protein regulatory network increased with the identification of Inhibitor of DNA binding (ID) proteins (14). The ID proteins harbor the HLH domain

needed for dimerization with E proteins but lack the DNA binding basic domain (b), creating a complex that is unable to bind the target DNA elements in the genome. Thus, ID proteins are powerful functional inhibitors of bHLH protein activity with important roles in hematopoiesis. This Research Topic includes a review article describing our current understanding of the function of ID proteins blood cell development (Singh et al.). The authors describe what has been learned from gain and loss of function experiments and provide insight into the intricate interplay between the different E and ID proteins in hematopoiesis.

The importance of E proteins in lymphocyte development was revealed by the targeted inactivation of the E47/E12 encoding *E2A*. These studies revealed a critical requirement for these E proteins at the earliest stages of lymphocyte development (15–18). This Research Topic contains a review by Aubrey et al. detailing how the complex interplay between E and ID proteins drive developmental trajectories in lymphopoiesis. The article provides a molecular insight to the mechanisms by which E proteins drive lymphoid lineage differentiation and control antigen receptor recombination to generate a functional B and T lymphocytes. An original research contribution by Roels et al. focuses on a multi-omics analysis resolving the functions of E proteins in human T-cell development. This work not only provides us with insight into the evolution of the immune system but also provides information that can be explored to better understand the role of E proteins in leukemia.

The E protein/ID protein axis is also of importance for the formation of innate lymphoid cells (ILCs) (19–25). Our current understanding of the interplay between E and ID proteins in commitment to the T lymphocyte and ILC fates is the focus of the review article by Pankow and Sun presented in this Research Topic. The role of E proteins in T cell development is complex with functional importance in early development (26, 27) as well as in the generation of effector populations in the adaptive immune response (28, 29). The article by Hidaka et al highlights this complexity by reviewing the role of E and ID proteins in early T cell development as well as in the generation of regulatory T cells.

Not only TCF3 (E2A) but also TCF12 (HEB) is reported to be of essential importance for normal T-cell development (30, 31). The use of alternative *Tcf12* transcriptional start sites and alternative splicing of the transcript results in the generation of HEBCan and HEBAlt proteins. Of note, the HEBAlt protein harbors a unique N-terminal domain as compared to the HEBCan protein, and has been reported to act as a driver of early T-cell development (32, 33). In this Research Topic, an original article by Yoganathan et al. reveals that a YYY motif in the HEBAlt specific region of the protein is targeted by Janus Kinase activity. This work proposes a direct connection between E protein function and extracellular signaling events in T-cell development. Janus kinase activity is not the only signaling pathway that is functionally integrated into the E protein/ID protein axis. The review by Hwang et al. explores the signaling networks involving E- and ID proteins that control the development of T-cells as well as T-cell activation.

Among the functionally integrated signaling pathways, Notch signaling is of special interest as it is both a driver of T-cell development (34, 35) and has an important role in T-cell transformation (36, 37). Notch signaling has been reported to result in the targeting of E proteins for degradation (38, 39) suggesting that E protein dose may be directly linked to malignant transformation. The original article by Veiga et al. reports the analysis of a set of most elegant complementary T lymphocyte acute lymphoblastic leukemia (T-ALL) models revealing a unique role for TCF12 in malignant transformation. Loss and gain of function experiments provide evidence showing that a reduced TCF12 dose is redundant to NOTCH activation in the transformation process. This would indicate that targeting of TCF12 by NOTCH signaling is a major contributor to the powerful oncogenic activity exerted by NOTCH in T-ALL. In a second original article in this Research Topic, Carr et al., tested the requirements for the HMG-transcription factor LEF1, a factor previously proposed to be a component of the regulatory network involving E proteins and Notch1, in T-ALL (40). Here, they revealed that E protein deficiency promotes leukemia through adaptive mechanisms involving LEF1 addiction or independence, based on the status of LEF1 expression at the time of transformation. These papers stress the complexity by which E proteins contribute to malignant transformation, the subject of the review article by Parriott et al.. This article discusses the mechanism by which E proteins directly contribute to malignant transformation as the targets of the oncogenic bHLH proteins TAL1 and LYL1 that are overexpressed in mouse and human T-ALL.

We believe that the articles presented within the frame of this Research Topic provide timely and novel knowledge, and serve as a valuable source of information for investigators in molecular and developmental immunology.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Ephrussi A, Church GM, Tonegawa S, Gilbert W. B linage-specific interactions of an immunoglobulin enhancer with cellular factors *in vivo*. *Science* (1985) 227:134–40. doi: 10.1126/science.3917574

2. Church GM, Ephrussi A, Gilbert W, Tonegawa S. Cell-type-specific contacts to immunoglobulin enhancers in nuclei. *Nature* (1985) 313:798–801. doi: 10.1038/313798a0

3. Murre C, McCaw PS, Baltimore D. A new DNA binding and dimersation motif in immunoglobulin enhancer binding, daughterless, myoD and myc proteins. *Cell* (1989) 56:777–83. doi: 10.1016/0092-8674(89)90682-X

4. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, et al. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* (1989) 58:537–44. doi: 10.1016/0092-8674(89)90434-0

5. Murre C, Voronova A, Baltimore D. B-cell- and myocyte-specific E2-box-binding factors contain E12/E47- like subunits. *Mol Cell Biol* (1991) 11:1156–60. doi: 10.1128/mcb.11.2.1156-1160.1991

6. Bain G, Gruenwald S, Murre C. E2A and E2-2 are subunits of b-cell-specific E2-box DNA-binding proteins. *Mol Cell Biol* (1993) 13:3522–9. doi: 10.1128/mcb.13.6.3522-3529.1993

7. Hu S-J, Olson EN, Kingston RE. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding activity of myogenic regulatory factors. *Mol Cell Biol* (1992) 12:1031–42. doi: 10.1128/mcb.12.3.1031-1042.1992

8. Henthorn P, Kiledjian M, Kadesch T. Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science* (1990) 247:467–70. doi: 10.1126/science.2105528

9. Murre C. Helix-loop-helix proteins and the advent of cellular diversity: 30 years of discovery. *Genes Dev* (2019) 33:6–25. doi: 10.1101/gad.320663.118

10. Bain G, Maandag ECR, te Riele HPJ, Feeney AJ, Sheehy A, Schlissel M, et al. Both E12 and E47 allow commitment to the b cell lineage. *Immunity* (1997) 6:145–54. doi: 10.1016/S1074-7613(00)80421-5

11. Zhuang Y, Barndt RJ, Pan L, Kelley R, Dai M. Functional replacement of the mouse E2A gene with a human HEB cDNA. *Mol Cell Biol* (1998) 18:3340–9. doi: 10.1128/MCB.18.6.3340

12. Zhuang Y, Cheng P, Weintraub H. B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, *E2A*, *E2-2* and HEB. *Mol Cell Biol* (1996) 16:2898–905. doi: 10.1128/MCB.16.6.2898

13. Sun X-H. Constitutive expression of the Id1 gene impairs mouse b cell development. *Cell* (1994) 79:893–900. doi: 10.1016/0092-8674(94)90078-7

14. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* (1990) 61:49–59. doi: 10.1016/0092-8674(90)90214-Y

15. Dias S, Mansson R, Gurbuxani S, Sigvardsson M, Kee BL. E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity* (2008) 29:217–27. doi: 10.1016/j.immuni.2008.05.015

16. Zhuang Y, Soriano P, Weintraub H. The helix-loop-helix gene E2A is requreid for b cell formation. *Cell* (1994) 79:875–84. doi: 10.1016/0092-8674(94)90076-0

17. Bain G, Maandag ECR, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, et al. E2A proteins are required for proper b cell development and initiation of immunoglobulin gene rearrangements. *Cell* (1994) 79:885–92. doi: 10.1016/0092-8674(94)90077-9

18. Yang Q, Kardava L, St Leger A, Martincic K, Varnum-Finney B, Bernstein ID, et al. E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors. *J Immunol* (2008) 181:5885–94. doi: 10.4049/jimmunol.181.9.5885

19. Verykokakis M, Krishnamoorthy V, Iavarone A, Lasorella A, Sigvardsson M, Kee BL. Essential functions for ID proteins at multiple checkpoints in invariant NKT cell development. *J Immunol* (2013) 191:5973–83. doi: 10.4049/jimmunol.1301521

20. D'Cruz LM, Stradner MH, Yang CY, Goldrath AW. E and id proteins influence invariant NKT cell sublineage differentiation and proliferation. *J Immunol* (2014) 192:2227–36. doi: 10.4049/jimmunol.1302904

21. Zook EC, Li ZY, Xu Y, de Pooter RF, Verykokakis M, Beaulieu A, et al. Transcription factor ID2 prevents e proteins from enforcing a naive T lymphocyte gene program during NK cell development. *Sci Immunol* (2018) 3(22):eaao2139. doi: 10.1126/sciimmunol.aao2139

22. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157:340–56. doi: 10.1016/j.cell.2014.03.030

23. Delconte RB, Shi W, Sathe P, Ushiki T, Seillet C, Minnich M, et al. The helix-Loop-Helix protein ID2 governs NK cell fate by tuning their sensitivity to interleukin-15. *Immunity* (2016) 44:103–15. doi: 10.1016/j.immuni.2015.12.007

24. Qian L, Bajana S, Georgescu C, Peng V, Wang HC, Adrianto I, et al. Suppression of ILC2 differentiation from committed T cell precursors by e protein transcription factors. *J Exp Med* (2019) 216:884–99. doi: 10.1084/jem.20182100

25. Peng V, Georgescu C, Bakowska A, Pankow A, Qian L, Wren JD, et al. E proteins orchestrate dynamic transcriptional cascades implicated in the suppression of the differentiation of group 2 innate lymphoid cells. *J Biol Chem* (2020) 295:14866–77. doi: 10.1074/jbc.RA120.013806

26. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol* (1997) 17:4782–91. doi: 10.1128/MCB.17.8.4782

27. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double- knockout mice. *Mol Cell Biol* (1997) 17:7317–27. doi: 10.1128/MCB.17.12.7317

28. Cannarile MA, Lind NA, Rivera R, Sheridan AD, Camfield KA, Wu BB, et al. Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nat Immunol* (2006) 7:1317–25. doi: 10.1038/ni1403

29. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nat Immunol* (2011) 12:1221–9. doi: 10.1038/ni.2158

30. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor HEB in thymocyte survival, tcra rearrangement and the development of natural killer T cells. *Nat Immunol* (2010) 11:240–9. doi: 10.1038/ni.1845

31. Braunstein M, Anderson MK. HEB-deficient T-cell precursors lose T-cell potential and adopt an alternative pathway of differentiation. *Mol Cell Biol* (2011) 31:971–82. doi: 10.1128/MCB.01034-10

32. Wang D, Claus CL, Vaccarelli G, Braunstein M, Schmitt TM, Zuniga-Pflucker JC, et al. The basic helix-loop-helix transcription factor HEBAlt is expressed in pro-T cells and enhances the generation of T cell precursors. *J Immunol* (2006) 177:109–19. doi: 10.4049/jimmunol.177.1.109

33. Wang D, Claus CL, Rajkumar P, Braunstein M, Moore AJ, Sigvardsson M, et al. Context-dependent regulation of hematopoietic lineage choice by HEBAlt. *J Immunol* (2010) 185:4109–17. doi: 10.4049/jimmunol.0901783

34. Schmitt TM, Zuniga-Pflucker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 *in vitro*. *Immunity* (2002) 17:749–56. doi: 10.1016/S1074-7613(02)00474-0

35. Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S, et al. Notch1 expression in early lymphopoiesis influences b versus T lineage determination. *Immunity* (1999) 11:299–308. doi: 10.1016/S1074-7613(00)80105-3

36. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. TAN-1, the human homolog of the drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* (1991) 66:649–61. doi: 10.1016/0092-8674(91)90111-B

37. Weng AP, Ferrando AA, Lee W, J.P.t. Morris LB, Sanchez-Irizarry C, Blacklow SC, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* (2004) 306:269–71. doi: 10.1126/science.1102160

38. Nie L, Xu M, Vladimirova A, Sun XH. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J* (2003) 22:5780–92. doi: 10.1093/emboj/cdg567

39. Ordentlich P, Lin A, Shen CP, Blaumueller C, Matsuno K, Artavanis-Tsakonas S, et al. Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol* (1998) 18:2230–9. doi: 10.1128/MCB.18.4.2230

40. Spaulding C, Reschly EJ, Zagort DE, Yashiro-Ohtani Y, Beverly LJ, Capobianco A, et al. Notch1 co-opts lymphoid enhancer factor 1 for survival of murine T-cell lymphomas. *Blood* (2007) 110:2650–8. doi: 10.1182/blood-2007-04-084202

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ORIGINAL RESEARCH
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Oncogenic and Tumor Suppressor Functions for Lymphoid Enhancer Factor 1 in *E2a*$^{-/-}$ T Acute Lymphoblastic Leukemia

Tiffany Carr[1], *Stephanie McGregor*[2†], *Sheila Dias*[3], *Mihalis Verykokakis*[3†], *Michelle M. Le Beau*[4], *Hai-Hui Xue*[5], *Mikael Sigvardsson*[6], *Elizabeth T. Bartom*[7] *and Barbara L. Kee*[1,2,3*]

[1] Committee on Immunology, The University of Chicago, Chicago, IL, United States, [2] Committee on Cancer Biology, The University of Chicago, Chicago, IL, United States, [3] Department of Pathology, The University of Chicago, Chicago, Chicago, IL, United States, [4] Department of Medicine, Section of Hematology/Oncology, The University of Chicago, Chicago, IL, United States, [5] Center for Discovery and Innovation, Hackensack University Medical Center, Nutley, NJ, United States, [6] Department of Molecular Hematology, Lund University, Lund, Sweden, [7] Department of Biochemistry and Molecular Genetics, Northwestern University, Chicago, IL, United States

T lymphocyte acute lymphoblastic leukemia (T-ALL) is a heterogeneous disease affecting T cells at multiple stages of their development and is characterized by frequent genomic alterations. The transcription factor LEF1 is inactivated through mutation in a subset of T-ALL cases but elevated LEF1 expression and activating mutations have also been identified in this disease. Here we show, in a murine model of T-ALL arising due to *E2a* inactivation, that the developmental timing of *Lef1* mutation impacts its ability to function as a cooperative tumor suppressor or oncogene. T cell transformation in the presence of LEF1 allows leukemic cells to become addicted to its presence. In contrast, deletion prior to transformation both accelerates leukemogenesis and results in leukemic cells with altered expression of genes controlling receptor-signaling pathways. Our data demonstrate that the developmental timing of *Lef1* mutations impact its apparent oncogenic or tumor suppressive characteristics and demonstrate the utility of mouse models for understanding the cooperation and consequence of mutational order in leukemogenesis.

Keywords: E2a, Lef1, leukemia, thymus, lymphocyte

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Masaki Miyazaki, Kyoto University, Japan
Phuong Cao Thi Ngoc, Lund University, Sweden

***Correspondence:**
Barbara L. Kee
bkee@bsd.uchicago.edu

†Present address:
Stephanie McGregor, Dept. of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI, United States
Mihalis Verykokakis, Biomedical Sciences Research Center (BSRC) "Aexander Fleming", Vari, Greece

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INTRODUCTION

T acute lymphoblastic leukemia (T-ALL) is an aggressive malignancy that accounts for approximately 10-15% of pediatric and 25% of adult leukemia (1). This disease arises in thymocytes leading to an enlarged thymus and respiratory distress; however, leukemic cells can disseminate through the blood and infiltrate tissues. Treatment involves intensive chemotherapy but 25% of pediatric cases and nearly 50% of adult cases show therapy resistance or relapse within 5 years (1). T-ALL is a consequence of transformation of cells at multiple stages of T cell differentiation (2, 3) and distinct subsets of T-ALL can be identified by their unique gene

expression signatures, genetic alterations, and response to therapy (1, 4, 5). Recent analysis further highlights that clonal evolution and progressive mutation contribute to disease evolution but much remains to be learned about the consequences of individual mutations and mutational order on transformation (6). Understanding the molecular pathogenesis of T-ALL is crucial for the improved development of prognostic markers and tailored therapeutic approaches.

The most broadly occurring mutations in T-ALL affect the Notch signaling pathway. Mutations in the *NOTCH1* gene occur in > 60% of cases (7). Mutations occur within the heterodimerization domain, resulting in ligand independent activation, and the intracellular PEST domain, resulting in stabilization of the transcriptionally active form (intracellular Notch1/ICN). In another 15% of T-ALL, mutations occur in *FBXW7*, which encodes an ubiquitin ligase involved in the PEST-domain dependent degradation of ICN (8–10). Further, epigenetic alterations affecting the *Notch1* gene impact the site of transcription initiation and splicing and result in ligand independent activation (11–13). Consistent with the oncogenic role of Notch1, ectopic expression of constitutively active forms of Notch1 in mouse T cell progenitors leads to their transformation (14).

Activation of the *TAL1* and *LYL1* genes is also associated with subsets of T-ALL (4). Tal1 and Lyl1 are basic helix-loop-helix proteins that bind DNA in association with the E protein transcription factors (15), which are critical for T cell development (16). While Tal1:E protein and Lyl1:E protein dimers have targets implicated in T-ALL (17–21), their ability to inhibit E protein homodimer formation is sufficient to promote T cell transformation as revealed by the development of T-ALL like disease in *E2a*$^{-/-}$ mice and in mice ectopically expressing inhibitors of E protein DNA binding (22–25). *E2a*$^{-/-}$ leukemias are characterized by recurrent mutations in the *Notch1* PEST domain and altered *Notch1* splicing and transcription initiation leading to ligand independence (11, 26).

A critical target of Notch1 in many cases of T-ALL is c-*myc* (27–29). However, in *E2a*$^{-/-}$ leukemias Notch1 signaling is essential but it regulates expression of *Lef1*, encoding a TCF1-related transcription factor that is an effector of the Wnt signaling pathway (26, 30, 31). LEF1 appears essential for the survival of *E2a*$^{-/-}$ leukemias since siRNA-mediated knockdown of *Lef1* causes cell cycle arrest and the death of leukemias *in vitro* (30). Other murine models of T-ALL, including those arising in *Tcf7*$^{-/-}$ and *Ikzf1*$^{-/-}$ mice also show increased expression of *Lef1* (32, 33). Recently, a study of childhood ALL, including 28 patients with T-ALL, revealed a positive prognostic value to high *LEF1* expression and a second study confirmed this for specific LEF1 isoforms (34, 35). However, in a study of adult T-ALL, 25% of patients had elevated expression of LEF1 that was associated with a poor prognosis (36). In 4 patients, mutations in *LEF1* (K86E and P106L) were found to augment the transcriptional capacity of LEF1. In contrast to these findings of increased LEF1 expression or function, a subset of human T-ALL (18-27%) have inactivating mutations within the *LEF1* gene (5, 37). These observations suggest that LEF1 can function as both a pro- and anti-leukemia factor but a molecular understanding of the basis for these distinct functions is currently unknown.

To gain insight into the role of LEF1 in T-ALL we investigated its requirement in the generation of *E2a*$^{-/-}$ T cell leukemias. We demonstrate that *E2a*$^{-/-}$ leukemias that arose in mice sufficient for LEF1 became dependent on this transcription factor for their survival. However, deletion of *Lef1* prior to T cell transformation resulted in significant alterations in T cell development and a reduced latency to leukemic morbidity. Leukemic cells arising in the latter context resembled *E2a*$^{-/-}$ leukemias in that they had mutations in Notch1 and dependence on the Notch signaling pathway as well as frequent trisomy of chromosome 15, and hence increased c-*myc* expression. However, they differed from *E2a*$^{-/-}$ leukemias in that they had a CD4^{lo}CD8^{lo} phenotype with increased peripheral cell numbers at the time of sacrifice. Cell lines generated from these leukemias reveal differences in expression of multiple genes associated with monocarboxylic transport and hedgehog signaling that could also impact T cell receptor signaling. Our study describes novel models for studying LEF1 function in T-ALL and indicate that LEF1 is a modulator of leukemic transformation providing both addictive and inhibitory functions depending on its availability during transformation.

METHODS AND MATERIALS

Mice

Mice were backcrossed onto an FvB/NJ background for at least 8 generations. All experiments were performed in compliance with the University of Chicago Institutional Animal Care and Use Committee. *E2a*$^{-/-}$, *Lef1*$^{f/f}$, and Lck-Cre mice and genotyping protocols were described previously (38). FvBn/J mice and Lck-Cre mice were purchased from the Jackson Laboratory.

Flow Cytometry

Thymocytes or splenocytes were dissected and dispersed using frosted glass slides followed by filtration through a 100 mM cell strainer. Cells were stained at a concentration of 2 x 10^7 cells/ml in FACS buffer (PBS + 5% FCS +.02% azide) after incubation with FcBlock. Intracellular staining was performed using the FoxP3/Transcription Factor Staining Kit. Antibodies were purchased from BioLegend, eBiosciences or Fischer Scientific and specific antibody clones and fluorochromes are available upon request. Antibodies used: Lef1, CD4, CD8, CD117, CD25, CD44, TCRb, CD11b, CD11c, DX5, B220, Gr1. Data was acquired on an LSRII or Fortessa and analyzed using FlowJo (TreeStar).

Cell Lines

Leukemia cell lines were generated by culturing thymic cells from moribund mice in OPTI-MEM media containing 10% FCS, 2-mercaptoethanol and Pen/Strep/Glu for greater than 2 weeks. All established lines were frozen in 5% DMSO/50%FCS in liquid nitrogen for long term storage.

In Vitro Culture

OP9-DL1 stromal cells were maintained in OPTI-MEM and plated 1 day before used to achieve a near confluent monolayer of cells. Multipotent progenitors were isolated as Ter119-Gr1- cells from e13 fetal liver and cultured on OP9-DL1 in the presence of 5 ng/ml Flt3 ligand, IL-7 and CD117 ligand.

Retroviral Transduction

The retroviral vectors MigR1, MigR1-Cre, MigR1-DNMAML were described previously (39). Retroviral plasmid DNA was isolated using CsCl. Retroviral supernatants were produced by transfecting plasmid DNA into Phoenix cells using Ca_2PO_4 precipitation and cells were transduced with retrovirus as previously described (40).

RNA Extraction, Microarray, Sequencing and Analysis

RNA was extracted using Trizol Reagent. For microarray analysis, RNA was converted to cDNA that was used to probe Affymetrix MOE 430_2 arrays as previously described (41). Raw array data were normalized with RMAexpress (http://rmaexpress.bmbolstad.com/) and analyzed by dChip (http://www.biostat.harvard.edu/complab/dchip/). Probe set annotation was obtained from Affymetrix. For RNA-seq analysis, RNA was sequenced on a Next-Seq500 and analyzed as described (42). Raw sequence reads were trimmed using Trimmomatic v0.33 (TRAILING:30 MINLEN:20) and then aligned to mouse genome assembly mm10 with TopHat v 2.1.0. Reads were assigned to genes using the htseq-count tool from HTSeq v 0.6.1 and gene annotations from Ensembl release 78. The R package EdgeR was used to normalize the gene counts and to calculate differential expression statistics for each gene for each pairwise comparison of sample groups. Metascape analysis was performed on differential gene expression lists (https://metascape.org) (43). Genes were considered differentially expressed at Log2FC with an adj. p-value of <0.01.

Quantitative PCR

RNA was reverse transcribed with Superscript III (Invitrogen). Quantitative RT-PCR was performed in an iCycler (Bio-Rad Laboratories) with SYBR Green (Bio-Rad Laboratories). Expression values were normalized to Hprt and were calculated by the ΔC_T method. Primer sequences are available on request.

Spectral Karyotyping Analysis

To characterize the cytogentic pattern of T cell leukemias from $E2a^{-/-}Lef1^{\Delta/\Delta}$ mice, SKY analysis was performed using the ASI SkyPaintTM assay for mouse chromosomes as described previously (44) on cell lines or fresh leukemic cells from moribund mice with leukemia (10 metaphase cells were analyzed per case). Karyotype results are in **Table S2**.

Western Blot

Total protein extracts were prepared and analyzed by Western blot analysis as described previously (30). Primary antibodies used were anti-Notch1 antibody (V1744) reactive with the cleaved cytoplasmic domain (Cell Signaling Technology) and anti-actin (Abcam).

Data Sharing Statement

For original data please contact bkee@bsd.uchicago.edu. RNA-sequencing data can be accessed in the Gene Expression Omnibus under GSE186420. Microarray data is under GSE196391.

RESULTS

LEF1 Is Required for the Survival of E2A-Deficient T Cell Leukemias

Our previous studies revealed that Notch1 is mutated in $E2a^{-/-}$ T cell leukemias and required for their survival (26). We identified *Lef1* as a target of the Notch pathway in these cells and demonstrated that siRNA directed against *Lef1* reduced the viability of these leukemic cells (30). Here, using flow cytometry, we found that LEF1 and TCF1 protein are detected in $E2a^{-/-}$ leukemias and that LEF1, but not TCF1, was reduced after treatment of cells with a γ-secretase inhibitor, which antagonizes Notch signaling (**Figures 1A, B**). To rigorously demonstrate that LEF1 was required for growth of these leukemias, we created $E2a^{-/-}$ mice that were homozygous for alleles of *Lef1* with loxp sites flanking the DNA binding domain (32). We generated 2 lines from the leukemias arising in these mice and used a retrovirus producing Cre to delete the DNA binding domain of LEF1 (**Figure 1C**). The retrovirus produced GFP in addition to Cre and therefore we could track Cre expressing cells by their expression of GFP (**Figure 1C, D**). LEF1 was lost from a subset of cells after transduction with MigR1-Cre and the frequency of LEF1 negative cells mirrored the frequency of GFP^+ cells (**Figure 1D**). QPCR analysis of sorted GFP^+ cells from MigR1 or MigR1-Cre transduced cells revealed decreased *Lef1* mRNA after transduction of $E2a^{-/-}Lef1^{f/f}$ but not $E2a^{-/-}$ leukemias with MigR1-Cre (**Figure 1E**). We tracked the fate of cells with deletion of *Lef1* by following the frequency of GFP^+ cells. GFP^+ cells in MigR1-Cre expressing $E2a^{-/-}Lef1^{f/f}$ cells declined steadily over time consistent with the loss of cells that lacked LEF1 (**Figure 1F**). An $E2a^{-/-}$ leukemia line that was heterozygous for the $Lef1^f$ allele ($E2a^{-/-}Lef1^{f/+}$) and transduced with MigR1-Cre also showed reduced representation of GFP^+ cells with time in culture, although not to the degree of $E2A^{-/-}Lef1^{f/f}$ leukemias (**Figure 1F**). In contrast, the same cell lines transduced with MigR1, which does not promote deletion of *Lef1*, demonstrated a stable frequency of GFP^+ cells over time (**Figures 1D–F**). Similarly, $E2a^{-/-}$ lines transduced with either MigR1 or MigR1-Cre showed stable GFP expression (**Figure 1F**). To further examine the impact of LEF1-deletion on these leukemic cells, we analyzed the transcriptome of GFP^+ cells 72 hours after transduction with MigR1 or MigR1-Cre. We found decreased expression of multiple signaling-associate genes (*Id3*, *Syk*, *Sgk*, *Rasgrp1*) and increased expression of *Cdkn1a*, encoding the cell cycle inhibitor p21 (**Figure 1G** and **Table S1**). The increased expression of p21 could contribute to the reduced expansion of LEF1-deleted

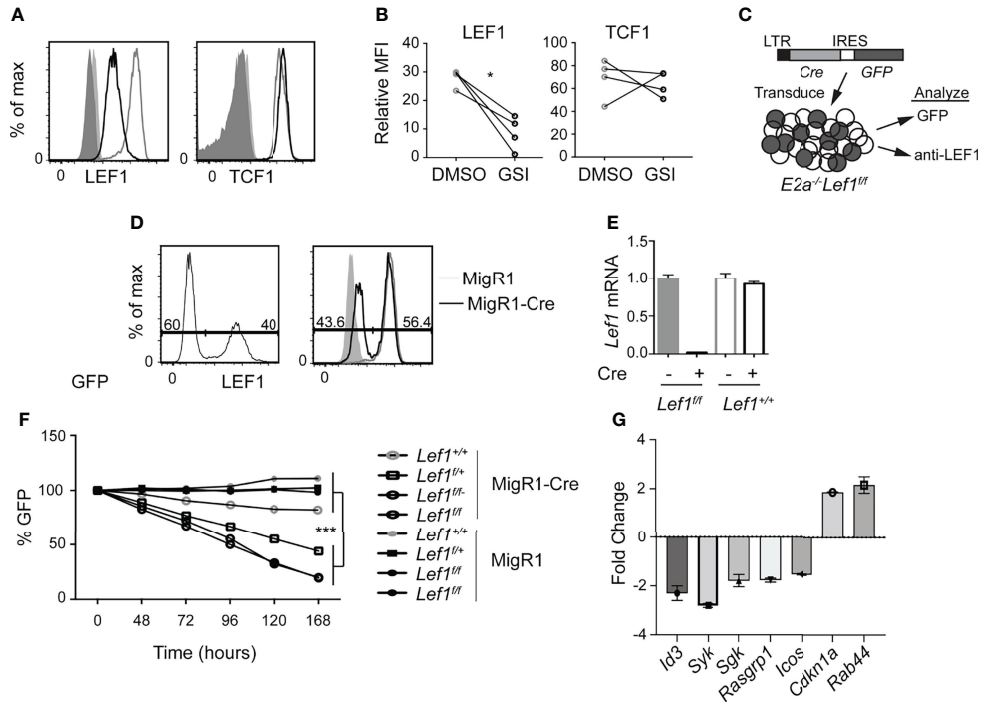


FIGURE 1 | LEF1 is required for the survival of established *E2a*$^{-/-}$ T cell leukemias. **(A)** Flow cytometry for LEF1 (left) or TCF1 (right) in an *E2a*$^{-/-}$ T cell leukemia line treated with DMSO (grey) or the Notch1 inhibitor GSI (black). Shaded histograms are isotype control stainings. n = 4 **(B)** Summary of the MFI for LEF1 or TCF1 in multiple *E2a*$^{-/-}$ leukemia lines. *p < 0.05, Students t-test. **(C)** Schematic representation of experiment to inactivate *Lef1* in *E2a*$^{-/-}$*Lef1*$^{f/f}$ T cell leukemias. **(D)** Expression of GFP (left) or LEF1 (right) in *E2a*$^{-/-}$*Lef1*$^{f/f}$ leukemias transduced with MigR1-Cre (black). For LEF1 staining, MigR1 transduced cells (grey) and isotype controls (shaded histograms) are also shown. **(E)** qRT-PCR analysis for *Lef1* mRNA in GFP^{+} cells isolated from *E2a*$^{-/-}$ leukemias with *Lef1*$^{f/f}$ or *Lef1*$^{+/+}$ 96 hours after transduction with MigR1 or MigR1-Cre. Error bars represent standard deviation. **(F)** The relative percent of GFP expressing cells with time in culture after retroviral transduction of *E2a*$^{-/-}$*Lef1*$^{+/+}$, *E2a*$^{-/-}$*Lef1*$^{f/+}$ or *E2a*$^{-/-}$*Lef1*$^{f/f}$ leukemias with MigR1 or MigR1-Cre retrovirus. Data are representative of n = 4 **(A, B)**, n = 2 **(D, E)** and n = 3 **(F)** experiments. ***p < 0.005. Students t-test on *E2a*$^{-/-}$*Lef1*$^{F/F}$ and *E2a*$^{-/-}$*Lef1*$^{F/+}$ compared to *E2a*$^{-/-}$*Lef1*$^{+/+}$ leukemias at t = 168 hours. **(G)** RNA from a GFP^{+} *E2A*$^{-/-}$*Lef1*$^{F/F}$ leukemia transduced with MigR1 or MigR1-Cre was analyzed at t = 72 hours by microarray. The graph depicts the FC between MigR1 and MigR1-Cre transduced cells for selected genes and 95% confidence.

leukemias. These data demonstrate that LEF1 is required for the maintenance of *E2a*$^{-/-}$ T cell leukemia lines *in vitro* and are consistent with our previous studies using *Lef1* siRNA (30).

LEF1 Is Increased in *E2a*$^{-/-}$ DN3 Thymocytes and Promotes Their Differentiation

To determine whether the increased expression of LEF1 occurs in pre-leukemic mice, we examined the Lineage^{-} population of the thymus for expression of LEF1. As expected, there were fewer DN3 thymocytes in *E2a*$^{-/-}$ mice compared to control with an increased frequency of CD117^{lo}CD25^{int} cells, previously shown to be innate lymphoid cells (**Figure 2A**) (45, 46). The *E2a*$^{-/-}$ DN3 cells expressed substantially more LEF1 than control DN3 thymocytes (**Figure 2B**). By qRT-PCR we found increased expression of *Lef1* mRNA in *E2a*$^{-/-}$ DN3 thymocytes (**Figure 2C**). DN3 thymocytes fail to develop from *E2a*$^{-/-}$ multipotent progenitors cultured *in vitro* on OP9-DL1 (45, 47), indicating that these cells are compromised with respect to T cell differentiation. To determine whether LEF1 might provide an advantage to these cells, we used a retrovirus to force multipotent progenitor (MPP) cells to ectopically express LEF1. *E2a*$^{-/-}$ MPPs transduced with the MigR1 retrovirus, which produce GFP only, failed to generate DN3 cells *in vitro*, as expected (**Figures 2D, E**). In contrast, *E2a*$^{-/-}$ MPPs transduced with LEF1 producing retrovirus generated DN3 cells and an increased frequency of DN2 cells. Notably, even control MPPs transduced with LEF1 producing retrovirus showed increased generation of DN2 and DN3 cells (**Figures 2D, E**). This propensity to promote differentiation of control and *E2a*$^{-/-}$ MPPs was not dependent on the presence of the β-catenin interaction domain (CAT) as a retrovirus producing a mutant form of LEF1 lacking the CAT domain also supported differentiation (**Figures 2D, E**). These data lead us to hypothesize that the increased expression of LEF1 in *E2a*$^{-/-}$ DN3 thymocytes aids in their differentiation from more immature progenitors and that LEF1's essential functions are independent of its interaction with β-catenin.

T Cell Specific Deletion of *Lef1* in *E2a*$^{-/-}$ Mice Abrogates DN3 Development

To test the hypothesis that LEF1 is essential for the development of *E2a*$^{-/-}$ DN3 cells and for leukemic transformation,

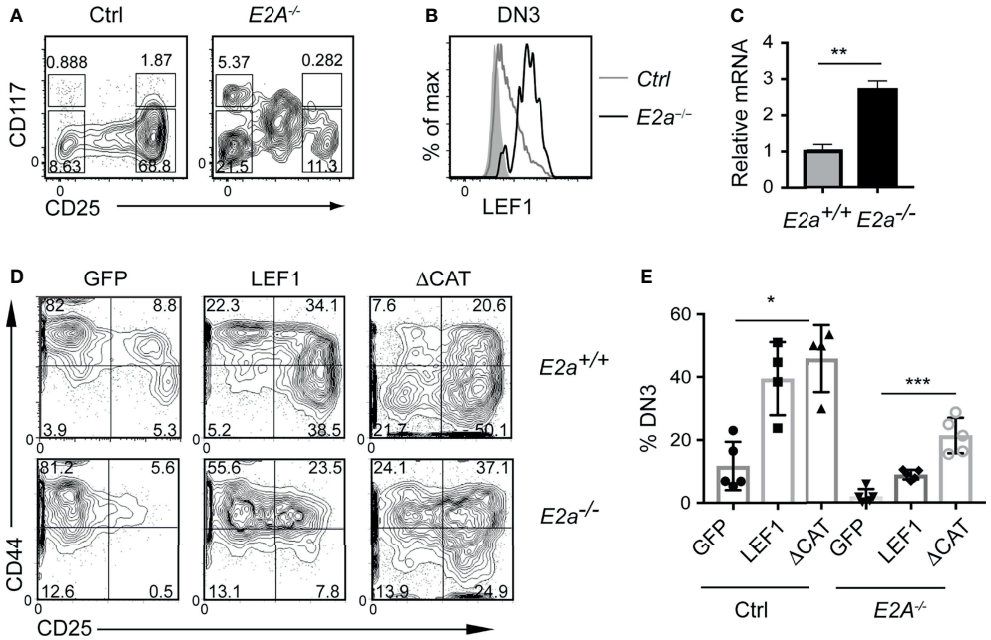


FIGURE 2 | LEF1 is highly expressed in *E2a*$^{-/-}$ DN3 cells and promotes their development from MPPs. **(A)** Flow cytometry showing CD117 and CD25 on Lineage$^-$ (CD4, CD8, CD11b, Ter119) thymocytes from Ctrl (left) and *E2a*$^{-/-}$ (right) mice at 5 weeks of age. **(B)** LEF1 expression in DN3 thymocytes from Ctrl (grey) and *E2a*$^{-/-}$ (black) mice. **(C)** Summary of *Lef1* mRNA relative to *Hprt* mRNA in Ctrl (grey) and *E2a*$^{-/-}$ (black) DN3 thymocytes. Data are representative of mice at 5-6 weeks of age. **p < 0.01 Student's t-test. **(D)** Flow cytometry of MPPs isolated from Ctrl (top) or *E2a*$^{-/-}$ (bottom) embryos (16 days p.c.) cultured *in vitro* for 10 days after retroviral transduction with MigR1 (GFP), MigR1-LEF1 or MigR1-LEF1ΔCAT. Data are gated on GFP$^+$ cells. **(E)** Summary of the %DN3 cells in the Lineage$^-$ population of MPPs cultured *in vitro* as in **(D)** from the indicated strains. Data are representative of **(A, B)** n = 3, **(C)** n = 2, **(D, E)** n = 4-6 experiments. *p < 0.05, **p < 0.01 and ***p < 0.005 ANOVA.

we generated Lck-Cre *E2a*$^{-/-}$*Lef1*$^{f/f}$ mice, which express Cre starting at the DN2 stage. Lck-Cre$^+$ *E2a*$^{-/-}$*Lef1*$^{f/f}$ mice (DKO) and *E2a*$^{-/-}$ mice had similar numbers of thymocytes and Lineage$^-$ thymocytes (**Figures 3A–C**). However, the Lineage$^-$ population of DKO mice showed a reduced frequency of DN3 thymocytes (**Figures 3B, D**). The number of DN3 thymocytes was also significantly decreased in DKO compared to *E2a*$^{-/-}$ mice (**Figure 3E**). In contrast Lck-Cre$^+$ *Lef1*$^{f/f}$ (*Lef1*$^{\Delta/\Delta}$) mice had thymocyte and Lineage$^-$ thymocyte numbers, as well as DN3 frequencies that were similar to Control mice (**Figure 3D**), indicating that *Lef1* deletion did not have a major impact on these cells.

Since the number of thymocytes was similar in *E2a*$^{-/-}$ and DKO mice we examined the phenotype of more mature thymocytes. As previously reported, *E2a*$^{-/-}$ thymocytes had a reduced frequency of CD4$^+$CD8$^+$ (DP) thymocytes with an increased frequency of CD4$^+$ and CD8$^+$ cells. The CD4 by CD8 profile of DKO thymocytes revealed a slightly increased frequency of CD4$^+$CD8$^+$ (DP) cells and a decreased frequency of CD4$^+$ of CD8$^+$ single positive thymocytes compared to *E2a*$^{-/-}$ mice (**Figure 4A**). *E2a*$^{-/-}$ mice also have an increased frequency of TCRβ$^+$ thymocytes, although the number of these cells is lower than in Ctrl mice (**Figures 4A, B**). The frequency and number of TCRβ$^+$ cells was reduced in DKO mice compared to *E2a*$^{-/-}$ mice, although it was still higher than in Ctrl or *Lef1*$^{\Delta/\Delta}$ mice (**Figures 4A, B**). These data suggest that deletion of LEF1 had a subtle but significant impact on the differentiation of thymocytes in the absence of *E2a*. Interestingly, a substantial portion of DKO Lineage$^+$ thymocytes, which are primarily DP cells, expressed CD25 (**Figures 4C, D**). The frequency of Lineage$^+$CD25$^+$ cells was also higher in *E2a*$^{-/-}$ thymocytes than in Ctrl or *Lef1*$^{\Delta/\Delta}$ thymocytes but was not significantly elevated in number (**Figures 4C, D**). Indeed, a direct comparison of DP thymocytes revealed a substantial increase CD25 on DKO compared to *E2a*$^{-/-}$ thymocytes (**Figure 4E**). Given that CD25 is a known Notch1 target gene (48), we tested the hypothesis that Notch1 was expressed in DP thymocytes from DKO mice. By QPCR analysis we observed mRNA for *Notch1* and the Notch1 target gene *Nrarp* in DP thymocytes from DKO but not *E2a*$^{-/-}$ or Ctrl mice (**Figure 4F**). These data indicate that Notch1 is activated in DKO DP thymocytes.

DKO Mice Develop T Cell Leukemia With Reduced Latency

To determine whether deletion of *Lef1* impacted the transformation potential of *E2a*$^{-/-}$ thymocytes, we allowed the mice to age and monitored them for signs of leukemia. Surprisingly, DKO mice became moribund with an average latency of 100 days (range 80-130 days) whereas the average latency for *E2a*$^{-/-}$ mice was 130 days (range 100-170 days), and notably, all mice that we followed developed disease (**Figure 5A**). At sacrifice, leukemia was confirmed by counting thymocyte

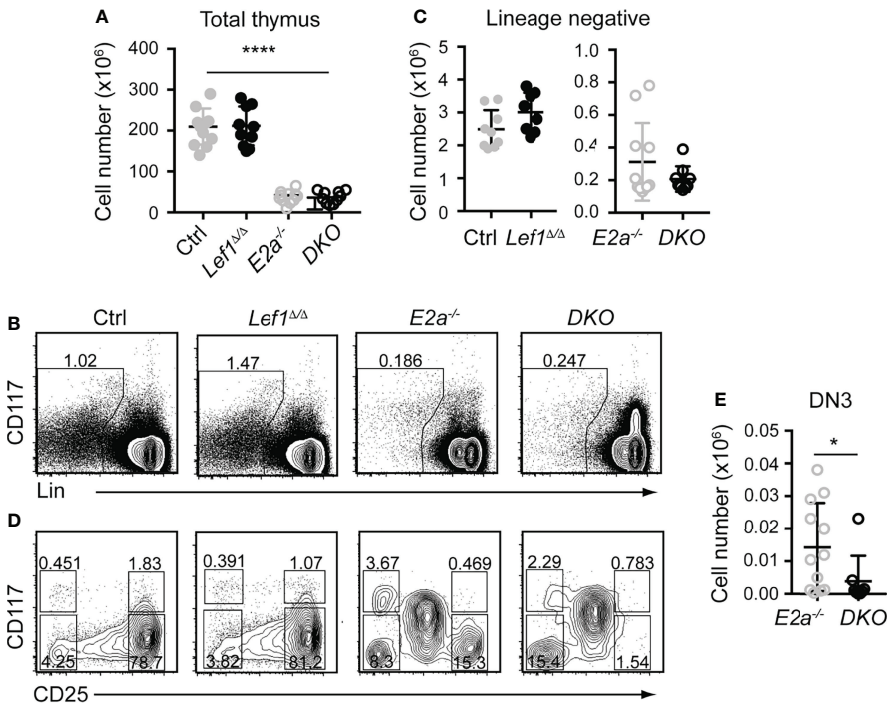


FIGURE 3 | T cell specific deletion of *Lef1* from *E2a*$^{-/-}$ mice does not impact thymocyte numbers but results in a loss of DN3 cells. **(A)** Total thymocyte numbers from mice of the indicated genotype. ****p < 0.001, Anova for multiple comparisons. **(B)** Flow cytometry showing Lineage markers (CD8, TCRβ, TCRγδ, CD11b, CD11c, NK1.1) and CD117 and the gating strategy for Lin$^-$ cells. **(C)** Lin$^-$ thymocyte numbers in mice of the indicated genotypes. **(D)** Flow cytometry showing CD25 and CD117 on Lin$^-$ thymocytes. **(E)** Summary of DN3 thymocyte numbers in mice of the indicated genotypes. Data is representative on more than 8 experiments *p < 0.05, Students t-test. Mice were between 5-7 weeks of age.

numbers and by flow cytometry of multiple tissues. Cell numbers were similar in the thymus of moribund *E2a*$^{-/-}$ or DKO mice, although the range was greater for the DKO cells (**Figure 5B**). However, splenic lymphocyte numbers were elevated in the moribund DKO as compared to *E2a*$^{-/-}$ mice (**Figure 5C**). Primary leukemic cells in DKO mice were frequently low for CD4 and CD8 whereas *E2a*$^{-/-}$ leukemias were CD4^{hi}CD8^{hi} or contained SP cells (**Figures 5D, E**). The DKO leukemias also expressed CD25 without CD44, a phenotype that is distinct from that of the majority of *E2a*$^{-/-}$ leukemias (**Figure 5E**). Taken together, these data demonstrate that, despite the reduced number of DN3 cells, DKO mice developed T cell leukemia with reduced latency compared to *E2a*$^{-/-}$ mice and these leukemias had a distinct surface receptor phenotype.

DKO Leukemias Have *Notch1* Mutations and Require Notch Signaling

E2a$^{-/-}$ leukemias have mutations in the *Notch1* gene and they are dependent on Notch signaling for their survival (26). To determine whether DKO leukemias also had mutations in the *Notch1* gene, we PCR amplified the 3' portion of *Notch1* and performed sequencing on 3 DKO cell lines that we established in culture. Notably, we found insertions that resulted in out of frame translation of the PEST domain of Notch1 in all of the DKO lines (**Figure 6A**). These mutations are predicted to stabilize ICN1 and, indeed, ICN1 protein could be detected in these leukemias by western blot analysis (**Figure 6B**). To determine whether these leukemias were dependent on Notch1 signaling we used retroviral transduction to ectopically express a dominant negative version of the Notch1 co-activator MAML in these cells (39). The frequency of cells expressing DN-MAML, identified by their expression of GFP, declined over time in culture regardless of whether they were *E2a*$^{-/-}$ or DKO leukemias (**Figure 6C**). In contrast, the frequency of GFP$^+$ cells remained stable in cultures transduced with the control MigR1 retrovirus (**Figure 6C**). Cytogenetic analysis using spectral karyotyping of three DKO primary leukemias revealed trisomy for chromosome 15 containing the *c-Myc* locus, a feature that is also observed in *E2a*$^{-/-}$ leukemias (**Figures 6C, D** and **Table S2**) (24). Consistent with this observation, *E2a*$^{-/-}$ and DKO cell lines had similar levels of *c-Myc* mRNA (**Figure 6E**). These data demonstrate that DKO leukemias, like *E2a*$^{-/-}$ leukemias, required Notch signaling for their survival and had mutations impacting the stability of ICN1 expression of *c-Myc*.

DKO Leukemias Have an Altered Transcriptome Implicating Monocarboxylic Acid Transport and Hedgehog Signaling

To gain further insight into the differences between *E2a*$^{-/-}$ and DKO leukemias we performed RNA-sequencing on multiple

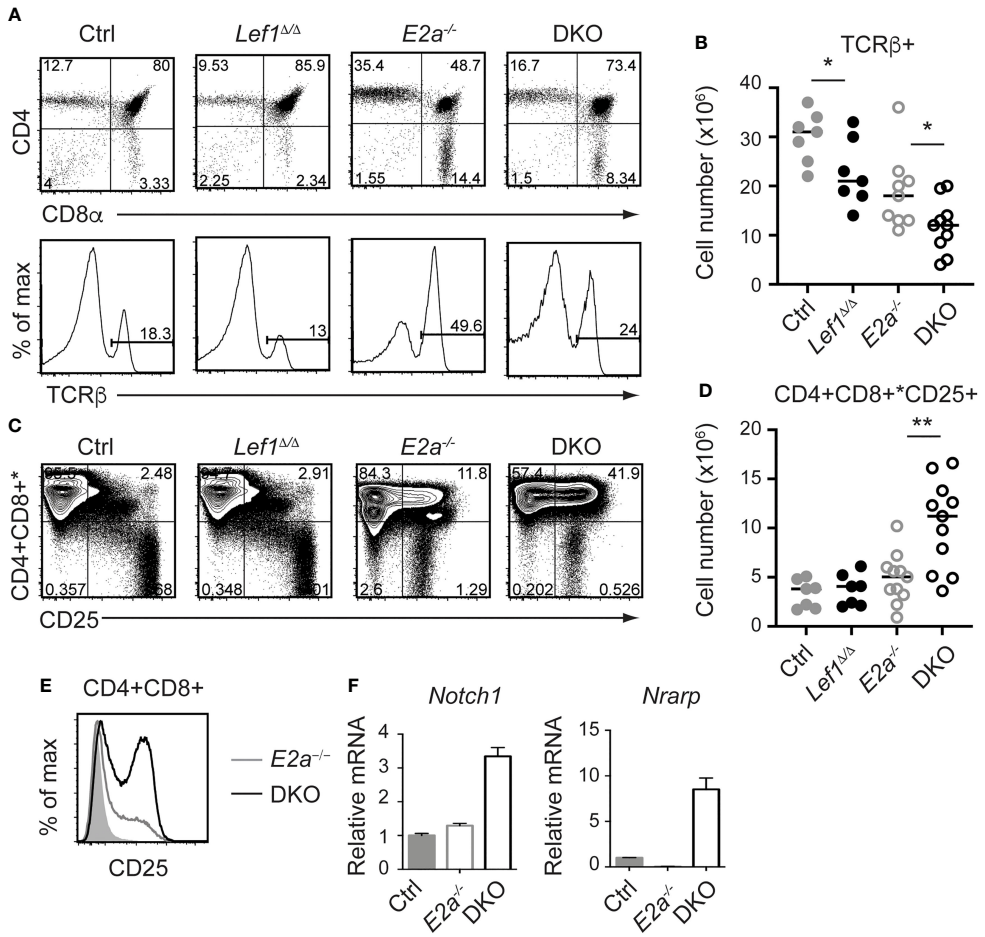
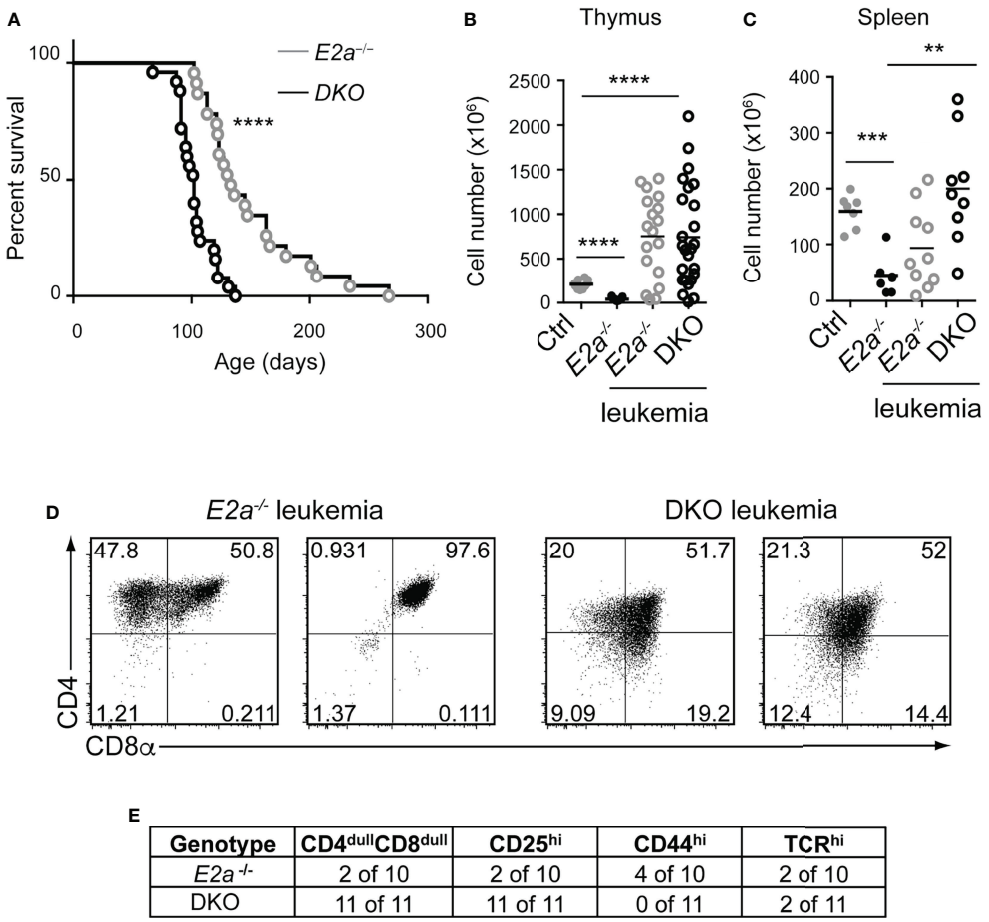


FIGURE 4 | LEF1 restrains expression of CD25, *Notch1*, and *Nrarp* in *E2a*$^{-/-}$ CD4^{+}CD8^{+} thymocytes. **(A)** Flow cytometry showing CD4 and CD8 (top) or TCRβ (bottom) on thymocytes from mice of the indicated genotype. **(B)** Summary of the number of TCRβ^{+} thymocytes in the indicated strains. **(C)** Flow cytometry showing expression of CD4 and CD8 (* indicates CD11b, CD11c, CD3e were also in this panel) and CD25. *p < 0.05, Student's t-test. **(D)** Total number of CD4^{+}CD8^{+*}CD25^{+} cells in mice of the indicated genotype. **p < 0.01, Student's t-test. **(E)** Flow cytometry of CD25 on CD4^{+}CD8^{+} thymocytes from WT (shaded), *E2a*$^{-/-}$ (grey) and DKO (black) mice. **(F)** qRT-PCR for *Notch1* and *Nrarp* on sorted CD4^{+}CD8^{+} thymocytes from mice of the indicated genotype. Data is representative of >6 mice **(A-D)** or 2 experiments **(E, F)**. Mice were 5-7 weeks of age.

E2a$^{-/-}$ and DKO leukemia lines. There was a large amount of variation in the gene programs of these leukemias but they were resolved into distinct populations by principle component analysis through PC1 and PC3 (**Table S3** and **Figure 7A**). We identified 89 genes that were generally decreased in DKO as compared to the *E2a*$^{-/-}$ lines and 70 genes that were increased (Log2FC, adj. p-value <0.01) (**Figure 7B**). *Tcf7* mRNA appeared to be increased in DKO leukemias but TCF1 protein, unlike LEF1 protein, was expressed similarly in *E2a*$^{-/-}$ and DKO lines when evaluated by flow cytometry (**Figure S1**). Analysis of the differentially expressed genes by Metascape revealed that the genes that decreased in the DKO leukemias were enriched for genes involved in IL-4 production whereas those that increased were enriched for genes in the monocarboxylic acid transport (MCT) and the Hedgehog signaling pathways (**Figures 7C, D**). Genes in the MCT pathway included *Fabp5*, *Pla2g12a*, *Syk* and *Hoxa13* (**Figures 7B, E**). Genes in the Hedgehog signaling pathway included *Axin2*, *Cdkn1a*, *Prkch* and *Rasgrp1* (**Figures 7B, F**). Taken together, these data indicate that DKO and *E2a*$^{-/-}$ leukemia lines share many common gene expression features but differ in a few key genes that could impact their metabolic requirements or response to external signals.

DISCUSSION

Over the past 10 years, analysis of gene expression and mutations in T-ALL have revealed both positive and negative associations with *LEF1* (34, 36, 37). Here, we investigated the role of LEF1 in T cell transformation in a murine model of T-ALL. We used conditional alleles of *Lef1* in *E2a*$^{-/-}$ T lymphocytes, that were deleted either before or after leukemic transformation and found that LEF1 can function as either an oncogene or a tumor suppressor depending on the context in which it is deleted.



E

Genotype	CD4^{dull}CD8^{dull}	CD25^{hi}	CD44^{hi}	TCR^{hi}
E2a$^{-/-}$	2 of 10	2 of 10	4 of 10	2 of 10
DKO	11 of 11	11 of 11	0 of 11	2 of 11

FIGURE 5 | DKO mice have an accelerated onset of T cell leukemia. **(A)** Kaplan-Meier plot of leukemia incidence in *E2a*$^{-/-}$ and DKO mice. ****p < 0.0001, Mantel-Cox Test **(B)** Total thymocyte numbers and **(C)** spleen lymphocyte numbers at time of morbidity in *E2a*$^{-/-}$ and DKO mice compared to age matched Ctrl and non-moribund *E2a*$^{-/-}$ mice. ** p< 0.01, ***p < 0.005, ****p < 0.001. Student's t-test for pairwise comparisons. **(D)** Flow cytometry showing CD4 and CD8 on two representative *E2a*$^{-/-}$ and DKO thymic leukemias. **(E)** Summary of phenotype of *E2a*$^{-/-}$ and DKO leukemias.

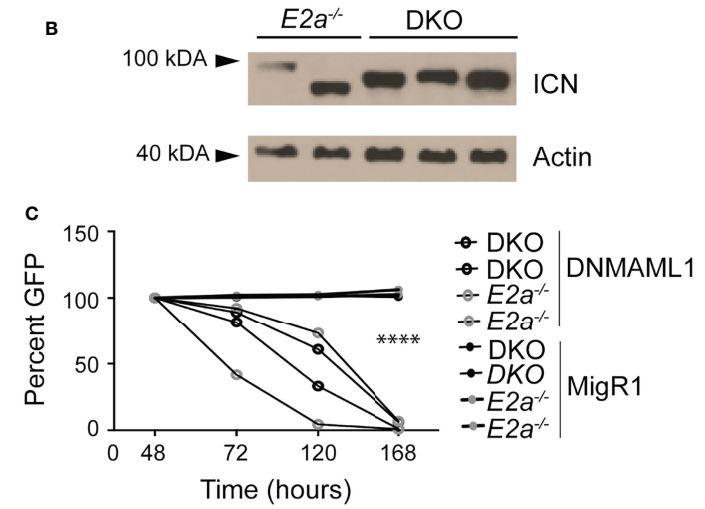
When LEF1 is present during the transformation process, leukemias can become addicted to its presence. In contrast, when *Lef1* is deleted prior to transformation, early T cell development is altered and there is a more rapid onset of leukemogenesis. We note that leukemias arising in DKO mice had some of the characteristics of human T-ALL with *LEF1* inactivating mutations including mutations in the Notch1 signaling pathway and gain of chromosome 15 (37), although these characteristics are shared with *E2a*$^{-/-}$ leukemias (24, 26). However, DKO leukemias had a more a rapid onset than *E2a*$^{-/-}$ leukemias mirroring the early development of T-ALL with *Lef1* inactivating mutations (37). In addition to providing insight into how LEF1 can be both a positive and negative regulator of leukemogenesis, our data indicate that the order of acquisition of specific mutations in T cell progenitors can impact the phenotype and latency of T-ALL.

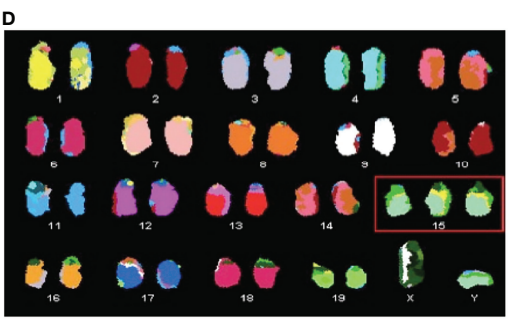
The leukemic cells that arise in DKO mice have very dim expression of CD4 and CD8 and express CD25, which distinguishes them from *E2a*$^{-/-}$ leukemias. Cell lines established from DKO mice also showed some differences in gene expression from *E2a*$^{-/-}$ leukemias. In particular, genes associated with the MCT and Hedgehog signaling pathway, were elevated relative to *E2a*$^{-/-}$ leukemias. Among these genes were *Rasgrp1, Pla2g12a* and *Syk*, which are also involved in T cell receptor signaling and their increased expression may be related to the dim expression of CD4 and CD8, which canonically identifies cells undergoing strong TCR signaling or negative selection (49). The increased expression of *Fabp5* also raises the possibility that DKO and *E2a*$^{-/-}$ leukemias may have different metabolic requirements. While there was quite a bit of variability in the gene programs of individual leukemia lines, these observations raise the possibility that leukemias with different mutation profiles may have unique susceptibilities that could be exploited for therapy.

Lef1 is increased in expression in multiple mouse models of T-ALL. *Tcf7*$^{-/-}$ mice also have increased *Lef1* in DN thymocytes but develop an ETP-like T-ALL (32). TCF1 was shown to repress *Lef1* and indeed, TCF1 binds to a cluster of sites in the *Lef1* gene and represses transcription from this region in a reporter

A

Genotype	Notch1 mutation	Location/truncation
E2a$^{-/-}$	G to T mutation	aa2260
E2a$^{-/-}$	T to C mutation	aa2886
DKO	CCCCCCG insertion	aa2399
DKO	G insertion	aa2361
DKO	CCCT insertion	aa2361





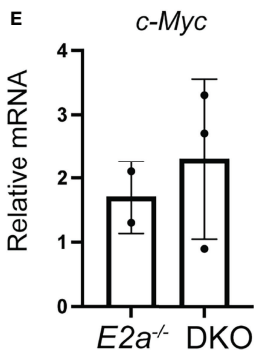


FIGURE 6 | DKO leukemias require Notch1 signaling and have amplification of chromosome 15. **(A)** Identification of mutations in the *Notch1* gene in two *E2a*$^{-/-}$ and 3 DKO leukemias. **(B)** Western Blot Analysis for cleaved ICN1 in the indicated leukemia lines. Actin is shown as a loading control. **(C)** Relative GFP expression in leukemias of the indicated genotype after transduction with MigR1 or MigR1-DNMAML. The frequency of GFP^{+} cells was assessed by flow cytometry at the indicated times after transduction. ****p < 0.0001 Students t-test on MigR1 versus DNMAML infected cells **(D)** Spectral karyotyping analysis of metaphase cells isolated from a DKO leukemia. **(E)** qRT-PCR analysis for *c-Myc* in 2 *E2a*$^{-/-}$ and 3 DKO leukemias.

construct transduced into DN thymocytes. TCF1 is expressed in *E2a*$^{-/-}$ thymocytes at levels indistinguishable from control mice indicating that the increased LEF1 is not a consequence of TCF1-deficiency (45, 46). We showed here that unlike LEF1, TCF1 is not regulated by Notch signaling in *E2a*$^{-/-}$ leukemias and therefore we hypothesize that Notch1 regulates *Lef1* directly and independent of TCF1. Deletion of *Lef1* in *Tcf7*$^{-/-}$ thymocytes had surprisingly little impact on DN thymocyte development but prevented β-selection and abrogated leukemogenesis (32). In contrast, deletion of *Lef1* in *E2a*$^{-/-}$ mice did not prevent leukemogenesis. Therefore, TCF1 appears sufficient in *E2a*$^{-/-}$ mice to support T cell transformation. TCF1 protein was expressed equivalently in *E2a*$^{-/-}$ and DKO leukemias suggesting that LEF1 does not promote leukemogenesis by repressing *Tcf7*. Given that *E2a*$^{-/-}$ leukemias are not ETP-like, we do not think that the major function of LEF1 is simply antagonism of TCF1. However, it is possible that high levels of LEF1 impact TCF1 function by competing with TCF1 for binding to TCF1/LEF1 binding sites, either promoting or inhibiting TCF1-like functions. In this scenario, LEF1 may

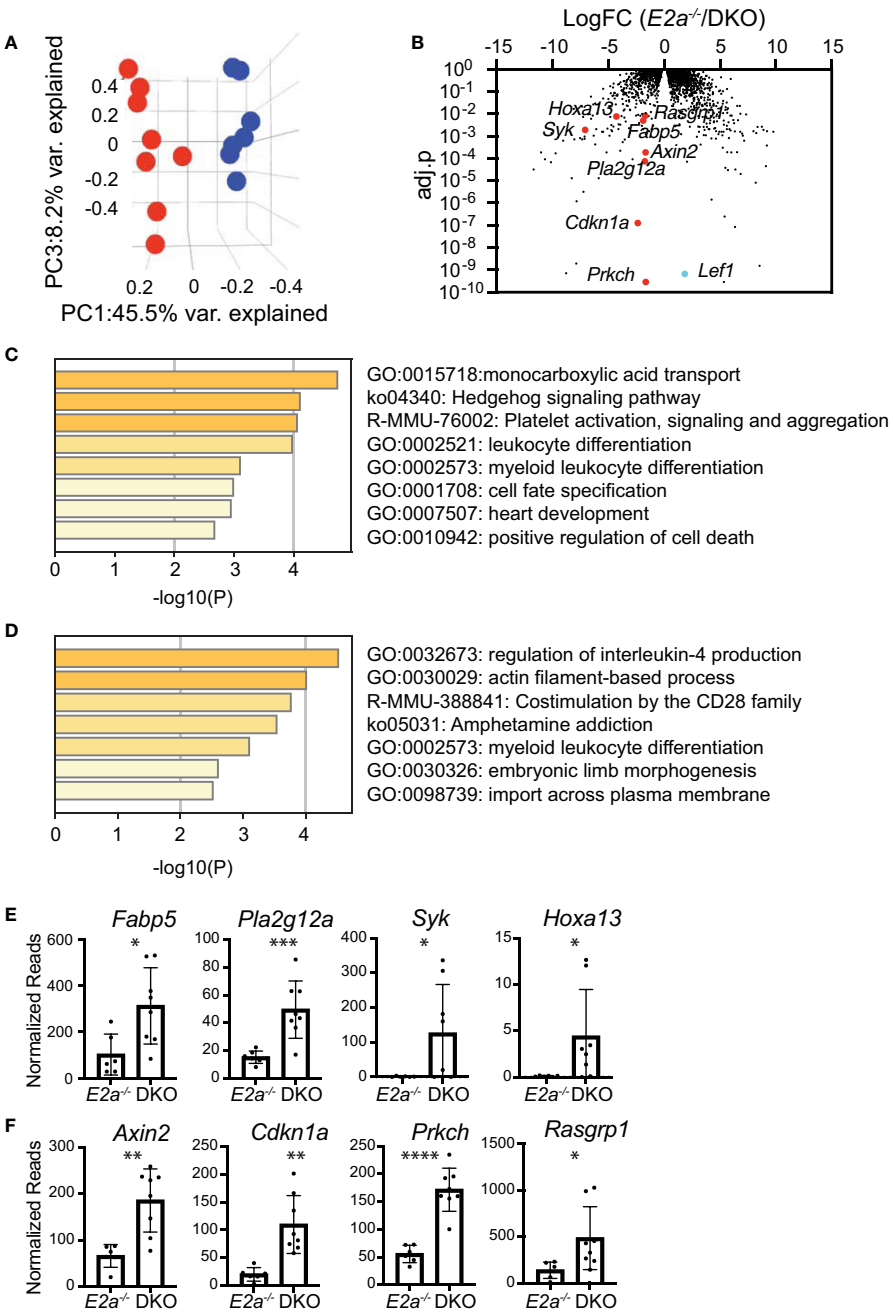


FIGURE 7 | RNA-sequencing of *E2a*$^{-/-}$ and DKO leukemia lines reveals differences in multiple genes associated with signaling and monocarboxylic acid transport. **(A)** Principal Component Analysis of *E2a*$^{-/-}$ (blue) and DKO (red) leukemias based on differential gene expression in RNA-seq analysis. adj.p based on FDR. **(B)** Volcano plot showing Log2FC by adj.p-value comparing RNA-seq from *E2a*$^{-/-}$ and DKO leukemias. *Lef1* and genes on interest that were higher in DKO as compared to *E2a*$^{-/-}$ leukemias are indicated. **(C)** Pathway analysis for genes that are increased in DKO compared to *E2a*$^{-/-}$ leukemias. **(D)** Pathway analysis of genes that are decreased in DKO compared to *E2a*$^{-/-}$ leukemias. **(E)** Normalized reads for individual *E2a*$^{-/-}$ and DKO leukemia samples for selected genes in the monocarboxylic acid transport pathway or **(F)** the Hedgehog Signaling Pathway. *p < 0.05, **p < 0.01, ***p < 0.005, and ****p < 0.0001 by student t-test of individual normalized reads.

antagonize TCF1 functions that prevent transformation while promoting, or leaving intact, TCF1 functions that support T cell differentiation. Indeed, we note that *Cdkn1a* is increased in both DKO leukemias and *E2a*$^{-/-}$*Lef1*$^{F/F}$ leukemias after deletion of *Lef1* compared to *E2a*$^{-/-}$ leukemias suggesting that *Cdkn1a* could be a TCF1 target that is repressed by LEF1. In contrast, *Syk* and *Rasgrp1* are increased in DKO compared to *E2A*$^{-/-}$ leukemias but decreased after deletion of *Lef1* from *E2a*$^{-/-}$*Lef1*$^{F/F}$ leukemias.

Thus, we could speculate that *Syk* and *Rasgrp1* are positively regulated by LEF1 or TCF1, gaining a dependence on high levels of LEF1 in *E2a*$^{-/-}$Lef1F/F leukemias and TCF1 in DKO leukemias. Further studies are required to understand the mechanisms by which LEF1 and TCF1 function in these different contexts.

We anticipated that LEF1 would be required for the differentiation of ETPs to the DN3 stage in the absence of E2A and our *in vitro* experiments supported this hypothesis. Indeed, deletion of *Lef1* in *E2a*$^{-/-}$ mice resulted in a loss of DN3 thymocytes. However, total thymocyte numbers were not impacted by deletion of *Lef1* suggesting that LEF1 was not required for T cell development in the absence of *E2a*. Given that we observed increased CD25 and Notch1 signaling in DP thymocytes after deletion of *Lef1*, we hypothesize that LEF1 prevented differentiation of DN3 cells into DP cells. Whether these cells are truly DP thymocytes or DN3 thymocytes that express CD4 and CD8 remains to be investigated. Thus, the altered latency of transformation in DKO mice could be related to differences in the intrinsic susceptibility of thymocytes at different stages of differentiation to transformation in the absence of E2A or to an altered environment in which the transforming progenitors reside.

Taken together, our data demonstrate that LEF1 impacts the developmental trajectory of *E2a*$^{-/-}$ T cell progenitors and can act as a tumor suppressor or oncogene depending on its availability during the transformation process. Moreover, our data support the utility of mouse models for understanding the cooperativity and consequence of mutational order on leukemogenesis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE186420 and GSE196391.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of Chicago.

AUTHOR CONTRIBUTIONS

TC, SM, SD, and MV designed and performed experiments and interpreted data. ML supervised the SKY analysis, HX provided the *Lef1*$^{F/F}$ mice, MS supervised the microarray experiments, EB performed bioinformatics analysis, BK designed and performed experiments, interpreted data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.845488/full#supplementary-material

Supplementary Figure 1 | Expression of LEF1 and TCF1 in *E2a*$^{-/-}$ and DKO leukemias. Normalized Reads for Lef1 **(A)** and Tcf7 **(B)** from RNA-sequencing data using RNA isolated from *E2a*$^{-/-}$ or DKO leukemia lines. Each dot represents the normalized reads from on line. Flow cytometry for **(C)** LEF1 and **(D)** TCF1 in Ctrl thymocytes (left panels) or an *E2a*$^{-/-}$ (middle panels) or DKO (right panels) leukemia. The shaded histogram is isotype control staining. ***p < 0.005.

REFERENCES

1. Belver L, Ferrando A. The Genetics and Mechanisms of T Cell Acute Lymphoblastic Leukaemia. *Nat Rev Cancer* (2016) 16:494–507. doi: 10.1038/nrc.2016.63
2. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, et al. Proposals for the Immunological Classification of Acute Leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). *Leukemia* (1995) 9:1783–6.
3. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-Cell Precursor Leukaemia: A Subtype of Very High-Risk Acute Lymphoblastic Leukaemia. *Lancet Oncol* (2009) 10:147–56. doi: 10.1016/S1470-2045(08)70314-0
4. Ferrando AA, Look AT. Gene Expression Profiling in T-Cell Acute Lymphoblastic Leukemia. *Semin Hematol* (2003) 40:274–80. doi: 10.1016/S0037-1963(03)00195-1
5. Noronha EP, Marques LVC, Andrade FG, Thuler LCS, Terra-Granado E, Pombo-de-Oliveira MS. Brazilian Collaborative Study Group of Acute. The Profile of Immunophenotype and Genotype Aberrations in Subsets of Pediatric T-Cell Acute Lymphoblastic Leukemia. *Front Oncol* (2019) 9:316. doi: 10.3389/fonc.2019.00316
6. Ferrando AA, Lopez-Otin C. Clonal Evolution in Leukemia. *Nat Med* (2017) 23:1135–45. doi: 10.1038/nm.4410
7. Weng AP, Ferrando AA, Lee W, t. Morris JP, Silverman LB, Sanchez-Irizarry C, et al. Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. *Science* (2004) 306:269–71. doi: 10.1126/science.1102160
8. Malyukova A, Brown S, Papa R, O'Brien R, Giles J, Trahair TN, et al. FBXW7 Regulates Glucocorticoid Response in T-Cell Acute Lymphoblastic Leukaemia by Targeting the Glucocorticoid Receptor for Degradation. *Leukemia* (2013) 27:1053–62. doi: 10.1038/leu.2012.361

9. Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, et al. The SCFFBW7 Ubiquitin Ligase Complex as a Tumor Suppressor in T Cell Leukemia. *J Exp Med* (2007) 204:1825–35. doi: 10.1084/jem.20070872
10. King B, Trimarchi T, Reavie L, Xu L, Mullenders J, Ntziachristos P, et al. The Ubiquitin Ligase FBXW7 Modulates Leukemia-Initiating Cell Activity by Regulating MYC Stability. *Cell* (2013) 153:1552–66. doi: 10.1016/j.cell.2013.05.041
11. Gomez-del Arco P, Kashiwagi M, Jackson AF, Naito T, Zhang J, Liu F, et al. Alternative Promoter Usage at the Notch1 Locus Supports Ligand-Independent Signaling in T Cell Development and Leukemogenesis. *Immunity* (2010) 33:685–98. doi: 10.1016/j.immuni.2010.11.008
12. Ashworth TD, Pear WS, Chiang MY, Blacklow SC, Mastio J, Xu L, et al. Deletion-Based Mechanisms of Notch1 Activation in T-ALL: Key Roles for RAG Recombinase and a Conserved Internal Translational Start Site in Notch1. *Blood* (2010) 116:5455–64. doi: 10.1182/blood-2010-05-286328
13. Jeannet R, Mastio J, Macias-Garcia A, Oravecz A, Ashworth T, Geimer Le Lay AS, et al. Oncogenic Activation of the Notch1 Gene by Deletion of Its Promoter in Ikaros-Deficient T-ALL. *Blood* (2010) 116:5443–54. doi: 10.1182/blood-2010-05-286658
14. Allman D, Karnell FG, Punt JA, Bakkour S, Xu L, Myung P, et al. Separation of Notch1 Promoted Lineage Commitment and Expansion/Transformation in Developing T Cells. *J Exp Med* (2001) 194:99–106. doi: 10.1084/jem.194.1.99
15. de Pooter RF, Kee BL. E Proteins and the Regulation of Early Lymphocyte Development. *Immunol Rev* (2010) 238:93–109. doi: 10.1111/j.1600-065X.2010.00957.x
16. Anderson MK. At the Crossroads: Diverse Roles of Early Thymocyte Transcriptional Regulators. *Immunol Rev* (2006) 209:191–211. doi: 10.1111/j.0105-2896.2006.00352.x
17. Liau WS, Tan SH, Ngoc PCT, Wang CQ, Tergaonkar V, Feng H, et al. Aberrant Activation of the GIMAP Enhancer by Oncogenic Transcription Factors in T-Cell Acute Lymphoblastic Leukemia. *Leukemia* (2017) 31:1798–807. doi: 10.1038/leu.2016.392
18. Mansour MR, Sanda T, Lawton LN, Li X, Kreslavsky T, Novina CD, et al. The TAL1 Complex Targets the FBXW7 Tumor Suppressor by Activating Mir-223 in Human T Cell Acute Lymphoblastic Leukemia. *J Exp Med* (2013) 210:1545–57. doi: 10.1084/jem.20122516
19. Sanda T, Lawton LN, Barrasa MI, Fan ZP, Kohlhammer H, Gutierrez A, et al. Core Transcriptional Regulatory Circuit Controlled by the TAL1 Complex in Human T Cell Acute Lymphoblastic Leukemia. *Cancer Cell* (2012) 22:209–21. doi: 10.1016/j.ccr.2012.06.007
20. Nagel S, Ehrentraut S, Tomasch J, Lienenklaus S, Schneider B, Geffers R, et al. Transcriptional Activation of Prostate Specific Homeobox Gene NKX3-1 in Subsets of T-Cell Lymphoblastic Leukemia (T-ALL). *PLoS One* (2012) 7:e40747. doi: 10.1371/journal.pone.0040747
21. Kusy S, Gerby B, Goardon N, Gault N, Ferri F, Gerard D, et al. NKX3.1 is a Direct TAL1 Target Gene That Mediates Proliferation of TAL1-Expressing Human T Cell Acute Lymphoblastic Leukemia. *J Exp Med* (2010) 207:2141–56. doi: 10.1084/jem.20100745
22. Kim D, Peng XC, Sun XH. Massive Apoptosis of Thymocytes in T-Cell-Deficient Id1 Transgenic Mice. *Mol Cell Biol* (1999) 19:8240–53. doi: 10.1128/MCB.19.12.8240
23. Morrow MA, Mayer EW, Perez CA, Adlam M, Siu G. Overexpression of the Helix-Loop-Helix Protein Id2 Blocks T Cell Development at Multiple Stages. *Mol Immunol* (1999) 36:491–503. doi: 10.1016/S0161-5890(99)00071-1
24. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2A Deficiency Leads to Abnormalities in Alphabeta T-Cell Development and to Rapid Development of T-Cell Lymphomas. *Mol Cell Biol* (1997) 17:4782–91. doi: 10.1128/MCB.17.8.4782
25. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High Incidence of T-Cell Tumors in E2A-Null Mice and E2A/Id1 Double-Knockout Mice. *Mol Cell Biol* (1997) 17:7317–27. doi: 10.1128/MCB.17.12.7317
26. Reschly EJ, Spaulding C, Vilimas T, Graham WV, Brumbaugh RL, Aifantis I, et al. Notch1 Promotes Survival of E2A-Deficient T Cell Lymphomas Through Pre-T Cell Receptor-Dependent and -Independent Mechanisms. *Blood* (2006) 107:4115–21. doi: 10.1182/blood-2005-09-3551
27. Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, et al. C-Myc Is an Important Direct Target of Notch1 in T-Cell Acute Lymphoblastic Leukemia/Lymphoma. *Genes Dev* (2006) 20:2096–109. doi: 10.1101/gad.1450406
28. Sharma VM, Calvo JA, Draheim KM, Cunningham LA, Hermance N, Beverly L, et al. Notch1 Contributes to Mouse T-Cell Leukemia by Directly Inducing the Expression of C-Myc. *Mol Cell Biol* (2006) 26:8022–31. doi: 10.1128/MCB.01091-06
29. Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, et al. NOTCH1 Directly Regulates C-MYC and Activates a Feed-Forward-Loop Transcriptional Network Promoting Leukemic Cell Growth. *Proc Natl Acad Sci USA* (2006) 103:18261–6. doi: 10.1073/pnas.0606108103
30. Spaulding C, Reschly EJ, Zagort DE, Yashiro-Ohtani Y, Beverly LJ, Capobianco A, et al. Notch1 Co-Opts Lymphoid Enhancer Factor 1 for Survival of Murine T-Cell Lymphomas. *Blood* (2007) 110:2650–8. doi: 10.1182/blood-2007-04-084202
31. Steinke FC, Xue HH. From Inception to Output, Tcf1 and Lef1 Safeguard Development of T Cells and Innate Immune Cells. *Immunol Res* (2014) 59:45–55. doi: 10.1007/s12026-014-8545-9
32. Yu S, Zhou X, Steinke FC, Liu C, Chen SC, Zagorodna O, et al. The TCF-1 and LEF-1 Transcription Factors Have Cooperative and Opposing Roles in T Cell Development and Malignancy. *Immunity* (2012) 37:813–26. doi: 10.1016/j.immuni.2012.08.009
33. Geimer Le Lay AS, Oravecz A, Mastio J, Jung C, Marchal P, Ebel C, et al. The Tumor Suppressor Ikaros Shapes the Repertoire of Notch Target Genes in T Cells. *Sci Signal* (2014) 7:ra28. doi: 10.1126/scisignal.2004545
34. Jia M, Zhao HZ, Shen HP, Cheng YP, Luo ZB, Li SS, et al. Overexpression of Lymphoid Enhancer-Binding Factor-1 (LEF1) Is a Novel Favorable Prognostic Factor in Childhood Acute Lymphoblastic Leukemia. *Int J Lab Hematol* (2015) 37:631–40. doi: 10.1111/ijlh.12375
35. Erbilgin Y, Hatirnaz Ng O, Can I, Firtina S, Kucukcankurt F, Karaman S, et al. Prognostic Evidence of LEF1 Isoforms in Childhood Acute Lymphoblastic Leukemia. *Int J Lab Hematol* (2021) 43(5):1093–103. doi: 10.1111/ijlh.13513
36. Guo X, Zhang R, Liu J, Li M, Song C, Dovat S, et al. Characterization of LEF1 High Expression and Novel Mutations in Adult Acute Lymphoblastic Leukemia. *PLoS One* (2015) 10:e0125429. doi: 10.1371/journal.pone.0125429
37. Gutierrez A, Sanda T, Ma W, Zhang J, Grebliunaite R, Dahlberg S, et al. Inactivation of LEF1 in T-Cell Acute Lymphoblastic Leukemia. *Blood* (2010) 115:2845–51. doi: 10.1182/blood-2009-07-234377
38. Carr T, Krishnamoorthy V, Yu S, Xue HH, Kee BL, Verykokakis M. The Transcription Factor Lymphoid Enhancer Factor 1 Controls Invariant Natural Killer T Cell Expansion and Th2-Type Effector Differentiation. *J Exp Med* (2015) 212:793–807. doi: 10.1084/jem.20141849
39. Maillard I, Weng AP, Carpenter AC, Rodriguez CG, Sai H, Xu L, et al. Mastermind Critically Regulates Notch-Mediated Lymphoid Cell Fate Decisions. *Blood* (2004) 104:1696–702. doi: 10.1182/blood-2004-02-0514
40. Kee BL. Id3 Induces Growth Arrest and Caspase-2-Dependent Apoptosis in B Lymphocyte Progenitors. *J Immunol* (2005) 175:4518–27. doi: 10.4049/jimmunol.175.7.4518
41. Dias S, Mansson R, Gurbuxani S, Sigvardsson M, Kee BL. E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* (2008) 29:217–27. doi: 10.1016/j.immuni.2008.05.015
42. Jacobsen JA, Woodard J, Mandal M, Clark MR, Bartom ET, Sigvardsson M, et al. EZH2 Regulates the Developmental Timing of Effectors of the Pre-Antigen Receptor Checkpoints. *J Immunol* (2017) 198:4682–91. doi: 10.4049/jimmunol.1700319
43. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape Provides a Biologist-Oriented Resource for the Analysis of Systems-Level Datasets. *Nat Commun* (2019) 10:1523. doi: 10.1038/s41467-019-09234-6
44. Le Beau MM, Espinosa R3rd, Davis EM, Eisenbart JD, Larson RA. And XXXE. D. Green. Cytogenetic and Molecular Delineaton of a Region of Chroosome 7 Commonly Deleted in Malignant Myeloid Diseases. *Blood* (1996) 88:1930–5.
45. Xu W, Carr T, Ramirez K, McGregor S, Sigvardsson M, Kee BL. E2A Transcription Factors Limit Expression of Gata3 to Facilitate T Lymphocyte Lineage Commitment. *Blood* (2013) 121:1534–42. doi: 10.1182/blood-2012-08-449447
46. Miyazaki M, Miyazaki K, Chen K, Jin Y, Turner J, Moore AJ, et al. The E-Id Protein Axis Specifies Adaptive Lymphoid Cell Identity and Suppresses

Thymic Innate Lymphoid Cell Development. *Immunity* (2017) 46:818–34.e814. doi: 10.1016/j.immuni.2017.04.022
47. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E Proteins and Notch Signaling Cooperate to Promote T Cell Lineage Specification and Commitment. *J Exp Med* (2006) 203:1329–42. doi: 10.1084/jem.20060268
48. Gothert JR, Brake RL, Smeets M, Duhrsen U, Begley CG, Izon DJ. NOTCH1 Pathway Activation Is an Early Hallmark of SCL T Leukemogenesis. *Blood* (2007) 110:3753–62. doi: 10.1182/blood-2006-12-063644
49. Gascoigne NR, Palmer E. Signaling in Thymic Selection. *Curr Opin Immunol* (2011) 23:207–12. doi: 10.1016/j.coi.2010.12.017

Conflict of Interest: BK is on the Scientific Advisory Board for Century Therapeutics.

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Monoallelic *Heb/Tcf12* Deletion Reduces the Requirement for NOTCH1 Hyperactivation in T-Cell Acute Lymphoblastic Leukemia

Diogo F. T. Veiga[1,2†], Mathieu Tremblay[1†], Bastien Gerby[1,3], Sabine Herblot[1,4], André Haman[1], Patrick Gendron[1], Sébastien Lemieux[1,5], Juan Carlos Zúñiga-Pflücker[6], Josée Hébert[1,7,8,9], Joseph Paul Cohen[10,11] and Trang Hoang[1]*

[1] Department of Pharmacology and Physiology, Université de Montréal, Institute for Research in Immunology and Cancer, QC, Canada, [2] Department of Translational Medicine, School of Medical Sciences, University of Campinas, Campinas, Brazil, [3] Centre de Recherches en Cancérologie de Toulouse (CRCT), Université de Toulouse, Institut National de la Santé et de la Recherche Médicale (INSERM), UMR-1037, Université Toulouse III Paul Sabatier (UPS), Toulouse, France, [4] Unité de recherche en hémato-oncologie Charles-Bruneau, Centre de Recherche du CHU Sainte-Justine, Montréal, Canada, [5] Department of Biochemistry and Molecular Medicine, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, QC, Canada, [6] Department of Immunology, University of Toronto, and Sunnybrook Research Institute, Toronto, ON, Canada, [7] Institut universitaire d'hémato-oncologie et de thérapie cellulaire, Hôpital Maisonneuve-Rosemont, Montréal, QC, Canada, [8] Quebec Leukemia Cell Bank, Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Montréal, QC, Canada, [9] Department of Medicine, Université de Montréal, Montréal, QC, Canada, [10] Department of Computer Science and Operations Research, Université de Montréal, Montreal, QC, Canada, [11] Université de Montréal, Montreal, QC, Canada

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***Correspondence:**
Trang Hoang
trang.hoang@umontreal.ca

†These authors have contributed equally to this work and share first authorship

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Early T-cell development is precisely controlled by E proteins, that indistinguishably include HEB/TCF12 and E2A/TCF3 transcription factors, together with NOTCH1 and pre-T cell receptor (TCR) signalling. Importantly, perturbations of early T-cell regulatory networks are implicated in leukemogenesis. NOTCH1 gain of function mutations invariably lead to T-cell acute lymphoblastic leukemia (T-ALL), whereas inhibition of E proteins accelerates leukemogenesis. Thus, NOTCH1, pre-TCR, E2A and HEB functions are intertwined, but how these pathways contribute individually or synergistically to leukemogenesis remain to be documented. To directly address these questions, we leveraged *Cd3e*-deficient mice in which pre-TCR signaling and progression through β-selection is abrogated to dissect and decouple the roles of pre-TCR, NOTCH1, E2A and HEB in SCL/TAL1-induced T-ALL, *via* the use of *Notch1* gain of function transgenic (*Notch1IC*^{tg}) and *Tcf12*$^{+/-}$ or *Tcf3*$^{+/-}$ heterozygote mice. As a result, we now provide evidence that both HEB and E2A restrain cell proliferation at the β-selection checkpoint while the clonal expansion of SCL-LMO1-induced pre-leukemic stem cells in T-ALL is uniquely dependent on *Tcf12* gene dosage. At the molecular level, HEB protein levels are decreased *via* proteasomal degradation at the leukemic stage, pointing to a reversible loss of function mechanism. Moreover, in *SCL-LMO1*-induced T-ALL, loss of one *Tcf12* allele is sufficient to bypass pre-TCR signaling which is required for *Notch1* gain of function mutations and for progression to T-ALL. In contrast, *Tcf12* monoallelic deletion does not accelerate *Notch1IC*-induced T-ALL, indicating that *Tcf12* and *Notch1* operate in the same pathway. Finally, we identify a tumor suppressor gene set downstream of HEB, exhibiting significantly lower expression

levels in pediatric T-ALL compared to B-ALL and brain cancer samples, the three most frequent pediatric cancers. In summary, our results indicate a tumor suppressor function of HEB/TCF12 in T-ALL to mitigate cell proliferation controlled by NOTCH1 in pre-leukemic stem cells and prevent NOTCH1-driven progression to T-ALL.

Keywords: SCL/TAL1, LMO1, HEB/TCF12, E2A/TCF3, NOTCH1, T-cell acute lymphoblastic leukemia, tumor suppressor genes

1 INTRODUCTION

Thymocyte reprogramming into self-renewing cells is a mandatory event in T-cell leukemogenesis, induced by aberrantly expressed oncogenic transcription factors (1–6). This initiating event sets a pre-leukemic state, while progression to overt leukemia requires additional collaborating events within pathways that control cell fate in the thymus, to evolve through layers of selective pressure (7–10).

The first acquisition of full T-lineage identity is marked by successful rearrangement of the T cell receptor (*Tcr*) β locus catalyzed by recombination-activating gene 1 (RAG1) and RAG2 at the CD4/CD8 double negative DN2-DN3 transitional stages (**Figure 1A**). The β-selection checkpoint is controlled by the pre-TCR resulting from the pairing of the successfully rearranged TCR β chain with the invariant pre-Tα chain and the CD3 signaling complex to trigger a burst of cell proliferation and survival, leading to differentiation of DN thymocytes to the $CD4^+CD8^+$ double-positive (DP) stage. Both the pre-TCR and NOTCH1 have obligatory functions at this first checkpoint (11–13). Gain of function mutations of *NOTCH1* are found in more than 55% of childhood T-ALL (14), leading to the well-accepted notion that *NOTCH1* is a major oncogenic event in T-ALL (15–17). The acquisition of *Notch1* mutations in T-ALL absolutely requires pre-TCR/CD3 signaling (4) and involves recombination activating enzymes (RAG1/2) (18). Additionally, the NOTCH1 pathway can also be hyperactive as a consequence of loss of function mutations of FBXW7, the E3 ligase that degrades MYC (19), an essential downstream target of NOTCH1 (20, 21). Nonetheless, the *NOTCH-MYC-FBXW7* triad appears to be genetically unaltered in ~1/3 T-ALL cases, raising the question whether additional genes or pathways may contribute to T-ALL progression.

Comprehensive high throughput sequencing have unravelled the genomic landscape of T-ALL in children (22–24) and adults (25), uncovering a low mutation burden in leukemias compared to solid tumors (26, 27). These studies identified recurring mutations within genes and pathways that control cell fate in thymocytes, confirming the dominant presence of NOTCH1 as a driver mutation. Unlike NOTCH1, oncogenic transcription factors in T-ALL are not mutated but aberrantly expressed in the T lineage driven by chromosomal translocations. These oncogenic transcription factors belong to two families, the basic helix-loop-helix family (SCL/TAL1, TAL2, LYL1) and associated partners (LMO1, LMO2), as well as homeodomain proteins (TLX1, TLX3, HOXA) (reviewed in (28, 29). Transgenic mice in which oncogene expression is driven in the thymus develop T-ALL with variable latency, indicating the necessary acquisition of collaborating events. This is illustrated by the loss of *Bcl11b* function, through transcription repression by the *TLX1* oncogene or through mono-allelic deletion in mouse models (30) and in 9% of human T-ALL (31).

Similar to BCL11B (32), both E2A (E12 and E47 (33)), and HEB (HEBCan and HEBAlt (34)) are essential for the commitment of progenitor thymocytes to the T-cell lineage (35, 36) by governing a gene expression program that is critical for T-cell development [(37), reviewed in (38)] and includes T-cell specific genes such as *Ptcra* and *Cd4* (39, 40), as well as cell cycle genes such as *Cdkn1a* (37, 41). Moreover, E2A is antiproliferative in thymocytes (42–44) and E2A-deficient mice develop T-cell lymphomas (42, 45), indicating that E2A has tumor suppressor functions, much like BCL11B. Nonetheless and unlike *BCL11B*, neither *E2A/TCF3* nor *HEB/TCF12* was found mutated or affected by copy number variations in human T-ALL (10, 25), raising the possibility of non-genetic inactivation of E2A or HEB that has so far escaped genomic studies. E protein activity can be inhibited by direct heterodimerization with Id proteins, members of the HLH family that lack DNA binding domains (37) or by the SCL and LYL1 oncoproteins (39, 40, 46, 47). Nonetheless, inhibition of E protein by SCL is insufficient for T-cell leukemogenesis which requires transcription activation of a stemness gene expression program by the SCL-LMO1 complex (2) or LMO2 (48, 49). Finally, O'Neil et al. have previously shown a genetic collaboration between *Tcf12* or *Tcf3*-deficiency and *SCL/TAL1* in accelerating T-ALL onset (47). Because *Heb* deficiency would cause reduced pre-TCR expression (50) and decreased cell proliferation (51), it remains to be documented how this would accelerate T-ALL onset. In summary, while it is well recognized that E2A can be a tumor suppressor in mouse models, it is not clear whether E2A or HEB is inactivated in human T-ALL and how inactivation may occur, given the essential and dosage-dependent role of E2A and HEB in the T lineage (50–52).

Given the intricate interaction between NOTCH1, pre-TCR signaling and E proteins, we elected to use $Cd3e^{-/-}$ mice as a powerful genetic model to dissect and decouple the roles of NOTCH1, pre-TCR and HEB in leukemia progression, specifically in DN3 thymocytes, previously shown to be the cell of origin of SCL-LMO1 (2, 4) and LMO2 (48) -induced T-ALL. Thus, by abrogating β-selection and analyzing *Notch1* gain of function and *Tcf12* loss of function individually, our results unravel a strong selective pressure for down regulation of HEB protein levels driven either by NOTCH1 and/or by pre-TCR signaling as a requirement for progression from the pre-leukemic state to overt T-ALL.

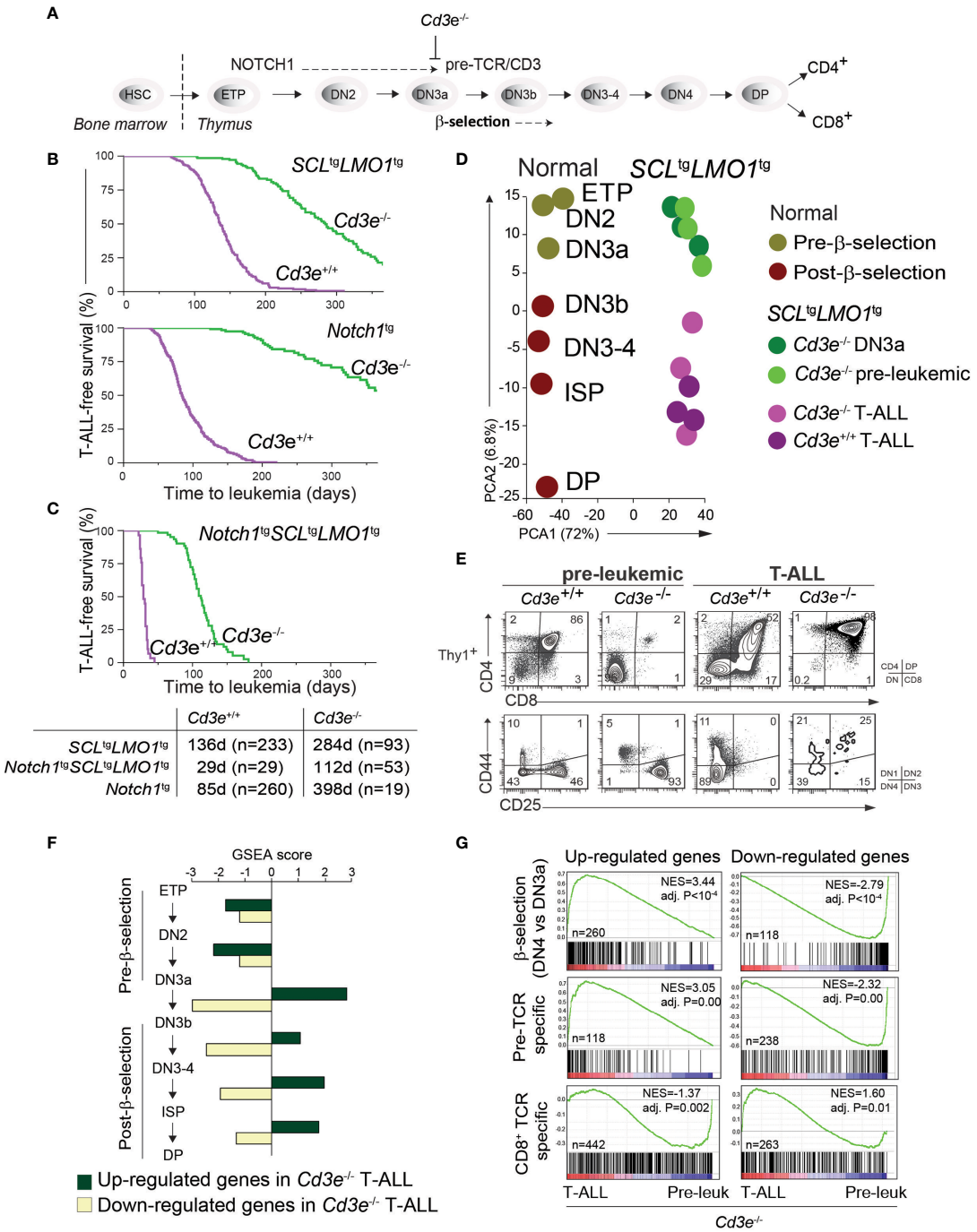

FIGURE 1 | Pre-TCR signaling is functionally important for T-ALL progression. **(A)** Pre-TCR signalling and thymocytes development. **(B, C)** Kaplan-Meyer survival curves comparing disease development in the pre-TCR proficient (*Cd3e*$^{+/+}$) and deficient (*Cd3e*$^{-/-}$) backgrounds in three models of T-ALL. **(D)** Principal component analysis of the transcriptomes of normal thymocyte subsets compared to SCL-LMO1-induced pre-leukemic and leukemic (T-ALL) thymocytes. **(E)** FACS phenotypes of *SCL*^{tg}*LMO1*^{tg} thymocytes from *Cd3e*$^{+/+}$ and *Cd3e*$^{-/-}$ backgrounds at pre-leukemic and leukemic stages. **(F)** Gene set enrichment analysis (GSEA) correlates disease progression to stages of thymocyte differentiation. Up-regulated and down-regulated gene sets were computed at each stage of normal thymocyte development from ETP to DP using microarray data from the Immgen project (http://www.immgen.org/), and enrichment was tested during disease progression (pre-leukemia to leukemia). Dark green bars denote enrichment of the up-regulated signature, and yellow bars denote enrichment of down-regulated signatures. **(G)** GSEA analysis of β-selection, pre-TCR specific and CD8+ TCR specific gene signatures during disease progression. Left panels show the enrichment tests for up-regulated gene signatures, and right panels show enrichment of genes decreased by β-selection, pre-TCR and CD8+ TCR.

2 MATERIALS AND METHODS

2.1 Mouse Models and Cell Lines

All animals were kept on a C57BL6/J strain background and maintained in pathogen-free conditions according to institutional animal care and use guidelines. Lck-NotchIC9 (*Notch1*^{tg}) (53), SIL-SCL (A (5)3SCL; *SCL*^{tg}) (54), *Lck-LMO1* (*LMO1*^{tg}) (55), *Cd3e*$^{-/-}$ (56), *E2a/Tcf3*$^{+/-}$ (57) and *Heb/Tcf12*$^{+/-}$ (52) were described previously. Kaplan-Meier survival and statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software, Inc.). T-ALL susceptibility was computed from areas under the curve (AUC) of Kaplan–Meier survival curves using Prism (1-AUC). Generation of human xenograft T-ALL blasts (14H025 and 14H148) were described previously (58). Human blast and primary murine thymocytes were cultured in MEM Alpha culture medium (Thermo Fisher) supplemented with 10% FBS, 10mM HEPES, 1mM sodium pyruvate, 55 μM β-mercaptoethanol, 2 mM glutamax, 5 ng/mL human FLT-3 Ligand, 5 ng/mL murine IL-7 and 20 ng/mL murine SCF. The DN T-cell line AD10.1 and Jurkat were cultured as previously described (50). KOPT-K1 and P12-ICHIKAWA cell lines were obtained from the DSMZ collection, Germany and maintained in RPMI-1640 culture media supplemented with 10% Fetal Bovine Serum (FBS).

2.2 Flow Cytometry Analysis and Cell Sorting

Single-cell suspensions were prepared from thymi or thymoma of mice. Flow cytometry analysis and cell sorting were done as described previously (39) using antibodies against Thy1.2, CD4, CD8, CD25 and CD44, using propidium iodide to exclude dead cells. Multiparametric flow cytometry analysis was performed on a FORTESSA flow cytometer, and cell sorting was performed on FACSAria (BD Biosciences, San Jose, CA). Cell cycle analysis using DAPI staining was performed using ModFit (Verity Software Software, USA).

2.3 RT-PCR and *Notch1* Sequencing

For *Cdnk1*a gene expression analysis, DN thymocytes from wt and *Heb/Tcf12*$^{-/-}$ newborn mice were sorted by flow cytometry and cDNAs were prepared as described previously (39). Southern blots of the amplicons were revealed by hybridization using an internal ^{32}P-labeled oligonucleotide fragment (primer sequences are listed in **Supplementary Table l**). The ribosomal *Rps16* expression was used as a control for cDNA quality and quantity.

For *Notch1* sequencing, cDNA was prepared from total RNAs as described previously (39). Amplification of *Notch1* exons 26, 27, and 34 from leukemias cDNA were Sanger sequenced in both directions. Quantitative gene expression analysis was performed on StepOne system (Life Technologies) using specific primers and Advanced qPCR mix (Wisent). Primer sequences used for specific mRNA amplification are listed in **Supplemental Table S1**.

2.4 Western Blot Analysis

Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors. Protein extracts were resolved on bis-acrylamide gel, transferred on PVDF membranes and hybridized with anti-HEB and anti-E2A (Santa-Cruz Biotechnology Inc., CA) and anti-tubulin-β (Sigma) and anti-ERK (Cell Signaling) as a loading control.

2.5 ChIP Assays

Chromatin immunoprecipitation were performed on either *Cd3e*$^{-/-}$ primary thymocytes or AD10.1 extracts as previously described (50). Quantitative PCR was performed on StepOne system (Life Technologies) using specific primers using Advanced qPCR mix (Wisent). Oligonucleotide sequences used for promoter amplification are shown in **Supplementary Table 1**.

2.6 Microarray Analysis

Total RNAs were prepared from freshly isolated thymocytes from *Cd3e*$^{-/-}$ (control DN3 thymocytes), *Cd3*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} T-ALL and pre-leukemic (3-week-old), and *Cd3e*$^{+/+}$*SCL*^{tg}*LMO1*^{tg} leukemic mice using the RNeasy extraction kit (Qiagen, Mississauga, ON). cDNA synthesis, labeling and hybridization onto Affymetrix Mouse Genome 430A 2.0 arrays were performed at the Ottawa Health Research Institute (Ottawa, ON) as described (2). Raw data were normalized using the RMA procedure implemented in the Affy package from Bioconductor (59).

2.7 Gene Set Enrichment Analysis

We obtained raw microarray data for normal thymocyte populations generated by the Immgen project from GEO (accession number GSE15907). Data were normalized using the RMA procedure implemented in the Affy package from Bioconductor (59). We derived "transition" signatures for each differentiation step (i.e. ETP to DN2, DN2 to DN3a, etc.), which contained genes whose expression levels present with at least a 2-fold change (up or down-regulated) in the transition (gene signatures provided in **Supplementary File 1**). In **Figure 1F**, gene set enrichment analysis (60) was applied to detect transition signatures that are enriched in the transcriptome of *Cd3e*$^{-/-}$ *SCL*^{tg}*LMO1*^{tg} and *Cd3e*$^{+/+}$*SCL*^{tg}*LMO1*^{tg} leukemic cells.

Pre-TCR specific signatures (**Figure 1G**, **Supplementary File 1**) included genes that increased or decreased at least 2-fold during the DN3a-DN3b transition, and are not regulated by the αβTCR in peripheral CD8$^+$ T cells stimulated by antigen (naive versus activated CD8+ T cells). Conversely, TCR-specific signatures (**Figure 1G**, **Supplementary File 1**) included genes exclusively regulated by the αβTCR in activated CD8$^+$ T cells.

2.8 Regulator Analysis Using ChIP-Seq Datasets

We collected genome-wide chromatin occupancy data for 7 transcription factors implicated in pre-TCR signalling (11 ChIP-seq experiments in total, **Figure 2A**) from Wang et al. (61), Miyazaki et al. (37), and the HemoChIP project (62). ChIP-seq data obtained for E2A (DN3 and DN4) (37) and NOTCH1 (G4A2 and T6E murine cell lines) (61) were processed according to the following steps: (i) sequence reads were mapped to the mouse genome mm9 using Bowtie with default parameters (maximum 2 mismatches); and (ii) peak coordinates were

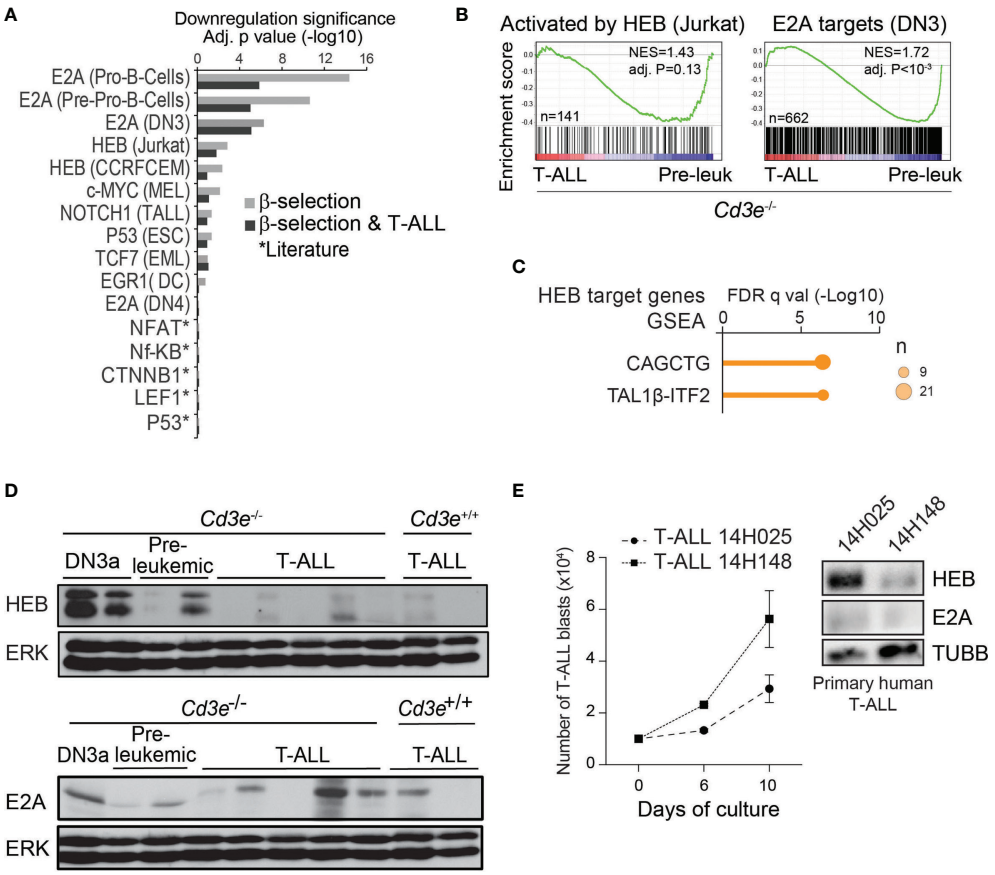

FIGURE 2 | T-ALL progression associated with an inhibition of E proteins targets and downregulation of HEB protein. **(A)** Regulator analysis identified transcription factors associated to transcriptional repression during T-ALL progression and β-selection. Regulators are rank-ordered according to their enrichment scores. The bars display the downregulation significance (log10 adjusted P, Fisher's exact test). Targets were extracted from ChIP-seq datasets (cell types in parenthesis). **(B)** GSEA analysis of genes activated by HEB and E2A targets during progression from pre-leukemic to leukemic state in *Cd3e*-deficient *SCL*^{tg}*LMO1*^{tg} T-ALL. **(C)** Binding site motif enrichment (MSig) within the proximal promoter regions of HEB target genes down-regulated between *Cd3e*$^{-/-}$ leukemic and pre-leukemic cells. **(D)** HEB and E2A protein levels during T-ALL progression measured by Western blotting. **(E)** Growth in vitro for primary T-ALL samples (left panel). Western blot analysis of theindicated T-ALL samples (right panel).

determined by the MACS tool, using the cutoff P < 10^{-9}. Peak coordinates for the HemoChIP dataset mapped to the mouse genome mm9 were downloaded from http://hscl.cimr.cam.ac.uk/ChIP-Seq_Compendium/ChIP- Seq_Compendium2.html. Last, all peaks were associated to their closest transcription start sites in the mouse genome using PeakAnalyzer v.1.4 tool (63). Lists of targets bound by transcription factors included all genes containing at least one binding site for the regulator (**Supplementary File 1**). We tested enrichment of targets using the Fisher's exact test (**Figure 2A**).

2.9 Exome Sequencing and Data Analysis

DNA was extracted from *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} leukemias (n=4) and control *Cd3e*$^{-/-}$ thymocytes (n=2), followed by targeted exome enrichment was performed using the mouse Nimblegen SeqCap EZ kit from Roche. Sequencing was performed in the Illumina HiSeq2000 at the IRIC Genomics platform. Low quality bases (quality below 20) in paired-end reads were trimmed off using the Trimmomatic tool (64), duplicate reads were removed using Picard (http://broadinstitute.github.io/picard), and alignment to the mouse genome (mm10) was performed with bwa (65). The resulting depth of coverage was 20X for at least 85% of the captured exome across all samples. Exome variants in T-ALL samples were predicted using Strelka (66), using *Cd3e*$^{-/-}$ thymocytes as matching controls, and annotation was performed using ANNOVAR (67). SIFT scores to determine deleterious variants were computed using the Variant Effect Predictor tool (68).

2.10 RNA-Sequencing and Data Analysis

RNA extracted from *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} leukemias (n=4) and control *Cd3e*$^{-/-}$ thymocytes (n=2) was prepared using the TruSeq RNA kit (Illumina) and sequenced in the Illumina HiSeq2000 in the IRIC Genomics platform. Low quality bases (quality below 20) in paired-end reads were trimmed off using the Trimmomatic, and processed reads were aligned to the mouse

genome mm10 using Tophat2/Bowtie2 v2.0.7 (69). Gene levels were quantified (FPKM values) based on the UCSC reference genes annotation using cuffdiff v.2.1.1.

RNA-seq data from pediatric tumors including a minimum of 313 T-ALL, 720 B-ALL and 350 Brain tumor samples were accessible for data analysis *via* the St. Jude PeCan data portal (https://pecan.stjude.cloud) (70) using Protein paint to capture RNA expression from the above ALL datasets (71).

3 RESULTS

3.1 Functional Importance of the Pre-TCR in Disease Progression

The first critical event in leukemogenesis is the reprogramming of DN3 thymocytes into pre-leukemic stem cells (pre-LSCs) by the SCL-LMO1 oncogenes (2). These two oncogenes target the DN3 population (4, 72) but are not sufficient *per se* for progression to T-ALL. While the initiating reprogramming event is pre-TCR independent (2), progression to T-ALL requires both pre-TCR and NOTCH1 signaling (4), thus emulating the requirement for cooperative signaling between the two important pathways for normal thymocyte differentiation (11). To define the precise contribution of each pathway to disease progression, we took a genetic approach to quantitatively estimate T-ALL progression using disease penetrance and the time to leukemia onset as endpoints to measure leukemogenesis. T-ALL induced by $SCL^{tg}LMO1^{tg}$ or $Notch1^{tg}$ separately is affected by the absence of pre-TCR/CD3 signaling (**Figure 1B**) as previously reported (4, 72, 73). In contrast, *SCL-LMO1* together with the hyperactive *Notch1* allele (*ICN1*, hereafter $Notch1^{tg}$), induce T-ALL with full penetrance in the presence or in the absence of *Cd3e* (**Figure 1C**). Nonetheless, in *Cd3e*-proficient mice, pre-TCR/CD3 signaling accelerates the disease to 29 days, compared to 112 days in *Cd3e*-deficient mice. These results indicate that $Notch1^{tg}$ drives the penetrance of T-ALL while the pre-TCR determines the time to leukemia. Of note, T-ALL induced by the three oncogenes together in the absence of *Cd3e* reproduce the disease induced by the two transcription factor oncogenes *SCL* and *LMO1* in a *Cd3e*-proficient background (**Figure S1**). Therefore, in the context of T-ALL induced by the *SCL* and *LMO1* oncogenes, the *Notch1* transgene controls disease penetrance while pre-TCR signaling accelerates disease onset.

3.2 Re-Activation of a Pre-TCR-Driven Proliferation Signature in the Absence of CD3 Signaling Associated With Disease Progression

While pre-TCR signaling has been known to be important for leukemogenesis (74, 75) and more specifically for SCL-LMO1-induced T-ALL (4, 76), the contribution of the pre-TCR and downstream molecular effectors remain to be uncovered. T-ALL that still develops in the absence of CD3 or RAG, completely lacked the typical *Notch1* gain of function mutations (4), providing us with a unique genetic tool to dissect the contribution of these pathways to T-ALL.

We therefore conducted a transcriptomic analysis that capitalized on our identification of DN3a as the cell of origin of T-ALL, and in $Cd3e^{-/-}$ mice in which pre-TCR signaling is abrogated, causing thymocyte differentiation blockade at the DN3a stage. This allows for a stringent comparison between the pre-leukemic and the leukemic state to define the molecular signature of progression. During the pre-leukemic stage, $Cd3e^{-/-}$ $SCL^{tg}LMO1^{tg}$ thymocytes are blocked at the DN3a stage, as expected from the absence of CD3.

We next compared the transcriptomes of pre-leukemic (n=3) and of $SCL^{tg}LMO1^{tg}$ leukemic cells (n=6) (**Figure 1D**). We applied principal component analysis (PCA) to compare the transcriptomes of normal thymocyte populations (obtained from the Immgen project) with pre-leukemic and leukemic samples. The first component reflected the distinct Affymetrix chips used for profiling whereas the second PCA component organized the transcriptomes according to their differentiation trajectories, from ETP to DP cells. Overall, PCA showed that pre-leukemic samples were comparable to the $Cd3\epsilon^{-/-}$ DN3a thymocytes. Strikingly, progression to T-ALL correlates with the expression profiles of thymocytes that have undergone β-selection, despite the absence of pre-TCR signaling in $Cd3\epsilon^{-/-}$ mice. Consistent with PCA, we observed that leukemic cells acquired a post-β-selection phenotype to become DN3b-DP cells (98% of the thymic mass), compared to 2% at the pre-leukemic stage, despite the complete lack of normal pre-TCR function in $Cd3e^{-/-}$ mice (**Figure 1E**).

We also correlated disease progression with gene signatures of thymocyte differentiation using gene set enrichment analysis (GSEA). We observed that only signatures associated to post-β-selection thymocytes (DN3b to DN4 cells) were positively correlated with T-ALL progression (**Figure 1F**). The strongest correlation was associated with the DN3a-DN3b transition, exactly at the stage where the pre-TCR/CD3 triggers a burst of cell proliferation following a productive TCRβ rearrangement and the formation of a functional pre-TCR/CD3 complex.

CD3 signaling is important for pre-TCR function and β-selection, but also for TCR signaling. To distinguish the contribution of these two pathways to T-ALL progression, we applied GSEA to analyse gene signatures of antigen-independent (pre-TCR) and antigen-dependent (TCR) T-cell stimulation in T-ALL progression. Pre-TCR-induced genes (DN3b-DN4) correlated positively with leukemia progression (**Figure 1G**, adj. $P < 10^{-4}$). In contrast, the TCR gene signature did not correlate positively with T-ALL progression induced by SCL-LMO1 (**Figure 1G**).

In summary, our results indicate that leukemic cells display gene signatures of post-β-selection thymocytes, suggesting that a pre-TCR/CD3-like proliferation has occurred even in the absence of a functional pre-TCR ($Cd3e^{-/-}$ background). Moreover, progression to T-ALL overlaps specifically with pre-TCR-driven gene signature. This CD3-independent activation of the pre-TCR molecular signature indicates a strong selective pressure during leukemic progression for pathways that normally control the β-selection checkpoint.

3.3 T-ALL Progression Associated With an Inhibition of E Proteins

3.3.1 Genomic Analyses Identify the Down-Regulation of E2A and HEB Targets During Normal β-Selection and the Progression From Pre-Leukemic to Leukemic Stages

Several transcription factors have been implicated in pre-TCR signaling and/or β-selection (reviewed in (77)). To determine their potential contribution to T-ALL progression, we first performed a systematic regulator analysis based on published ChIP-seq datasets (62).

This analysis predicted that targets of E proteins, E2A and HEB, are down-regulated during β-selection and T-ALL progression (**Figure 2A**, Fisher's exact test). In addition, from data obtained with shRNA knock-down of *HEB* (78),we identified a list of 389 genes activated by HEB in Jurkat cells, (i.e. fold-change > 1.5, t-test $P < 0.05$). GSEA indicated that these HEB targets (**Figure 2B**, left panel) as well as E2A-bound genes (**Figure 2B**, right panel) are down-regulated when comparing *Cd3e*$^{-/-}$ leukemic and pre-leukemic cells. Last, well known E-Box motifs CAGCTG and TAL1β-ITF2 (79) were found to be enriched in HEB target genes (**Figure 2C**).

Overall, these analyses indicate that inhibition of E protein activity may be important both at the β-selection checkpoint and during T-ALL progression.

3.3.2 HEB Protein Levels Are Down Regulated in T-ALL

Mice lacking *E2a*/*Tcf3* develop lymphomas, suggesting that E2A is a tumor suppressor (42, 45). Nonetheless, *TCF3* mRNA is highly expressed in human T-ALL (9, 80) and the *TCF3* gene is neither deleted nor mutated, raising the question how E2A acts as a tumor suppressor. Previous work showed that pre-TCR signaling inhibits E2A activity *via* upregulation of *Id3* (81). However, the very low levels of *ID3* in most human T-ALL samples (**Figure S2A**) and in murine *SCL*^{tg}*LMO1*^{tg} T-ALL (**Figure S2B**) do not support a role for ID3 in sequestering HEB or E2A in T-ALL.

Next, we investigated *Heb* expression at the mRNA and protein levels in T-ALL progression. *Heb/Tcf12* mRNA levels were equally high in control, pre-leukemic and leukemic cells (**Figure S2C**). In contrast, we found by western blotting that HEB protein was almost absent in murine leukemic cells, contrasting with high expression levels in normal DN3a thymocytes and variable levels in pre-leukemic thymocytes (**Figure 2D**, upper panel). E2A protein levels also decreased with progression to T-ALL, albeit to a lesser extent (**Figure 2D**, lower panel) while mRNA levels remained elevated (**Figure S2C**). Moreover, we observed that HEB levels steadily increased in Jurkat cells treated with the proteasome inhibitor MG132 (**Figure S2D**), indicating that HEB levels are regulated by proteasomal degradation in leukemic cells. We next inspected E protein levels in two primary T-ALL patient samples. HEB protein levels were undetected in the sample with higher proliferation in culture, whereas E2A was undetectable in both samples (**Figure 2E**). Taken together, these results indicate a strong selective pressure for HEB protein down-regulation during progression to T-ALL.

3.4 HEB Restricts Cell Proliferation at the β-Selection Checkpoint and Acts a Tumor Suppressor in T-ALL

To directly address the role of *Heb* or *E2a*, we analyzed thymocyte numbers in *E2a/Tcf3*$^{+/-}$ or *Heb/Tcf12*$^{+/-}$ mice in the context of the *SCL*^{tg}*LMO1*^{tg} mice or their wild type (*wt*) littermates. In adult *wt* mice, removal of one *Heb* allele or one *E2a* allele did not significantly affect thymocyte numbers at the DN3 to DP stages (**Figure 3A**), although *Heb* monoallelic deletion resulted in modest but significantly increased cell numbers within populations undergoing β-selection, i.e. DN3b and DN3-4 (**Figure S3**), concurring with the view that *Heb* enforces a proliferation checkpoint at this stage (44, 82). We and others previously showed that the *SCL-LMO1* or *LMO2* oncogenes expand the DN3 populations due to increased self-renewal capacity (2, 4). Interestingly, *Heb* haploinsufficiency further increased the expansion of the DN3 and DN4 populations induced by the *SCL-LMO1* oncogenes (**Figure 3A**). To directly address the antiproliferative role of HEB, we compared S/G2/M phase progression in *Heb/Tcf12*$^{+/-}$ and *Heb/Tcf12*$^{+/+}$ DN3 thymocytes from *Cd3e*$^{-/-}$ mice. In absence of pre-TCR signaling, while loss of one allele of *Heb* increased the proportion of proliferating cells, we found that the *SCL-LMO1* oncogenes decreased the proportion of cycling DN3 thymocytes, consistent with a role for *SCL* in quiescence control (83). In this context, removing one *Heb* allele re-established proliferating DN3 thymocytes to normal proportions (**Figure 3B**). Therefore, in the absence of pre-TCR signaling, the SCL and LMO1 oncogenes revealed *Heb* haplo-insufficiency in cell cycle control, indicating and that HEB anti-proliferative function is required to control oncogenic stress at the β-selection checkpoint. In addition to the previously reported role for *Id3*-mediated inhibition of E proteins during β-selection in steady state (84), our data indicate a distinctive requirement for HEB in stress response.

The E proteins and Id axis has a well-established tumor-suppressor function (42, 45). We therefore addressed the question whether both HEB and E2A have a tumor suppressor function in the context of SCL-LMO1-induced T-ALL (**Figure 3C**). In *Cd3e*-proficient mice, loss of one *E2a* allele caused a modest decrease in latency from 122 days in littermate controls to 114 days (**Figure 3C**, left panel). In contrast, deletion of one *Heb* allele accelerated the time of onset to 83 days compared to 130 days in littermate controls (**Figure 3C**, right panel). Together, our results indicate a tumor suppressor function for *Heb* which acts in a gene-dosage dependent manner in T-ALL induced by SCL and LMO1.

3.5 Monoallelic *Heb/Tcf12* Deletion Accelerates *SCL*^{tg}*LMO1*^{tg}-Induced T-ALL Without Affecting *Notch1*^{tg}-Induced T-ALL

In *Cd3e*-deficient mice expressing the *SCL* and *LMO1* oncogenes, inactivation of a single *Heb* allele bypassed pre-TCR signalling to increase the proportion and numbers of DN4 cells during the

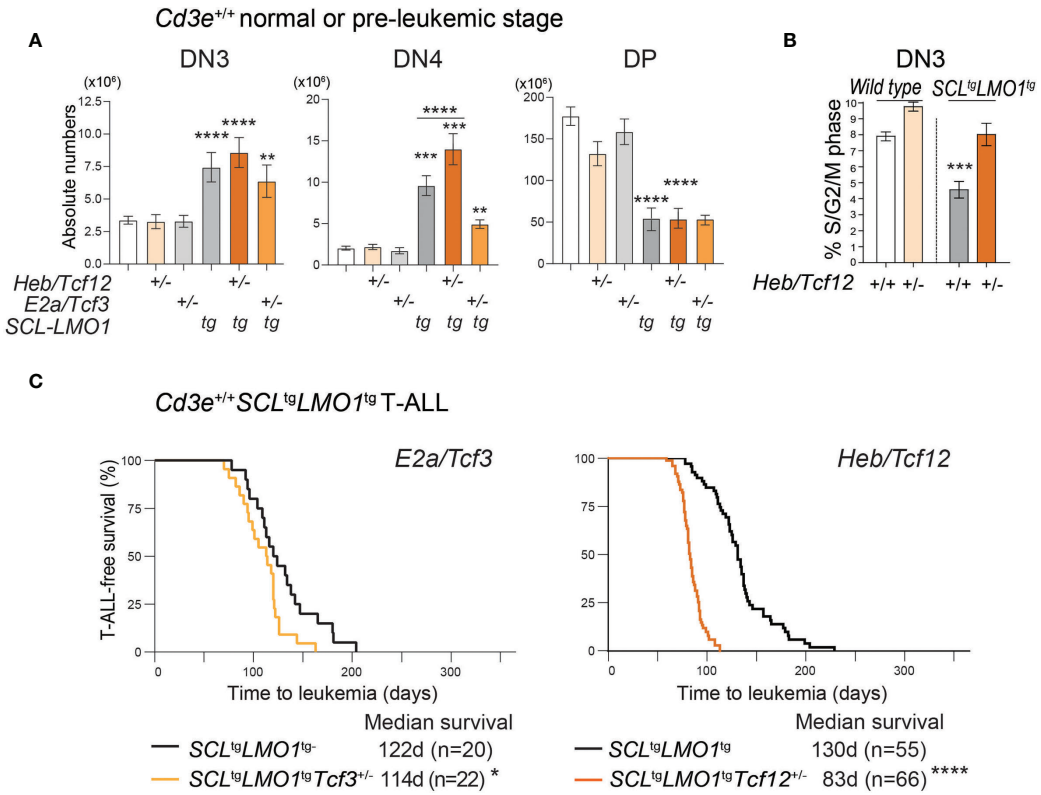

FIGURE 3 | HEB restricts cell proliferation at the b-selection checkpoint and acts as a tumor suppressor in T-ALL. **(A)** Effect of loss of one allele of *Heb/Tcf12* or *E2a/Tcf3* on the absolute numbers of thymocytes within the indicated subsets in a *wt* or *SCL*^{tg}*LMO1*^{tg} *background.* Shown are the average ± SD of at least 9 mice per group (5-6 weeks). Where not specified P value is compared to wild type control: **p = 0.0039, ***p < 0.002 and ****p < 0.0001. **(B)** Monoallelic Heb/Tcf12 deletion increased S/G2/M phase in Cd3e-deficientpre-leukemic DN3 thymocytes. Cell cycle analysis of Cd3e-deficient thymocytes in Heb/ Tcf12+/+ andHeb/Tcf12+/- backgrounds. *p value <0.01). **(C)** Kaplan-Meyer survival curves comparing *E2a/Tcf3*$^{+/+}$ with *E2a/Tcf3*$^{+/-}$ backgrounds (left panel) as well as *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds in Cd3e-proficient *SCL*^{tg}*LMO1*^{tg} leukemias. In both panels, the +/+ genotypes are wild type littermates of +/- mice. N represents the numbers of mice and the median survival in days was computed from the survival curves. *p = 0.039 and ****p < 0.0001.

pre-leukemic stage (**Figures 4A, B** and **Figure S4**). In addition, monoallelic *Heb* deletion allowed a minor population of SCL-LMO1 expressing thymocytes to progress to the DP stage (1.4%, **Figures 4A, B**). Last, progression to the leukemic stage is associated with a transition to a post-β-selection phenotype in *Cd3e*-deficient *SCL*^{tg}*LMO1*^{tg} mice, which consistently increased in *Heb/Tcf12*$^{+/-}$ T-ALL (**Figures 4C, D**).

We next assessed the impact of *Heb* gene dosage on disease penetrance and time to leukemia. Strikingly, decreased *Heb* (*Heb/Tcf12*$^{+/-}$) compensates for the absence of pre-TCR signaling in *Cd3e*$^{-/-}$ mice and allowed *SCL*^{tg}*LMO1*^{tg} T-ALL to become fully penetrant, in addition to accelerating disease onset by 107 days (**Figures 5A, B**). Hence, in *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} mice in which disease penetrance was 80%, loss of one *Heb* allele recapitulated the effect of the *Notch1* oncogene on restoring full disease penetrance as shown in **Figure 1C**. Since *Notch1*^{tg}-induced T-ALL is also dependent on pre-TCR function (**Figures 1C** and **5C, D**), we next addressed the importance of *Heb* in T-ALL induced by *Notch1*^{tg} in pre-TCR proficient and pre-TCR deficient mice. In contrast to *SCL* and *LMO1*, decreased *Heb* gene dosage did not affect *Notch1*^{tg}-induced T-ALL in *Cd3e*$^{-/-}$ or in *Cd3e*$^{+/+}$ mice (**Figures 5C–F**), indicating that *Heb* and *Notch1* operate in the same genetic pathway in T-ALL, as suggested during normal differentiation (36). In summary, *Heb* and *Notch1* inversely control the penetrance of T-ALL induced by the SCL and LMO1 oncogenes whereas the pre-TCR determines the DN-DP transition during the pre-leukemic stage and the time of disease onset.

Since the *Notch1* oncogene is sufficient to bypass pre-TCR signaling to confer full leukemic penetrance in *Cd3e*$^{-/-}$ *SCL*^{tg}*LMO1*^{tg} mice (**Figure 1B**), we addressed the question whether the increased penetrance caused by monoallelic *Heb* deletion shown in **Figure 5A** could be due to the acquisition of *Notch1* gain of function mutations. We analyzed a cohort of mice with *Heb/Tcf12*$^{+/-}$ and *Heb/Tcf12*$^{+/+}$ T-ALL for the presence of *Notch1* mutations (**Figures 5E** and **S5**). As expected, 19 of 20 *SCL*^{tg}*LMO1*^{tg} T-ALL in *Cd3e*$^{+/+}$*Heb/Tcf12*$^{+/+}$ mice exhibit *Notch1* gain of function mutations (4, 85) which affect the PEST domain whereas *Cd3e*-deficient *SCL*^{tg}*LMO1*^{tg}*Heb/Tcf12*$^{+/+}$ T-ALL completely lacked *Notch1* mutation as reported (4). In

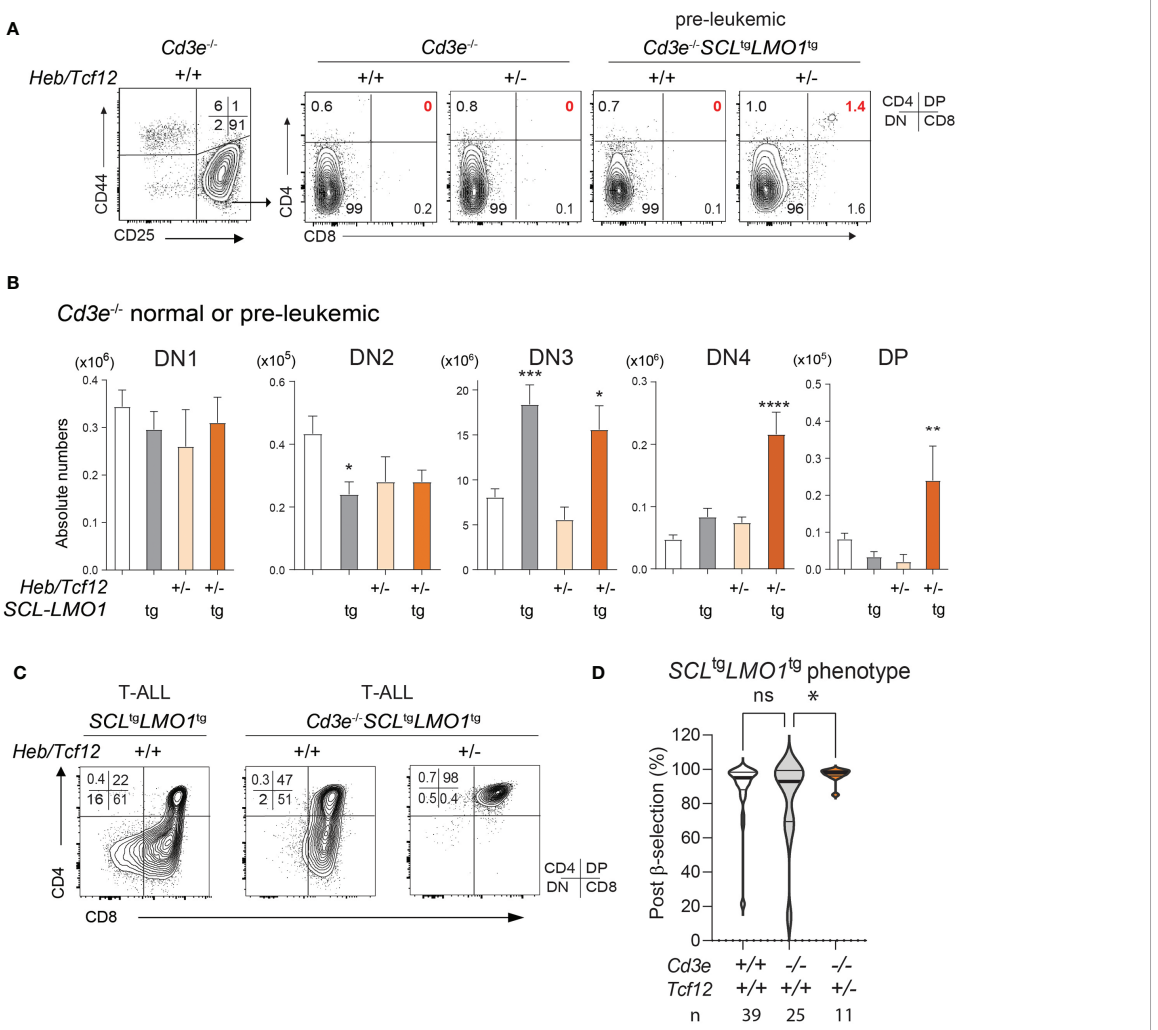

FIGURE 4 | The SCL and LMO1 oncogenes favor the DN-DP transition at the leukemic stage despite the absence of pre-TCR/CD3 signaling: synergy with decreased *Heb* gene dosage. **(A)** FACS phenotypes of normal *Cd3e*$^{-/-}$ or pre-leukemic *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} thymocytes from *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds. Flow cytometry profiles for the CD4– CD8– DN populations are shown in **Figure S4**. **(B)** Absolute numbers of thymocytes within the indicated subsets in normal *Cd3e*$^{-/-}$ or pre-leukemic *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} in *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds. Shown are the average ± SD of at least 5 mice per group, taken at 4 weeks. Note the significant increase in the post-β selection DN4 and DP populations in *SCL*^{tg}*LMO1*^{tg}*Heb/Tcf12*$^{+/-}$ mice. **(C)** FACS phenotypes of *Cd3e*-proficient or deficient *SCL*^{tg}*LMO1*^{tg} leukemias from *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds. **(D)** T-ALL with a post-β-selection phenotype. Shown are the percentages of cells from each T-ALL with post-β-selection surface phenotypes (DN3-4, DN4, ISP, SP). n represents the numbers of mice analysed. * adj p=0.02. pvalue < 0.05; **p value < 0.005; ***p value < 0.0005; ****p value < 0.0001, ns, Non significant.

comparison, only 1 of 7 *Heb/Tcf12*$^{+/-}$ T-ALLs acquired *Notch1* mutations (**Figure 5E** and **Figure S5**). Therefore, the increased aggressiveness of *Heb/Tcf12*$^{+/-}$ T-ALL is unlikely due to *Notch1* gain of function mutations.

These results establish *Heb* as a tumor suppressor that normally enforces a proliferative checkpoint during β-selection to suppress oncogene-induced T-ALL in a gene dosage-dependent manner. Unlike classical tumor suppressors, the human *HEB* gene is not affected at the genomic level in T-ALL (10, 25). Here, we show that HEB is regulated at the protein level, pointing to a distinctive loss of function mechanism.

3.6 A Threshold-Dependent Role for *Cdkn1a* Downstream of HEB

3.6.1 Exome Sequencing of *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} T-ALLs Reveals Loss of Function Mutations in HEB-Bound Genes

Since *HEB/TCF12* is not deleted nor inactivated by deleterious mutations in human T-ALL, we addressed the possibility that down-regulation or genetic inactivation of HEB targets with tumor suppressor function could also be a mechanism associated to and/or selected for during disease progression. We performed exome sequencing of *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg}

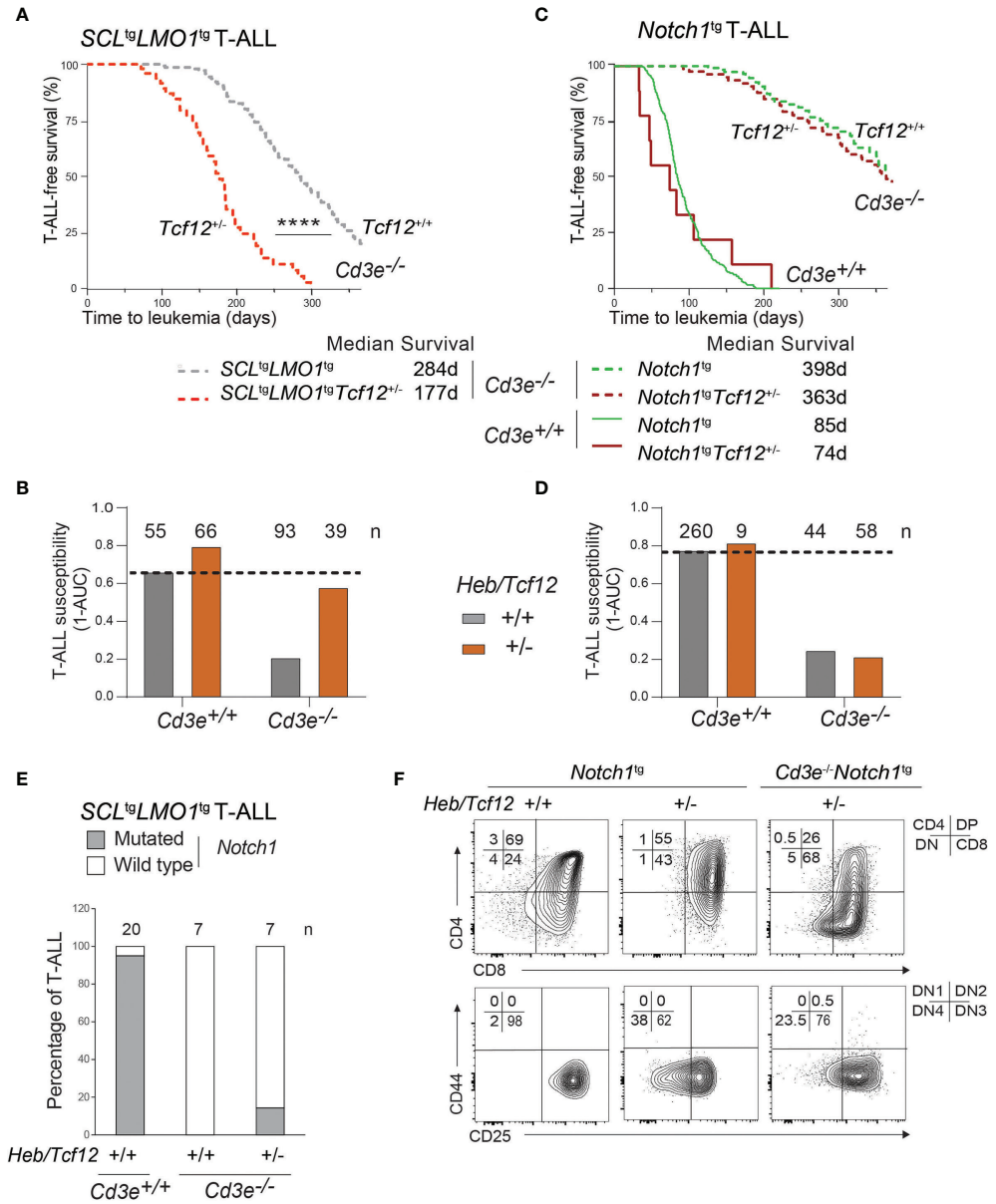

FIGURE 5 | The *SCL* and *LMO1* oncogenes but not the *Notch1/IC9* oncogene reveal *Heb/Tcf12* as a haplo-insufficient tumor suppressor in T-ALL. **(A)** Kaplan-Meyer survival curves comparing *Cd3e*-deficient *SCL*^{tg}*LMO1*^{tg} leukemias in *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds. Shown is the median survival in days. **(B)** T-ALL susceptibility of *SCL*^{tg}*LMO1*^{tg} mice in *Cd3e*-proficient (*Cd3e*$^{+/+}$, **Figure 3C**) or deficient (*Cd3e*$^{-/-}$, **Figure 5A**) backgrounds was calculated from the area under the curve (AUC) of the above Kaplan-Meyer graph over 365 days. Shown on top are the numbers of mice per group. **(C, D)** Kaplan-Meyer survival curves **(C)** and T-ALL susceptibility **(D)** comparing *Notch1/IC9*-induced leukemias in *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds that are *Cd3e*-deficient or *Cd3e*-proficient. **(E)** Presence of *Notch1* activating mutations in *Cd3e*-proficient but not *Cd3e*-deficient *SCL*^{tg}*LMO1*^{tg} T-ALL. Shown are the percentage of T-ALL samples with an activating mutation in the *Notch1* locus in *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds. Shown on top are the numbers of leukemias sequenced in each group. Only one *Heb/Tcf12*$^{+/-}$ *Cd3e*-deficient *SCL*^{tg}*LMO1*^{tg} T-ALL harbours a mutation affecting the PEST domain of *Notch1*. **(F)** Leukemic phenotypes of *Cd3e*-proficient or *Cd3e*-deficient *Notch1*^{tg} leukemias from *Heb/Tcf12*$^{+/+}$ and *Heb/Tcf12*$^{+/-}$ backgrounds **** pvalue<0.0001.

leukemias (n=4) and control *Cd3e*$^{-/-}$thymocytes to identify genetic alterations involved in leukemia development in the absence of pre-TCR signaling. The software Strelka (66) was applied to discover somatic SNVs and short indels using the paired tumor-control configuration. After filtering out known polymorphisms reported in the SNP database v.138, we obtained 85 non-synonymous SNVs predicted to be deleterious by the Variant Effect Predictor tool (68). In addition, Strelka predicted 19 stop gains, 10 stop loss, 2 frameshift deletions and 1 frameshift insertion (**Supplementary File 2**).

We investigated the gene set affected by the above mutations (102 genes in total, **Figure 6A**) using the MSig database

enrichment tool (http://software.broadinstitute.org/gsea/msigdb/annotate.jsp). We found a strong enrichment (adjusted $P = 1.2x10^{-4}$) for genes containing the E-box motif CAGGTG in their proximal promoters, suggesting they could be regulated by E proteins E2A and HEB (**Figure 6B** and **Supplementary File 2**). The AP4 E-box motif CAGCTG was also found to be enriched in promoters of mutated genes (35 genes, adjusted $P = 1.2x10^{-4}$).

To confirm binding, we performed ChIP in the AD10 DN cell line and found by PCR that HEB occupies the promoter regions of 29 of the mutated genes within 2 kb of the transcriptional start site, in addition to *Cdkn1a*, validated here as a HEB target (**Figure S6A**). Binding enrichment was at least five-fold over a negative control and for 3 genes, the enrichment was almost as high as that observed with *Ptcra* which was amplified as a positive control (**Figure S6A**). In contrast, *Csf3r* which was amplified as a negative control did not show any enrichment.

3.6.2 Downstream HEB Targets With Putative Tumor Suppressor Function in Human T-ALL

We next addressed whether HEB-bound genes with loss of function mutations in murine T-ALL might be tumor suppressors in the human disease. We included *Cdkn1a* in this analysis because *Cdkn1a* is a well-documented HEB and E2A target gene (41, 44, 86) and because of strong genetic evidence for *Cdkn1a* tumor suppressor function in mice harboring one additional *Cdkn1a* allele (87). Out of 102 genes with deleterious mutations (for a total of 103 including *Cdkn1a*), 35 are HEB-bound genes (34.3%) according to previously published HEB ChIP-seq data with two human TAL1/SCL T-cell lines (78) and ChIP-PCR validation from this study (**Figure S6A**). Of note, 7 of these 35 HEB-bound genes (20%) are known tumor suppressors (https://bioinfo.uth.edu/TSGene, **Figure 6A**). We checked gene expression in blood and brain samples from normal tissues in the GTEx portal (https://www.gtexportal.org) and all seven genes were expressed in normal cells and therefore retained for further analysis. When we applied the same filtering strategy to E2A instead of HEB, only 10 of 103 genes are E2A-bound (9.7%) and 4 of the 10 genes are annotated as TSGenes (**Figures 6A, E, H**). All four genes are present in the seven HEB-bound TSGenes.

These 7 HEB-targets and TS genes were not found to be recurrently mutated in T-ALL (10). Of note, T-ALL can be classified into distinct molecular subgroups based on chromosomal translocations and gene signatures (9, 23, 88). Since our transgenic model is representative of the SCL/TAL1 molecular subgroup, we next addressed the question whether these seven HEB target TSGenes would be expressed at lower levels in the TAL1 subgroup compared to the other T-ALL subgroups, using the dataset published by Liu et al. (10). Gene expression levels were remarkably comparable between TAL1 and non-TAL1 subgroups and were consistently low compared to *TCF12* (**Figure 6B**). We then searched the Pediatric Cancer Genome Project (https://www.stjude.cloud) (89) that covers more diversified cancer types, in order to compare expression levels for these seven genes in T-ALL (10), B-ALL (90) and brain tumors (**Figure 6D**). Strikingly, *CDKN1A* expression is five-and four-fold lower in T-ALL compared to B-ALL and brain tumors, respectively (**Figure 6D**). In addition, four other TSGenes are expressed at two- to eight-fold lower levels in T-ALL compared to brain tumors and/or B-ALL samples: *NEDD4L*, *PLCB3*, *APC* and *ZMYND11* (**Figure 6D**). These expression patterns contrast sharply with those of *LCK* which is highest in T-ALL as expected (**Figure S6C**), *RUNX1* which is equally expressed in T- and B-ALL but not in brain tumors, or JAK1, encoding a non-receptor tyrosine kinase, which is higher in B-ALL compared to the other two groups, and finally *NAXE*, encoding a metabolic enzyme which is expressed in all three types of pediatric cancers with modest but significantly lower levels in B-ALL (**Figure S6B**). Overall, low expression levels of HEB target TSGenes in primary human T-ALL samples concur with decreased HEB function in T-ALL compared to B-ALL and brain tumors, the three most frequent pediatric cancers, and a tumor suppressor function for HEB in pediatric T-ALL. Of note, these potential TSGenes are rarely mutated or deleted in T-ALL. Rather, these TSGenes are expressed at much lower levels in T-ALL compared to the other pediatric tumors.

3.6.3 *Cdkn1a* Deletion Accelerates *SCL-LMO1*-Induced T-ALL

Cdkn1a, a typical target of E2A and Id (41, 86, 91, 92) is part of the gene set that is downregulated at the β-selection checkpoint (**Figure S6C**) (93). $p21^{Cdkn1a}$ mediates G1 arrest by inhibiting CDK1 and CDK2 and loss of *CDKN1A* is a predictor of poor outcome in renal cell carcinoma (94). In agreement with our results (**Figurse 6C, D**), *CDKN1A* was found to be very low in human T-ALL (95). We therefore addressed the functional implication of *Cdkn1a* in this mouse model of SCL/TAL1 human T-ALL. We first confirmed that both E2A and HEB occupy the *Cdkn1a* promoter in primary DN thymocytes (**Figure 6E**, left panel). Moreover, *Cdkn1a* expression was nearly abrogated in *Heb/Tcf12*-deficient DN thymocytes (**Figure 6E**, right panel), indicating that HEB is a major transcriptional regulator of *Cdkn1a* at this developmental stage in the thymus. Treatment of thymocytes with phorbol 12-myristate 13-acetate (PMA) leads to activation of protein kinase C (PKC) which phosphorylates RAF and activates the ERK-MAPK pathway, and thus can be used to emulate pre-TCR/TCR signalling (96). We observed that *Cdkn1a* levels were significantly down-regulated in DN3 cells incubated with PMA for 6 hours (**Figure S6D**), consistent with the view that pre-TCR signals down-regulate p21 through the ERK-MAPK pathway (84, 96). In mouse leukemias, *Cdkn1a* expression was decreased in pre-LSCs compared to wild-type DN3a thymocytes and was further reduced in leukemic cells, in both $Cd3e^{+/+}$ and $Cd3e^{-/-}$ leukemias (**Figure 6F**). Finally, we observed that T-ALL onset was accelerated by 37 days in *Cdkn1a*-deficient mice (**Figure 6G**), compared to the 53 day acceleration found in $Heb/Tcf12^{+/-}$ mice, confirming a tumor-suppressor function for *Cdkn1a* (**Figure 6G**). Of note, *Cdkn1a* was haplosufficient in this genetic assay (**Figure 6G**), indicating that a reduction threshold must be attained for leukemic progression to occur. These results concur with the stepwise decrease in *Cdkn1a* in pre-LSCs and in leukemic blasts at time of overt leukemia, indicating that p21 is a

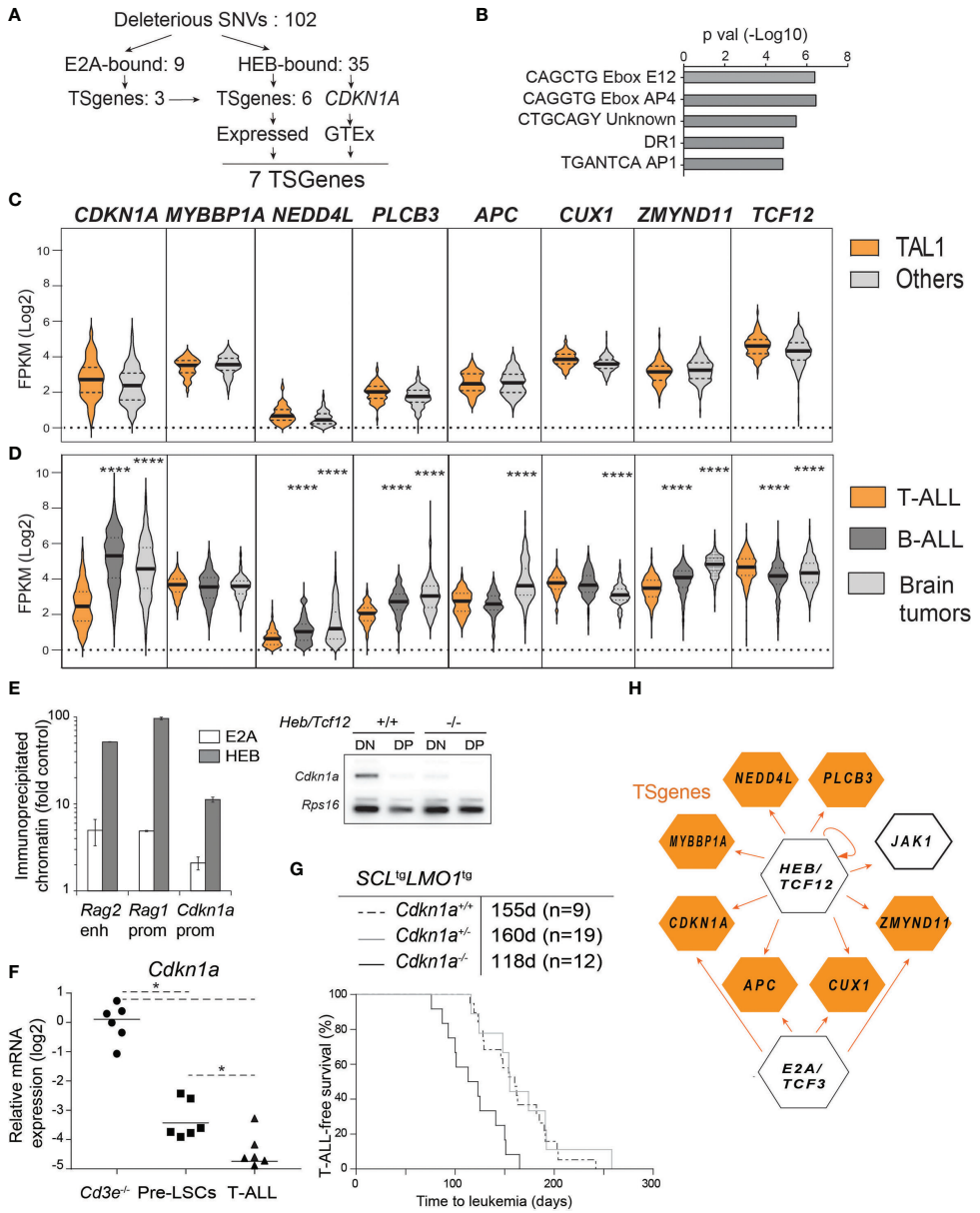

FIGURE 6 | HEB controls a tumor suppressor network in T-ALL. **(A)** Strategy of tumor suppressor identification amongst HEB target genes that are mutated in *Cd3e*$^{-/-}$*SCL*^{tg}*LMO1*^{tg} leukemic cells. **(B)** Enrichment in E boxes within the proximal promoter regions of HEB target genes. **(C, D)** Expression levels of the indicated TSGenes in TAL1+ vs other molecular subgroups in pediatric T-ALL dataset from Liu et al. **(C)** and in T-ALL, B-ALL and brain tumors from the Pediatric Cancer Genome Project cohort **(D)**. **(E)** Chromatin immunoprecipitation of *Cdkn1a* promoter with HEB and E2A in DN thymocytes. The *Rag1* promoter and *Rag2* enhancer are included as positive controls (left panel) and RT-PCR analysis of *Cdkn1a* expression in DN and DP thymocytes in *wt* and *Heb/Tcf12* knockout mice. Amplified bands were revealed by hybridization with an internal ^{32}P-labeled oligonucleotide fragment (right panel). **(F)** Quantitative RT-PCR analysis of *Cdkn1a* expression in *Cd3e*$^{-/-}$ DN3 cells, pre-LSCs and leukemic cells. **(G)** Kaplan-Meyer survival curves of *SCL*^{tg}*LMO1*^{tg} T-ALL with partial and complete loss of *Cdkn1a*. **(H)** A tumor suppressor network downstream of HEB or E2A validated by chromatin immunoprecipitation. HEB is autoregulatory but not E2A. *p value < 0.01; ****p value < 0.0001

threshold-dependent tumor suppressor in T-ALL. In summary, our genetic approach indicates that in the absence of a hyperactive NOTCH1 and of pre-TCR signaling, progression to acute leukemia in *SCL-LMO1*-induced T-ALL involves the downregulation of a tumor suppressor network implicating HEB and p21^{CDKN1A} (**Figure 6H**).

4 DISCUSSION

In the present study, we provide genetic evidence for differing requirements of NOTCH1, pre-TCR signaling and HEB in driving progression to T-ALL. By quantifying two disease endpoints, T-ALL penetrance and disease latency, i.e. the

median time to overt leukemia, we show that in the context of the *SCL* and *LMO1* oncogenes, the hyperactive *Notch1* oncogene on its own determines disease penetrance even in the absence of pre-TCR signaling. Despite the apparent sufficiency of the three oncogenes, *SCL*, *LMO1* and *Notch1*, an active pre-TCR accelerates the time of onset to 29 days, which is the time required for a single leukemic stem cell to induce T-ALL upon transplantation as shown previously (4). Therefore, SCL and LMO1 acting in synergy with two essential signaling pathways in thymocyte development are sufficient to transform a normal DN3a thymocyte into a fully transformed leukemia initiating cell. Moreover, our data indicate that *Notch1* and *Heb* operate in the same genetic pathway, and mono-allelic deletion of *Heb* can recapitulate the capacity of the *Notch1* oncogene to cause a fully penetrant disease in the absence of pre-TCR signaling.

4.1 Pre-TCR Signaling and Lower HEB Levels as Major Drivers of T-ALL Progression

While pre-TCR signaling has been shown to modulate the aggressiveness of T-ALL in several mouse models, the pre-TCR is dispensable for T-ALL induced by *E47*- or *Trp53*-deficiency (97, 98). Hence, the importance of the pre-TCR in T cell transformation induced by oncogenic events remained to be clarified. We propose that the importance of pre-TCR signaling depends on the primary oncogenic transcription factor. Previous transcriptome analyses of pediatric T-ALL identified five molecular subgroups and showed that the TCR signaling pathway is significantly enriched in the TAL1 group while other KEGG pathways related genes were more generally distributed within the other molecular subgroups (9). We now provide evidence that β-selection but not antigen-specific TCR signals are collaborating events. Indeed, the pre-TCR drives cell proliferation, which is required not only for clonal expansion but also for DN-DP differentiation (99) and thymocyte survival (100). A pre-T cell receptor lacking the TCR beta variable domain causes an expansion of the DP population that precedes overt T cell leukemia, suggesting that abnormal pre-TCR function can be oncogenic (101). In addition, the pre-TCR signal is important for *Notch1/ICN1*, *Notch3*- and *TEL-JAK2*-induced leukemias (74, 102, 103), indicating that developmental processes required for normal thymocyte development can be implicated in the pathogenesis of T-ALL (75). In the present study, even though T-ALL can develop in the absence of *Cd3e* and of *Notch1* mutations, our transcriptomic comparison of preleukemic cells with fully transformed leukemic cells indicate a reactivation of β-selection during the progression to T-ALL. The signal strength providing progression to CD4+CD8+ DP T-ALL can either originate from a hyperactive *Notch1* allele or the deletion of a single *Heb* allele. Our data provide compelling evidence for the importance of signal strength of NOTCH1 or HEB in driving the DN to DP transition associated with leukemogenesis.

The pre-TCR complex induces Id expression and consequently inhibition of E protein activity suggesting that the pre-TCR functions upstream of E proteins (81). Nonetheless, *E2a*-deficient mice develop T-cell lymphomas (42, 45), associated with *Notch1* mutations within the PEST domain (104), suggesting that *E2a* is not downstream of *Notch1*. Rather, the latter observations suggest that *Notch1* and *E2a* operate in parallel pathways. Moreover, *Heb* may function parallel to or downstream of the pre-TCR (51). Since NOTCH1 and pre-TCR functions are cooperative during β-selection (11), and both are believed to affect E protein function (81, 105, 106), pre-TCR signaling becomes a confounding factor in assessing the contribution of NOTCH1 and of E proteins in leukemogenesis. Using *Cd3e*$^{-/-}$ mice in which pre-TCR function is abrogated, we now show that oncogenic *Notch1* controls disease penetrance in *SCL-LMO1*-induced T-ALL while the pre-TCR signal governs the time of leukemia onset. Last, our data indicate that *Heb* but not *E2a* operate in the same pathway as *Notch1* since monoallelic deletion of *Heb* does not affect *Notch1*-induced T-ALL but accelerates the disease induced by SCL-LMO1 in *Cd3e*-sufficient mice. Finally, loss of one *Heb* allele in *Cd3e*-deficient mice compensates in part the *Notch1* oncogene to restore full penetrance to T-ALL induced by *SCL* and *LMO1*.

4.2 A Matter of Gene Dosage: Inactivation of HEB Targets in T-ALL by Down-Regulation or Deleterious Mutations.

We and others previously showed that the SCL transcription factor heterodimerizes with E2A or HEB, thereby inhibiting target gene expression such as *Cd4* and *Ptcra* and thymocyte differentiation (39, 40, 50). Nonetheless, E47 deficiency promotes the aberrant development of *Rag1* null thymocytes and appearance of DP cells (84), similar to the appearance of a minor population of DP cells reported here in *Cd3e*-deficient thymocytes lacking one *Heb* allele.

E proteins are negatively regulated by ID proteins, another class of bHLH protein. At the β-selection checkpoint, the activation of RAS extracellular-signal-related kinase (ERK)–MAP kinase pathway upregulates ID proteins that bind to E proteins preventing their transcriptional activity (84). E2A inhibits proliferation and differentiation at the β-selection checkpoint in the absence of pre-TCR expression (84), suggesting that inhibition of E2A *via* Id3 would enforce the two distinctive functional outputs of pre-TCR signaling in favoring both cell proliferation and differentiation. However, Id2 and Id3 suppress lymphomagenesis (107), suggesting a necessary balance of E protein/ID levels during thymocyte differentiation. Partial redundancy may explain that both *E2a* and *Heb* are haplosufficient for thymocyte differentiation during steady-state conditions. Nonetheless, the oncogenic stress induced by SCL and LMO1 in thymocytes reveal *Heb* haploinsufficiency at the DN to DP transition, more specifically at the β-selection checkpoint revealing at the same time that *E2a* cannot compensate for *Heb* in stress-response, despite their quasi-redundancy during steady-state conditions.

Our results also bring out the importance of monitoring protein levels in primary tumors, since inactivation of tumor suppressors *via* protein downregulation could represent a distinct mechanism driving T-ALL.

Master regulators of hematopoietic lineages have been implicated in tumor suppressor function, as reported for C/EBPα (108, 109) and SPI1/PU.1 in acute myeloblastic leukemia (AML) (110) or PAX5 loss of function in B-ALL (90, 111). While

E2A/TCF3 is annotated as a tumor suppressor gene (TSGene 2.0) with direct experimental and clinical evidence, *HEB/TCF12* tumor suppressor function has been overlooked, despite the critical role of HEB in the T lineage (reviewed in (4, 112)). Due to a non-redundant function to enforce T lineage (36) and at multiple stages of T cell differentiation, we propose that the essentiality of *HEB* precludes the possibility of identifying loss of function mutations in hematopoietic malignancies. Our study reveals the possibility of temporary loss of function *via* downregulation of HEB protein levels. Moreover, our analysis of the PCGP cohort identifies decreased gene expression levels in T-ALL compared to other pediatric cancers for at least five TSGenes that are HEB targets, regardless of the T-ALL molecular subgroup (9). In addition to well documented genetic alterations of tumor suppressor genes, our study indicates that reduced expression levels of multiple tumor suppressor genes could also contribute to the process of tumorigenesis, as illustrated here for *CDKN1A*.

4.3 *HEB* Controls a Tumor Suppressor Network in T-ALL that Includes *CDKN1A*

Cdkn1a is a well known E2A and HEB target (44) and tumor suppressor gene (113). More recently, the $Cdkn1a^{SUPER}$ mouse is shown to be more resistant to transformation and exhibit a strong cancer protection phenotype, establishing a direct gene dosage-dependent tumor suppressor function for *CDKN1A* (87). Our observations indicate that *Cdkn1a* is down regulated by the *SCL-LMO1* oncogenes during the pre-leukemic stage and further down regulated during progression to T-ALL. In *Cd3e*-deficient T-ALL, our data point to the down-regulation of *Cdkn1a* as part of the selective pressure to activate molecular mechanisms underlying the β-selection process and drive progression to T-ALL. Supporting this hypothesis, *CDKN1A* gene expression levels in the PCGP cohort is on average six- to seven-fold lower in pediatric T-ALL compared to B-ALL, AML or brain tumors. In addition to mRNA downregulation, $p21^{Cdkn1a}$ can also be phosphorylated and degraded *via* ubiquitin-dependent and ubiquitin-independent proteolysis (113). Much like *HEB*, the *CDKN1A* gene is not subject to copy number variations nor deleterious mutations in hematopoietic malignancies. Rather, CDKN1A levels can be downregulated at the mRNA level, as illustrated here, or at the level of protein stability.

HEB and E2A exert an anti-proliferative function in thymocytes prior to *Tcrb* rearrangement, required for the formation of a functional pre-TCR (44), consistent with a tumor suppressor function reported here for HEB. In addition to a well-timed restriction in cell proliferation, it remains possible that tumor suppression involves additional molecular functions secured either by HEB or HEB downstream targets, as reported for TP53 (114). In addition to *CDKN1A*, HEB and E2A co-occupy three TSG loci, *APC*, a haplo-insufficient tumor suppressor, *ZMYND11* a chromatin reader and tumor suppressor in breast cancer (115) and *CUX1*, a homeodomain transcription regulator and haploinsufficient tumor suppressor (116). HEB is also found to occupy three other TSGenes, *MYBPP1A*, potentially involved in nucleolar stress, *NEDD4L*, an E3 ligase with tumor suppressor function (117) and *PLCB3*, encoding phospholipase C beta 3 (phosphatidylinositol-specific), involved in G-protein-linked receptor-mediated signal transduction (**Figure 6H**). These additional targets may account for the more prominent role of HEB as a tumor suppressor in response to the oncogenic stress induced by SCL and LMO1, exactly at the β-selection checkpoint controlled by HEB. Beyond the SCL/TAL1 molecular group, five HEB target-TSGenes are down regulated in T-ALL compared to other pediatric tumors. In T-ALL, we propose that HEB controls a network of tumor suppressor genes to mitigate the oncogenic stress occurring at the β-selection checkpoint. Unlike classical tumor suppressors, these genes are not inactivated by genomic deletion or deleterious mutations but are down-regulated at the mRNA or at the protein levels. This mechanism cannot be detected by assessing DNA copy number variation or whole-genome sequencing, as exemplified by the total absence of *HEB* copy number loss or point mutations in T-ALL. In this context, future large-scale proteomics studies in primary tumors will be able to address whether downregulation of HEB protein is a recurrent event in human T-ALL.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://www.ncbi.nlm.nih.gov/geo, accession ID: GSE198506. RNA-seq data from pediatric tumors used for analysis in this study were obtained from the St. Jude Cloud (https://www.stjude.cloud).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique de la recherche clinique, Université de Montréal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DV, MT, BG and TH conceived and designed the work. DV, MT, BG, SH and AH conducted the studies and/or contributed to the acquisition of data. DV, MT, BG, SH, PG, SL, JZ-P, JH and JPC contributed to the interpretation of the data and/or data analysis. JH, BG and AH contributed to experiments with primary human leukemic cells. DV, MT, and TH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.867443/full#supplementary-material

REFERENCES

1. Hoang T, Lambert JA, Martin R. SCL/TAL1 in Hematopoiesis and Cellular Reprogramming. *Curr Top Dev Biol* (2016) 118:163–204. doi: 10.1016/bs.ctdb.2016.01.004
2. Gerby B, Tremblay CS, Tremblay M, Rojas-Sutterlin S, Herblot S, Hebert J, et al. SCL, LMO1 and Notch1 Reprogram Thymocytes Into Self-Renewing Cells. *PLoS Genet* (2014) 10(12):e1004768. doi: 10.1371/journal.pgen.1004768
3. McCormack MP, Shields BJ, Jackson JT, Nasa C, Shi W, Slater NJ, et al. Requirement for Lyl1 in a Model of Lmo2-Driven Early T-Cell Precursor ALL. *Blood* (2013) 122(12):2093–103. doi: 10.1182/blood-2012-09-458570
4. Tremblay M, Tremblay CS, Herblot S, Aplan PD, Hebert J, Perreault C, et al. Modeling T-Cell Acute Lymphoblastic Leukemia Induced by the SCL and LMO1 Oncogenes. *Genes Dev* (2010) 24(11):1093–105. doi: 10.1101/gad.1897910
5. Cleveland SM, Smith S, Tripathi R, Mathias EM, Goodings C, Elliott N, et al. Lmo2 Induces Hematopoietic Stem Cell-Like Features in T-Cell Progenitor Cells Prior to Leukemia. *Stem Cells* (2013) 31(5):882–94. doi: 10.1002/stem.1345
6. Fregona V, Bayet M, Gerby B. Oncogene-Induced Reprogramming in Acute Lymphoblastic Leukemia: Towards Targeted Therapy of Leukemia-Initiating Cells. *Cancers* (2021) 13(21):5511. doi: 10.3390/cancers13215511
7. Greaves M, Maley CC. Clonal Evolution in Cancer. *Nature* (2012) 481 (7381):306–13. doi: 10.1038/nature10762
8. Oshima K, Khiabanian H, da Silva-Almeida AC, Tzoneva G, Abate F, Ambesi-Impiombato A, et al. Mutational Landscape, Clonal Evolution Patterns, and Role of RAS Mutations in Relapsed Acute Lymphoblastic Leukemia. *Proc Natl Acad Sci* (2016) 113(40):11306–11. doi: 10.1073/pnas.1608420113
9. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene Expression Signatures Define Novel Oncogenic Pathways in T Cell Acute Lymphoblastic Leukemia. *Cancer Cell* (2002) 1(1):75–87. doi: 10.1016/S1535-6108(02)00018-1
10. Liu Y, Easton J, Shao Y, Maciaszek J, Wang Z, Wilkinson MR, et al. The Genomic Landscape of Pediatric and Young Adult T-Lineage Acute Lymphoblastic Leukemia. *Nat Genet* (2017) 49(8):1211–8. doi: 10.1038/ng.3909
11. Ciofani M, Schmitt TM, Ciofani A, Michie AM, Cuburu N, Aublin A, et al. Obligatory Role for Cooperative Signaling by Pre-TCR and Notch During Thymocyte Differentiation. *J Immunol* (2004) 172(9):5230–9. doi: 10.4049/jimmunol.172.9.5230
12. Maillard I, Tu L, Sambandam A, Yashiro-Ohtani Y, Millholland J, Keeshan K, et al. The Requirement for Notch Signaling at the Beta-Selection Checkpoint *In Vivo* is Absolute and Independent of the Pre-T Cell Receptor. *J Exp Med* (2006) 203(10):2239–45. doi: 10.1084/jem.20061020
13. Ciofani M, Zúñiga-Pflücker JC. Notch Promotes Survival of Pre–T Cells at the β-Selection Checkpoint by Regulating Cellular Metabolism. *Nat Immunol* (2005) 6(9):881–8. doi: 10.1038/ni1234
14. Weng AP, Ferrando AA, Lee W, Morris J, Silverman LB, Sanchez-Irizarry C, et al. Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. *Science* (2004) 306(5694):269–71. doi: 10.1126/science.1102160
15. Roy M, Pear WS, Aster JC. The Multifaceted Role of Notch in Cancer. *Curr Opin Genet Dev* (2007) 17(1):52–9. doi: 10.1016/j.gde.2006.12.001
16. Kushwah R, Guezguez B, Lee JB, Hopkins CI, Bhatia M. Pleiotropic Roles of Notch Signaling in Normal, Malignant, and Developmental Hematopoiesis in the Human. *EMBO Rep* (2014) 15(11):1128–38. doi: 10.15252/embr.201438842
17. Tatarek J, Cullion K, Ashworth T, Gerstein R, Aster JC, Kelliher MA. Notch1 Inhibition Targets the Leukemia-Initiating Cells in a Tal1/Lmo2 Mouse Model of T-ALL. *Blood* (2011) 118(6):1579–90. doi: 10.1182/blood-2010-08-300343
18. Ashworth TD, Pear WS, Chiang MY, Blacklow SC, Mastio J, Xu L, et al. Deletion-Based Mechanisms of Notch1 Activation in T-ALL: Key Roles for RAG Recombinase and a Conserved Internal Translational Start Site in Notch1. *Blood* (2010) 116(25):5455–64. doi: 10.1182/blood-2010-05-286328
19. King B, Trimarchi T, Reavie L, Xu L, Mullenders J, Ntziachristos P, et al. The Ubiquitin Ligase FBXW7 Modulates Leukemia-Initiating Cell Activity by Regulating MYC Stability. *Cell* (2013) 153(7):1552–66. doi: 10.1016/j.cell.2013.05.041
20. Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, et al. C-Myc is an Important Direct Target of Notch1 in T-Cell Acute Lymphoblastic Leukemia/Lymphoma. *Genes Dev* (2006) 20(15):2096–109. doi: 10.1101/gad.1450406
21. Yashiro-Ohtani Y, Wang H, Zang C, Arnett KL, Bailis W, Ho Y, et al. Long-Range Enhancer Activity Determines Myc Sensitivity to Notch Inhibitors in T Cell Leukemia. *Proc Natl Acad Sci USA* (2014) 111(46):E4946–53. doi: 10.1073/pnas.1407079111
22. De Keersmaecker K, Atak ZK, Li N, Vicente C, Patchett S, Girardi T, et al. Exome Sequencing Identifies Mutation in CNOT3 and Ribosomal Genes RPL5 and RPL10 in T-Cell Acute Lymphoblastic Leukemia. *Nat Genet* (2013) 45(2):186–90. doi: 10.1038/ng.2508
23. Homminga I, Pieters R, Langerak AW, de Rooi JJ, Stubbs A, Verstegen M, et al. Integrated Transcript and Genome Analyses Reveal NKX2-1 and MEF2C as Potential Oncogenes in T Cell Acute Lymphoblastic Leukemia. *Cancer Cell* (2011) 19(4):484–97. doi: 10.1016/j.ccr.2011.02.008
24. Cauwelier B, Cave H, Gervais C, Lessard M, Barin C, Perot C, et al. Clinical, Cytogenetic and Molecular Characteristics of 14 T-ALL Patients Carrying the TCRbeta-HOXA Rearrangement: A Study of the Groupe Francophone De Cytogenetique Hematologique. *Leukemia* (2007) 21(1):121–8. doi: 10.1038/sj.leu.2404410
25. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, et al. COSMIC: The Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res* (2018) 47(D1):D941–D7. doi: 10.1093/nar/gky1015
26. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational Landscape and Significance Across 12 Major Cancer Types. *Nature* (2013) 502(7471):333–9. doi: 10.1038/nature12634
27. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer Genome Landscapes. *Science* (2013) 339(6127):1546–58. doi: 10.1126/science.1235122
28. Look AT. Oncogenic Transcription Factors in the Human Acute Leukemias. *Science* (1997) 278(5340):1059–64. doi: 10.1126/science.278.5340.1059

29. Van Vlierberghe P, Ferrando A. The Molecular Basis of T Cell Acute Lymphoblastic Leukemia. *J Clin Invest* (2012) 122(10):3398–406. doi: 10.1172/JCI61269
30. De Keersmaecker K, Real PJ, Gatta GD, Palomero T, Sulis ML, Tosello V, et al. The TLX1 Oncogene Drives Aneuploidy in T Cell Transformation. *Nat Med* (2010) 16(11):1321–7. doi: 10.1038/nm.2246
31. Gutierrez A, Kentsis A, Sanda T, Holmfeldt L, Chen SC, Zhang J, et al. The BCL11B Tumor Suppressor is Mutated Across the Major Molecular Subtypes of T-Cell Acute Lymphoblastic Leukemia. *Blood* (2011) 118 (15):4169–73. doi: 10.1182/blood-2010-11-318873
32. Li L, Leid M, Rothenberg EV. An Early T Cell Lineage Commitment Checkpoint Dependent on the Transcription Factor Bcl11b. *Science* (2010) 329(5987):89–93. doi: 10.1126/science.1188989
33. Murre C, McCaw PS, Baltimore D. A New DNA Binding and Dimerization Motif in Immunoglobulin Enhancer Binding, Daughterless, MyoD, and Myc Proteins. *Cell* (1989) 56(5):777–83. doi: 10.1016/0092-8674(89)90682-X
34. Wang D, Claus CL, Vaccarelli G, Braunstein M, Schmitt TM, Zúñiga-Pflücker JC, et al. The Basic Helix-Loop-Helix Transcription Factor HEBAlt is Expressed in Pro-T Cells and Enhances the Generation of T Cell Precursors. *J Immunol* (2006) 177(1):109–19. doi: 10.4049/jimmunol.177.1.109
35. Barndt RJ, Dai M, Zhuang Y. Functions of E2A-HEB Heterodimers in T-Cell Development Revealed by a Dominant Negative Mutation of HEB. *Mol Cell Biol* (2000) 20(18):6677–85. doi: 10.1128/MCB.20.18.6677-6685.2000
36. Braunstein M, Anderson MK. HEB-Deficient T-Cell Precursors Lose T-Cell Potential and Adopt an Alternative Pathway of Differentiation. *Mol Cell Biol* (2011) 31(5):971–82. doi: 10.1128/MCB.01034-10
37. Miyazaki M, Rivera RR, Miyazaki K, Lin YC, Agata Y, Murre C. The Opposing Roles of the Transcription Factor E2A and its Antagonist Id3 That Orchestrate and Enforce the Naive Fate of T Cells. *Nat Immunol* (2011) 12 (10):992–1001. doi: 10.1038/ni.2086
38. Belle I, Zhuang Y. E Proteins in Lymphocyte Development and Lymphoid Diseases. *Curr Top Dev Biol* (2014) 110:153–87. doi: 10.1016/B978-0-12-405943-6.00004-X
39. Herblot S, Steff AM, Hugo P, Aplan PD, Hoang T. SCL and LMO1 Alter Thymocyte Differentiation: Inhibition of E2A-HEB Function and Pre-T Alpha Chain Expression. *Nat Immunol* (2000) 1(2):138–44. doi: 10.1038/77819
40. Chervinsky DS, Zhao XF, Lam DH, Ellsworth M, Gross KW, Aplan PD. Disordered T-Cell Development and T-Cell Malignancies in SCL LMO1 Double-Transgenic Mice: Parallels With E2A-Deficient Mice. *Mol Cell Biol* (1999) 19(7):5025–35. doi: 10.1128/MCB.19.7.5025
41. Herblot S, Aplan PD, Hoang T. Gradient of E2A Activity in B-Cell Development. *Mol Cell Biol* (2002) 22(3):886–900. doi: 10.1128/MCB.22.3.886-900.2002
42. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2A Deficiency Leads to Abnormalities in Alphabeta T-Cell Development and to Rapid Development of T-Cell Lymphomas. *Mol Cell Biol* (1997) 17(8):4782–91. doi: 10.1128/MCB.17.8.4782
43. Park ST, Nolan GP, Sun XH. Growth Inhibition and Apoptosis Due to Restoration of E2A Activity in T Cell Acute Lymphoblastic Leukemia Cells. *J Exp Med* (1999) 189(3):501–8. doi: 10.1084/jem.189.3.501
44. Wojciechowski J, Lai A, Kondo M, Zhuang Y. E2A and HEB are Required to Block Thymocyte Proliferation Prior to Pre-TCR Expression. *J Immunol* (2007) 178(9):5717–26. doi: 10.4049/jimmunol.178.9.5717
45. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High Incidence of T-Cell Tumors in E2A-Null Mice and E2A/Id1 Double-Knockout Mice. *Mol Cell Biol* (1997) 17(12):7317–27. doi: 10.1128/MCB.17.12.7317
46. Hsu HL, Wadman I, Baer R. Formation of *In Vivo* Complexes Between the TAL1 and E2A Polypeptides of Leukemic T Cells. *Proc Natl Acad Sci USA* (1994) 91(8):3181–5. doi: 10.1073/pnas.91.8.3181
47. O'Neil J, Shank J, Cusson N, Murre C, Kelliher M. TAL1/SCL Induces Leukemia by Inhibiting the Transcriptional Activity of E47/HEB. *Cancer Cell* (2004) 5(6):587–96. doi: 10.1016/j.ccr.2004.05.023
48. McCormack MP, Young LF, Vasudevan S, de Graaf CA, Codrington R, Rabbitts TH, et al. The Lmo2 Oncogene Initiates Leukemia in Mice by Inducing Thymocyte Self-Renewal. *Science* (2010) 327(5967):879–83. doi: 10.1126/science.1182378
49. Smith S, Tripathi R, Goodings C, Cleveland S, Mathias E, Hardaway JA, et al. LIM Domain Only-2 (LMO2) Induces T-Cell Leukemia by Two Distinct Pathways. *PLoS One* (2014) 9(1):e85883. doi: 10.1371/journal.pone.0085883
50. Tremblay M, Herblot S, Lecuyer E, Hoang T. Regulation of pT Alpha Gene Expression by a Dosage of E2A, HEB, and SCL. *J Biol Chem* (2003) 278 (15):12680–7. doi: 10.1074/jbc.M209870200
51. Barndt R, Dai MF, Zhuang Y. A Novel Role for HEB Downstream or Parallel to the Pre-TCR Signaling Pathway During Alpha Beta Thymopoiesis. *J Immunol* (1999) 163(6):3331–43.
52. Zhuang Y, Cheng P, Weintraub H. B-Lymphocyte Development is Regulated by the Combined Dosage of Three Basic Helix-Loop-Helix Genes, E2A, E2-2, and HEB. *Mol Cell Biol* (1996) 16(6):2898–905. doi: 10.1128/MCB.16.6.2898
53. Robey E, Chang D, Itano A, Cado D, Alexander H, Lans D, et al. An Activated Form of Notch Influences the Choice Between CD4 and CD8 T Cell Lineages. *Cell* (1996) 87(3):483–92. doi: 10.1016/S0092-8674(00)81368-9
54. Aplan PD, Jones CA, Chervinsky DS, Zhao X, Ellsworth M, Wu C, et al. An Scl Gene Product Lacking the Transactivation Domain Induces Bony Abnormalities and Cooperates With LMO1 to Generate T-Cell Malignancies in Transgenic Mice. *EMBO J* (1997) 16(9):2408–19. doi: 10.1093/emboj/16.9.2408
55. McGuire EA, Rintoul CE, Sclar GM, Korsmeyer SJ. Thymic Overexpression of Ttg-1 in Transgenic Mice Results in T-Cell Acute Lymphoblastic Leukemia/Lymphoma. *Mol Cell Biol* (1992) 12(9):4186–96. doi: 10.1128/mcb.12.9.4186-4196.1992
56. Malissen M, Gillet A, Ardouin L, Bouvier G, Trucy J, Ferrier P, et al. Altered T Cell Development in Mice With a Targeted Mutation of the CD3-Epsilon Gene. *EMBO J* (1995) 14(19):4641–53. doi: 10.1002/j.1460-2075.1995.tb00146.x
57. Zhuang Y, Kim CG, Bartelmez S, Cheng P, Groudine M, Weintraub H. Helix-Loop-Helix Transcription Factors E12 and E47 are Not Essential for Skeletal or Cardiac Myogenesis, Erythropoiesis, Chondrogenesis, or Neurogenesis. *Proc Natl Acad Sci USA* (1992) 89(24):12132–6. doi: 10.1073/pnas.89.24.12132
58. Gerby B, Veiga D, Krosl J, Ouellette J, Fares I, Tremblay M, et al. High-Throughput Screening in Niche-Based Assay Identifies Compounds to Target Pre-Leukemic Stem Cells. *J Clin Invest* (2016) 126(12):4569–84. doi: 10.1172/JCI86489
59. Gautier L, Cope L, Bolstad BM, Irizarry RA. Affy–Analysis of Affymetrix GeneChip Data at the Probe Level. *Bioinformatics* (2004) 20(3):307–15. doi: 10.1093/bioinformatics/btg405
60. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles. *Proc Natl Acad Sci USA* (2005) 102(43):15545–50. doi: 10.1073/pnas.0506580102
61. Wang H, Zou J, Zhao B, Johannsen E, Ashworth T, Wong H, et al. Genome-Wide Analysis Reveals Conserved and Divergent Features of Notch1/RBPJ Binding in Human and Murine T-Lymphoblastic Leukemia Cells. *Proc Natl Acad Sci USA* (2011) 108(36):14908–13. doi: 10.1073/pnas.1109023108
62. Hannah R, Joshi A, Wilson NK, Kinston S, Gottgens B. A Compendium of Genome-Wide Hematopoietic Transcription Factor Maps Supports the Identification of Gene Regulatory Control Mechanisms. *Exp Hematol* (2011) 39(5):531–41. doi: 10.1016/j.exphem.2011.02.009
63. Salmon-Divon M, Dvinge H, Tammoja K, Bertone P. PeakAnalyzer: Genome-Wide Annotation of Chromatin Binding and Modification Loci. *BMC Bioinform* (2010) 11:415. doi: 10.1186/1471-2105-11-415
64. Bolger AM, Lohse M, Usadel B. Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* (2014) 30(15):2114–20. doi: 10.1093/bioinformatics/btu170
65. Li H, Durbin R. Fast and Accurate Short Read Alignment With Burrows-Wheeler Transform. *Bioinformatics* (2009) 25(14):1754–60. doi: 10.1093/bioinformatics/btp324
66. Saunders CT, Wong WS, Swamy S, Becq J, Murray LJ, Cheetham RK. Strelka: Accurate Somatic Small-Variant Calling From Sequenced Tumor-Normal Sample Pairs. *Bioinformatics* (2012) 28(14):1811–7. doi: 10.1093/bioinformatics/bts271
67. Wang K, Li M, Hakonarson H. ANNOVAR: Functional Annotation of Genetic Variants From High-Throughput Sequencing Data. *Nucleic Acids Res* (2010) 38(16):e164. doi: 10.1093/nar/gkq603

68. McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the Consequences of Genomic Variants With the Ensembl API and SNP Effect Predictor. *Bioinformatics* (2010) 26(16):2069–70. doi: 10.1093/bioinformatics/btq330
69. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: Accurate Alignment of Transcriptomes in the Presence of Insertions, Deletions and Gene Fusions. *Genome Biol* (2013) 14(4):R36. doi: 10.1186/gb-2013-14-4-r36
70. McLeod C, Gout AM, Zhou X, Thrasher A, Rahbarinia D, Brady SW, et al. St. Jude Cloud: A Pediatric Cancer Genomic Data-Sharing Ecosystem. *Cancer Discov* (2021) 11(5):1082–99. doi: 10.1158/2159-8290.CD-20-1230
71. Zhou X, Edmonson MN, Wilkinson MR, Patel A, Wu G, Liu Y, et al. Exploring Genomic Alteration in Pediatric Cancer Using ProteinPaint. *Nat Genet* (2016) 48(1):4–6. doi: 10.1038/ng.3466
72. Chervinsky DS, Lam DH, Melman MP, Gross KW, Aplan PD. Scid Thymocytes With TCRbeta Gene Rearrangements are Targets for the Oncogenic Effect of SCL and LMO1 Transgenes. *Cancer Res* (2001) 61(17):6382–7.
73. Campese AF, Garbe AI, Zhang F, Grassi F, Screpanti I, von Boehmer H. Notch1-Dependent Lymphomagenesis is Assisted by But Does Not Essentially Require Pre-TCR Signaling. *Blood* (2006) 108(1):305–10. doi: 10.1182/blood-2006-01-0143
74. Bellavia D, Campese AF, Checquolo S, Balestri A, Biondi A, Cazzaniga G, et al. Combined Expression of Ptalpha and Notch3 in T Cell Leukemia Identifies the Requirement of preTCR for Leukemogenesis. *Proc Natl Acad Sci USA* (2002) 99(6):3788–93. doi: 10.1073/pnas.062050599
75. Aifantis I, Raetz E, Buonamici S. Molecular Pathogenesis of T-Cell Leukaemia and Lymphoma. *Nat Rev Immunol* (2008) 8(5):380–90. doi: 10.1038/nri2304
76. Fasseu M, Aplan PD, Chopin M, Boissel N, Bories JC, Soulier J, et al. P16ink4a Tumor Suppressor Gene Expression and CD3epsilon Deficiency But Not Pre-TCR Deficiency Inhibit TAL1-Linked T-Lineage Leukemogenesis. *Blood* (2007) 110(7):2610–9. doi: 10.1182/blood-2007-01-066209
77. Bailis W, Pear WS. "The Molecular Basis of T Cell Development and How Epigenetic/Transcriptional Deregulation Leads to T-ALL". In: C Bonifer and NP Cockerill, editors. *Transcriptional and Epigenetic Mechanisms Regulating Normal and Aberrant Blood Cell Development*, vol. p . Berlin, Heidelberg: Springer Berlin Heidelberg (2014). p. 267–93.
78. Sanda T, Lawton LN, Barrasa MI, Fan ZP, Kohlhammer H, Gutierrez A, et al. Core Transcriptional Regulatory Circuit Controlled by the TAL1 Complex in Human T Cell Acute Lymphoblastic Leukemia. *Cancer Cell* (2012) 22(2):209–21. doi: 10.1016/j.ccr.2012.06.007
79. Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A, et al. The LIM-Only Protein Lmo2 is a Bridging Molecule Assembling an Erythroid, DNA-Binding Complex Which Includes the TAL1, E47, GATA-1 and Ldb1/NLI Proteins. *EMBO J* (1997) 16(11):3145–57. doi: 10.1093/emboj/16.11.3145
80. Simon C, Chagraoui J, Krosl J, Gendron P, Wilhelm B, Lemieux S, et al. A Key Role for EZH2 and Associated Genes in Mouse and Human Adult T-Cell Acute Leukemia. *Genes Dev* (2012) 26(7):651–6. doi: 10.1101/gad.186411.111
81. Xi H, Schwartz R, Engel I, Murre C, Kersh GJ. Interplay Between RORgammat, Egr3, and E Proteins Controls Proliferation in Response to Pre-TCR Signals. *Immunity* (2006) 24(6):813–26. doi: 10.1016/j.immuni.2006.03.023
82. Engel I, Murre C. E2A Proteins Enforce a Proliferation Checkpoint in Developing Thymocytes. *EMBO J* (2004) 23(1):202–11. doi: 10.1038/sj.emboj.7600017
83. Lacombe J, Herblot S, Rojas-Sutterlin S, Haman A, Barakat S, Iscove NN, et al. Scl Regulates the Quiescence and the Long-Term Competence of Hematopoietic Stem Cells. *Blood* (2010) 115(4):792–803. doi: 10.1182/blood-2009-01-201384
84. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early Thymocyte Development is Regulated by Modulation of E2A Protein Activity. *J Exp Med* (2001) 194(6):733–45. doi: 10.1084/jem.194.6.733
85. Lin YW, Nichols RA, Letterio JJ, Aplan PD. Notch1 Mutations are Important for Leukemic Transformation in Murine Models of Precursor-T Leukemia/Lymphoma. *Blood* (2006) 107(6):2540–3. doi: 10.1182/blood-2005-07-3013
86. Schwartz R, Engel I, Fallahi-Sichani M, Petrie HT, Murre C. Gene Expression Patterns Define Novel Roles for E47 in Cell Cycle Progression, Cytokine-Mediated Signaling, and T Lineage Development. *Proc Natl Acad Sci USA* (2006) 103(26):9976–81. doi: 10.1073/pnas.0603728103
87. Torgovnick A, Heger JM, Liaki V, Isensee J, Schmitt A, Knittel G, et al. The Cdkn1a(SUPER) Mouse as a Tool to Study P53-Mediated Tumor Suppression. *Cell Rep* (2018) 25(4):1027–39.e6. doi: 10.1016/j.celrep.2018.09.079
88. Soulier J, Clappier E, Cayuela JM, Regnault A, Garcia-Peydro M, Dombret H, et al. HOXA Genes are Included in Genetic and Biologic Networks Defining Human Acute T-Cell Leukemia (T-ALL). *Blood* (2005) 106(1):274–86. doi: 10.1182/blood-2004-10-3900
89. Downing JR, Wilson RK, Zhang J, Mardis ER, Pui CH, Ding L, et al. The Pediatric Cancer Genome Project. *Nat Genet* (2012) 44(6):619–22. doi: 10.1038/ng.2287
90. Gu Z, Churchman ML, Roberts KG, Moore I, Zhou X, Nakitandwe J, et al. PAX5-Driven Subtypes of B-Progenitor Acute Lymphoblastic Leukemia. *Nat Genet* (2019) 51(2):296–307. doi: 10.1038/s41588-018-0315-5
91. Funato N, Ohtani K, Ohyama K, Kuroda T, Nakamura M. Common Regulation of Growth Arrest and Differentiation of Osteoblasts by Helix-Loop-Helix Factors. *Mol Cell Biol* (2001) 21(21):7416–28. doi: 10.1128/MCB.21.21.7416-7428.2001
92. Prabhu S, Ignatova A, Park ST, Sun XH. Regulation of the Expression of Cyclin-Dependent Kinase Inhibitor P21 by E2A and Id Proteins. *Mol Cell Biol* (1997) 17(10):5888–96. doi: 10.1128/MCB.17.10.5888
93. Mingueneau M, Kreslavsky T, Gray D, Heng T, Cruse R, Ericson J, et al. The Transcriptional Landscape of Alphabeta T Cell Differentiation. *Nat Immunol* (2013) 14(6):619–32. doi: 10.1038/ni.2590
94. Ohashi R, Angori S, Batavia AA, Rupp NJ, Ajioka Y, Schraml P, et al. Loss of CDKN1A mRNA and Protein Expression Are Independent Predictors of Poor Outcome in Chromophobe Renal Cell Carcinoma Patients. *Cancers* (2020) 12(2):465. doi: 10.3390/cancers12020465
95. Davies C, Hogarth LA, Dietrich PA, Bachmann PS, Mackenzie KL, Hall AG, et al. P53-Independent Epigenetic Repression of the P21waf1 Gene in T-Cell Acute Lymphoblastic Leukemia. *J Biol Chem* (2011) 286(43):37639–50. doi: 10.1074/jbc.M111.272336
96. Bain G, Cravatt CB, Loomans C, Alberola-Ila J, Hedrick SM, Murre C. Regulation of the Helix-Loop-Helix Proteins, E2A and Id3, by the Ras-ERK MAPK Cascade. *Nat Immunol* (2001) 2(2):165–71. doi: 10.1038/84273
97. Engel I, Murre C. Disruption of Pre-TCR Expression Accelerates Lymphomagenesis in E2A-Deficient Mice. *Proc Natl Acad Sci USA* (2002) 99(17):11322–7. doi: 10.1073/pnas.162373999
98. Liao MJ, Zhang XX, Hill R, Gao J, Qumsiyeh MB, Nichols W, et al. No Requirement for V(D)J Recombination in P53-Deficient Thymic Lymphoma. *Mol Cell Biol* (1998) 18(6):3495–501. doi: 10.1128/MCB.18.6.3495
99. Kreslavsky T, Gleimer M, Miyazaki M, Choi Y, Gagnon E, Murre C, et al. β-Selection-Induced Proliferation Is Required for αβ T Cell Differentiation. *Immunity* (2012) 37(5):840–53. doi: 10.1016/j.immuni.2012.08.020
100. Wong GW, Knowles GC, Mak TW, Ferrando AA, Zuniga-Pflucker JC. HES1 Opposes a PTEN-Dependent Check on Survival, Differentiation, and Proliferation of TCRbeta-Selected Mouse Thymocytes. *Blood* (2012) 120(7):1439–48. doi: 10.1182/blood-2011-12-395319
101. Jacobs H, Ossendorp F, de Vries E, Ungewiss K, von Boehmer H, Borst J, et al. Oncogenic Potential of a Pre-T Cell Receptor Lacking the TCR Beta Variable Domain. *Oncogene* (1996) 12(10):2089–99.
102. dos Santos NR, Rickman DS, de Reynies A, Cormier F, Williame M, Blanchard C, et al. Pre-TCR Expression Cooperates With TEL-JAK2 to Transform Immature Thymocytes and Induce T-Cell Leukemia. *Blood* (2007) 109(9):3972–81. doi: 10.1182/blood-2006-09-048801
103. Allman D, Karnell FG, Punt JA, Bakkour S, Xu L, Myung P, et al. Separation of Notch1 Promoted Lineage Commitment and Expansion/Transformation in Developing T Cells. *J Exp Med* (2001) 194(1):99–106. doi: 10.1084/jem.194.1.99
104. Reschly EJ, Spaulding C, Vilimas T, Graham WV, Brumbaugh RL, Aifantis I, et al. Notch1 Promotes Survival of E2A-Deficient T Cell Lymphomas

Through Pre-T Cell Receptor-Dependent and -Independent Mechanisms. *Blood* (2006) 107(10):4115–21. doi: 10.1182/blood-2005-09-3551
105. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E Proteins and Notch Signaling Cooperate to Promote T Cell Lineage Specification and Commitment. *J Exp Med* (2006) 203(5):1329–42. doi: 10.1084/jem.20060268
106. Yashiro-Ohtani Y, He Y, Ohtani T, Jones ME, Shestova O, Xu L, et al. Pre-TCR Signaling Inactivates Notch1 Transcription by Antagonizing E2A. *Genes Dev* (2009) 23(14):1665–76. doi: 10.1101/gad.1793709
107. Miyazaki M, Miyazaki K, Chen S, Chandra V, Wagatsuma K, Agata Y, et al. The E-Id Protein Axis Modulates the Activities of the PI3K-AKT-Mtorc1-Hif1a and C-Myc/p19Arf Pathways to Suppress Innate Variant TFH Cell Development, Thymocyte Expansion, and Lymphomagenesis. *Genes Dev* (2015) 29(4):409–25. doi: 10.1101/gad.255331.114
108. Girard N, Tremblay M, Humbert M, Grondin B, Haman A, Labrecque J, et al. RARalpha-PLZF Oncogene Inhibits C/EBPalpha Function in Myeloid Cells. *Proc Natl Acad Sci USA* (2013) 110(33):13522–7. doi: 10.1073/pnas.1310067110
109. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, et al. Dominant-Negative Mutations of CEBPA, Encoding CCAAT/enhancer Binding Protein-Alpha (C/EBPalpha), in Acute Myeloid Leukemia. *Nat Genet* (2001) 27(3):263–70. doi: 10.1038/85820
110. Mueller BU, Pabst T, Osato M, Asou N, Johansen LM, Minden MD, et al. Heterozygous PU.1 Mutations are Associated With Acute Myeloid Leukemia. *Blood* (2002) 100(3):998–1007. doi: 10.1182/blood-2002-12-3903
111. Familiades J, Bousquet M, Lafage-Pochitaloff M, Bene MC, Beldjord K, De Vos J, et al. PAX5 Mutations Occur Frequently in Adult B-Cell Progenitor Acute Lymphoblastic Leukemia and PAX5 Haploinsufficiency is Associated With BCR-ABL1 and TCF3-PBX1 Fusion Genes: A GRAALL Study. *Leukemia* (2009) 23(11):1989–98. doi: 10.1038/leu.2009.135
112. Braunstein M, Anderson MK. HEB in the Spotlight: Transcriptional Regulation of T-Cell Specification, Commitment, and Developmental Plasticity. *Clin Dev Immunol* (2012) 2012:678705. doi: 10.1155/2012/678705
113. Abbas T, Dutta A. P21 in Cancer: Intricate Networks and Multiple Activities. *Nat Rev Cancer* (2009) 9(6):400–14. doi: 10.1038/nrc2657
114. Li T, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, et al. Tumor Suppression in the Absence of P53-Mediated Cell-Cycle Arrest, Apoptosis, and Senescence. *Cell* (2012) 149(6):1269–83. doi: 10.1016/j.cell.2012.04.026
115. Wen H, Li Y, Xi Y, Jiang S, Stratton S, Peng D, et al. ZMYND11 Links Histone H3.3K36me3 to Transcription Elongation and Tumour Suppression. *Nature* (2014) 508(7495):263–8. doi: 10.1038/nature13045
116. Ramdzan ZM, Nepveu A. CUX1, a Haploinsufficient Tumour Suppressor Gene Overexpressed in Advanced Cancers. *Nat Rev Cancer* (2014) 14 (10):673–82. doi: 10.1038/nrc3805
117. Xie S, Xia L, Song Y, Liu H, Wang ZW, Zhu X. Insights Into the Biological Role of NEDD4L E3 Ubiquitin Ligase in Human Cancers. *Front Oncol* (2021) 11:774648. doi: 10.3389/fonc.2021.774648

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REVIEW
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E Protein Transcription Factors as Suppressors of T Lymphocyte Acute Lymphoblastic Leukemia

Geoffrey Parriott[1] *and Barbara L. Kee*[1,2,3]*

[1] Committee on Immunology, University of Chicago, Chicago, IL, United States, [2] Committee on Cancer Biology, University of Chicago, Chicago, IL, United States, [3] Department of Pathology, University of Chicago, Chicago, IL, United States

T Lymphocyte Acute Lymphoblastic Leukemia (ALL) is an aggressive disease arising from transformation of T lymphocytes during their development. The mutation spectrum of T-ALL has revealed critical regulators of the growth and differentiation of normal and leukemic T lymphocytes. Approximately, 60% of T-ALLs show aberrant expression of the hematopoietic stem cell-associated helix-loop-helix transcription factors TAL1 and LYL1. TAL1 and LYL1 function in multiprotein complexes that regulate gene expression in T-ALL but they also antagonize the function of the E protein homodimers that are critical regulators of T cell development. Mice lacking E2A, or ectopically expressing TAL1, LYL1, or other inhibitors of E protein function in T cell progenitors, also succumb to an aggressive T-ALL-like disease highlighting that E proteins promote T cell development and suppress leukemogenesis. In this review, we discuss the role of E2A in T cell development and how alterations in E protein function underlie leukemogenesis. We focus on the role of TAL1 and LYL1 and the genes that are dysregulated in *E2a*$^{-/-}$ T cell progenitors that contribute to human T-ALL. These studies reveal novel mechanisms of transformation and provide insights into potential therapeutic targets for intervention in this disease.

Keywords: Leukemia, E protein, TAL1, LYL1, murine, T lymphocyte

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Utpal Dave,
Indiana University, United States

***Correspondence:**
Barbara L. Kee
bkee@bsd.uchicago.edu

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INTRODUCTION

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive disease that accounts for 15% of pediatric and 25% of adult leukemia cases (1). These leukemias can be grouped into subtypes based on their unique gene expression profiles and response to therapy with subtypes mirroring known stages of T cell development (2–4). Consistent with these heterogeneous phenotypes, mutations in, or altered expression of, genes encoding multiple transcription factors (*cMYC*, *IKZF1*, *GATA3*, *TCF7*, *LEF1*), epigenetic regulators (*EZH2* and *PHF6*), cytokine receptors (*IL7R* and *FLT3*), cell cycle regulators (*CDKN2A* and *CDKN2B*), and signaling proteins (PTEN) have been identified in subsets of human T-ALL (5–7). Despite this heterogeneity, there are oncogenic pathways that are dysregulated broadly across multiple T-ALL subtypes. In particular, mutations that impact the Notch signaling pathway are present in approximately 80% of T-ALL (8). Approximately 60% of T-ALL cases also have mutations that augment expression of the transcription factors T acute

lymphoblastic leukemia antigen 1 (TAL1), also called Stem Cell Leukemia (SCL), and lymphocytic leukemia antigen 1 (LYL1) (9). The high frequency of NOTCH1, TAL1 and LYL1 dysregulation indicate that these pathways impact key processes underlying T cell homeostasis at multiple stage of development and make them attractive targets for therapeutic intervention. Indeed, NOTCH signaling inhibitors have been designed and tested in clinical trials and show some utility for treatment of T-ALL (10). Unfortunately, the broad expression of NOTCH1 has limited the utility of NOTCH1 inhibition as a single agent for treatment of T-ALL, although recently described inhibitors have had some success (10). Nonetheless, ongoing studies taking advantage of partial Notch signaling inhibition combined with targeting other essential pathways is a promising approach for future therapeutics (11). A better understanding of the mechanisms leading to leukemogenesis and the sensitivity of leukemias to inhibition of oncogenic pathways will help to develop novel therapeutics to intervene in this disease.

TAL1 and LYL1 are basic helix-loop-helix (bHLH) proteins that bind DNA in association with the E protein transcription factors (12). The E proteins form homodimers that are critical for B and T lymphocyte development and dimerization with TAL1 or LYL1 alters E protein DNA binding specificity and promotes interactions with unique transcriptional complexes (13–15). Therefore, while TAL1:E protein or LYL1:E protein dimers in T-ALL regulate leukemia-associated genes, expression of TAL1 and LYL1 can also interrupt the function of E protein homodimers. There is substantial evidence in mice to suggest that inhibition of E protein function is sufficient to promote T cell progenitor transformation (16–19). These mouse models recapitulate features of their human T-ALL counterparts such as recurrent mutations in the *Notch1* gene and a requirement for Notch signaling for their survival (20–22). In this review we will discuss the mechanisms driving the genesis and maintenance of T-ALL with a focus on the insights gained through studies in *E2a*-deficieint mice.

E2A PROTEINS IN LYMPHOPOIESIS

The *Tcf3* (*E2a)* gene encodes 2 bHLH proteins (E12 and E47) through alternative splicing of exons encoding the bHLH domain (13). The HLH domain is involved in dimerization with other HLH proteins and the basic region is largely responsible for DNA binding, although some DNA contacts are made with the HLH domain (12). The bHLH domains of E12 and E47 share approximately 80% identity and they bind the same DNA motif, although with differing affinity, and interact with the same proteins (23, 24). There are two additional genes encoding for E proteins in humans and mice, *TCF12* (HEB) and *TCF4* (E2-2), that each code for two E box binding proteins through alternative transcription start sites, resulting in proteins with differing activation domains but identical bHLH domains (25). Other proteins that dimerize with E proteins include the Class IV HLH proteins (ID1-4), which lack a DNA binding domain and therefore prevent E proteins from stably binding DNA, and Class II bHLH proteins, which are largely cell type specific (i.e. MYOD in muscle cells and TAL1 in hematopoietic stem cells) (12). E proteins are broadly and constitutively expressed and are generally found in complexes with tissue-restricted Class II proteins. However, E proteins function as homodimers in lymphocytes; E2A homodimers predominating in B lymphocytes and dimers of E2A and HEB being prevalent in T lymphocytes (13, 25). Consistent with this, $E2a^{-/-}$ mice have severe defects in lymphopoiesis with a complete lack of B lymphocytes and a 3-5X decrease in thymocyte numbers prior to the onset of leukemogenesis (16, 17, 26, 27). *Tcf12*-deficiency or *Tcf4*-deficiency also impacts T cell development but to date, neither of these deficiencies is sufficient to promote T-ALL like disease (25, 28–30).

An advantage of studying T-ALL in mice is that we can track progenitors prior to the onset of disease and discern the impact of these mutations on T cell development as well as on leukemogenesis (**Figure 1**). TAL1, and by analogy its E protein partners, plays a critical role in specification of hematopoietic stem cells (HSCs) from hemangiogenic endothelium but TAL1 is not essential for HSC survival after HSC development (31, 32). However, post HSC specification, TAL1 plays important roles in megakaryocyte differentiation and erythropoiesis (33, 34). LYL1 is not essential for HSC specification, although it becomes essential when TAL1 is limiting, indicating that these proteins have some redundant functions (35). ChIP-seq experiments with TAL1 and LYL1 in HSC-like cell lines revealed extensive overlap in their binding sites indicating that these proteins regulate an overlapping set of genes (36). In $E2a^{-/-}$ mice, HSC specification is intact but lymphopoiesis is impacted at the stage when HSCs become specified to the lymphoid fate with fewer lympho-myeloid primed progenitors (LMPPs) and a failure to initiate expression of multiple lymphoid genes (37, 38). In LMPPs E2A likely functions in cooperation with LYL1 since $Lyl1^{-/-}$ mice have a strikingly similar phenotype to $E2a^{-/-}$ mice at this stage (39). In contrast, TAL1 antagonizes T lymphocyte specification within the HSC and LMPP populations (40). Therefore, despite the similarity in TAL1 and LYL1 structure and their overlapping function in HSC specification, these proteins function in an opposing manner to regulate lymphoid specification. Given the roles of E2A, TAL1 and LYL1 in T-ALL, we anticipate that understanding how these proteins control lymphocyte development will provide insights into the mechanisms that drive lymphopoietic alterations and transformation.

E2A proteins are required for proper expression of *Notch1* at the inception of T cell development (37, 41). Consistent with this, when $E2A^{-/-}$ multipotent progenitors are cultured under T cell differentiation conditions *in vitro* they fail to generate T cells unless they are transduced with a NOTCH1 producing retroviral vector (42). $E2a^{-/-}$ DN2 thymocytes struggle to enter the T cell lineage and fail to control the expression of GATA3, which is substantially elevated in $E2a^{-/-}$ DN2 and DN3 thymocytes (43). This elevated expression of GATA3 contributes to diversion of these cells toward the innate lymphoid lineages, which is particularly evident when *E2a* and *Heb* are both deleted or when ID1 is over expressed in T cell progenitors (43–46).

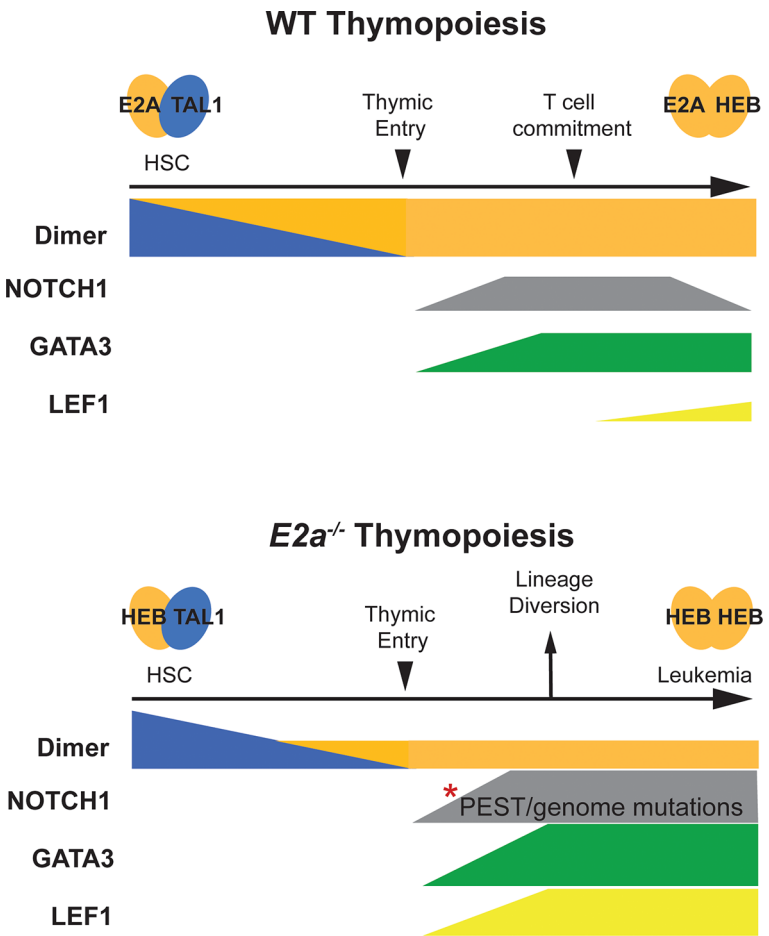

FIGURE 1 | Schematic representation of T cell development in WT and *E2a*$^{-/-}$ mice. T cell development in WT mice is depicted in the top half of the figure. Tal1:E2A (or E protein) dimers are prevalent in HSCs and decline as progenitors differentiate toward the T cell lineage. At the time of thymic commitment E2a:HEB dimers dominate. NOTCH1 and GATA3 start to be expressed upon thymic entry, along with TCF1 (not depicted) under the influence of NOTCH1 ligands in the thymus. LEF1 expression initiates with commitment to the T lymphocyte lineage but remains low throughout T cell development. T cell development in *E2a*$^{-/-}$ mice is depicted on the lower half of the figure. Here, Tal1:HEB or E2-2 dimers control early hematopoiesis resulting in a reduced number of thymic seeding progenitors that can only express HEB : HEB or E2-2 dimers after extinction of TAL1 expression. GATA3 expression is high and diverts progenitors toward the ILC/NK fate. Mutations in the *Notch1* gene accumulate and LEF1 is expressed at very high levels. Combined, these alterations in NOTCH1, GATA3 and LEF1 contribute to T cell transformation.

Indeed, heterozygous deletion of *Gata3* restores differentiation of *E2a*$^{-/-}$ DN2 cells into the T cell lineage (43). Ectopic expression of GATA3 under the control of the CD2 promoter is able to promote T cell transformation suggesting that this failure to repress *Gata3* could be a key event in the generation of *E2a*$^{-/-}$ leukemias (47). In established *E2a*$^{-/-}$ leukemia lines re-expression of E2A proteins alters the transcription of numerous genes including *Gata3*, which is indirectly regulated by E2A-mediated induction of GFI1B (48). Whether GFI1B, alone or in combination with the related transcription factor GFI1, functions to dampen *Gata3* expression at the inception of T cell development remains to be fully explored but it is notable that both *Gfi1b*$^{-/-}$ and *Gfi1*$^{-/-}$ mice have defects in T cell development that overlap with those of *E2a*$^{-/-}$ mice (49, 50). During B cell development *Gata3* is repressed by EBF1 suggesting that GFI1/GFI1B and EBF1 might play similar roles in progenitors prior to their entry into the T and B cell developmental pathway with EBF1 leading to more severe, or sustained, repression of *Gata3* (51).

The few T cell progenitors that develop from *E2a*$^{-/-}$ DN2 thymocytes highly express LEF1, an effector of the Wnt signaling pathway, and LEF1 is required for the survival of *E2a*$^{-/-}$ leukemias (52, 53). LEF1 is not essential for T cell development owing to the high expression of the related transcription factor TCF1 in T cell progenitors (54). *Tcf7* (encoding TCF1) is regulated by NOTCH1 and plays a major role in T cell lineage specification (55, 56). TCF1 is expressed in *E2a*$^{-/-}$ thymocytes despite the increased expression of LEF1; nonetheless, LEF1 impacts *E2A*$^{-/-}$ T cell development. Indeed, deletion of *Lef1* from *E2a*$^{-/-}$ T cell progenitors results in a profound loss of DN3 thymocytes while, surprisingly, not affecting overall T cell numbers (53). These findings suggest

that LEF1 plays a role in controlling the maturation of *E2a*$^{-/-}$ T cells. *Lef1* mRNA is elevated in multiple mouse models that develop T-ALL, and as described later in this review, LEF1 can play both oncogenic and tumor suppressor roles in these models depending on the timing of its expression (53, 57–60). In the following sections we will discuss the known contributions of the E2A interacting proteins TAL1 and LYL1 and the genes dysregulated in *E2a*$^{-/-}$ thymocytes to human and murine T-ALL.

TAL1 AND LYL1

TAL1 was identified as a gene involved in the t(1:14) and t(1;7) chromosomal translocations in T-ALL, which place *TAL1* under the control of the *TCRA/TCRD* or *TCRB* locus, respectively (61–63). LYL1 was also identified through a chromosomal translocation in T-ALL in which *LYL1* on chromosome 19 is juxtaposed to the *TCRB* constant regions on Chromosome 7 (64). These translocations are found in approximately 3-7% of TAL1/LYL1^{+} T-ALL cases, however, there are frequent alterations at the *TAL1* locus in T-ALL including deletions such as *TAL1*^{d}, which arises from a site-specific DNA recombination event causing a 90kb deletion upstream of *TAL1* (62). These deletions place the coding region of the *TAL1* gene downstream of regulatory elements in the SCL interrupting locus (*STIL*). The *STIL* regulatory elements are constitutively active in thymocytes, resulting in ectopic TAL1 expression. These *TAL1* upstream deletions are specific for T-ALL cells and are most likely caused by erroneous V(D)J recombinase activity (65, 66). Alterations in the *TAL1* gene that result in ectopic T lymphocyte expression of TAL1 are now recognized to be present in as many as 60% of T-ALL (1). While these genomic alterations account for a majority of T-ALL associated *TAL1* expression, a subset of patients have ectopic TAL1 expression without these alterations. Studies into the mechanisms of *TAL1* deregulation in these patients revealed small insertions (<20bp) in a region 8kb upstream of *TAL1* that create a *de novo* MYB binding site that results in strong enhancer activity in these leukemias (67). Chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) experiments showed that MYB binds to this novel site along with chromatin remodelers and other components of the DNA transcriptional machinery. Deletion of the novel MYB binding site abrogated MYB binding and significantly reduced TAL1 expression. MYB is highly expressed in thymocytes and MYB is often dysregulated in cancer and thus this mutation can lead to robust *TAL1* transcription in leukemic cells (68). Taken together, these findings outline multiple mechanisms leading to the errant expression of TAL1 in T-ALL (**Figure 2**).

TAL1 positive leukemias frequently express CD4 and CD8 and have a cortical phenotype similar to what is observed in *E2a*$^{-/-}$ mice (2, 16, 17, 69). In mice, expression of TAL1 under the control of the Lck promoter, which drives gene expression early in T cell development, is sufficient to predispose mice in T-ALL-like disease (70). That TAL1 functions through sequestration of

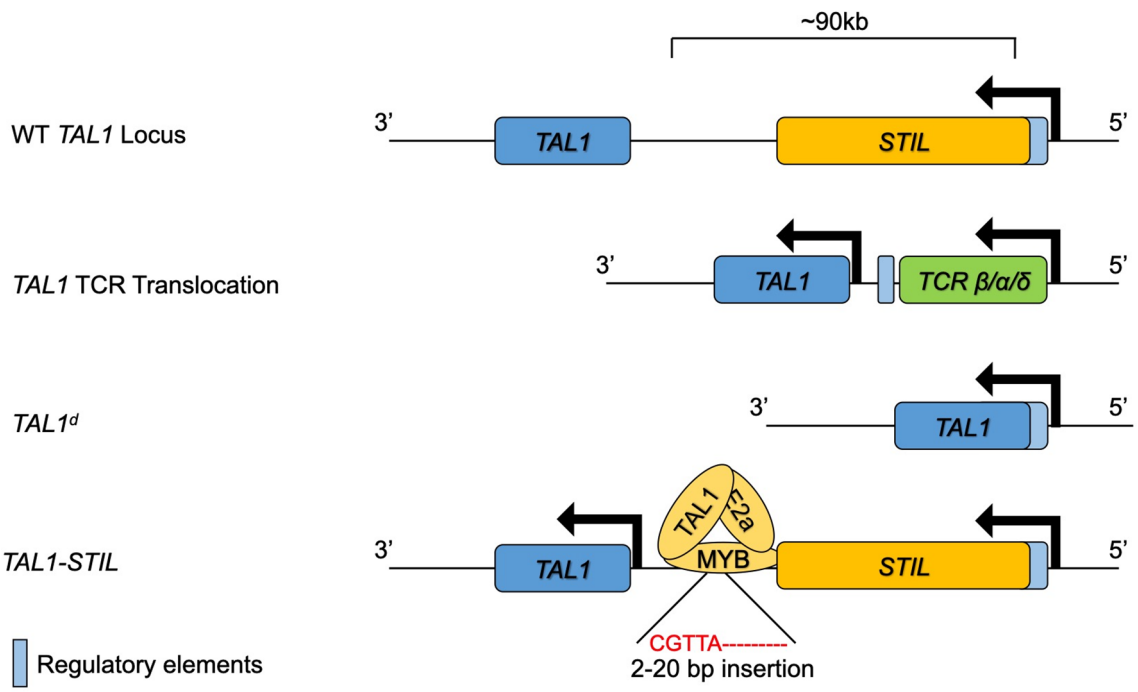

FIGURE 2 | The *TAL1* genomic locus and mutations leading to ectopic expression in T lymphocytes and T-ALL. The WT *TAL1* locus is depicted downstream of the *STIL* gene. *TAL1* transcription is increased in T lymphocytes through genomic translocation into the *TCRB/A/D* locus, through genomic deletions bringing the *STIL* enhancer close to *TAL1* or through insertions that create a novel *MYB* binding enhancer upstream of *TAL1*.

E protein is implied by studies that showed that TAL1-driven leukemia is not dependent on the DNA binding ability of TAL1 (18). Moreover, transgenic expression of ID1 or ID2, which prevent E proteins from binding to DNA, also predisposes mice to develop a T-ALL- like disease (19, 71). It is also notable that Lck-TAL1 promotes leukemia in a dose-dependent manner that is augmented by deletion of the E proteins encoded by *Heb/Tcf12* indicating that E protein dose is a major determinant of leukemogenesis in this model (18). Analogous to what is observed in Lck-TAL1 transgenic mice, ectopic expression of LYL1 in T cell progenitors blocks the formation of E-protein homodimers, suppresses the expression of E2A-dependent genes, and leads to T-ALL like disease (69, 72). These findings suggest that at least a part of the mechanism through which TAL1 and LYL1 promote leukemogenesis is through inhibition of E protein homodimer function. However, $LYL1^+$ and $TAL1^+$ leukemias have unique gene expression profiles and $LYL1^+$ leukemias tend to be related to immature CD4-CD8- T cell progenitors (2). These observations suggest that LYL1 and TAL1 have unique functions or that they are expressed in different cellular contexts, either distinct stages of development or stages of transformation. Interestingly, nearly 30% of pediatric $TAL1^+$ T-ALL patients have heterozygous loss-of-function mutations in *USP7*, a deubiquitinating enzyme that interacts with E proteins, and other leukemia-associated proteins, and is associated with decreased E protein target gene expression and increased cell growth (73). Therefore, there appears to be multiple mechanisms contributing to reduced E protein function in T-ALL.

While inhibition of E protein function is sufficient to predispose T cell progenitors to transformation, TAL1 and LYL1 may contribute to transformation through their participation in transcriptional complexes that activate or inhibit gene expression. In hematopoietic progenitors and T-ALL, both TAL1 and LYL1 bind DNA in large complexes that include the LMO (LIM only), LIM domain binding (LDB1), and GATA protein families (74–78). Indeed, LMO proteins are critical members of the TAL1 complex, acting as bridging factors that connect TAL1 to other DNA binding proteins like GATA1/2/3 (79). This is of particular interest because LMO proteins are overexpressed in approximately 10% of T-ALLs and these leukemias frequently have *TAL1* overexpression (1). In a subset of pediatric (3.7%) and adult (5.5%) T-ALL the *LMO2* gene contains intronic indels that result in *de novo* binding sites for the leukemia-associated transcription factors MYB, ETS1 or RUNX1 and thus dysregulated LMO2 expression (80). Interestingly, both TAL1 and LMO1 or LMO2 are required to induce reporter activity in T-ALL cell lines (81). Experiments in human T-ALL cell lines have been vital to elucidating the core components of the TAL1 complex in leukemia. ChIP-seq in these lines has identified E2A, GATA3, LMO1/LMO2, RUNX1, and MYB as co-bound to TAL1 bound regions suggesting multi-transcription factor complex formation (82). Using siRNA to knock down TAL1 or other members of the TAL1 complex, Sanda et al. identified genes regulated by this complex in leukemias (83). Interestingly, expression of the genes that make up the TAL1 complex is decrease upon TAL1 siRNA knockdown. This finding suggests a positive feed-forward mechanism where the oncogenic TAL1 complex promotes expression of itself, in addition to promoting expression of other known oncogenes. TAL1-dependent genes include *MYB*, which positively regulates cell cycle and anti-apoptotic genes, *TRIB2*, which supports the survival of T-ALL cell lines, and *ARID5*, a gene associated with a variety of leukemias (83–85). Of note, the oncogenic microRNA miR-223 is also decreased after reducing TAL1 and ChIP-seq revealed that the TAL1 complex binds to a putative enhancer near this gene (86).

There is evidence indicating that LYL1 forms oncogenic complexes similar to TAL1. Indeed, Jurkat cells forced to express LYL1, LMO2 and LDB1 induced robust target gene expression that was dependent on LMO2 and LDB1 (77). Further, LMO2 is frequently overexpressed in TAL1 expressing leukemias but in LMO2 transgenic mice TAL1 is dispensable for leukemogenesis (87). In contrast, deletion of *Lyl1* significantly increases leukemia latency in LMO2 transgenic mice suggesting that LYL1 supports transformation. Microarray analysis revealed that LMO2 expressing thymocytes have higher *Lyl1* expression compared to wild type thymocytes indicating a potential feed-forward mechanism reminiscent of the mechanism seen in TAL1 expressing leukemias. Consistent with this idea, the *LYL1* promoter contains ETS and GATA binding sites, which promote the expression of *LYL1* in HSCs and both ETS1 and GATA3 are implicated in T-ALL (88–90). Taken together, these data suggest that LYL1 may function in a manner analogous to TAL1 during T cell progenitor transformation.

NOTCH1

NOTCH1 is constitutively activated in a majority of T-ALL, including those that overexpress *TAL1*, and in leukemias from $E2a^{-/-}$ mice (20, 91). NOTCH1 functions as both a surface receptor and transcription factor that is essential for T cell development (8, 92). The ligands for NOTCH1 are members of the Jagged and Delta-like family, with DELTA-LIKE 4 (DLL4) being the most important in the thymus (93). NOTCH1 is translated as a single protein that is cleaved in the Golgi to create extracellular and intracellular components that are held together in the membrane by heterodimerization domains (HD) (8). Upon ligand binding the extracellular portion undergoes a conformational change that allows cleavage by a disintegrin and metalloprotease, which exposes a cleavage site for γ-secretase, which then cleaves and liberates the intracellular domain of NOTCH1 (called ICN). The ICN translocates to the nucleus, where it complexes with the DNA bound transcriptional repressor CBF1/RBP-Jκ and recruits Mastermind (MAML) proteins to initiate the transcription of multiple genes that promote T cell specification (8). Activation of NOTCH1 is transient owing to the presence of a PEST sequence at the 3' end that is recognized by the FBW7 ubiquitin ligase and targets ICN for proteasomal degradation (94). Targets of the ICN/CBF1/MAML complex in murine T cells include *Hes1* and *Tcf7*, both of all of which play critical roles in T cell development (56, 95, 96).

Interestingly, Notch signaling also shuts down transcription of *Ccr9*, which encodes a chemokine receptor involved in thymic homing of LMPPs (97, 98). Repression of *Ccr9* may trap lymphoid progenitors in the cortex of the thymus as they undergo commitment to the T cell lineage. Repression of *Ccr9* may also explain why ectopic expression of ICN in murine HSCs promotes T cell transformation without accumulation of these leukemic cells in the thymus (99).

NOTCH1 was identified as an oncogene in T-ALL by its involvement in a t(7;9)(q34;q34.3) translocation that placed the 3' end of the *NOTCH1* gene under control of the *TCRB* locus, resulting in constitutive activation of NOTCH1 in T cell progenitors (100). This translocation is present in approximately 2% of leukemias, however, it is now appreciated that > 60% of all human T-ALLs have mutations in *NOTCH1* (8). These mutations cluster in the heterodimerization domain (HD) and in the PEST domain. Mutations affecting the HD domains prevent the association of the extracellular and intracellular portions of NOTCH1 thus allowing for spontaneous γ-secretase mediated cleavage to produce active ICN. The PEST domain mutations promote stabilization of ICN by removing the phosphorylation sites that lead to docking of FBW7. These mutations are not mutually exclusive, with ~20% of human leukemias having mutations in both domains (101). Additional mutations have been identified that inactivate FBW7 resulting in the constitutive stabilization of ICN (8, 102, 103). The TAL1 complex also represses *FBWX7* through miRNA-223 suggesting that there may be numerous mechanisms contributing to stabilization of NOTCH1 in T-ALL (86).

Leukemias arising in *E2a*$^{-/-}$ mice have mutations in the PEST domain of NOTCH1 but no mutations have been identified in the HD domain (20). How then is NOTCH1 activated in these leukemias? Insight into this question came when it was revealed that alternative transcription initiation sites are used at the *Notch1* gene in *Ikzf1*$^{-/-}$ and *E2a*$^{-/-}$ leukemias (104, 105). Multiple transcripts were identified that initiate from a cryptic promoter upstream of exon 26 leading to a protein that lacks the extracellular domain of NOTCH1. This cyptic promoter can be activated by deletion of the promoter upstream of exon 1, which occurs through a RAG1-dependent mechanism (106). Surprisingly, in *Ikzf1*$^{-/-}$ mice, deletion of the first exon of *Notch1* did not impact T cell development, unlike what is seen in *Ikzf1*$^{+/+}$ mice, due to use of this alternative mechanism for transcribing *Notch1* in the absence of IKAROS. *Ikzf1*$^{-/-}$ thymocytes have increased histone acetylation near IKAROS binding sites located near the alternative *Notch1* promoter raising the possibility that IKAROS represses the use of this alternative mechanism through epigenetic modification (104, 105). Potential E2A binding sites are also present within the alternate promoter and these alternative NOTCH1 isoforms are expressed in *E2a*$^{-/-}$ leukemias indicating that E proteins may cooperate with IKAROS to repress alternative promoter use (104). While E2A may repress the alternative promoter, E2A promotes *Notch1* expression in thymic seeding progenitors (42). This deficiency in NOTCH1 could provide a strong selective pressure for NOTCH1 mutation or altered transcription initiation site used to support T cell development. Genomic deletions have also been found in mouse leukemic cells that result in splicing of Exon 1 to downstream exons and again result in proteins that lack the extracellular domain and are constitutively active but dependent on γ-secretase (8, 106).

The mechanisms by which NOTCH1 promotes leukemogenesis have been studied intensively. Interestingly, Notch signaling can impact expression of E2A, at least in mice, where it has been shown that mitogen activated protein kinase phosphorylation of E2A leads to NOTCH1-dependent ubiquitination and proteasomal degradation of E2A (107, 108). Whether this mechanism contributes to ICN induced leukemogenesis in mice or humans requires further investigation. In human T-ALL, a major target of Notch signaling is *c*-MYC, which itself is oncogenic in T lymphocyte progenitors (8, 109). ICN binds to an enhancer 140 Mb downstream of c-*MYC*, whose activity correlates with responsiveness to NOTCH1 inhibitors (110). Moreover, mutation of this enhancer prevents leukemogenesis by ectopic expression of ICN demonstrating that it is an essential target. This enhancer is also regulated by NOTCH3 in NOTCH3-dependent leukemias (111). It is likely that there are many essential targets of NOTCH1 in T-ALL. Indeed, in *E2a*$^{-/-}$ leukemias c-MYC expression is stably amplified through trisomy at chromosome 15 and therefore does not require ICN for expression yet these leukemias are still dependent on Notch1 signaling (16, 52).

ChIP-seq analysis for ICN has revealed multiple novel targets of NOTCH1 in leukemia (112). ICN bound regions are in close proximity to RUNX, ETS, and ZNF143 binding motifs and these regions have extensive histone acetylation and H3K4me3 chromatin modifications, indicative of open chromatin and active gene transcription (60, 112). Thus, it is possible that NOTCH1 promotes accessibility to target gene regulatory regions, which allows other T cell specific transcription factors or DNA transcriptional machinery to bind and promote gene expression. Consistent with this idea, NOTCH1 was required for recruitment of RUNX1 and MYB to enhancers located within the *TCRG* and *TCRB* locus (113).

Many ICN target genes, including *DTX1*, *IGF1R*, *IL7R*, and *GIMAP*, have been identified by evaluating changes in gene expression after treatment of leukemias with γ-secretase inhibitors (60, 109). Importantly, many of these genes are co-regulated by T cell specific factors like RUNX and ETS1. Deletion of RUNX1 in DN2/3 thymocytes impairs IL7R expression (114), and expression of dominant-negative RUNX1 and NOTCH1 inhibitors (RUNT and DN-MAML, respectively) suppressed *IL7R* mRNA expression (60). Further, ETS1 binds to multiple NOTCH1 occupied sites in T-ALL (89). Indeed, mice overexpressing NOTCH1 fail to develop leukemia when lacking functional ETS1 suggesting that both of these factors are required for leukemia initiation. ETS1 is frequently over expressed in human T-ALL samples and cell lines indicating that ETS1 may act in concert with NOTCH1 in the human disease as well. Indeed, shRNA–mediated knockdown of ETS1 in human T-ALL lines

promoted cell death and significantly down-regulated expression of the oncogenes c-*MYC* and *IGFR1*, as well as other NOTCH1 target genes like *HES1* and *DELTEX1* (89). Understanding of the spectrum of genes induced by NOTCH1 and identifying co-regulators may reveal mechanisms that could be targeted for treatment of T-ALL.

GATA3

GATA3 is essential for T cell specification and in its absence multipotent progenitors fail to generate committed T lymphocytes (115–117). Ectopic expression of GATA3 can also derail T cell development and force T cell progenitors down alternative lineages, such as the mast cell lineage (118). However, transgenic expression of GATA3, under the control of the CD2 promoter, which drives expression in all lymphocytes, predisposes mice to develop T-ALL-like disease with trisomy of chromosome 15 and activation of NOTCH1, similar to what is seen in *E2a*$^{-/-}$ leukemias, although with longer latency (47, 119). GATA3 is elevated in *E2a*$^{-/-}$ T cell progenitors and has a negative impact on the ability of DN2 cells to generate T lineage-restricted cells (43). These findings support a role for GATA3 in T cell leukemogenesis and implicate it as a potential contributing factor to T cell transformation in *E2a*$^{-/-}$ mice even though this has not been formally demonstrated. Indeed, in non-ETP-ALL GATA3 expression is elevated compared to T cells from healthy donors and defines a stem-like progenitor (120). The mechanism by which GATA3 promotes thymocyte transformation and leukemia survival is not well understood. One potential mechanism involves GATA3's association with TAL1 as a member of the oncogenic TAL1 complex. Indeed, siRNA knockdown of GATA3 in T-ALL cells represses transcription of TAL1 target genes suggesting that GATA3 is required for proper TAL1 complex function (83). GATA3 and other members of the TAL1 oncogenic complex also bind to the NOTCH1-regulated enhancer downstream of c-*Myc* (90, 110). Mutating the GATA3 binding sites in this enhancer impacted nucleosome eviction and chromatin accessibility, resulting in decreased c-MYC expression and abrogated leukemia development in mice (90). These observations indicate that GATA3 cooperates with TAL1 and NOTCH1 to promote transformation through regulation of c-*MYC*.

In contrast to these cases of increased GATA3 expression, 5% of T-ALL patients have silencing mutations in the *GATA3* gene (1). Consistent with this, another study found that 33% of patients in their cohort with the ETP-ALL subtype had reduced GATA3 expression associated with increased methylation throughout the *GATA3* gene (120). Thus, GATA3 may play multiple distinct roles in T-ALL development, suppressing ETP-ALL or promoting T-ALL at later stages. Decreased GATA3 expression in ETP-ALL is consistent with GATA3's function in promoting T cell lineage differentiation as *GATA3* silencing could contribute to a developmental block at the ETP stage that supports transformation. It also seems likely that GATA3 is not a driver mutation and its function may be dependent on the spectrum of additional mutations that occur during transformation.

LEF1/TCF1

The NOTCH1 target gene *Tcf7*, encoding the protein TCF1, is also implicated as a suppressor of T cell transformation (57, 121). TCF1 is a member of the HMG box family of proteins along with the closely related protein LEF1. Both TCF1 and LEF1 can promote transcription in response to canonical WNT signaling activation or repress transcription through recruitment of the Groucho related co-repressors such as TLE3 (122, 123). In the absence of TCF1, thymocytes have a developmental block at the ETP, DN2, and ISP stages whereas mice lacking LEF1 have no obvious defects in DN thymocytes (54–56, 124). Combined deletion of *Tcf7* and *Lef1* exacerbates the phenotype seen in *Tcf7*-deficient mice, leading to a nearly complete block in T cell development (54). This observation indicates that TCF1 and LEF1 have overlapping functions and that LEF1 partially compensates for the loss of TCF1. In addition to the defects seen in T lymphopoiesis, approximately 50% of *Tcf7*$^{-/-}$ mice develop T-ALL (57, 121). *Tcf7*$^{-/-}$ leukemias are heterogeneous; phenotypically resembling DN3, DN4, and DP thymocytes. Despite this cell surface phenotype, RNA profiling revealed that the transcriptome of *Tcf7*$^{-/-}$ T-ALLs is related to that of human ETP-ALLs, which is consistent with the early requirement for TCF1 in T cell development (57). *Tcf7*$^{-/-}$ leukemias have activated NOTCH signaling and inhibiting this pathway with GSI at least partially impacts their viability (121). Further, *Tcf7*$^{-/-}$ leukemias highly express ID2 and LEF1, particularly in a subset of T cell progenitors with a gene signature predictive of high leukemic potential, suggesting that that suppression of E protein activity may be a feature of transformation in this model (57, 58). Indeed, *Tcf7*$^{-/-}$*Id2*$^{-/-}$ mice showed an increased latency of leukemogenesis consistent with this hypothesis.

Like *Tcf7*$^{-/-}$ leukemias, *E2a*$^{-/-}$ leukemias have high expression of *Lef1* and LEF1 is required for the survival and proliferation of these leukemias (52). LEF1 is an oncogene in acute myeloid leukemia and in multiple forms of B lymphocyte leukemia and it is suppressed by TCF1 (125–128). Ectopic expression of LEF1 in HSCs induced acute myeloid leukemia-like or B cell ALL-like disease in mice, demonstrating LEF1's oncogenic potential (127). In an adult cohort of T-ALL patients, high LEF1 expression was associated with increased expression of the oncogenes encoding c-MYC and CYCLIN D1 suggesting that LEF1 is positively associated with T cell leukemia (129). Moreover, 4 unique mutations that augment LEF1 function were found in these patients. In contrast, approximately 11% of pediatric T-ALL patients were found to have inactivating mutations in the *LEF1* gene (7, 130). These mutations consist of deletions or truncation mutations, both resulting in lower LEF1 function. These conflicting findings suggest that LEF1 can play multiple roles in T cell leukemia. Indeed, while *E2a*$^{-/-}$ leukemias are dependent on LEF1, inactivation of *Lef1* in *E2a*$^{-/-}$ mice prior to transformation did not prevent transformation; rather, it reduced leukemia latency and resulted in leukemias with a

unique gene expression program compared to *E2a*$^{-/-}$ leukemias (53). Taken together, these experiments reveal that the timing of genetic alterations in the evolution of T-ALL can determine latency, phenotype and genetic susceptibilities within these cells.

CONCLUSIONS

T-ALL is a heterogeneous disease that is associated with mutations in numerous T cell specific transcription factors, epigenetic regulators, and signaling pathways. Despite this heterogeneity, many of the mutations seen in patients impact the function of E proteins and their target genes. Recent studies looking at the clonal evolution of T-ALL have further indicated that TAL1 upregulation is a founding event in the human disease, preceding mutations in NOTCH1 (131). Thus, understanding how reduced E protein function impacts T cell development and leukemogenesis is highly relevant to the human disease. The *E2a*$^{-/-}$ mouse model has revealed connections between T cell developmental alterations and the transformation process. For example, *Notch1*, *Gata3*, and *Lef1/Tcf7* expression are altered early in T cell development in *E2a*$^{-/-}$ mice and contribute to transformation. The pathways regulated by these proteins are promising candidates for therapeutic intervention in T-ALL. Indeed, inhibitors of NOTCH1 activation or function have been in clinical trials, however many of these inhibitors have adverse side effects due to the many cell types that rely on Notch signaling (11, 132). Novel NOTCH1 inhibitors that prevent the formation of the ICN transcriptional activation complex show decreased off target effects *in vivo* compared to inhibitors that target NOTCH1 activation, but still induce mild intestinal toxicity (133). Further investigation of oncogenic and tumor suppressive pathways in murine T-ALL models, including GATA3 and TCF1/LEF1, and their application to human leukemia may identify novel targets that alone, or in combination with other targets, will have fewer side effects without sacrificing anti-leukemia efficacy.

AUTHOR CONTRIBUTIONS

GP and BK wrote the manuscript and approved the final version. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Belver L, Ferrando A. The Genetics and Mechanisms of T Cell Acute Lymphoblastic Leukaemia. *Nat Rev Cancer* (2016) 16(8):494–507. doi: 10.1038/nrc.2016.63
2. Ferrando AA, Look AT. Gene Expression Profiling in T-Cell Acute Lymphoblastic Leukemia. *Semin Hematol* (2003) 40(4):274–80. doi: 10.1016/s0037-1963(03)00195-1
3. Noronha EP, Marques LVC, Andrade FG, Thuler LCS, Terra-Granado E, Pombo-de-Oliveira MS, et al. The Profile of Immunophenotype and Genotype Aberrations in Subsets of Pediatric T-Cell Acute Lymphoblastic Leukemia. *Front Oncol* (2019) 9:316. doi: 10.3389/fonc.2019.00316
4. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-Cell Precursor Leukaemia: A Subtype of Very High-Risk Acute Lymphoblastic Leukaemia. *Lancet Oncol* (2009) 10(2):147–56. doi: 10.1016/S1470-2045(08)70314-0
5. Van Vlierberghe P, Ferrando A. The Molecular Basis of T Cell Acute Lymphoblastic Leukemia. *J Clin Invest* (2012) 122(10):3398–406. doi: 10.1172/JCI61269
6. Iacobucci I, Kimura S, Mullighan CG. Biologic and Therapeutic Implications of Genomic Alterations in Acute Lymphoblastic Leukemia. *J Clin Med* (2021) 10(17):3792–816. doi: 10.3390/jcm10173792
7. Girardi T, Vicente C, Cools J, De Keersmaecker K. The Genetics and Molecular Biology of T-All. *Blood* (2017) 129(9):1113–23. doi: 10.1182/blood-2016-10-706465
8. Aster JC, Blacklow SC, Pear WS. Notch Signalling in T-Cell Lymphoblastic Leukaemia/Lymphoma and Other Haematological Malignancies. *J Pathol* (2011) 223(2):262–73. doi: 10.1002/path.2789
9. Sanda T, Leong WZ. Tal1 as a Master Oncogenic Transcription Factor in T-Cell Acute Lymphoblastic Leukemia. *Exp Hematol* (2017) 53:7–15. doi: 10.1016/j.exphem.2017.06.001
10. Ferreira A, Aster JC. Notch Signaling in Cancer: Complexity and Challenges on the Path to Clinical Translation. *Semin Cancer Biol* (2021) S1044-579X(21)00109-7. doi: 10.1016/j.semcancer.2021.04.008
11. Zheng R, Li M, Wang S, Liu Y. Advances of Target Therapy on Notch1 Signaling Pathway in T-Cell Acute Lymphoblastic Leukemia. *Exp Hematol Oncol* (2020) 9(1):31. doi: 10.1186/s40164-020-00187-x
12. Massari ME, Murre C. Helix-Loop-Helix Proteins: Regulators of Transcription in Eucaryotic Organisms. *Mol Cell Biol* (2000) 20(2):429–40. doi: 10.1128/MCB.20.2.429-440.2000
13. Kee BL. E and Id Proteins Branch Out. *Nat Rev Immunol* (2009) 9(3):175–84. doi: 10.1038/nri2507
14. Hsu HL, Cheng JT, Chen Q, Baer R. Enhancer-Binding Activity of the Tal-1 Oncoprotein in Association With the E47/E12 Helix-Loop-Helix Proteins. *Mol Cell Biol* (1991) 11(6):3037–42. doi: 10.1128/mcb.11.6.3037-3042.1991
15. Miyamoto A, Cui X, Naumovski L, Cleary ML. Helix-Loop-Helix Proteins Lyl1 and E2a Form Heterodimeric Complexes With Distinctive DNA-Binding Properties in Hematolymphoid Cells. *Mol Cell Biol* (1996) 16(5):2394–401. doi: 10.1128/MCB.16.5.2394
16. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2a Deficiency Leads to Abnormalities in Alphabeta T-Cell Development and to Rapid Development of T-Cell Lymphomas. *Mol Cell Biol* (1997) 17(8):4782–91. doi: 10.1128/mcb.17.8.4782
17. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High Incidence of T-Cell Tumors in E2a-Null Mice and E2a/Id1 Double-Knockout Mice. *Mol Cell Biol* (1997) 17(12):7317–27. doi: 10.1128/mcb.17.12.7317
18. O'Neil J, Shank J, Cusson N, Murre C, Kelliher M. Tal1/Scl Induces Leukemia by Inhibiting the Transcriptional Activity of E47/Heb. *Cancer Cell* (2004) 5(6):587–96. doi: 10.1016/j.ccr.2004.05.023

19. Kim D, Peng XC, Sun XH. Massive Apoptosis of Thymocytes in T-Cell-Deficient Id1 Transgenic Mice. *Mol Cell Biol* (1999) 19(12):8240–53. doi: 10.1128/mcb.19.12.8240
20. Reschly EJ, Spaulding C, Vilimas T, Graham WV, Brumbaugh RL, Aifantis I, et al. Notch1 Promotes Survival of E2a-Deficient T Cell Lymphomas Through Pre-T Cell Receptor-Dependent and -Independent Mechanisms. *Blood* (2006) 107(10):4115–21. doi: 10.1182/blood-2005-09-3551
21. Tatarek J, Cullion K, Ashworth T, Gerstein R, Aster JC, Kelliher MA. Notch1 Inhibition Targets the Leukemia-Initiating Cells in a Tal1/Lmo2 Mouse Model of T-All. *Blood* (2011) 118(6):1579–90. doi: 10.1182/blood-2010-08-300343
22. Sharma VM, Draheim KM, Kelliher MA. The Notch1/C-Myc Pathway in T Cell Leukemia. *Cell Cycle* (2007) 6(8):927–30. doi: 10.4161/cc.6.8.4134
23. Murre C, McCaw PS, Baltimore D. A New DNA Binding and Dimerization Motif in Immunoglobulin Enhancer Binding, Daughterless, Myod, and Myc Proteins. *Cell* (1989) 56(5):777–83. doi: 10.1016/0092-8674(89)90682-x
24. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, et al. Interactions Between Heterologous Helix-Loop-Helix Proteins Generate Complexes That Bind Specifically to a Common DNA Sequence. *Cell* (1989) 58(3):537–44. doi: 10.1016/0092-8674(89)90434-0
25. Braunstein M, Anderson MK. Heb in the Spotlight: Transcriptional Regulation of T-Cell Specification, Commitment, and Developmental Plasticity. *Clin Dev Immunol* (2012) 2012:678705. doi: 10.1155/2012/678705
26. Zhuang Y, Soriano P, Weintraub H. The Helix-Loop-Helix Gene E2a Is Required for B Cell Formation. *Cell* (1994) 79(5):875–84. doi: 10.1016/0092-8674(94)90076-0
27. Bain G, Maandag EC, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, et al. E2a Proteins Are Required for Proper B Cell Development and Initiation of Immunoglobulin Gene Rearrangements. *Cell* (1994) 79(5):885–92. doi: 10.1016/0092-8674(94)90077-9
28. Barndt RJ, Dai M, Zhuang Y. Functions of E2a-Heb Heterodimers in T-Cell Development Revealed by a Dominant Negative Mutation of Heb. *Mol Cell Biol* (2000) 20(18):6677–85. doi: 10.1128/MCB.20.18.6677-6685.2000
29. Wikstrom I, Forssell J, Penha-Goncalves MN, Bergqvist I, Holmberg D. A Role for E2-2 at the Dn3 Stage of Early Thymopoiesis. *Mol Immunol* (2008) 45(11):3302–11. doi: 10.1016/j.molimm.2008.02.012
30. Bergqvist I, Eriksson M, Saarikettu J, Eriksson B, Corneliussen B, Grundstrom T, et al. The Basic Helix-Loop-Helix Transcription Factor E2-2 Is Involved in T Lymphocyte Development. *Eur J Immunol* (2000) 30(10):2857–63. doi: 10.1002/1521-4141(200010)30:10<2857::AID-IMMU2857>3.0.CO;2-G
31. Schlaeger TM, Mikkola HK, Gekas C, Helgadottir HB, Orkin SH. Tie2cre-Mediated Gene Ablation Defines the Stem-Cell Leukemia Gene (Scl/Tal1)-Dependent Window During Hematopoietic Stem-Cell Development. *Blood* (2005) 105(10):3871–4. doi: 10.1182/blood-2004-11-4467
32. Mikkola HK, Klintman J, Yang H, Hock H, Schlaeger TM, Fujiwara Y, et al. Haematopoietic Stem Cells Retain Long-Term Repopulating Activity and Multipotency in the Absence of Stem-Cell Leukaemia Scl/Tal-1 Gene. *Nature* (2003) 421(6922):547–51. doi: 10.1038/nature01345
33. Gekas C, Rhodes KE, Gereige LM, Helgadottir H, Ferrari R, Kurdistani SK, et al. Mef2c Is a Lineage-Restricted Target of Scl/Tal1 and Regulates Megakaryopoiesis and B-Cell Homeostasis. *Blood* (2009) 113(15):3461–71. doi: 10.1182/blood-2008-07-167577
34. Chagraoui H, Kassouf M, Banerjee S, Goardon N, Clark K, Atzberger A, et al. Scl-Mediated Regulation of the Cell-Cycle Regulator P21 Is Critical for Murine Megakaryopoiesis. *Blood* (2011) 118(3):723–35. doi: 10.1182/blood-2011-01-328765
35. Souroullas GP, Salmon JM, Sablitzky F, Curtis DJ, Goodell MA. Adult Hematopoietic Stem and Progenitor Cells Require Either Lyl1 or Scl for Survival. *Cell Stem Cell* (2009) 4(2):180–6. doi: 10.1016/j.stem.2009.01.001
36. Tijssen MR, Cvejic A, Joshi A, Hannah RL, Ferreira R, Forrai A, et al. Genome-Wide Analysis of Simultaneous Gata1/2, Runx1, Fli1, and Scl Binding in Megakaryocytes Identifies Hematopoietic Regulators. *Dev Cell* (2011) 20(5):597–609. doi: 10.1016/j.devcel.2011.04.008
37. Dias S, Mansson R, Gurbuxani S, Sigvardsson M, Kee BL. E2a Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* (2008) 29(2):217–27. doi: 10.1016/j.immuni.2008.05.015
38. Yang Q, Kardava L, St Leger A, Martincic K, Varnum-Finney B, Bernstein ID, et al. E47 Controls the Developmental Integrity and Cell Cycle Quiescence of Multipotential Hematopoietic Progenitors. *J Immunol* (2008) 181(9):5885–94. doi: 10.4049/jimmunol.181.9.5885
39. Zohren F, Souroullas GP, Luo M, Gerdemann U, Imperato MR, Wilson NK, et al. The Transcription Factor Lyl-1 Regulates Lymphoid Specification and the Maintenance of Early T Lineage Progenitors. *Nat Immunol* (2012) 13(8):761–9. doi: 10.1038/ni.2365
40. de Pooter RF, Dias S, Chowdhury M, Bartom ET, Okoreeh MK, Sigvardsson M, et al. Cutting Edge: Lymphomyeloid-Primed Progenitor Cell Fates Are Controlled by the Transcription Factor Tal1. *J Immunol* (2019) 202(10):2837–42. doi: 10.4049/jimmunol.1801220
41. Pereira de Sousa A, Berthault C, Granato A, Dias S, Ramond C, Kee BL, et al. Inhibitors of DNA Binding Proteins Restrict T Cell Potential by Repressing Notch1 Expression in Flt3-Negative Common Lymphoid Progenitors. *J Immunol* (2012) 189(8):3822–30. doi: 10.4049/jimmunol.1103723
42. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E Proteins and Notch Signaling Cooperate to Promote T Cell Lineage Specification and Commitment. *J Exp Med* (2006) 203(5):1329–42. doi: 10.1084/jem.20060268
43. Xu W, Carr T, Ramirez K, McGregor S, Sigvardsson M, Kee BL. E2a Transcription Factors Limit Expression of Gata3 to Facilitate T Lymphocyte Lineage Commitment. *Blood* (2013) 121(9):1534–42. doi: 10.1182/blood-2012-08-449447
44. Miyazaki M, Miyazaki K, Chen K, Jin Y, Turner J, Moore AJ, et al. The E-Id Protein Axis Specifies Adaptive Lymphoid Cell Identity and Suppresses Thymic Innate Lymphoid Cell Development. *Immunity* (2017) 46(5):818–34 e4. doi: 10.1016/j.immuni.2017.04.022
45. Wang HC, Qian L, Zhao Y, Mengarelli J, Adrianto I, Montgomery CG, et al. Downregulation of E Protein Activity Augments an Ilc2 Differentiation Program in the Thymus. *J Immunol* (2017) 198(8):3149–56. doi: 10.4049/jimmunol.1602009
46. Qian L, Bajana S, Georgescu C, Peng V, Wang HC, Adrianto I, et al. Suppression of Ilc2 Differentiation From Committed T Cell Precursors by E Protein Transcription Factors. *J Exp Med* (2019) 216(4):884–99. doi: 10.1084/jem.20182100
47. Nawijn MC, Ferreira R, Dingjan GM, Kahre O, Drabek D, Karis A, et al. Enforced Expression of Gata-3 During T Cell Development Inhibits Maturation of Cd8 Single-Positive Cells and Induces Thymic Lymphoma in Transgenic Mice. *J Immunol* (2001) 167(2):715–23. doi: 10.4049/jimmunol.167.2.715
48. Xu W, Kee BL. Growth Factor Independent 1b (Gfi1b) Is an E2a Target Gene That Modulates Gata3 in T-Cell Lymphomas. *Blood* (2007) 109(10):4406–14. doi: 10.1182/blood-2006-08-043331
49. Yucel R, Karsunky H, Klein-Hitpass L, Moroy T. The Transcriptional Repressor Gfi1 Affects Development of Early, Uncommitted C-Kit+ T Cell Progenitors and Cd4/Cd8 Lineage Decision in the Thymus. *J Exp Med* (2003) 197(7):831–44. doi: 10.1084/jem.20021417
50. Doan LL, Porter SD, Duan Z, Flubacher MM, Montoya D, Tsichlis PN, et al. Targeted Transcriptional Repression of Gfi1 by Gfi1 and Gfi1b in Lymphoid Cells. *Nucleic Acids Res* (2004) 32(8):2508–19. doi: 10.1093/nar/gkh570
51. Banerjee A, Northrup D, Boukarabila H, Jacobsen SE, Allman D. Transcriptional Repression of Gata3 Is Essential for Early B Cell Commitment. *Immunity* (2013) 38(5):930–42. doi: 10.1016/j.immuni.2013.01.014
52. Spaulding C, Reschly EJ, Zagort DE, Yashiro-Ohtani Y, Beverly LJ, Capobianco A, et al. Notch1 Co-Opts Lymphoid Enhancer Factor 1 for Survival of Murine T-Cell Lymphomas. *Blood* (2007) 110(7):2650–8. doi: 10.1182/blood-2007-04-084202
53. Carr T, McGregor S, Dias S, Verykokakis M, LeBeau MM, Xue HH, et al. Oncogenic and Tumor Suppressor Functions for Lymphoid Enhancer Factor I in a Murine Model of T Acute Lymphoblastic Leukemia. *Front Immunol* (2022) 13:845488. doi: 10.3389/fimmu.2022.845488
54. Okamura RM, Sigvardsson M, Galceran J, Verbeek S, Clevers H, Grosschedl R. Redundant Regulation of T Cell Differentiation and Tcralpha Gene Expression by the Transcription Factors Lef-1 and Tcf-1. *Immunity* (1998) 8(1):11–20. doi: 10.1016/s1074-7613(00)80454-9
55. Weber BN, Chi AW, Chavez A, Yashiro-Ohtani Y, Yang Q, Shestova O, et al. A Critical Role for Tcf-1 in T-Lineage Specification and Differentiation. *Nature* (2011) 476(7358):63–8. doi: 10.1038/nature10279
56. Germar K, Dose M, Konstantinou T, Zhang J, Wang H, Lobry C, et al. T-Cell Factor 1 Is a Gatekeeper for T-Cell Specification in Response to Notch

Signaling. *Proc Natl Acad Sci USA* (2011) 108(50):20060–5. doi: 10.1073/pnas.1110230108
57. Yu S, Zhou X, Steinke FC, Liu C, Chen SC, Zagorodna O, et al. The Tcf-1 and Lef-1 Transcription Factors Have Cooperative and Opposing Roles in T Cell Development and Malignancy. *Immunity* (2012) 37(5):813–26. doi: 10.1016/j.immuni.2012.08.009
58. Wang F, Qi Z, Yao Y, Yu G, Feng T, Zhao T, et al. Exploring the Stage-Specific Roles of Tcf-1 in T Cell Development and Malignancy at Single-Cell Resolution. *Cell Mol Immunol* (2021) 18(3):644–59. doi: 10.1038/s41423-020-00527-1
59. Geimer Le Lay AS, Oravecz A, Mastio J, Jung C, Marchal P, Ebel C, et al. The Tumor Suppressor Ikaros Shapes the Repertoire of Notch Target Genes in T Cells. *Sci Signal* (2014) 7(317):ra28. doi: 10.1126/scisignal.2004545
60. Wang H, Zang C, Taing L, Arnett KL, Wong YJ, Pear WS, et al. Notch1-Rbpj Complexes Drive Target Gene Expression Through Dynamic Interactions With Superenhancers. *Proc Natl Acad Sci USA* (2014) 111(2):705–10. doi: 10.1073/pnas.1315023111
61. Fitzgerald TJ, Neale GA, Raimondi SC, Goorha RM. C-Tal, a Helix-Loop-Helix Protein, Is Juxtaposed to the T-Cell Receptor-Beta Chain Gene by a Reciprocal Chromosomal Translocation: T(1;7)(P32;Q35). *Blood* (1991) 78 (10):2686–95. doi: 10.1182/blood.V78.10.2686.2686
62. Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanan G, et al. Site-Specific Recombination of the Tal-1 Gene Is a Common Occurrence in Human T Cell Leukemia. *EMBO J* (1990) 9(10):3343–51. doi: 10.1002/j.1460-2075.1990.tb07535.x
63. Begley CG, Aplan PD, Davey MP, Nakahara K, Tchorz K, Kurtzberg J, et al. Chromosomal Translocation in a Human Leukemic Stem-Cell Line Disrupts the T-Cell Antigen Receptor Delta-Chain Diversity Region and Results in a Previously Unreported Fusion Transcript. *Proc Natl Acad Sci USA* (1989) 86 (6):2031–5. doi: 10.1073/pnas.86.6.2031
64. Mellentin JD, Smith SD, Cleary ML. Lyl-1, a Novel Gene Altered by Chromosomal Translocation in T Cell Leukemia, Codes for a Protein With a Helix-Loop-Helix DNA Binding Motif. *Cell* (1989) 58(1):77–83. doi: 10.1016/0092-8674(89)90404-2
65. Breit TM, Mol EJ, Wolvers-Tettero IL, Ludwig WD, van Wering ER, van Dongen JJ. Site-Specific Deletions Involving the Tal-1 and Sil Genes Are Restricted to Cells of the T Cell Receptor Alpha/Beta Lineage: T Cell Receptor Delta Gene Deletion Mechanism Affects Multiple Genes. *J Exp Med* (1993) 177(4):965–77. doi: 10.1084/jem.177.4.965
66. Aplan PD, Lombardi DP, Ginsberg AM, Cossman J, Bertness VL, Kirsch IR. Disruption of the Human Scl Locus by "Illegitimate" V-(D)-J Recombinase Activity. *Science* (1990) 250(4986):1426–9. doi: 10.1126/science.2255914
67. Mansour MR, Abraham BJ, Anders L, Berezovskaya A, Gutierrez A, Durbin AD, et al. Oncogene Regulation. An Oncogenic Super-Enhancer Formed Through Somatic Mutation of a Noncoding Intergenic Element. *Science* (2014) 346(6215):1373–7. doi: 10.1126/science.1259037
68. Liu X, Xu Y, Han L, Yi Y. Reassessing the Potential of Myb-Targeted Anti-Cancer Therapy. *J Cancer* (2018) 9(7):1259–66. doi: 10.7150/jca.23992
69. Zhong Y, Jiang L, Hiai H, Toyokuni S, Yamada Y. Overexpression of a Transcription Factor Lyl1 Induces T- and B-Cell Lymphoma in Mice. *Oncogene* (2007) 26(48):6937–47. doi: 10.1038/sj.onc.1210494
70. Kelliher MA, Seldin DC, Leder P. Tal-1 Induces T Cell Acute Lymphoblastic Leukemia Accelerated by Casein Kinase Iialpha. *EMBO J* (1996) 15 (19):5160–6. doi: 10.1002/j.1460-2075.1996.tb00900.x
71. Morrow MA, Mayer EW, Perez CA, Adlam M, Siu G. Overexpression of the Helix-Loop-Helix Protein Id2 Blocks T Cell Development at Multiple Stages. *Mol Immunol* (1999) 36(8):491–503. doi: 10.1016/s0161-5890(99)00071-1
72. Lukov GL, Rossi L, Souroullas GP, Mao R, Goodell MA. The Expansion of T-Cells and Hematopoietic Progenitors as a Result of Overexpression of the Lymphoblastic Leukemia Gene, Lyl1 Can Support Leukemia Formation. *Leuk Res* (2011) 35(3):405–12. doi: 10.1016/j.leukres.2010.07.023
73. Shaw TI, Dong L, Tian L, Qian C, Liu Y, Ju B, et al. Integrative Network Analysis Reveals Usp7 Haploinsufficiency Inhibits E-Protein Activity in Pediatric T-Lineage Acute Lymphoblastic Leukemia (T-All). *Sci Rep* (2021) 11(1):5154. doi: 10.1038/s41598-021-84647-2
74. Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A, et al. The Lim-Only Protein Lmo2 Is a Bridging Molecule Assembling an Erythroid, DNA-Binding Complex Which Includes the Tal1, E47, Gata-1 and Ldb1/Nli Proteins. *EMBO J* (1997) 16(11):3145–57. doi: 10.1093/emboj/16.11.3145
75. Schlaeger TM, Schuh A, Flitter S, Fisher A, Mikkola H, Orkin SH, et al. Decoding Hematopoietic Specificity in the Helix-Loop-Helix Domain of the Transcription Factor Scl/Tal-1. *Mol Cell Biol* (2004) 24(17):7491–502. doi: 10.1128/MCB.24.17.7491-7502.2004
76. Wilson NK, Miranda-Saavedra D, Kinston S, Bonadies N, Foster SD, Calero-Nieto F, et al. The Transcriptional Program Controlled by the Stem Cell Leukemia Gene Scl/Tal1 During Early Embryonic Hematopoietic Development. *Blood* (2009) 113(22):5456–65. doi: 10.1182/blood-2009-01-200048
77. Layer JH, Christy M, Placek L, Unutmaz D, Guo Y, Dave UP. Ldb1 Enforces Stability on Direct and Indirect Oncoprotein Partners in Leukemia. *Mol Cell Biol* (2020) 40(12):e00652–19. doi: 10.1128/MCB.00652-19
78. Layer JH, Alford CE, McDonald WH, Dave UP. Lmo2 Oncoprotein Stability in T-Cell Leukemia Requires Direct Ldb1 Binding. *Mol Cell Biol* (2016) 36 (3):488–506. doi: 10.1128/MCB.00901-15
79. Hoang T, Lambert JA, Martin R. Scl/Tal1 in Hematopoiesis and Cellular Reprogramming. *Curr Top Dev Biol* (2016) 118:163–204. doi: 10.1016/bs.ctdb.2016.01.004
80. Rahman S, Magnussen M, Leon TE, Farah N, Li Z, Abraham BJ, et al. Activation of the Lmo2 Oncogene Through a Somatically Acquired Neomorphic Promoter in T-Cell Acute Lymphoblastic Leukemia. *Blood* (2017) 129(24):3221–6. doi: 10.1182/blood-2016-09-742148
81. Ono Y, Fukuhara N, Yoshie O. Tal1 and Lim-Only Proteins Synergistically Induce Retinaldehyde Dehydrogenase 2 Expression in T-Cell Acute Lymphoblastic Leukemia by Acting as Cofactors for Gata3. *Mol Cell Biol* (1998) 18(12):6939–50. doi: 10.1128/MCB.18.12.6939
82. Tan TK, Zhang C, Sanda T. Oncogenic Transcriptional Program Driven by Tal1 in T-Cell Acute Lymphoblastic Leukemia. *Int J Hematol* (2019) 109 (1):5–17. doi: 10.1007/s12185-018-2518-z
83. Sanda T, Lawton LN, Barrasa MI, Fan ZP, Kohlhammer H, Gutierrez A, et al. Core Transcriptional Regulatory Circuit Controlled by the Tal1 Complex in Human T Cell Acute Lymphoblastic Leukemia. *Cancer Cell* (2012) 22 (2):209–21. doi: 10.1016/j.ccr.2012.06.007
84. Ciciro Y, Sala A. Myb Oncoproteins: Emerging Players and Potential Therapeutic Targets in Human Cancer. *Oncogenesis* (2021) 10(2):19. doi: 10.1038/s41389-021-00309-y
85. Tan SH, Yam AW, Lawton LN, Wong RW, Young RA, Look AT, et al. Trib2 Reinforces the Oncogenic Transcriptional Program Controlled by the Tal1 Complex in T-Cell Acute Lymphoblastic Leukemia. *Leukemia* (2016) 30 (4):959–62. doi: 10.1038/leu.2015.195
86. Mansour MR, Sanda T, Lawton LN, Li X, Kreslavsky T, Novina CD, et al. The Tal1 Complex Targets the Fbxw7 Tumor Suppressor by Activating Mir-223 in Human T Cell Acute Lymphoblastic Leukemia. *J Exp Med* (2013) 210 (8):1545–57. doi: 10.1084/jem.20122516
87. McCormack MP, Shields BJ, Jackson JT, Nasa C, Shi W, Slater NJ, et al. Requirement for Lyl1 in a Model of Lmo2-Driven Early T-Cell Precursor All. *Blood* (2013) 122(12):2093–103. doi: 10.1182/blood-2012-09-458570
88. Chan WY, Follows GA, Lacaud G, Pimanda JE, Landry JR, Kinston S, et al. The Paralogous Hematopoietic Regulators Lyl1 and Scl Are Coregulated by Ets and Gata Factors, But Lyl1 Cannot Rescue the Early Scl-/- Phenotype. *Blood* (2007) 109(5):1908–16. doi: 10.1182/blood-2006-05-023226
89. McCarter AC, Gatta GD, Melnick A, Kim E, Sha C, Wang Q, et al. Combinatorial Ets1-Dependent Control of Oncogenic Notch1 Enhancers in T-Cell Leukemia. *Blood Cancer Discov* (2020) 1(2):178–97. doi: 10.1158/2643-3230.bcd-20-0026
90. Belver L, Yang AY, Albero R, Herranz D, Brundu FG, Quinn SA, et al. Gata3-Controlled Nucleosome Eviction Drives Myc Enhancer Activity in T-Cell Development and Leukemia. *Cancer Discov* (2019) 9(12):1774–91. doi: 10.1158/2159-8290.CD-19-0471
91. O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH, et al. Activating Notch1 Mutations in Mouse Models of T-All. *Blood* (2006) 107(2):781–5. doi: 10.1182/blood-2005-06-2553
92. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, et al. Deficient T Cell Fate Specification in Mice With an Induced Inactivation of Notch1. *Immunity* (1999) 10(5):547–58. doi: 10.1016/s1074-7613(00)80054-0

93. Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, Pierres M, et al. Delta-Like 4 Is the Essential, Nonredundant Ligand for Notch1 During Thymic T Cell Lineage Commitment. *J Exp Med* (2008) 205(11):2515–23. doi: 10.1084/jem.20080829
94. Crusio KM, King B, Reavie LB, Aifantis I. The Ubiquitous Nature of Cancer: The Role of the Scf(Fbw7) Complex in Development and Transformation. *Oncogene* (2010) 29(35):4865–73. doi: 10.1038/onc.2010.222
95. De Obaldia ME, Bell JJ, Wang X, Harly C, Yashiro-Ohtani Y, DeLong JH, et al. T Cell Development Requires Constraint of the Myeloid Regulator C/Ebp-Alpha by the Notch Target and Transcriptional Repressor Hes1. *Nat Immunol* (2013) 14(12):1277–84. doi: 10.1038/ni.2760
96. Harly C, Kenney D, Ren G, Lai B, Raabe T, Yang Q, et al. The Transcription Factor Tcf-1 Enforces Commitment to the Innate Lymphoid Cell Lineage. *Nat Immunol* (2019) 20(9):1150–60. doi: 10.1038/s41590-019-0445-7
97. Krishnamoorthy V, Carr T, de Pooter RF, Emanuelle AO, Gounari F, Kee BL. Repression of Ccr9 Transcription in Mouse T Lymphocyte Progenitors by the Notch Signaling Pathway. *J Immunol* (2015) 194(7):3191–200. doi: 10.4049/jimmunol.1402443
98. Zlotoff DA, Sambandam A, Logan TD, Bell JJ, Schwarz BA, Bhandoola A. Ccr7 and Ccr9 Together Recruit Hematopoietic Progenitors to the Adult Thymus. *Blood* (2010) 115(10):1897–905. doi: 10.1182/blood-2009-08-237784
99. Allman D, Karnell FG, Punt JA, Bakkour S, Xu L, Myung P, et al. Separation of Notch1 Promoted Lineage Commitment and Expansion/Transformation in Developing T Cells. *J Exp Med* (2001) 194(1):99–106. doi: 10.1084/jem.194.1.99
100. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. Tan-1, the Human Homolog of the Drosophila Notch Gene, Is Broken by Chromosomal Translocations in T Lymphoblastic Neoplasms. *Cell* (1991) 66(4):649–61. doi: 10.1016/0092-8674(91)90111-b
101. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating Mutations of Notch1 in Human T Cell Acute Lymphoblastic Leukemia. *Science* (2004) 306(5694):269–71. doi: 10.1126/science.1102160
102. Thompson BJ, Buonamici S, Sulis ML, Palomero T, Vilimas T, Basso G, et al. The Scffbw7 Ubiquitin Ligase Complex as a Tumor Suppressor in T Cell Leukemia. *J Exp Med* (2007) 204(8):1825–35. doi: 10.1084/jem.20070872
103. O'Neil J, Grim J, Strack P, Rao S, Tibbitts D, Winter C, et al. Fbw7 Mutations in Leukemic Cells Mediate Notch Pathway Activation and Resistance to Gamma-Secretase Inhibitors. *J Exp Med* (2007) 204(8):1813–24. doi: 10.1084/jem.20070876
104. Gomez-del Arco P, Kashiwagi M, Jackson AF, Naito T, Zhang J, Liu F, et al. Alternative Promoter Usage at the Notch1 Locus Supports Ligand-Independent Signaling in T Cell Development and Leukemogenesis. *Immunity* (2010) 33(5):685–98. doi: 10.1016/j.immuni.2010.11.008
105. Jeannet R, Mastio J, Macias-Garcia A, Oravecz A, Ashworth T, Geimer Le Lay AS, et al. Oncogenic Activation of the Notch1 Gene by Deletion of Its Promoter in Ikaros-Deficient T-All. *Blood* (2010) 116(25):5443–54. doi: 10.1182/blood-2010-05-286658
106. Ashworth TD, Pear WS, Chiang MY, Blacklow SC, Mastio J, Xu L, et al. Deletion-Based Mechanisms of Notch1 Activation in T-All: Key Roles for Rag Recombinase and a Conserved Internal Translational Start Site in Notch1. *Blood* (2010) 116(25):5455–64. doi: 10.1182/blood-2010-05-286328
107. Nie L, Perry SS, Zhao Y, Huang J, Kincade PW, Farrar MA, et al. Regulation of Lymphocyte Development by Cell-Type-Specific Interpretation of Notch Signals. *Mol Cell Biol* (2008) 28(6):2078–90. doi: 10.1128/MCB.00844-07
108. Nie L, Xu M, Vladimirova A, Sun XH. Notch-Induced E2a Ubiquitination and Degradation Are Controlled by Map Kinase Activities. *EMBO J* (2003) 22(21):5780–92. doi: 10.1093/emboj/cdg567
109. Chiang MY, Wang Q, Gormley AC, Stein SJ, Xu L, Shestova O, et al. High Selective Pressure for Notch1 Mutations That Induce Myc in T-Cell Acute Lymphoblastic Leukemia. *Blood* (2016) 128(18):2229–40. doi: 10.1182/blood-2016-01-692855
110. Herranz D, Ambesi-Impiombato A, Palomero T, Schnell SA, Belver L, Wendorff AA, et al. A Notch1-Driven Myc Enhancer Promotes T Cell Development, Transformation and Acute Lymphoblastic Leukemia. *Nat Med* (2014) 20(10):1130–7. doi: 10.1038/nm.3665
111. Choi SH, Severson E, Pear WS, Liu XS, Aster JC, Blacklow SC. The Common Oncogenomic Program of Notch1 and Notch3 Signaling in T-Cell Acute Lymphoblastic Leukemia. *PloS One* (2017) 12(10):e0185762. doi: 10.1371/journal.pone.0185762
112. Wang H, Zou J, Zhao B, Johannsen E, Ashworth T, Wong H, et al. Genome-Wide Analysis Reveals Conserved and Divergent Features of Notch1/Rbpj Binding in Human and Murine T-Lymphoblastic Leukemia Cells. *Proc Natl Acad Sci USA* (2011) 108(36):14908–13. doi: 10.1073/pnas.1109023108
113. Rodriguez-Caparros A, Garcia V, Casal A, Lopez-Ros J, Garcia-Mariscal A, Tani-Ichi S, et al. Notch Signaling Controls Transcription *Via* the Recruitment of Runx1 and Myb to Enhancers During T Cell Development. *J Immunol* (2019) 202(8):2460–72. doi: 10.4049/jimmunol.1801650
114. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR. The Role of the Runx Transcription Factors in Thymocyte Differentiation and in Homeostasis of Naive T Cells. *J Exp Med* (2007) 204(8):1945–57. doi: 10.1084/jem.20070133
115. Garcia-Ojeda ME, Klein Wolterink RG, Lemaitre F, Richard-Le Goff O, Hasan M, Hendriks RW, et al. Gata-3 Promotes T-Cell Specification by Repressing B-Cell Potential in Pro-T Cells in Mice. *Blood* (2013) 121(10):1749–59. doi: 10.1182/blood-2012-06-440065
116. Van de Walle I, Dolens AC, Durinck K, De Mulder K, Van Loocke W, Damle S, et al. Gata3 Induces Human T-Cell Commitment by Restraining Notch Activity and Repressing Nk-Cell Fate. *Nat Commun* (2016) 7:11171. doi: 10.1038/ncomms11171
117. Scripture-Adams DD, Damle SS, Li L, Elihu KJ, Qin S, Arias AM, et al. Gata-3 Dose-Dependent Checkpoints in Early T Cell Commitment. *J Immunol* (2014) 193(7):3470–91. doi: 10.4049/jimmunol.1301663
118. Taghon T, Yui MA, Rothenberg EV. Mast Cell Lineage Diversion of T Lineage Precursors by the Essential T Cell Transcription Factor Gata-3. *Nat Immunol* (2007) 8(8):845–55. doi: 10.1038/ni1486
119. van Hamburg JP, de Bruijn MJ, Dingjan GM, Beverloo HB, Diepstraten H, Ling KW, et al. Cooperation of Gata3, C-Myc and Notch in Malignant Transformation of Double Positive Thymocytes. *Mol Immunol* (2008) 45(11):3085–95. doi: 10.1016/j.molimm.2008.03.018
120. Fransecky L, Neumann M, Heesch S, Schlee C, Ortiz-Tanchez J, Heller S, et al. Silencing of Gata3 Defines a Novel Stem Cell-Like Subgroup of Etp-All. *J Hematol Oncol* (2016) 9(1):95. doi: 10.1186/s13045-016-0324-8
121. Tiemessen MM, Baert MR, Schonewille T, Brugman MH, Famili F, Salvatori DC, et al. The Nuclear Effector of Wnt-Signaling, Tcf1, Functions as a T-Cell-Specific Tumor Suppressor for Development of Lymphomas. *PloS Biol* (2012) 10(11):e1001430. doi: 10.1371/journal.pbio.1001430
122. Staal FJ, Clevers HC. Wnt Signaling in the Thymus. *Curr Opin Immunol* (2003) 15(2):204–8. doi: 10.1016/s0952-7915(03)00003-7
123. Brantjes H, Roose J, van De Wetering M, Clevers H. All Tcf Hmg Box Transcription Factors Interact With Groucho-Related Co-Repressors. *Nucleic Acids Res* (2001) 29(7):1410–9. doi: 10.1093/nar/29.7.1410
124. Verbeek S, Izon D, Hofhuis F, Robanus-Maandag E, te Riele H, van de Wetering M, et al. An Hmg-Box-Containing T-Cell Factor Required for Thymocyte Differentiation. *Nature* (1995) 374(6517):70–4. doi: 10.1038/374070a0
125. Staal FJ, Clevers H. Tales of the Unexpected: Tcf1 Functions as a Tumor Suppressor for Leukemias. *Immunity* (2012) 37(5):761–3. doi: 10.1016/j.immuni.2012.10.008
126. Feder K, Edmaier-Schroger K, Rawat VPS, Kirsten N, Metzeler K, Kraus JM, et al. Differences in Expression and Function of Lef1 Isoforms in Normal Versus Leukemic Hematopoiesis. *Leukemia* (2020) 34(4):1027–37. doi: 10.1038/s41375-019-0635-1
127. Petropoulos K, Arseni N, Schessl C, Stadler CR, Rawat VP, Deshpande AJ, et al. A Novel Role for Lef-1, a Central Transcription Mediator of Wnt Signaling, in Leukemogenesis. *J Exp Med* (2008) 205(3):515–22. doi: 10.1084/jem.20071875
128. Santiago L, Daniels G, Wang D, Deng FM, Lee P. Wnt Signaling Pathway Protein Lef1 in Cancer, as a Biomarker for Prognosis and a Target for Treatment. *Am J Cancer Res* (2017) 7(6):1389–406.
129. Guo X, Zhang R, Liu J, Li M, Song C, Dovat S, et al. Characterization of Lef1 High Expression and Novel Mutations in Adult Acute Lymphoblastic Leukemia. *PloS One* (2015) 10(5):e0125429. doi: 10.1371/journal.pone.0125429
130. Gutierrez A, Sanda T, Ma W, Zhang J, Grebliunaite R, Dahlberg S, et al. Inactivation of Lef1 in T-Cell Acute Lymphoblastic Leukemia. *Blood* (2010) 115(14):2845–51. doi: 10.1182/blood-2009-07-234377
131. Furness CL, Mansur MB, Weston VJ, Ermini L, van Delft FW, Jenkinson S, et al. The Subclonal Complexity of Stil-Tal1+ T-Cell Acute Lymphoblastic

Leukaemia. *Leukemia* (2018) 32(9):1984–93. doi: 10.1038/s41375-018-0046-8
132. Moore G, Annett S, McClements L, Robson T. Top Notch Targeting Strategies in Cancer: A Detailed Overview of Recent Insights and Current Perspectives. *Cells* (2020) 9(6):1503–649. doi: 10.3390/cells9061503
133. Lehal R, Zaric J, Vigolo M, Urech C, Frismantas V, Zangger N, et al. Pharmacological Disruption of the Notch Transcription Factor Complex. *Proc Natl Acad Sci USA* (2020) 117(28):16292–301. doi: 10.1073/pnas.1922606117

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REVIEW
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The E-Id Axis Instructs Adaptive Versus Innate Lineage Cell Fate Choice and Instructs Regulatory T Cell Differentiation

*Reiko Hidaka, Kazuko Miyazaki and Masaki Miyazaki**

Laboratory of Immunology, Institute for Life and Medical Sciences, Kyoto University, Kyoto, Japan

Immune responses are primarily mediated by adaptive and innate immune cells. Adaptive immune cells, such as T and B cells, evoke antigen-specific responses through the recognition of specific antigens. This antigen-specific recognition relies on the V(D)J recombination of immunoglobulin (*Ig*) and T cell receptor (*TCR*) genes mediated by recombination-activating gene (*Rag*)1 and *Rag2* (*Rag1/2*). In addition, T and B cells employ cell type-specific developmental pathways during their activation processes, and the regulation of these processes is strictly regulated by the transcription factor network. Among these factors, members of the basic helix-loop-helix (bHLH) transcription factor mammalian E protein family, including E12, E47, E2-2, and HEB, orchestrate multiple adaptive immune cell development, while their antagonists, Id proteins (Id1-4), function as negative regulators. It is well established that a majority of T and B cell developmental trajectories are regulated by the transcriptional balance between E and Id proteins (the E-Id axis). E2A is critically required not only for B cell but also for T cell lineage commitment, whereas Id2 and Id3 enforce the maintenance of naïve T cells and naïve regulatory T (Treg) cells. Here, we review the current knowledge of E- and Id-protein function in T cell lineage commitment and Treg cell differentiation.

Keywords: T cell versus ILCs, Rag gene expression, E-Id axis, T-lineage commitment, Treg differentiation

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Oklahoma Medical Research Foundation, United States

***Correspondence:**
Masaki Miyazaki
mmiyazaki@infront.kyoto-u.ac.jp

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INTRODUCTION

Innate immune cells and adaptive lymphocytes cooperatively evoke immune responses aimed at protecting our bodies from invasion of the pathogens. Innate immune cells, such as macrophages, neutrophils, and dendritic cells, are activated by pattern recognition receptors (PRRs) that recognize microbial components. On the other hand, adaptive lymphocyte T and B cells recognize specific antigens through diverse antigen receptors. This specific immune response relies on the V(D)J recombination of the immunoglobulin (*Ig*) and T cell receptor (*TCR*) genes mediated by the recombination-activating gene (*Rag1/2*). The assembly of the *TCR* and *Ig* genes from the arrays of variable (V), diversity (D), and joining (J) gene segments is initiated by a Rag1 and Rag2 protein complex, which recognizes and cleaves the recombination signal sequences (RSSs) flanking the V, D, and J segments of the *Ig* and *TCR* genes (1, 2). The expression of the *Rag1/2* genes is stringently

controlled. These genes are expressed only in T and B progenitor/precursor cells, meaning that *Rag1/2* expression is a hallmark of the adaptive lymphocyte lineage.

Common lymphoid progenitors (CLPs) can give rise to T cells, B cells, innate lymphoid cells (ILCs) including natural killer (NK) cells, and dendritic cells (DCs). Once lymphoid progenitors from the fetal liver or bone marrow (BM) migrate into the thymus, they receive Notch1 receptor signaling through the interaction with Delta-like 4 (DL4)-expressing thymic epithelial cells and commit to the T cell lineage (3–6). After T cell lineage commitment, TCRβ and/or TCRγ/δ V(D)J gene recombination is initiated in immature $CD4^-CD8^-$ (double negative; DN) cells. DN cells are divided into multiple distinct stages distinguished by surface expression of CD44 and CD25 (DN1-4). In DN1 cells, early T cell progenitors (ETPs) are defined by $CD25^-CD44^+KIT^{hi}$ expression, and committed T progenitor (pro-T) cells start expressing CD25 (DN2) since CD25 is a direct target of Notch signaling. Following the success of productive TCRβ recombination in DN3 cells ($CD44^-CD25^+$), DN3 cells start proliferating and differentiating into DN4 cells and further into $CD4^+CD8^+$ (double positive; DP) cells (T precursor (pre-T) cells). Recombination of the TCRγ/δ gene occurs concurrently with TCRβ recombination in DN2-3 cells (7). Upon reaching the DP stage, thymocytes exit the cell cycle (resting DP cells) and start TCRα VJ recombination (8, 9). DP cells that succeed in the production of a functional TCRα/β undergo positive and negative selection, which permits the developmental progression of T cells that have acquired a TCR with moderate affinity for self-antigens associated with major histocompatibility complex (MHC) class I (for CD8 single-positive (CD8SP) cells) or class II (for CD4SP cells) (10). The population of CD4SP cells that react more strongly with self-antigens associated with the MHC in the thymus differentiates into distinct regulatory T cells (Tregs), which specifically express the transcription factor (TF) Foxp3 and play an indispensable role in suppressing autoimmunity and excessive immune responses (11). On the other hand, innate type of T cells also arise from DP cells, which are selected by CD1 for invariant natural killer T (iNKT) cells and by MHC-related protein MR1 for mucosal-associated invariant T (MAIT) cells (12, 13). In these processes, sequential expression of an ensemble of TFs specifies the lineage-specific gene expression program and function through the regulation of the enhancer repertoire and activities (14, 15). However, the precise molecular mechanisms of how lineage-specific TFs synergistically regulate enhancer activities and how these factors cooperatively orchestrate the changes in chromatin architecture for appropriate gene expression remain unclear.

E proteins are basic helix-loop-helix (bHLH) TFs involved in multiple hematopoietic developmental processes, and mammalian E proteins include E12, E47 (from the *E2A*;*Tcf3* gene), E2-2 (*Tcf4*), and HEB (*Tcf12*). E proteins bind to the E-box motif (CANNTG) within the cis-regulatory element (CRE, enhancer region) of the target genes by forming homodimers or heterodimers. In contrast, Id proteins contain an HLH dimerization domain but lack the basic region that is required for DNA binding and form heterodimers with E proteins, antagonizing the DNA binding activity of E proteins and functioning as negative regulators of E proteins (16–18). Id proteins include Id1, Id2, Id3 and Id4, and hematopoietic cells primarily express Id2 and Id3. It is well established that the E and Id protein axis (the E-Id axis) regulates developmental trajectories of adaptive lymphocytes (19–21). The E2A gene encodes the E12 and E47 proteins, and E47 primarily regulates B cell lineage commitment, along with Ebf1, Pax5, and Foxo1 (22, 23). For T cell lineage commitment, E2A acts in pro-T cells along with HEB to establish a T cell-specific gene expression program and to suppress ILC development (24–28). HEB is also required for iNKT cell development from DP cells (29), and HEB and E2A play an important role in positive selection of DP cells (30). In contrast, *Id3* is upregulated by pre-TCR and γδ TCR signaling through ERK-MAPK, Egr1, and NFAT and plays a central role in αβ/γδ T cell fate and maturation (31–33). Furthermore, a recent report revealed the importance of the Notch-E2A-Tcf1 axis in αβ versus γδT cell lineage bifurcation and γδT cell function (34). In addition, E2-2 is critically required for interferon-producing plasmacytoid DC (pDC) development, while Id2 regulates antigen-presenting classical DC (cDC) development by neutralizing E2-2 activity (35–37). Furthermore, Id2 is well known as a critical regulator of the development of all ILC subsets, including ILC1-3s, NK cells, and lymphoid tissue inducer (LTi) cells (38, 39).

Many reviews describing the role of the E-Id axis have focused on the lineage commitment of T and B cells and DCs and on development of conventional T cells, NK cells, γδT cells, and iNKT cells. In this review we focus on the roles of the E-Id axis in T cell lineage commitment, including adaptive versus innate lymphoid cells, and during Treg cell differentiation.

ADAPTIVE VERSUS INNATE LYMPHOID CELLS

ILCs are a family of lymphocytes that do not have diversified antigen recognition receptors, such as Ig and TCR, and that primarily reside in various tissues and respond to infection, injury and damage (40). ILCs modulate immune responses and contribute to the maintenance of tissue homeostasis by sustaining appropriate immune responses at mucosal barriers and by enhancing immune responses through secretion of inflammatory cytokines. Functional similarities regulated by a common set of specific TFs may suggest that ILCs are the innate counterparts of T cells. ILCs can be segregated into distinct classes according to effector cytokine secretion and expression of specific TFs. ILC1s, including NK cells, are characterized by secretion of interferon-γ (IFN- γ) and expression of the specific TF T-bet. ILC2s express the TF Gata3 and Th2 cytokines (interleukin-4 (IL-4), IL-5, and IL-13). ILC3s, including LTi-like cells, express Rorγt and secrete IL-17/IL-22 and lymphotoxin (40, 41). Therefore, ILC1s, ILC2s, and ILC3s are counterparts of CD4 helper T_H1, T_H2, and T_H17 cells, respectively, while NK cells mirror CD8 cytotoxic T cells. As well as adaptive T and B

lymphocytes, ILCs develop from common lymphoid progenitors (CLPs), and lineage commitment into ILCs is regulated by sequential expression of an ensemble of TFs, including Nfil3, Tox, Id2, Tcf1, and Gata3 (42–48). In addition, PLZF in ILC precursors (ILCp), Bcl11b and Rorα in ILC2s, and Runx3 in ILC1s/3s are required for this process (49–52). In particular, it is well known that Gata3, Tcf1, and Bcl11b are also required for early T cell development (3, 53). These observations clearly show close similarities between ILC and T cell lineages not only in effector function but also in their development, and a combination of these shared TFs determines effector functions in each lineage of ILCs after passing the developmental bifurcation of adaptive and innate lymphoid lineage commitment. However, how these shared TFs play their distinct roles in early T cell and ILC development remains to be clarified. Therefore, it is important to understand what events result in the differences between T cells and ILCs during their development.

ILCs are derived from CLPs in the fetal liver (FL) and adult bone marrow (BM), and differentiate into functional mature ILCs in the resident tissues, while CD4 helper T cells and CD8 cytotoxic T cells mature in the thymus. The frequencies of ILCs, including mature Id2- and Gata3-expressing ILC2s and PLZF-expressing ILCps, are considerably low in the thymus of normal adult mice (54), because the majority of thymocytes in adult thymus are developing T cells. Consistent with a report that Rag1/2-mediated TCR recombination is dispensable for ILC development (55, 56), we and another group observed both the absence of D-J and V-DJ recombination of the TCRβ gene in ILC2s from wild-type lung tissue and aberrant ILC2s in the thymus from E2A/HEB-deficient mice (28, 57). According to these observations, the cell fate of the T versus ILC lineage must be principally determined by the thymic microenvironment. Notch signaling is one of the most likely external or environmental factors that distinguish T cells from the ILC lineage. In the absence of DL4 in thymic stromal cells, aberrant ILC2s are observed in the thymus, and constitutive Notch signaling completely blocks the ILC lineage *in vivo*. However, the proliferation of committed ILC precursors require mild to moderate Notch signaling, and short exposure to a Notch ligand combined with a high amount of IL-7 in CLPs leads to ILC2 generation *in vitro* (6, 58). Interestingly, recent studies have revealed an unexpectedly close relationship between T cells and ILCs (57, 59). Specifically, ILCps in BM express high levels of TCRβ constant region transcripts, and a proportion of tissue-resident ILC2s have undergone TCRγ gene recombination and express high levels of mRNAs of TCRβ and TCRγ4 constant regions (Cβ1/2 and Cγ4); however, the frequency of these TCRγ gene recombination is low, compared to that in γδT cells, and the recombination in these cells are nonfunctional (28, 57). Consistent with this observation, a high level of mRNA expression and broad chromatin accessibility in the TCRβ constant region with little or no expression of any TCR Vβ region in E2A/HEB-deficient ETPs, which tend toward an aberrant ILC lineage, were detected (28). According to these observations, T precursor cells that fail to properly undergo TCR recombination, especially TCRγ/δ recombination, may be able to convert their cell lineage into ILCs (56, 57). However, the numbers of mature ILC2s and PLZF-expressing ILCps in *Rag2*-deficient thymuses remain low; this phenomenon cannot explain why TCRγ/δ genes, but not TCRβ D-J gene, recombination are observed in ILCs, although TCRβ D-J and TCRγ/δ recombination occurs concurrently in the DN2 stage (28, 57). In contrast to these reports, the Sun group demonstrated that ILC2s in the thymus and lug from wild-type and *E2A/HEB* deletion (plck-Cre) mice, but not from *Id1*-transgenic (*Id1-Tg*) mice, exhibited TCRβ D-J and V-DJ gene recombination, which are detected by Southern blotting, and estimated that around 10% of ILC2s performed these recombination (60). In this report, even committed DN3 cells have a potential to differentiate into ILC2s *in vitro*, suggesting the lineage conversion of T cells to ILCs (60). Although these phenomena remain puzzling, T cells and ILCs are very close counterparts, and Rag1/2-mediated TCRβ recombination and its expression seem to be functional hallmarks of physiological T cell lineage commitment *in vivo*. A recent study provided an important clue regarding the mystery of the checkpoint for T cells and ILC2s in the thymus (61). During embryogenesis, functional ILC2s differentiate from ETPs in the fetal thymus, and these ILC2s preferentially migrate to mucosal tissues and reside for a long period. In this time-restricted thymic ILC2 development, specific TF RORα is the key factor that promotes ILC2 development and simultaneously suppresses the T cell lineage program by inducing *Id2* expression, leading to E2A function antagonism (61, 62). This study demonstrated that ILC2 development in E2A/HEB-deficient mice does not represent simple aberrant ILC development and instead may be an implication of the physiological embryonic thymocyte development toward the ILC2 lineage. Although *Id2* expression is a critical regulator of the ILC lineage, *Id2* deletion in E2A/HEB deficiency leads to thymic ILC development as well as E2A/HEB deficiency, and transient *Id2* expression induced by doxycycline can induce aberrant ILC2 development in adult thymus. Thus, T cell and ILC lineages may simply depend on the magnitude of E protein activity, and Id2 may function as a lineage switch for ILCs (28). Therefore, we conclude that after the enhancer repertoire associated with each lineage regulated by the E-Id axis is established, an ensemble of shared TFs, such as Tcf1, Bcl11b, and Gata3, instructs the lineage-specific gene expression programs in both T cells and ILCs (**Figure 1**). Indeed, Bcl11b binds to different sites in a lineage-specific manner associated with cell type-specific protein complexes (63). Interestingly, some members of these factors are dynamically recruited to the regulatory regions not only in a lineage-specific manner but also in a developmental stage-specific manner (64).

However, it remains unclear whether the loss of E protein activity in ETPs induces only ILC lineage commitment or also leads to the expansion of ILC precursors or mature ILCs. Since Id2 is continuously expressed at high levels after ILC lineage commitment, the magnitude of E protein activity may control not only the ILC versus T lineage commitment but also the expansion or activation of ILCs after the commitment, which is

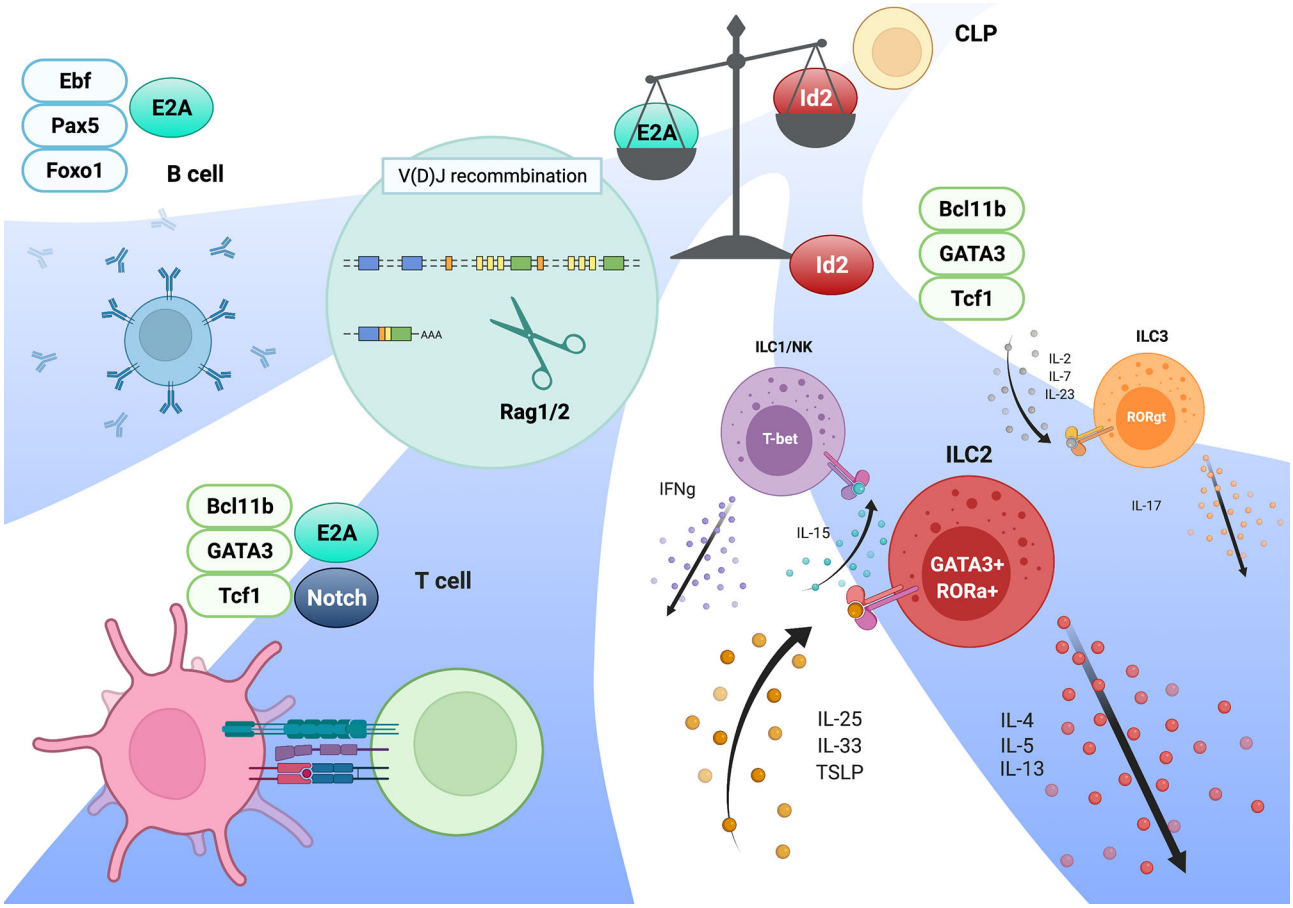

FIGURE 1 | Model of adaptive and innate lymphocytes lineages mediated by the E-Id axis. The magnitude of E protein transcriptional activity determines the lineage commitments of adaptive versus innate lymphocytes. Following this process, an ensemble of TFs specific for each lineages validates lineage-specific gene expression program, along with E proteins in T and B cells. This figure was created with BioRender.com.

antagonized by Id2. Because E2A functions both as an initiator upon T cell lineage commitment and as a gatekeeper at β-selection (65), the loss of E protein activity in ILCs may play a role in the activation or expansion of ILCs.

How is the E-Id axis regulated? While E2A and HEB mRNA expression levels are relatively consistent throughout the thymocyte development (ImmGen data; https://www.immgen.org/), the E2A protein level is high in ETPs and is the highest in DN2 cells; this level is downregulated in resting DP cells, as revealed by E2A-GFP fusion knock-in mouse analysis, indicating the presence of posttranslational regulation of the E2A protein (66–68). On the other hand, *Id3* is upregulated by TCR signaling, including pre- and γδ-TCR, during thymocyte development and remains at a high level in peripheral naïve T and Treg cells (32, 67, 69). In peripheral T cells, TCR stimulation induces E2A protein expression, which is required for rapid memory-precursor formation of CD8 T cells, while Id2 and 3 function as regulators of CD8 T cell responses (70). Surprisingly, differential *Id2* and *Id3* expression in CD4 T cells during viral infection regulates T_H1 or T_{FH} cell development, respectively (71). During ILC lineage commitment, *Id2* is initially upregulated in PLZF-expressing ILC precursors in which E2A protein is already downregulated, and this induction of *Id2* expression is associated with the IL7R expression level, suggesting the involvement of cytokine signaling in *Id2* expression (28). Consistently, the cis-regulatory element of the *Id2* gene, which expresses the long noncoding RNA Rroid, controls ILC1 function by regulating Stat5 deposition at the *Id2* promoter region; however, this locus is dispensable for *Id2* expression in other ILCs (72). Therefore, *Id2* expression in ILC lineages, which is probably mediated by cytokine signaling, is required not only for ILC lineage commitment but also for ILC maintenance.

RAG1 AND *RAG2* GENE EXPRESSION MEDIATED BY E PROTEINS

As we discussed in the introduction, *Rag1/2* gene expression discriminates between adaptive and innate lymphoid lineages. This indicates that TFs responsible for *Rag1/2* expression are

critical regulators of T and B lineage commitment (73). There are two waves of Rag1/2 expression during T and B cell development (74). The first wave of *Rag1/2* expression is required for the assembly of IgH and TCRβ genes in pro-B and pro-T cells, respectively. After the selection of pre-TCR (TCRβ) or pre-BCR (IgH), Rag1 expression is transiently downregulated during the transition from the progenitors to precursors. In the precursor stage, Rag1/2 are re-expressed for IgL and TCRα gene recombination. Following the positive and negative selection of the TCR or BCR, the *Rag1/2* genes are suppressed in mature naïve T and B cells and are never expressed for further recombination of the *TCR* and *Ig* genes. During these developmental processes, *Rag1/2* gene expression is tightly regulated, and other types of immune cells never express the *Rag1/2* genes. However, the molecular mechanisms of *Rag1/2* gene expression remained to be determined. Both *in vivo* and *in vitro* studies have attempted to define the enhancer regions and TFs responsible for *Rag1/2* expression (75). Both T and B progenitor/precursor cells express *Rag1/2* and require distinct enhancers of these genes. The deletion of *Erag* (*Enhancer of Rag*), which is located at 23 kb upstream of the *Rag2* promoter, resulted in a significant reduction in *Rag1/2* expression and partial developmental defects during B cell development, without affecting thymocyte development (76). A study has reported that this *Erag* region is positively regulated by Foxo1 and negatively regulated by Gfi1b, Ebf1, and c-Myb (77–80). In contrast, an anti-silencer element (*ASE*), which is 8 kb in length and located 73 kb upstream of the *Rag2* promoter, is required for *Rag1/2* gene expression in DN3 and DP cells but not in developing B cells (81). In ChIP-seq data, most of T cell TFs includng E2A, Bcl111b, Tcf1, Gata3, Runx1, and Ikaros bound to ASE regions, while B cell TFs such as E2A, Pax5, and Irf4, but not Ebf1, bound to Erag region (82, 84).

The Krangel group demonstrated that the chromatin organizer mediates the interaction between *ASE* and *Rag1/2* promoters to promote optimal expression of the *Rag1/2* genes in DP cells and suggested that the *ASE* and *Rag1* promoter regions function as a chromatin hub (82). Furthermore, this group proved that Gata3 and E2A regulate the *ASE* region, and *Rag1* promoter activity relies on Runx1 and E2A binding in the VL3-3M2DP thymocyte cell line (83). A study also identified T or B cell-specific enhancer elements that drive *Rag1/2* expression using the E2A ChIP-seq and ATAC-seq data from pro-T and pro-B cells to clarify the regulatory mechanisms of adaptive versus innate lineage choice. Two B cell-specific enhancers (*Rag* B cell enhancer 1 and 2; *R1B* (5 kb upstream of the *Rag1* promoter) and *R2B* (partially overlapping with *Erag*)) and one T cell-specific enhancer (*Rag*-T cell enhancer (*R-TEn*)) were identified (84). A common E2A-binding region near the *Rag1* promoter (*R1pro*), which is shared between T and B cells, was also identified. *R1B*/*R2B* and *R-TEn* uniquely bind to the *Rag1*/*Rag2* promoter regions and form distinct chromatin structures in developing T and B cells, respectively. Deletion of both *R1B* and *R2B* in mice resulted in a severe developmental block at the pro-B stage, but not in T-cell development, resulting from drastic impairments in Rag-mediated IgH gene recombination, whereas single deletion of either *R1B* or *R2B* resulted in mild-to-moderate defects in B cell development that also occurred in *Erag* deletion mice (76, 84). This finding suggests enhancer redundancy in *Rag1/2* expression in B cells. In contrast, *R-TEn* deletion resulted in severe developmental defects in β-selection of DN3 cells and positive selection of DP cells without affecting B cell development (84). These results raised the question of what TF regulates these *Rag* gene enhancer regions.

E2A is especially notable among TFs responsible for adaptive lymphocyte development because *Rag1/2* gene expression was significantly reduced in E2A-deficient lymphoid-primed multipotent progenitors (LMPPs) and T progenitor cells (28, 85, 86). A mutation of the E-box motifs in the *R-TEn* (*R-TEn-E-box-mutant*), which blocks E-protein binding without affecting the recruitment of other TFs to this enhancer, directly proves that the E2A/E protein regulates this enhancer. *R-TEn-E-box-mutant* mice showed developmental defects in β-selection and positive selection, resulting from a severe reduction in *Rag1/2* gene expression in DN3 and DP cells. Furthermore, genome structures, chromatin accessibility, histone H3 lysine K27 acetylation (H3K27ac), and cohesin recruitment were completely lost only at the *Rag* gene locus, indicating that the E2A/E protein binding to the enhancer region induces and promotes cell type-specific superenhancer (SE) formation (84). How does the E2A/E protein induce SE formation? bHLH TFs, such as E2A, were reported to interact with the histone acetyltransferase (HAT) CBP/P300 and SAGA proteins through the PECT motif within the activation domain 1 (AD1) of the E protein and recruit these coactivators to enhancer regions, thus inducing and promoting H3K27 acetylation (87–91). Active enhancers are accompanied by high levels of H3K27ac, CBP/P300, chromatin remodeler Brg1, and RNA polymerase II (PolII) to facilitate the recruitment of cohesin-loader NIPBL and the cohesin complex, which induce large-scale structural changes of the chromatin and may switch the locus from transcriptionally repressive (B) to permissive (A) compartments (92, 93). Simultaneously, E2A and other specific TFs also recruit the ten-eleven translocation (TET) family proteins to the enhancers to remove DNA methylation of the CpG islands in enhancers, which is associated with the SE function in developing and activated B cells (94, 95). SEs regulate certain genes that play characteristic roles in cell type-specific functions, thereby establishing cell identity (96, 97). Because the properties of SEs are based on highly cooperative interactions between cell type-specific TFs, transcriptional mediators, and RNA PolII and due to vulnerability to a perturbation of the key protein components (98), E2A functions in adaptive lymphocyte-specific enhancer regions as a pioneer and maintainer. Additionally, E2A plays an essential role in *Rag1* expression *in vivo* through the regulation of the promoter activity. Surprisingly, E-box motif mutations in the *Rag1*-promoter region (*R1pro-E-box-mutant*) alone in mice are sufficient to inhibit the *Rag1* gene expression, which leads to the developmental arrest at both the T and B cell progenitor stages, similar to those in *Rag1*-deficient mice. However, *Rag2*

expression and enhancer regions (*R-TEn* and *R1B/R2B*) are not affected in *R1pro-E-box-mutant* DN3 and pro-B cells (84). This result indicates that both cell type-specific enhancer and promoter regions independently rely on the recruitment of the E2A/E protein and that E protein-mediated interactions between enhancer and promoter regions determine adaptive lymphocyte-specific expression of the *Rag* gene. We summarised these regulatory regions in **Table 1**.

Overall, the binding of the E2A/E proteins to the E-box motifs in the cell type-specific cis-regulatory regions induces the recruitment of P300, other transcription mediators, the NIPBL/cohesin-complex, and chromatin organizers to orchestrate 3D structural changes of the genomes to initiate and maintain cell type-specific gene expression. In contrast, high expression levels of Id2 prevents *Rag* gene SE formation by antagonizing the E2A activity, and the *Rag* gene is sequestered in repressive chromatin (B) compartment (**Figure 2**). Curiously, sequence similarities of T and B cell-specific *Rag* gene enhanceres are conserved among mammals, birds and reptiles, but not in amphibians and fish. In addition, these conserved enhancer regions have been shown to harbor the E-box motifs conserved among these species (84). Thus, we propose that terrestrial animals evolutionarily acquired the gene regulatory mechanism mediated by the E proteins as enhancers to achieve higher *Rag* gene expression, which enables a diverse range of *TCR* and *Ig* gene recombination to protect against a wide range of the pathogens (99).

TREG CELLS AND THE ROLE OF THE E-ID AXIS

E and Id proteins play a central role in effector/memory and tissue-resident cytotoxic CD8 T cell differentiation and the activation of helper CD4 T cells, including T_H1 and follicular helper T (T_{FH}) cells (67, 71, 100–105). However, to our knowledge, no review papers have addressed the role of the E-Id axis in Treg cells. In this section, we focus on the roles of Id and E proteins in Treg cells. Treg cells play a central role in the maintenance of immune homeostasis by suppressing autoimmunity and excessive inflammatory responses and by tissue repair after inflammation. Naturally occurring Treg cells differentiate in the thymus (natural Treg (nTreg) or thymic Treg (tTreg) cells), which constitutively express TF Foxp3, while a population of Foxp3-expressing Treg cells develops from naïve CD4 T cells in the periphery (peripheral Treg (pTreg) cells) (106). In addition, naïve CD4 T cells can develop into Foxp3-expressing Treg cells *in vitro* by TCR stimulation in the presence of TGF-β plus IL-2 (induced Treg (iTreg) cells) (107). Treg cells show functional heterogeneity to regulate a variety of immune responses, and each subset of Treg cells has a specialized gene expression program. As well as conventional CD4 T cells, Treg cells differentiate into effector subsets, named effector Treg (eTreg) cells, accompanied by Blimp1 and Irf4 TFs, and express unique migratory chemokine receptors to home to the site of inflammation and higher suppressive molecules such as IL-10 and CTLA-4 to control tissue inflammation (108–110). For instance, T_H1-Treg cells express CXCR3, which is mediated by T-bet, to migrate into T_H1 inflammatory sites (111). In addition, follicular regulatory T (T_{FR}) cells, a specialized subset of Treg cells, regulate T_{FH} cell function and germinal center B-cell responses for the humoral immunity (112–114). More recently, specialized subsets of Treg cells in nonlymphoid tissues, such as adipose tissue, muscle tissue, lung tissue, and the central nervous system, have been shown to play an important role in tissue homeostasis and regenerative functions, and amphiregulin and Notch ligand Jagged1 from Treg cells contribute to tissue regeneration (115–118). This subset of Treg cells is often referred to as tissue-resident Treg (TR-Treg) cells. They are derived from effector Treg cells, which in turn are instructed by TF Batf (119) (**Figure 3**).

TABLE 1 | Description of Rag gene enhancer regions.

cis-regulatory element	Length/open	TF bindings by ChIP-seq data	Defects in deletion or mutant mouse	Rag1/Rag2 expression	Ref paper
anti-silence element (ASE)	8 kb		defects in thymocyte development (DN3, DP)	Rag1/2; down in DP cells	(79)
Enhancer of Rag (Erag)	1.7 kbp	E2A, Ets1, Ikaros	moderate defect in B cell development	Rag1/2; down in developing B cells	(74)
Rag-B cell enhancer 1 (R1B)	<1 kb	E2A, Ikaros, Irf4	mild defect in B cell development	moderate reduction of Rag1/2 expression	(80)
Rag-B cell enhancer 2 (R2B)	2 kb (partially overlapped with Erag)	E2A, Pax5, Ets1, Ikaros	moderate defect in B cell development	moderate reduction of Rag1/2 expression	(80)
R1B/R2B	R1B/R2B double deletion		developmental arrest at pro-B stage	drastic reduction of Rag1/2 expression in pro-B cells, but not in T cell	(80)
Rag-T cell enhancer (R-TEn)	2 kb (included in ASE)	Satb1, E2A, Ikaros, Bcl11b, Tcf1, Runx1, Gata3	defects in thymocyte development (DN3, DP)	Rag1/2; down in DN3a and DP cells	(80)
R-TEn peak 1	open in DN3/DP		defects in thymocyte development (DN3, DP)	Rag1/2; down in DN3a and DP cells	(80)
R-TEn peak 2	open in DP		no defect	normal	(80)
R-TEn peak1 E-box mutant	blocking E-protein binding to R-TEn		defects in thymocyte development (DN3, DP)	Rag1/2; down in DN3a and DP cells	(80)
Rag1 promoter E-box mutant	blocking E-protein binding to Rag1 promoter		developmental arrest at pro-B and DN3 stages	defects in Rag1, but not Rag2, expression in DN3a and pro-B cells	(80)

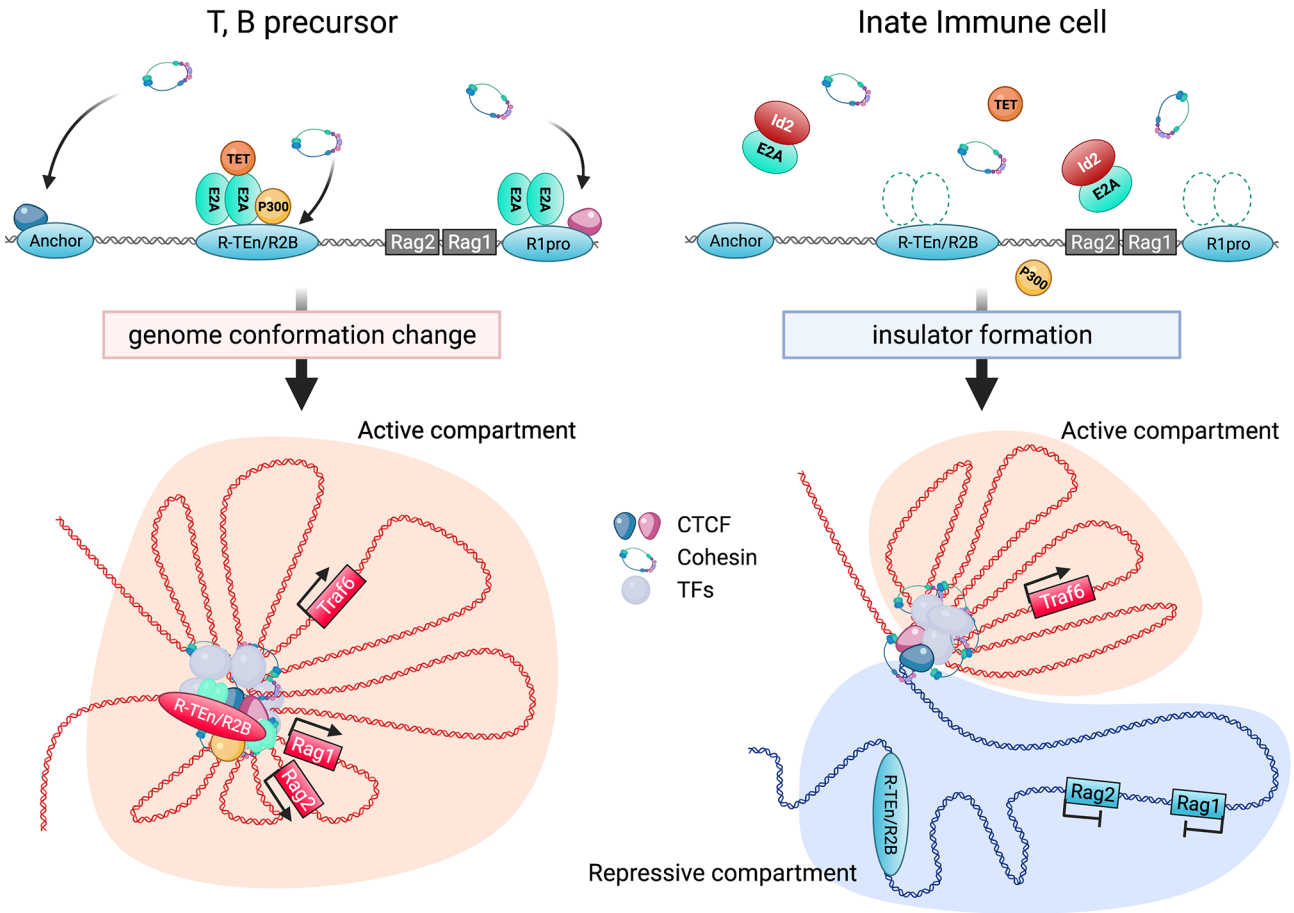

FIGURE 2 | Regulation of *Rag* gene locus by E2A and cis-regulatory elements. E2A binding to the specific enhancer (*R-TEn* and *R2B*) and *R1pro* regions induces the genome conformation changes to form adaptive lymphocyte-specific SE through the recruitment of P300, TET, and NIPBL-cohesin complex (left; developing T and B cells). In contrast, Id2 prevents E2A/E proteins from binding to these regulatory regions, leading to the insulator formation to sequester the *Rag* genes in repressive chromatin compartment in innate immune cells (right; macrophage etc). This figure was created with BioRender.com.

There are many previous studies about the role of the E-Id axis in Treg cell development and activation. The expression of *Id3* is high in naïve Treg cells and low in ICOS$^+$ effector Treg cells, and TCR stimulation in Treg cells downregulates *Id3*. In contrast, *Id2* levels are low in naïve Treg cells, and TCR stimulation induces the upregulation of *Id2 in vitro* (120, 121). It has been reported that *E2A/HEB* and *Id3* are involved in the development of tTreg cell and iTreg cells; drastically increased tTreg cells were observed in a study of *E2A/HEB*-deficient thymus, while decreased tTreg cells were detected in a study of *Id3*$^{-/-}$ thymus (122, 123). In addition, blocking the E protein by *Id1* overexpression in mice resulted in an increased frequency and number of tTreg cells due to the expansion of thymic Treg cells, while Foxp3 mRNA induced by TCR stimulation was significantly lower in naïve *Id1*-Tg CD4 T cells (124). However, the deletion of *E2A* and *HEB* in early stages blocks T cell lineage commitment, and their deletion in DP cells bypasses the TCR-mediated positive selection of DP cells, leading to the CD8SP stage accompanied by severe impairment of the CD4SP lineage (28, 125). In addition, *Id3* is required for MHC-restricted positive selection of DP cells (126). The combined loss of *Id2* and *Id3* results in blockage of the transition from CD69$^+$TCRβ$^{lo\ or\ -}$ DP to fully TCR-selected CD69$^+$TCRβ^{hi} DP cells at a young age; however, PLZF-expressing innate T_{FH} cells expand with limited TCR repertoires and occupy the CD4SP population in adults, suggesting that in the absence of *Id2* and *Id3*, conventional CD4 T cell development is severely affected (102). Therefore, it remains unclear whether changes in tTreg populations in these gene-deficient mice are reflected by the severely impaired CD4SP population and reduced strength of TCR signaling or whether E2A/HEB and Id3 are actually involved in the induction of Foxp3 expression or tTreg cell development. Furthermore, since Id3 enforces naïve T cell fate by antagonizing E2A activity and *Id3*-deficient CD4SP or CD8SP cells readily differentiate into IFN-γ-producing effector T cells,T_{FH} cells (CD4SP), or innate-like CD8 T cells in the thymus (67, 127), attenuated iTreg cell development in *Id3*$^{-/-}$ mice is more likely the result of fewer naïve CD4 T cells in the periphery. However, from the result that the deletion of *E2A/HEB* led to increased iTreg development *in vitro*, E protein activity is thought to be involved in iTreg cell

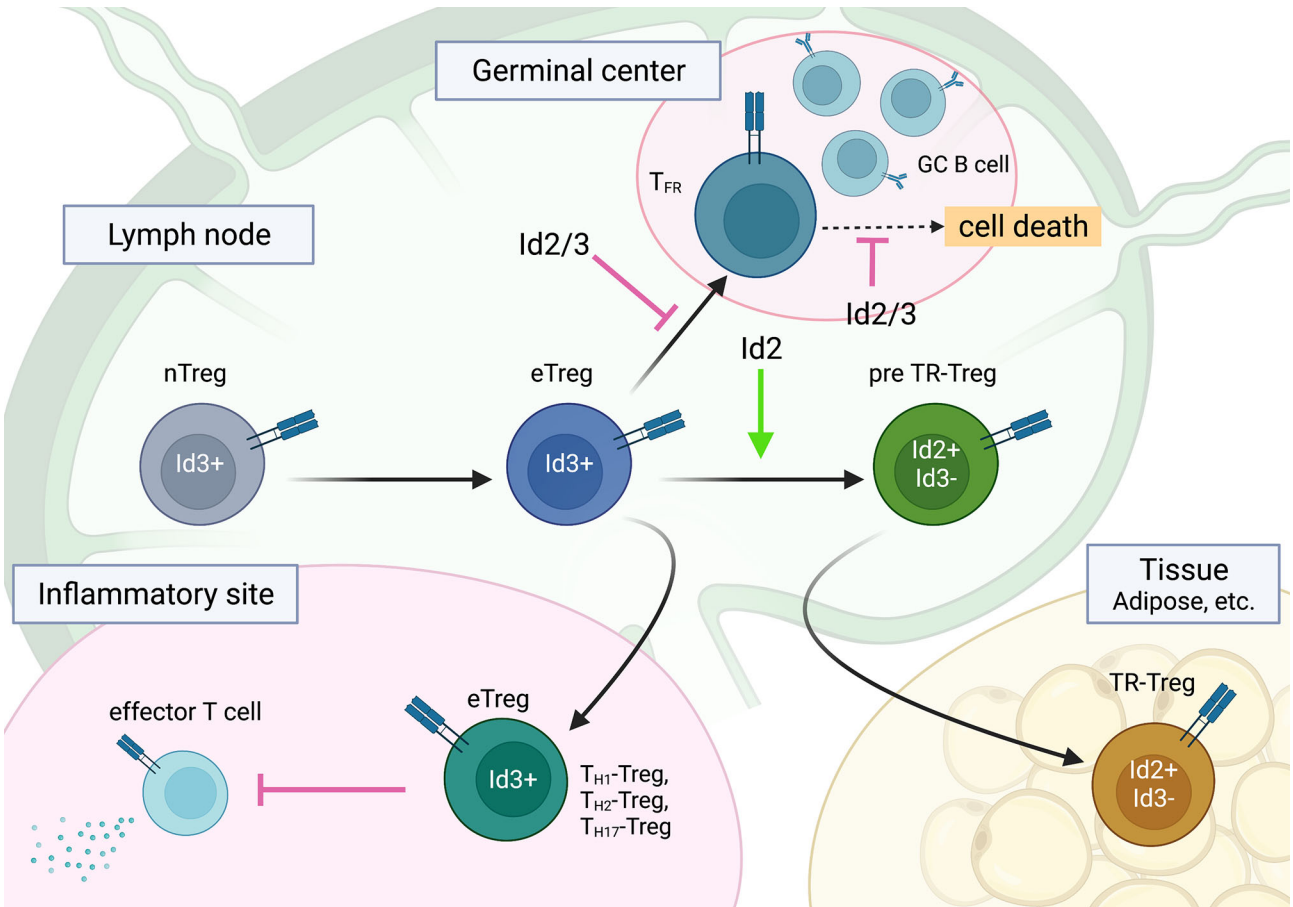

FIGURE 3 | The roles of Id2 and Id3 in Treg cell differentiation into subsets of effector Treg cells. Id2 and Id3 enforce the naïve state of Treg cells, especially in T_{FR} cells. A regulatory switch of Id3 to Id2 plays a role in TR-Treg cell differentiation and function. This figure was created with BioRender.com.

development (123). It was reported that E47 indirectly regulates Foxp3 expression through the regulation of Spi-B and SOCS3 in *Id3*-deficient Treg cells and that *Foxp3* mRNA in *Id2/Id3*-deficient Treg cells is comparable to that in control Treg cells, indicating that E2A does not regulate *Foxp3* gene expression (120, 128). In line with this, E2A occupancy around the *Foxp3* gene locus, by ChIP-seq analysis, was not detected in *Id2/Id3*-deficient DP cells (129).

Although the role of Id and E proteins in tTreg development is unclear, the E-Id axis plays an important role in Treg cell function. Indeed, Treg-specific deletion of *Id2* and *Id3* using Foxp3-Cre in mice leads to fatal inflammatory disease, which is characterized by spontaneous T_H2 inflammation in the lung, skin, and esophagus, similar to human atopic diseases such as bronchial asthma, atopic dermatitis, and eosinophilic esophagitis (120). *Id2/Id3* depletion in Treg cells induces CXCR5, which is a direct target of the E2A-Id3 axis in T_{FR} and T_{FH} cell development and preferentially migrates to B-cell follicles. However, *Id2/Id3*-deficiency in Treg cells has been shown to result in compromised maintenance of Treg cells mediated by TCR stimulation *in vitro*. This result suggests that Id proteins function as gatekeepers for eTreg and T_{FR} cells as well as CD4 T cells and control the maintenance of Treg cells. Although *Id2* and *Id3* compensate for each other in single KO Treg cells, Id2 and Id3 have distinct roles in Treg cell function. According to *Id3* expression with CD62L and CD44, the Campbell group demonstrated stepwise developmental stages toward TR-Treg cells; *Id3* was highly expressed in central naïve Treg cells and effector Treg cells, whereas $ICOS^{hi}$ $Id3^{lo}$ TR-Treg precursor cells expressed *Id2*, suggesting a regulatory switch from Id3 to Id2 in Treg cells (121, 130). This seems to be similar to tissue resident effector/memory CD8 T cells (100, 105). Interestingly, consistent with the Id switch in Treg cells, a loss of *Id2* expression in Treg cells results in decreased expression of TR-Treg cell-related functional molecules and leads to increased cell death of Treg cells, suggesting an Id2-dependent TR-Treg cell-specific program (131). Curiously, Treg cells lacking E2A and HEB exhibit effector phenotypes and increased stability, suggesting the linkage of E protein and TCR signaling in the gene signature of effector Treg cell development (132). In contrast, ectopic *Id2* expression in Treg cells in mice enhance Treg cell plasticity and lead to a reduction in Treg cells (133). Taken together, although

the underlying molecular mechanism remains to be determined, it now seems apparent that the E-Id axis orchestrates Treg cell differentiation toward the fate of T_{FR}, eTreg and TR-Treg cells and dictates function and plasticity in lymphoid and nonlymphoid tissues (**Figure 3**).

CONCLUSION

The E-Id transcriptional axis plays an important role in T/B cell lineage commitment, discrimination between T cells and ILCs, including *Rag* gene expression, and T/Treg cell function. However, it remains to be investigated how the E-Id axis orchestrates cell type-specific enhancer activities in conjunction with other TFs associated with T cell activation and TCR signaling. Future experiments are warranted to explore the role of the E-Id axis in T and B cell activation under the inflammatory conditions. These findings may have implications for health and immunological disorders.

AUTHOR CONTRIBUTIONS

RH, KM and MM wrote the manuscript and figures. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Schatz DG, Oettinger MA, Baltimore D. The V(D)J Recombination Activating Gene, RAG-1. *Cell* (1989) 59(6):1035–48. doi: 10.1016/0092-8674(89)90760-5
2. Schatz DG, Ji Y. Recombination Centres and the Orchestration of V(D)J Recombination. *Nat Rev Immunol* (2011) 11(4):251–63. doi: 10.1038/nri2941
3. Rothenberg EV. Programming for T-Lymphocyte Fates: Modularity and Mechanisms. *Genes Dev* (2019) 33(17-18):1117–35. doi: 10.1101/gad.327163.119
4. Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, Pierres M, et al. Delta-Like 4 is the Essential, Nonredundant Ligand for Notch1 During Thymic T Cell Lineage Commitment. *J Exp Med* (2008) 205(11):2515–23. doi: 10.1084/jem.20080829
5. Hozumi K, Mailhos C, Negishi N, Hirano K, Yahata T, Ando K, et al. Delta-Like 4 is Indispensable in Thymic Environment Specific for T Cell Development. *J Exp Med* (2008) 205(11):2507–13. doi: 10.1084/jem.20080134
6. Chea S, Schmutz S, Berthault C, Perchet T, Petit M, Burlen-Defranoux O, et al. Single-Cell Gene Expression Analyses Reveal Heterogeneous Responsiveness of Fetal Innate Lymphoid Progenitors to Notch Signaling. *Cell Rep* (2016) 14(6):1500–16. doi: 10.1016/j.celrep.2016.01.015
7. Capone M, Hockett RDJr., Zlotnik A. Kinetics of T Cell Receptor Beta, Gamma, and Delta Rearrangements During Adult Thymic Development: T Cell Receptor Rearrangements are Present in CD44(+)CD25(+) Pro-T Thymocytes. *Proc Natl Acad Sci USA* (1998) 95(21):12522–7. doi: 10.1073/pnas.95.21.12522
8. Kreslavsky T, Gleimer M, Von Boehmer H. Alphabeta Versus Gammadelta Lineage Choice at the First TCR-Controlled Checkpoint. *Curr Opin Immunol* (2010) 22(2):185–92. doi: 10.1016/j.coi.2009.12.006
9. Krangel MS. Mechanics of T Cell Receptor Gene Rearrangement. *Curr Opin Immunol* (2009) 21(2):133–9. doi: 10.1016/j.coi.2009.03.009
10. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and Negative Selection of the T Cell Repertoire: What Thymocytes See (and Don't See). *Nat Rev Immunol* (2014) 14(6):377–91. doi: 10.1038/nri3667
11. Hori S, Nomura T, Sakaguchi S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* (2003) 299(5609):1057–61. doi: 10.1126/science.1079490
12. Constantinides MG, Bendelac A. Transcriptional Regulation of the NKT Cell Lineage. *Curr Opin Immunol* (2013) 25(2):161–7. doi: 10.1016/j.coi.2013.01.003
13. Napier RJ, Adams EJ, Gold MC, Lewinsohn DM. The Role of Mucosal Associated Invariant T Cells in Antimicrobial Immunity. *Front Immunol* (2015) 6:344. doi: 10.3389/fimmu.2015.00344
14. Hosokawa H, Rothenberg EV. How Transcription Factors Drive Choice of the T Cell Fate. *Nat Rev Immunol* (2021) 21(3):162–76. doi: 10.1038/s41577-020-00426-6
15. Taniuchi I. CD4 Helper and CD8 Cytotoxic T Cell Differentiation. *Annu Rev Immunol* (2018) 36:579–601. doi: 10.1146/annurev-immunol-042617-053411
16. Murre C, Mccaw PS, Baltimore D. A New DNA Binding and Dimerization Motif in Immunoglobulin Enhancer Binding, Daughterless, MyoD, and Myc Proteins. *Cell* (1989) 56(5):777–83. doi: 10.1016/0092-8674(89)90682-x
17. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The Protein Id: A Negative Regulator of Helix-Loop-Helix DNA Binding Proteins. *Cell* (1990) 61(1):49–59. doi: 10.1016/0092-8674(90)90214-y
18. Murre C. Helix-Loop-Helix Proteins and the Advent of Cellular Diversity: 30 Years of Discovery. *Genes Dev* (2019) 33(1-2):6–25. doi: 10.1101/gad.320663.118
19. Kee BL. E and ID Proteins Branch Out. *Nat Rev Immunol* (2009) 9(3):175–84. doi: 10.1038/nri2507
20. Belle I, Zhuang Y. E Proteins in Lymphocyte Development and Lymphoid Diseases. *Curr Top Dev Biol* (2014) 110:153–87. doi: 10.1016/b978-0-12-405943-6.00004-x
21. Miyazaki K, Miyazaki M, Murre C. The Establishment of B Versus T Cell Identity. *Trends Immunol* (2014) 35(5):205–10. doi: 10.1016/j.it.2014.02.009
22. Lin YC, Jhunjhunwala S, Benner C, Heinz S, Welinder E, Mansson R, et al. A Global Network of Transcription Factors, Involving E2A, EBF1 and Foxo1, That Orchestrates B Cell Fate. *Nat Immunol* (2010) 11(7):635–43. doi: 10.1038/ni.1891
23. Lin YC, Benner C, Mansson R, Heinz S, Miyazaki K, Miyazaki M, et al. Global Changes in the Nuclear Positioning of Genes and Intra- and Interdomain Genomic Interactions That Orchestrate B Cell Fate. *Nat Immunol* (2012) 13(12):1196–204. doi: 10.1038/ni.2432
24. Wang HC, Qian L, Zhao Y, Mengarelli J, Adrianto I, Montgomery CG, et al. Downregulation of E Protein Activity Augments an ILC2 Differentiation Program in the Thymus. *J Immunol* (2017) 198(8):3149–56. doi: 10.4049/jimmunol.1602009
25. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E Proteins and Notch Signaling Cooperate to Promote T Cell Lineage Specification and Commitment. *J Exp Med* (2006) 203(5):1329–42. doi: 10.1084/jem.20060268
26. Braunstein M, Anderson MK. HEB-Deficient T-Cell Precursors Lose T-Cell Potential and Adopt an Alternative Pathway of Differentiation. *Mol Cell Biol* (2011) 31(5):971–82. doi: 10.1128/MCB.01034-10
27. Braunstein M, Anderson MK. HEB in the Spotlight: Transcriptional Regulation of T-Cell Specification, Commitment, and Developmental Plasticity. *Clin Dev Immunol* (2012) 2012:678705. doi: 10.1155/2012/678705

28. Miyazaki M, Miyazaki K, Chen K, Jin Y, Turner J, Moore AJ, et al. The E-Id Protein Axis Specifies Adaptive Lymphoid Cell Identity and Suppresses Thymic Innate Lymphoid Cell Development. *Immunity* (2017) 46(5):818–34:e4. doi: 10.1016/j.immuni.2017.04.022
29. D'cruz LM, Knell J, Fujimoto JK, Goldrath AW. An Essential Role for the Transcription Factor HEB in Thymocyte Survival, Tcra Rearrangement and the Development of Natural Killer T Cells. *Nat Immunol* (2010) 11(3):240–9. doi: 10.1038/ni.1845
30. Jones ME, Zhuang Y. Acquisition of a Functional T Cell Receptor During T Lymphocyte Development is Enforced by HEB and E2A Transcription Factors. *Immunity* (2007) 27(6):860–70. doi: 10.1016/j.immuni.2007.10.014
31. Bain G, Cravatt CB, Loomans C, Alberola-Ila J, Hedrick SM, Murre C. Regulation of the Helix-Loop-Helix Proteins, E2A and Id3, by the Ras-ERK MAPK Cascade. *Nat Immunol* (2001) 2(2):165–71. doi: 10.1038/84273
32. Koltsova EK, Ciofani M, Benezra R, Miyazaki T, Clipstone N, Zúñiga-Pflücker JC, et al. Early Growth Response 1 and NF-ATc1 Act in Concert to Promote Thymocyte Development Beyond the Beta-Selection Checkpoint. *J Immunol* (2007) 179(7):4694–703. doi: 10.4049/jimmunol.179.7.4694
33. Lauritsen JP, Wong GW, Lee SY, Lefebvre JM, Ciofani M, Rhodes M, et al. Marked Induction of the Helix-Loop-Helix Protein Id3 Promotes the Gammadelta T Cell Fate and Renders Their Functional Maturation Notch Independent. *Immunity* (2009) 31(4):565–75. doi: 10.1016/j.immuni.2009.07.010
34. Fahl SP, Contreras AV, Verma A, Qiu X, Harly C, Radtke F, et al. The E Protein-TCF1 Axis Controls γδ T Cell Development and Effector Fate. *Cell Rep* (2021) 34(5):108716. doi: 10.1016/j.celrep.2021.108716
35. Cisse B, Caton ML, Lehner M, Maeda T, Scheu S, Locksley R, et al. Transcription Factor E2-2 is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development. *Cell* (2008) 135(1):37–48. doi: 10.1016/j.cell.2008.09.016
36. Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B. Continuous Expression of the Transcription Factor E2-2 Maintains the Cell Fate of Mature Plasmacytoid Dendritic Cells. *Immunity* (2010) 33(6):905–16. doi: 10.1016/j.immuni.2010.11.023
37. Grajkowska LT, Ceribelli M, Lau CM, Warren ME, Tiniakou I, Nakandakari Higa S, et al. Isoform-Specific Expression and Feedback Regulation of E Protein TCF4 Control Dendritic Cell Lineage Specification. *Immunity* (2017) 46(1):65–77. doi: 10.1016/j.immuni.2016.11.006
38. Verykokakis M, Zook EC, Kee BL. ID'ing Innate and Innate-Like Lymphoid Cells. *Immunol Rev* (2014) 261(1):177–97. doi: 10.1111/imr.12203
39. Serafini N, Vosshenrich CA, Di Santo JP. Transcriptional Regulation of Innate Lymphoid Cell Fate. *Nat Rev Immunol* (2015) 15(7):415–28. doi: 10.1038/nri3855
40. Cherrier DE, Serafini N, Di Santo JP. Innate Lymphoid Cell Development: A T Cell Perspective. *Immunity* (2018) 48(6):1091–103. doi: 10.1016/j.immuni.2018.05.010
41. Klose CS, Artis D. Innate Lymphoid Cells as Regulators of Immunity, Inflammation and Tissue Homeostasis. *Nat Immunol* (2016) 17(7):765–74. doi: 10.1038/ni.3489
42. Yang Q, Li F, Harly C, Xing S, Ye L, Xia X, et al. TCF-1 Upregulation Identifies Early Innate Lymphoid Progenitors in the Bone Marrow. *Nat Immunol* (2015) 16(10):1044–50. doi: 10.1038/ni.3248
43. Seehus CR, Aliahmad P, de la Torre B, Iliev ID, Spurka L, Funari VA, et al. The Development of Innate Lymphoid Cells Requires TOX-Dependent Generation of a Common Innate Lymphoid Cell Progenitor. *Nat Immunol* (2015) 16(6):599–608. doi: 10.1038/ni.3168
44. Xu W, Domingues RG, Fonseca-Pereira D, Ferreira M, Ribeiro H, Lopez-Lastra S, et al. NFIL3 Orchestrates the Emergence of Common Helper Innate Lymphoid Cell Precursors. *Cell Rep* (2015) 10(12):2043–54. doi: 10.1016/j.celrep.2015.02.057
45. Klose CS, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of Type 1 ILCs From a Common Progenitor to All Helper-Like Innate Lymphoid Cell Lineages. *Cell* (2014) 157(2):340–56. doi: 10.1016/j.cell.2014.03.030
46. Seillet C, Rankin LC, Groom JR, Mielke LA, Tellier J, Chopin M, et al. Nfil3 is Required for the Development of All Innate Lymphoid Cell Subsets. *J Exp Med* (2014) 211(9):1733–40. doi: 10.1084/jem.20140145
47. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of Type 2 Innate Lymphoid Cells. *Immunity* (2012) 37(4):634–48. doi: 10.1016/j.immuni.2012.06.020
48. Yagi R, Zhong C, Northrup DL, Yu F, Bouladoux N, Spencer S, et al. The Transcription Factor GATA3 is Critical for the Development of All IL-7rα-Expressing Innate Lymphoid Cells. *Immunity* (2014) 40(3):378–88. doi: 10.1016/j.immuni.2014.01.012
49. Constantinides MG, Mcdonald BD, Verhoef PA, Bendelac A. A Committed Precursor to Innate Lymphoid Cells. *Nature* (2014) 508(7496):397–401. doi: 10.1038/nature13047
50. Ebihara T, Song C, Ryu SH, Plougastel-Douglas B, Yang L, Levanon D, et al. Runx3 Specifies Lineage Commitment of Innate Lymphoid Cells. *Nat Immunol* (2015) 16(11):1124–33. doi: 10.1038/ni.3272
51. Califano D, Cho JJ, Uddin MN, Lorentsen KJ, Yang Q, Bhandoola A, et al. Transcription Factor Bcl11b Controls Identity and Function of Mature Type 2 Innate Lymphoid Cells. *Immunity* (2015) 43(2):354–68. doi: 10.1016/j.immuni.2015.07.005
52. Wong SH, Walker JA, Jolin HE, Drynan LF, Hams E, Camelo A, et al. Transcription Factor Rorα is Critical for Nuocyte Development. *Nat Immunol* (2012) 13(3):229–36. doi: 10.1038/ni.2208
53. Hosokawa H, Romero-Wolf M, Yui MA, Ungerbäck J, Quiloan MLG, Matsumoto M, et al. Bcl11b Sets Pro-T Cell Fate by Site-Specific Cofactor Recruitment and by Repressing Id2 and Zbtb16. *Nat Immunol* (2018) 19(12):1427–40. doi: 10.1038/s41590-018-0238-4
54. Jones R, Cosway EJ, Willis C, White AJ, Jenkinson WE, Fehling HJ, et al. Dynamic Changes in Intrathymic ILC Populations During Murine Neonatal Development. *Eur J Immunol* (2018) 48(9):1481–91. doi: 10.1002/eji.201847511
55. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate Production of T(H)2 Cytokines by Adipose Tissue-Associated C-Kit(+)Sca-1(+) Lymphoid Cells. *Nature* (2010) 463(7280):540–4. doi: 10.1038/nature08636
56. Shin SB, Mcnagny KM. ILC-You in the Thymus: A Fresh Look at Innate Lymphoid Cell Development. *Front Immunol* (2021) 12:681110. doi: 10.3389/fimmu.2021.681110
57. Shin SB, Lo BC, Ghaedi M, Scott RW, Li Y, Messing M, et al. Abortive γδtcr Rearrangements Suggest ILC2s are Derived From T-Cell Precursors. *Blood Adv* (2020) 4(21):5362–72. doi: 10.1182/bloodadvances.2020002758
58. Koga S, Hozumi K, Hirano KI, Yazawa M, Terooatea T, Minoda A, et al. Peripheral Pdgfrα(+)Gp38(+) Mesenchymal Cells Support the Differentiation of Fetal Liver-Derived ILC2. *J Exp Med* (2018) 215(6):1609–26. doi: 10.1084/jem.20172310
59. Harly C, Cam M, Kaye J, Bhandoola A. Development and Differentiation of Early Innate Lymphoid Progenitors. *J Exp Med* (2018) 215(1):249–62. doi: 10.1084/jem.20170832
60. Qian L, Bajana S, Georgescu C, Peng V, Wang HC, Adrianto I, et al. Suppression of ILC2 Differentiation From Committed T Cell Precursors by E Protein Transcription Factors. *J Exp Med* (2019) 216(4):884–99. doi: 10.1084/jem.20182100
61. Ferreira ACF, Szeto ACH, Heycock MWD, Clark PA, Walker JA, Crisp A, et al. Rorα is a Critical Checkpoint for T Cell and ILC2 Commitment in the Embryonic Thymus. *Nat Immunol* (2021) 22(2):166–78. doi: 10.1038/s41590-020-00833-w
62. Kernfeld EM, Genga RMJ, Neherin K, Magaletta ME, Xu P, Maehr R. A Single-Cell Transcriptomic Atlas of Thymus Organogenesis Resolves Cell Types and Developmental Maturation. *Immunity* (2018) 48(6):1258–70.e6. doi: 10.1016/j.immuni.2018.04.015
63. Hosokawa H, Romero-Wolf M, Yang Q, Motomura Y, Levanon D, Groner Y, et al. Cell Type-Specific Actions of Bcl11b in Early T-Lineage and Group 2 Innate Lymphoid Cells. *J Exp Med* (2020) 217(1). doi: 10.1084/jem.20190972
64. Hosokawa H, Masuhara K, Koizumi M. Transcription Factors Regulate Early T Cell Development *via* Redeployment of Other Factors: Functional Dynamics of Constitutively Required Factors in Cell Fate Decisions. *Bioessays* (2021) 43(5):e2000345. doi: 10.1002/bies.202000345
65. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early Thymocyte Development is Regulated by Modulation of E2A Protein Activity. *J Exp Med* (2001) 194(6):733–45. doi: 10.1084/jem.194.6.733

66. Pan L, Hanrahan J, Li J, Hale LP, Zhuang Y. An Analysis of T Cell Intrinsic Roles of E2A by Conditional Gene Disruption in the Thymus. *J Immunol* (2002) 168(8):3923–32. doi: 10.4049/jimmunol.168.8.3923
67. Miyazaki M, Rivera RR, Miyazaki K, Lin YC, Agata Y, Murre C. The Opposing Roles of the Transcription Factor E2A and its Antagonist Id3 That Orchestrate and Enforce the Naive Fate of T Cells. *Nat Immunol* (2011) 12 (10):992–1001. doi: 10.1038/ni.2086
68. Teachenor R, Beck K, Wright LY, Shen Z, Briggs SP, Murre C. Biochemical and Phosphoproteomic Analysis of the Helix-Loop-Helix Protein E47. *Mol Cell Biol* (2012) 32(9):1671–82. doi: 10.1128/mcb.06452-11
69. Engel I, Murre C. The Function of E- and Id Proteins in Lymphocyte Development. *Nat Rev Immunol* (2001) 1(3):193–9. doi: 10.1038/35105060
70. D'cruz LM, Lind KC, Wu BB, Fujimoto JK, Goldrath AW. Loss of E Protein Transcription Factors E2A and HEB Delays Memory-Precursor Formation During the CD8+ T-Cell Immune Response. *Eur J Immunol* (2012) 42 (8):2031–41. doi: 10.1002/eji.201242497
71. Shaw LA, Bélanger S, Omilusik KD, Cho S, Scott-Browne JP, Nance JP, et al. Id2 Reinforces TH1 Differentiation and Inhibits E2A to Repress TFH Differentiation. *Nat Immunol* (2016) 17(7):834–43. doi: 10.1038/ni.3461
72. Mowel WK, Mccright SJ, Kotzin JJ, Collet MA, Uyar A, Chen X, et al. Group 1 Innate Lymphoid Cell Lineage Identity Is Determined by a Cis-Regulatory Element Marked by a Long Non-Coding RNA. *Immunity* (2017) 47(3):435–49.e8. doi: 10.1016/j.immuni.2017.08.012
73. Miyazaki K, Miyazaki M. The Interplay Between Chromatin Architecture and Lineage-Specific Transcription Factors and the Regulation of Rag Gene Expression. *Front Immunol* (2021) 12:659761. doi: 10.3389/fimmu.2021.659761
74. Wilson A, Held W, Macdonald HR. Two Waves of Recombinase Gene Expression in Developing Thymocytes. *J Exp Med* (1994) 179(4):1355–60. doi: 10.1084/jem.179.4.1355
75. Kuo TC, Schlissel MS. Mechanisms Controlling Expression of the RAG Locus During Lymphocyte Development. *Curr Opin Immunol* (2009) 21 (2):173–8. doi: 10.1016/j.coi.2009.03.008
76. Hsu LY, Lauring J, Liang HE, Greenbaum S, Cado D, Zhuang Y, et al. A Conserved Transcriptional Enhancer Regulates RAG Gene Expression in Developing B Cells. *Immunity* (2003) 19(1):105–17. doi: 10.1016/s1074-7613(03)00181-x
77. Amin RH, Schlissel MS. Foxo1 Directly Regulates the Transcription of Recombination-Activating Genes During B Cell Development. *Nat Immunol* (2008) 9(6):613–22. doi: 10.1038/ni.1612
78. Dengler HS, Baracho GV, Omori SA, Bruckner S, Arden KC, Castrillon DH, et al. Distinct Functions for the Transcription Factor Foxo1 at Various Stages of B Cell Differentiation. *Nat Immunol* (2008) 9(12):1388–98. doi: 10.1038/ni.1667
79. Schulz D, Vassen L, Chow KT, Mcwhirter SM, Amin RH, Möröy T, et al. Gfi1b Negatively Regulates Rag Expression Directly and *via* the Repression of Foxo1. *J Exp Med* (2012) 209(1):187–99. doi: 10.1084/jem.20110645
80. Timblin GA, Xie L, Tjian R, Schlissel MS. Dual Mechanism of Rag Gene Repression by C-Myb During Pre-B Cell Proliferation. *Mol Cell Biol* (2017) 37(12). doi: 10.1128/mcb.00437-16
81. Yannoutsos N, Barreto V, Misulovin Z, Gazumyan A, Yu W, Rajewsky N, et al. A Cis Element in the Recombination Activating Gene Locus Regulates Gene Expression by Counteracting a Distant Silencer. *Nat Immunol* (2004) 5 (4):443–50. doi: 10.1038/ni1053
82. Hao B, Naik AK, Watanabe A, Tanaka H, Chen L, Richards HW, et al. An Anti-Silencer- and SATB1-Dependent Chromatin Hub Regulates Rag1 and Rag2 Gene Expression During Thymocyte Development. *J Exp Med* (2015) 212(5):809–24. doi: 10.1084/jem.20142207
83. Naik AK, Byrd AT, Lucander ACK, Krangel MS. Hierarchical Assembly and Disassembly of a Transcriptionally Active RAG Locus in CD4(+)CD8(+) Thymocytes. *J Exp Med* (2019) 216(1):231–43. doi: 10.1084/jem.20181402
84. Miyazaki K, Watanabe H, Yoshikawa G, Chen K, Hidaka R, Aitani Y, et al. The Transcription Factor E2A Activates Multiple Enhancers That Drive Rag Expression in Developing T and B Cells. *Sci Immunol* (2020) 5(51). doi: 10.1126/sciimmunol.abb1455
85. Dias S, Månsson R, Gurbuxani S, Sigvardsson M, Kee BL. E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* (2008) 29(2):217–27. doi: 10.1016/j.immuni.2008.05.015
86. Xu W, Carr T, Ramirez K, Mcgregor S, Sigvardsson M, Kee BL. E2A Transcription Factors Limit Expression of Gata3 to Facilitate T Lymphocyte Lineage Commitment. *Blood* (2013) 121(9):1534–42. doi: 10.1182/blood-2012-08-449447
87. Bradney C, Hjelmeland M, Komatsu Y, Yoshida M, Yao TP, Zhuang Y. Regulation of E2A Activities by Histone Acetyltransferases in B Lymphocyte Development. *J Biol Chem* (2003) 278(4):2370–6. doi: 10.1074/jbc.M211464200
88. Eckner R, Yao TP, Oldread E, Livingston DM. Interaction and Functional Collaboration of P300/CBP and bHLH Proteins in Muscle and B-Cell Differentiation. *Genes Dev* (1996) 10(19):2478–90. doi: 10.1101/gad.10.19.2478
89. Massari ME, Grant PA, Pray-Grant MG, Berger SL, Workman JL, Murre C. A Conserved Motif Present in a Class of Helix-Loop-Helix Proteins Activates Transcription by Direct Recruitment of the SAGA Complex. *Mol Cell* (1999) 4(1):63–73. doi: 10.1016/s1097-2765(00)80188-4
90. Qiu Y, Sharma A, Stein R. P300 Mediates Transcriptional Stimulation by the Basic Helix-Loop-Helix Activators of the Insulin Gene. *Mol Cell Biol* (1998) 18(5):2957–64. doi: 10.1128/mcb.18.5.2957
91. Liang JJ, Peng H, Wang JJ, Liu XH, Ma L, Ni YR, et al. Relationship Between the Structure and Function of the Transcriptional Regulator E2A. *J Biol Res (Thessalon)* (2021) 28(1):15. doi: 10.1186/s40709-021-00146-5
92. Rowley MJ, Corces VG. Organizational Principles of 3D Genome Architecture. *Nat Rev Genet* (2018) 19(12):789–800. doi: 10.1038/s41576-018-0060-8
93. Zhu Y, Denholtz M, Lu H, Murre C. Calcium Signaling Instructs NIPBL Recruitment at Active Enhancers and Promoters via Distinct Mechanisms to Reconstruct Genome Compartmentalization. *Genes Dev* (2021) 35(1-2):65–81. doi: 10.1101/gad.343475.120
94. Lio CW, Zhang J, González-Avalos E, Hogan PG, Chang X, Rao A. Tet2 and Tet3 Cooperate With B-Lineage Transcription Factors to Regulate DNA Modification and Chromatin Accessibility. *Elife* (2016) 5. doi: 10.7554/eLife.18290
95. Lio CJ, Shukla V, Samaniego-Castruita D, González-Avalos E, Chakraborty A, Yue X, et al. TET Enzymes Augment Activation-Induced Deaminase (AID) Expression *via* 5-Hydroxymethylcytosine Modifications at the Aicda Superenhancer. *Sci Immunol* (2019) 4(34). doi: 10.1126/sciimmunol.aau7523
96. Hnisz D, Abraham BJ, Lee TI, Lau A, Saint-André V, Sigova AA, et al. Super-Enhancers in the Control of Cell Identity and Disease. *Cell* (2013) 155 (4):934–47. doi: 10.1016/j.cell.2013.09.053
97. Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, et al. Master Transcription Factors and Mediator Establish Super-Enhancers at Key Cell Identity Genes. *Cell* (2013) 153(2):307–19. doi: 10.1016/j.cell.2013.03.035
98. Hnisz D, Shrinivas K, Young RA, Chakraborty AK, Sharp PA. A Phase Separation Model for Transcriptional Control. *Cell* (2017) 169(1):13–23. doi: 10.1016/j.cell.2017.02.007
99. Yoshikawa G, Miyazaki K, Ogata H, Miyazaki M. The Evolution of Rag Gene Enhancers and Transcription Factor E and Id Proteins in the Adaptive Immune System. *Int J Mol Sci* (2021) 22(11). doi: 10.3390/ijms22115888
100. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, et al. The Transcriptional Regulators Id2 and Id3 Control the Formation of Distinct Memory CD8+ T Cell Subsets. *Nat Immunol* (2011) 12(12):1221–9. doi: 10.1038/ni.2158
101. Liu X, Chen X, Zhong B, Wang A, Wang X, Chu F, et al. Transcription Factor Achaete-Scute Homologue 2 Initiates Follicular T-Helper-Cell Development. *Nature* (2014) 507(7493):513–8. doi: 10.1038/nature12910
102. Miyazaki M, Miyazaki K, Chen S, Chandra V, Wagatsuma K, Agata Y, et al. The E–Id Protein Axis Modulates the Activities of the PI3K–AKT–mTORC1–Hif1a and C-Myc/p19Arf Pathways to Suppress Innate Variant TFH Cell Development, Thymocyte Expansion, and Lymphomagenesis. *Genes Dev* (2015) 29(4):409–25. doi: 10.1101/gad.255331.114
103. He R, Hou S, Liu C, Zhang A, Bai Q, Han M, et al. Follicular CXCR5-Expressing CD8(+) T Cells Curtail Chronic Viral Infection. *Nature* (2016) 537(7620):412–28. doi: 10.1038/nature19317
104. Omilusik KD, Nadjsombati MS, Shaw LA, Yu B, Milner JJ, Goldrath AW. Sustained Id2 Regulation of E Proteins is Required for Terminal

Differentiation of Effector CD8(+) T Cells. *J Exp Med* (2018) 215(3):773–83. doi: 10.1084/jem.20171584
105. Milner JJ, Toma C, He Z, Kurd NS, Nguyen QP, Mcdonald B, et al. Heterogenous Populations of Tissue-Resident CD8(+) T Cells Are Generated in Response to Infection and Malignancy. *Immunity* (2020) 52 (5):808–24.e7. doi: 10.1016/j.immuni.2020.04.007
106. Ohkura N, Sakaguchi S. Transcriptional and Epigenetic Basis of Treg Cell Development and Function: Its Genetic Anomalies or Variations in Autoimmune Diseases. *Cell Res* (2020) 30(6):465–74. doi: 10.1038/s41422-020-0324-7
107. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-Beta Induction of Transcription Factor Foxp3. *J Exp Med* (2003) 198 (12):1875–86. doi: 10.1084/jem.20030152
108. Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, et al. The Transcription Factors Blimp-1 and IRF4 Jointly Control the Differentiation and Function of Effector Regulatory T Cells. *Nat Immunol* (2011) 12(4):304–11. doi: 10.1038/ni.2006
109. Cretney E, Kallies A, Nutt SL. Differentiation and Function of Foxp3(+) Effector Regulatory T Cells. *Trends Immunol* (2013) 34(2):74–80. doi: 10.1016/j.it.2012.11.002
110. Wing JB, Sakaguchi S. Multiple Treg Suppressive Modules and Their Adaptability. *Front Immunol* (2012) 3:178. doi: 10.3389/fimmu.2012.00178
111. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The Transcription Factor T-Bet Controls Regulatory T Cell Homeostasis and Function During Type 1 Inflammation. *Nat Immunol* (2009) 10(6):595–602. doi: 10.1038/ni.1731
112. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular Regulatory T Cells Expressing Foxp3 and Bcl-6 Suppress Germinal Center Reactions. *Nat Med* (2011) 17(8):983–8. doi: 10.1038/nm.2426
113. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ Follicular Regulatory T Cells Control the Germinal Center Response. *Nat Med* (2011) 17(8):975–82. doi: 10.1038/nm.2425
114. Wing JB, Tekgüç M, Sakaguchi S. Control of Germinal Center Responses by T-Follicular Regulatory Cells. *Front Immunol* (2018) 9:1910. doi: 10.3389/fimmu.2018.01910
115. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, et al. PPAR-γ is a Major Driver of the Accumulation and Phenotype of Adipose Tissue Treg Cells. *Nature* (2012) 486(7404):549–53. doi: 10.1038/nature11132
116. Arpaia N, Green JA, Moltedo B, Arvey A, Hemmers S, Yuan S, et al. A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* (2015) 162 (5):1078–89. doi: 10.1016/j.cell.2015.08.021
117. Ali N, Zirak B, Rodriguez RS, Pauli ML, Truong HA, Lai K, et al. Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. *Cell* (2017) 169 (6):1119–29.e11. doi: 10.1016/j.cell.2017.05.002
118. Ito M, Komai K, Mise-Omata S, Iizuka-Koga M, Noguchi Y, Kondo T, et al. Brain Regulatory T Cells Suppress Astrogliosis and Potentiate Neurological Recovery. *Nature* (2019) 565(7738):246–50. doi: 10.1038/s41586-018-0824-5
119. Delacher M, Imbusch CD, Hotz-Wagenblatt A, Mallm JP, Bauer K, Simon M, et al. Precursors for Nonlymphoid-Tissue Treg Cells Reside in Secondary Lymphoid Organs and Are Programmed by the Transcription Factor BATF. *Immunity* (2020) 52(2):295–312.e11. doi: 10.1016/j.immuni.2019.12.002
120. Miyazaki M, Miyazaki K, Chen S, Itoi M, Miller M, Lu L-F, et al. Id2 and Id3 Maintain the Regulatory T Cell Pool to Suppress Inflammatory Disease. *Nat Immunol* (2014) 15(8):767–76. doi: 10.1038/ni.2928
121. Sullivan JM, Höllbacher B, Campbell DJ. Cutting Edge: Dynamic Expression of Id3 Defines the Stepwise Differentiation of Tissue-Resident Regulatory T Cells. *J Immunol* (2019) 202(1):31–6. doi: 10.4049/jimmunol.1800917
122. Maruyama T, Li J, Vaque JP, Konkel JE, Wang W, Zhang B, et al. Control of the Differentiation of Regulatory T Cells and T(H)17 Cells by the DNA-Binding Inhibitor Id3. *Nat Immunol* (2011) 12(1):86–95. doi: 10.1038/ni.1965
123. Gao P, Han X, Zhang Q, Yang Z, Fuss IJ, Myers TG, et al. Dynamic Changes in E-Protein Activity Regulate T Reg Cell Development. *J Exp Med* (2014) 211(13):2651–68. doi: 10.1084/jem.20132681
124. Liu C, Wang HC, Yu S, Jin R, Tang H, Liu YF, et al. Id1 Expression Promotes T Regulatory Cell Differentiation by Facilitating TCR Costimulation. *J Immunol* (2014) 193(2):663–72. doi: 10.4049/jimmunol.1302554
125. Wojciechowski J, Lai A, Kondo M, Zhuang Y. E2A and HEB are Required to Block Thymocyte Proliferation Prior to Pre-TCR Expression. *J Immunol* (2007) 178(9):5717–26. doi: 10.4049/jimmunol.178.9.5717
126. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte Selection is Regulated by the Helix-Loop-Helix Inhibitor Protein, Id3. *Immunity* (2000) 12(1):17–26. doi: 10.1016/s1074-7613(00)80155-7
127. Verykokakis M, Boos MD, Bendelac A, Kee BL. SAP Protein-Dependent Natural Killer T-Like Cells Regulate the Development of CD8(+) T Cells With Innate Lymphocyte Characteristics. *Immunity* (2010) 33(2):203–15. doi: 10.1016/j.immuni.2010.07.013
128. Rauch KS, Hils M, Lupar E, Minguet S, Sigvardsson M, Rottenberg ME, et al. Id3 Maintains Foxp3 Expression in Regulatory T Cells by Controlling a Transcriptional Network of E47, Spi-B, and SOCS3. *Cell Rep* (2016) 17 (11):2827–36. doi: 10.1016/j.celrep.2016.11.045
129. Roy S, Moore AJ, Love C, Reddy A, Rajagopalan D, Dave SS, et al. Id Proteins Suppress E2A-Driven Invariant Natural Killer T Cell Development Prior to TCR Selection. *Front Immunol* (2018) 9:42. doi: 10.3389/fimmu.2018.00042
130. Smigiel KS, Richards E, Srivastava S, Thomas KR, Dudda JC, Klonowski KD, et al. CCR7 Provides Localized Access to IL-2 and Defines Homeostatically Distinct Regulatory T Cell Subsets. *J Exp Med* (2014) 211(1):121–36. doi: 10.1084/jem.20131142
131. Frias ABJr., Hyzny EJ, Buechel HM, Beppu LY, Xie B, Jurczak MJ, et al. The Transcriptional Regulator Id2 Is Critical for Adipose-Resident Regulatory T Cell Differentiation, Survival, and Function. *J Immunol* (2019) 203(3):658–64. doi: 10.4049/jimmunol.1900358
132. Han X, Huang H, Gao P, Zhang Q, Liu X, Jia B, et al. E-Protein Regulatory Network Links TCR Signaling to Effector Treg Cell Differentiation. *Proc Natl Acad Sci USA* (2019) 116(10):4471–80. doi: 10.1073/pnas.1800494116
133. Hwang SM, Sharma G, Verma R, Byun S, Rudra D, Im SH. Inflammation-Induced Id2 Promotes Plasticity in Regulatory T Cells. *Nat Commun* (2018) 9(1):4736. doi: 10.1038/s41467-018-07254-2

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REVIEW
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Helix-Loop-Helix Proteins in Adaptive Immune Development

Megan Aubrey[†], *Zachary J. Warburg*[†] *and Cornelis Murre*[*]

Division of Biological Sciences, Section of Molecular Biology, University of California, San Diego, San Diego, CA, United States

The E/ID protein axis is instrumental for defining the developmental progression and functions of hematopoietic cells. The E proteins are dimeric transcription factors that activate gene expression programs and coordinate changes in chromatin organization. Id proteins are antagonists of E protein activity. Relative levels of E/Id proteins are modulated throughout hematopoietic development to enable the progression of hematopoietic stem cells into multiple adaptive and innate immune lineages including natural killer cells, B cells and T cells. In early progenitors, the E proteins promote commitment to the T and B cell lineages by orchestrating lineage specific programs of gene expression and regulating VDJ recombination of antigen receptor loci. In mature B cells, the E/Id protein axis functions to promote class switch recombination and somatic hypermutation. E protein activity further regulates differentiation into distinct CD4+ and CD8+ T cells subsets and instructs mature T cell immune responses. In this review, we discuss how the E/Id proteins define the adaptive immune system lineages, focusing on their role in directing developmental gene programs.

Keywords: HLH, E proteins, Id proteins, VDJ recombination, lymphopoiesis, hematopoiesis, T cell development, B cell development

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***Correspondence:**
Cornelis Murre
cmurre@ucsd.edu

[†]*These authors have contributed equally to this work and share first authorship*

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BACKGROUND

Decades of research have demonstrated the essential role of E proteins in mediating both innate and adaptive immune cell development and the wide implications of E protein activity in disease progression and immune response. In mammals, E proteins include E2A, E2-2 and HEB (1). E proteins are members of the helix-loop-helix (HLH) family of transcription factors. E proteins either homo- or heterodimerize with other HLH proteins to bind to E-box sites (CANNTG) through a basic region to modulate the expression of nearby and distal genes. This activity is opposed by Id proteins, which lack a basic DNA binding region and heterodimerize with E proteins to prevent them from binding to DNA. Together, E and Id proteins form an E-Id axis to instruct immune development.

This review discusses the role of the E-Id axis in adaptive immune development. These proteins are expressed in all mammalian cell types. They are regulated both transcriptionally and post-transcriptionally to orchestrate the development of an armamentarium of immune cell types and to establish a diverse immune repertoire. We focus on how appropriately timed differentiation to T and B cell fates is achieved while discussing how the development of alternative cell fates is suppressed.

E-ID AXIS IN EARLY HEMATOPOIESIS

Adaptive immune development begins in the fetal liver and in the bone marrow in adults, where E and ID proteins influence developmental decisions in hemopoietic stem cells (HSCs), which give rise to all blood cells (**Figure 1**). Differentiation to HSCs is achieved by the E protein SCL/TAL1, and maintained by E2A proteins and their repressive heterodimerizing HLH partners Lyl1 and Id1 (2–5). These factors also set the stage for the ratio of progenitors giving rise to B and T cells. E proteins oppose proliferation of HSCs, priming their expression to promote lymphoid-associated gene expression (6, 7). This activity promotes their differentiation into multipotent progenitors (MPPs) and further into lymphoid-primed MPPs (LMPPs), while preventing granulocyte-monocyte progenitor (GMP) development and partially restricting megakaryocyte-erythrocyte progenitor (MEP) development (6). As a result, E2A-deficient mice are associated with reduced HSCs and MPPs (3). Other E proteins, E2-2 and HEB, were found to be expendable at this early stage of development (8–10). Thus, E protein activity orchestrates a supportive transcriptional landscape for lymphocyte development in HSCs.

Id protein inhibition of E protein activity might play a role in generating a diverse immune repertoire from HSCs. E2A promotes HSC differentiation and represses proliferation by controlling the expression of p21 and Notch1 (11–14). E proteins further drive differentiation to LMPPs to common lymphoid progenitors (CLPs), which give rise to several cell fates including B cells, T cells, dendritic cells, innate lymphoid cells, and natural killer (NK) cells (10, 15). In the absence of E2A expression, fewer LMPPs progress to the CLP stage (6). The cells that do progress preferentially feed alternative lineages seeded by GMPs, MEPs, and Pre-MegE-progenitors (7). In the absence of E2A and HEB, CLPs are compromised in their ability to express an early lymphoid program (10). The capacity of CLPs to differentiate into NK, B or T lineages may be further divided by their expression of different surface markers (16–19). A recent study suggested that heterogeneous levels of E and Id proteins in CLPs may contribute to these unique differentiative capabilities (19). Thus, fine tuning of E protein activity in CLPs instructs immune cell fate.

EARLY B CELL DEVELOPMENT

E proteins orchestrate B cell development by defining signaling pathways in CLPs (**Figure 2**). E2A activity is required to activate Ebf1 and IL7 receptor protein (IL7Rα) in CLPs, which together with E2A activate Pax5 (20–23). E2A proteins also act in concert with Ebf1 to induce Foxo1 expression (24). Subsequently, E2A and HEB coordinate with Foxo1, Pax5 and Ebf1 to support the progression of CLPs through the B cell lineage (23, 25). Aberrant Id3 expression at

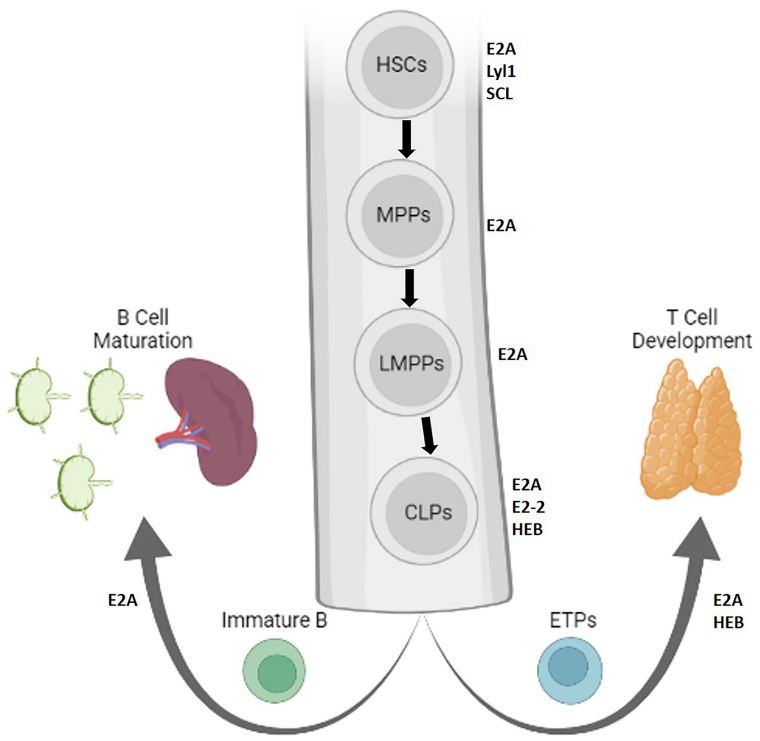

FIGURE 1 | Lymphopoiesis is directed by E protein activity in early stem cells. The role of E and Id proteins in early progenitors giving rise to B and T cells is depicted in the bone marrow. Protein factors that support stem cell maintenance or self-renewal are indicated adjacent to each cell, and bolded arrows represent lineage differentiation (Created with BioRender.com).

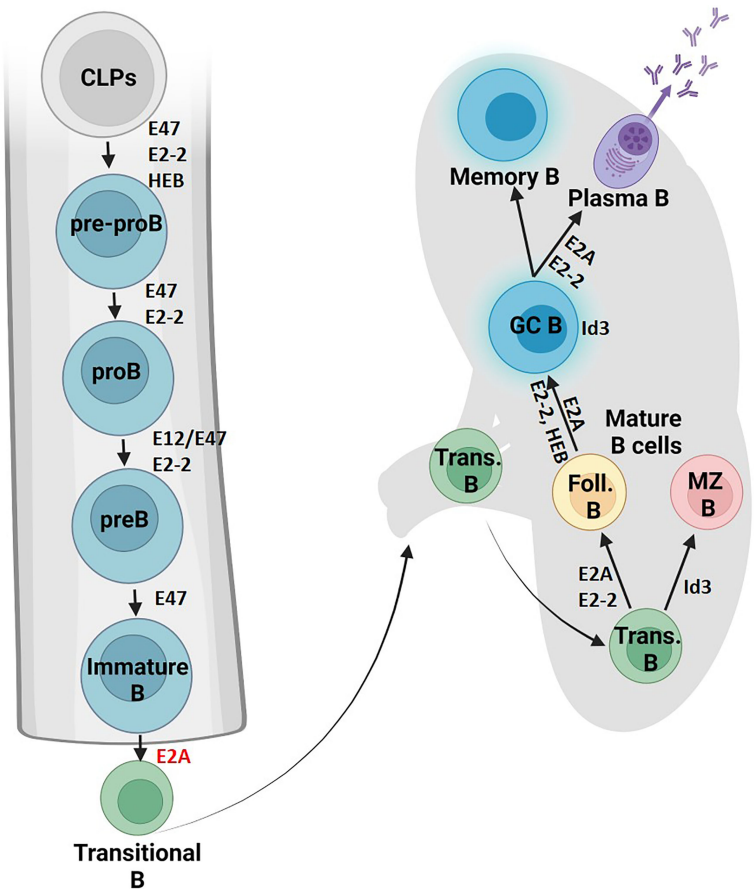

FIGURE 2 | B cell fate and differentiation is directed by E protein activity. The role of E proteins and their antagonists in B cell development is shown in the bone marrow and lymphoid organs. Protein factors supportive of differentiation or cell maintenance are indicated in black font next to the arrows or adjacent to the cell respectively. Protein factors with repressive functions are indicated in red font (Created with BioRender.com).

this earlier stage alternatively arrests B cell development and prevents IL7Rα induction, later inducing caspase-mediated apoptosis (26). Cytokine signaling from the TGF-β family represents one mechanism by which Id3 expression is regulated (27). Thus, E proteins promote B cell development from CLPs by priming a B-cell transcriptional network.

E proteins then stimulate the subset of CLPs primed with B cell lineage genes to progress into pre-pro-B cells. Studies have implicated that the E2A isoform E47 is required to get past this stage, while E12 is dispensable (28–31). E2-2 and HEB also both contribute to early B cell development (32). Notably, E2-2 mRNA expression is particularly high in pro-B cells and orchestrates the developmental maturation of these early B cell progenitors into pre-B cells (33).

In the B cells, E proteins are regulated by lineage specific post-transcriptional mechanisms. E47 homodimers, for example, are only detected at high levels in B cells (34). This cell specific homo-dimer activity may involve phosphorylation of specific residues of the E2A proteins (34, 35). Histone acetyltransferases including p300, CBP, and PCAF interact with E2A to mark the epigenetic chromatin landscape (36, 37). Further, miRNAs regulate E protein activity. A recent study identified miR-191 as a rheostatic regulator of B cell development to modulate E2A mRNA abundance from pro-B to immature B cells (38).

E proteins regulate gene expression by coordinating changes in nuclear architecture. E2A occupancy at the Ebf1 locus is associated with relocation away from the nuclear lamina in pro-B cells (39). E2A binding at or near enhancers or promoters was associated with deposition of active chromatin markers such as H3K4me1 with further activating epigenetic alterations between pre-pro-B to pro-B cells (40). Activated genes with E2A occupancy frequently contained coordinated DNA binding with Ebf1, Foxo1 and CTCF. Recent studies indicated that E2A occupancy is closely associated with recruitment of members of the cohesin complex (41). In parallel studies it was revealed that H3K27Ac marked enhancers are closely associated with recruitment of cohesin (42–44).

Collectively, these studies suggest that E2A may act, at least in part, by initiating loop extrusion across the enhancer landscape. Other mechanisms may also act with E2A to promote B cell

development. Notably, E2A recruits Tet2 and Tet3 to promote chromatin accessibility adjacent to E-box binding sites in pro-B cells (45). Future studies are warranted to determine how E2A mediated changes in DNA methylation and recruitment of cohesin are linked to induce lineage specific gene programs.

E proteins may also be essential in preventing the premature progression of B cells from pre-pro-B into pro-B cells. E2A enforces this checkpoint by binding an E-box element in a p21 regulatory region, which encodes a CDK inhibitor and induces cell cycle arrest (11). This checkpoint can be circumvented by repression of E protein activity by Id1-3 (11, 26). The downregulation of E protein activity by rapid induction of Id proteins occurs upon successful heavy chain V-DJ rearrangement in pro-B cells, and it will be important to establish whether and how alterations in Id3 protein levels modulate E2A activity to orchestrate antigen receptor assembly (28).

GENERATION OF B CELL DIVERSITY

Somatic recombination events in the B cell lineage generate a diverse antibody repertoire. B cells rearrange the variable regions of their immunoglobulin heavy chain (Igh) and immunoglobulin light chain (Igk or IgL) loci to produce mature B cells with unique antigen binding specificities. E2A regulates these recombination events by controlling appropriate expression of the Rag genes, as well as chromatin accessibility and 3D spatial organization of the Igh and Igk loci. Following these recombination events, B cells can undergo class switch recombination (CSR) and somatic hypermutation (SHM) to generate antigen receptors with higher affinities for their cognate antigens. E proteins regulate CSR and SHM by promoting chromatin accessibility at the targeted immunoglobulin (Ig) genes and by controlling the expression of key enzymes involved in these processes.

E2A Regulates Rag Expression in the B Cell Lineage

Recombination is catalyzed by the recombinase activating genes, Rag1 and Rag2 (46, 47). Rag1/2 expression peaks twice in the B cell linage, first in pro-B cells during Igh rearrangement, when E47 expression is high. Rag expression is then downregulated as cells pass through the pre-BCR checkpoint and transition to pre-B cells (48). E protein activity declines during this time, as pre-BCR signaling upregulates Id3 expression and E47 protein levels decline (49, 50). Rag and E47 protein levels are elevated again in pre-B cells undergoing Ig light chain rearrangements (48, 49). In E2A deficient mice, B cell development is blocked at the pre-pro-B cell stage and Igh rearrangements fail to initiate due to lack of Rag activity (31, 51). E2A regulates Rag expression in a dose dependent manner (49).

The Rag1/2 genes share a single genetic locus. An evolutionarily conserved B cell specific enhancer of Rag (*Erag*) contains E-box binding sites. Deletion of *Erag* in mice reduces Rag1/2 expression and compromises D_h-J_h and V_h-D_hJ_h recombination (52). Recent findings indicate that E2A directly regulates Rag1/2 gene expression in pro-B cells by binding to the Rag promoters and enhancer and orchestrating chromatin conformations that promote a transcriptionally active Rag locus (41). E2A also binds two additional B cell specific regulatory elements (R1B and R2B), which partially overlap with *Erag*. R1B and R2B orchestrate a B cell specific chromatin architecture at the Rag1/2 gene cluster. Deletion of these E2A binding elements resulted in reduced chromatin accessibility of the Rag1/2 genes, a loss of genomic interactions across the locus, reduced Rag1/2 expression, a significant developmental block at the pro-B cell stage, and severely compromised Igh recombination. Further, E2A directly regulates the Rag1 promoter in pro-B cells. Specific mutation of all 7 E-box sites in the Rag1 promoter (R1pro-E-box$^{mut/mut}$), resulted in a loss of chromatin accessibility at the Rag1 gene and reduced Rag1 gene expression. R1pro-E-box$^{mut/mut}$ mice are phenotypically similar to Rag$^{-/-}$ mice, have severely compromised Igh V-D_hJ_h recombination, and exhibit a developmental block at the pro-B cell stage (41).

Recombination of the Igh Locus

The immunoglobulin heavy chain locus is comprised of V_h, D_h, and J_h genes, which recombine in a step-wise manner. D_h to J_h recombination occurs first, and is followed by V_h to D_hJ_h recombination (53). E2A regulates $V_h(D_h)J_h$ recombination by promoting chromatin accessibility at the Igh locus. Early studies found that ectopic expression of either E2A gene product (E12 or E47) along with Rag1/2 in non-B lineage cells is sufficient to initiate Igh germline transcription (GLT) and D_h to J_h recombination (but not V_h to D_hJ_h recombination) (30, 54, 55). E2A initiates and maintains Pax5 expression in pro-B cells and cooperates with Pax5 to promote further chromatin accessibility and allow V_h to D_hJ_h recombination (29). Ectopic expression of Pax5 with Rag1/2 and E2A in non-lymphoid cells is sufficient to induce V_h to D_hJ_h recombination (56). Interestingly, enforced Pax5 expression restores Rag expression and D_h-J_h recombination at the heavy chain locus in Vav-CRE E2A$^{fl/fl}$ mice, even though the Rag1 promoter is directly regulated by E2A (29, 41).

E2A is also essential for Igh locus contraction (57, 58). Prior to $V_h(D_h)J_h$ recombination, the Igh locus repositions from the lamina to the nuclear interior and undergoes contraction to bring V_h and D_hJ_h genes into close physical proximity (59). This compaction allows V_h and D_hJ_h genes to adopt a wider spectrum of configurations in pro-B cells, to promote a higher diversity of V_h genes in the antibody repertoire (58). E2A directly binds to PAIR elements, regulatory elements in V_h region that facilitate locus contraction (60–63). The role of E2A binding at PAIR elements is not yet clear but it likely involves recruitment of the cohesin machinery to initiate loop extrusion across the Igh locus.

Expression of a pre-B cell receptor (pre-BCR) composed of a rearranged Igh protein and a surrogate light chain (SCL) is a developmental checkpoint that monitors for successful rearrangement of an Igh allele. Pre-BCR signaling indicating a productive Igh chain has recombined enforces allelic exclusion of the Igh locus. Pre-BCR mediated regulation of E2A might be important for downregulation of the SLC genes, as well as other

pre-BCR co-receptors and downstream signaling proteins (64, 65).

Recombination of the Igk Locus

The roles of E2A in orchestrating recombination of the immunoglobulin kappa (Igk) locus have been extensively studied. The E2A proteins were initially identified in a screen for factors that bind sites across the kappa locus intronic enhancer (iEκ) (66). Analogous to its role in Igh recombination, E2A promotes chromatin accessibility at the Igk locus. Ectopic E2A expression, along with Rag1/2, is sufficient to induce Igk germline transcription and V_k to J_k rearrangements in non-lymphoid cells (55). While forced Pax5 expression restored D_h-J_h recombination at the Igh locus in $E2A^{-/-}$ mice, it did not rescue V_k-J_k rearrangements, indicating a unique role for E2A in promoting Igk locus assembly (29).

E2A proteins directly bind sites across the Igk locus to recruit the histone acetyltransferases CBP and p300 (67, 68). E2A may increase the rearrangement frequencies of V_k genes by promoting their transcription. Promoters bound by E2A or that contain E2A binding sites are associated with strong promoters and are expressed at significantly higher frequencies compared to overall V_k genes (39, 67, 69). Another possible mechanism may involve recruitment of cohesin to instruct loop extrusion at enhancers across the Igh locus akin to that described above for the Igh locus. The Igk locus contains an ensemble of enhancers including, the intronic enhancer (iEκ) and the 3' enhancer (κE3'), that regulate V_k-J_k rearrangement (70–72). E2A binding to iEκ is essential for enhancer activation and regulates the appropriate developmental timing of Igk recombination (73–75). Mutation of two of the three E-box sites in iEκ resulted in the same reduction in Igk rearrangement as that with deletion of the entire iEκ enhancer (73). Before initiating light chain rearrangements, large cycling pre-B cells attenuate their IL-7/STAT5 signaling, cell cycle exit and transition into resting small pre-B cells. IL-7/STAT5 signaling negatively regulates Igk recombination by antagonizing E2A binding at iEκ (74, 75). Similar mechanisms regulate the activation of kE3'. Developmental control of the kE3'enhancer involves both active stimulation by PU.1, IRF4, and E2A and repression by STAT5. STAT5 signaling reduces kE3' activity in pro-B cells, possibly by blocking PU.1 recruitment to the enhancer, as STAT5 and PU.1 competitively bind to the enhancer (76). Pre-BCR signaling induced IRF4 promotes kE3' activation by cooperatively binding to the enhancer with E2A and by rendering kE3' activity insensitive to STAT5 (76–79). E2A and PU.1 recruit the TET proteins to kE3' where they promote increased chromatin accessibility by facilitating DNA demethylation (45). Proper developmental timing of Igk locus demethylation appears critical for appropriate Igk recombination. Proximal V_k gene promoters and kE3' were hypomethylated in mice in which the *de novo* methyltransferases Dnmt3a and Dnmt3b were deleted. These mice undergo premature Igk rearrangements, have increased Igk rearrangement frequencies, and over-utilize their most proximal V_k genes (80).

The Igk locus is poised for V_kJ_k recombination in pro-B cells, where it already exhibits signs of chromatin accessibility and has already undergone large scale locus contraction. The Igk locus repositions to the permissive compartment and contracts at the pre-pro-B to pro-B developmental cell transition. During this transition, the intronic enhancer (iE_k) forms extensive contacts with V_k genes across the locus that are associated with E2A occupancy. These changes in chromatin conformation are accompanied by increased Igk transcription, widespread H3K4 demethylation, and E2A binding across the locus (39, 81, 82). In response to pre-BCR signaling, the locus further contracts. E2A occupancy at the locus increases and kE3' forms stronger chromatin interactions with the V_k region (**Figure 3**). Interactions between kE3' and Igk flanking regions are reduced, while interactions between kE3' and V_K genes that are located close to E2A binding sites increase. There are strong positive correlations between presence of E2A binding sites, V_k gene usage, and long-range chromatin interaction frequencies between V_k genes and the kappa regulatory elements, which suggest that E2A is a key factor in Igk locus contraction (82).

In conclusion, much has been learned about the roles of E2A in orchestrating Igk locus rearrangement. In our view the most appealing mechanism is that the E2A proteins bind enhancer elements across the Igk locus to deposit H3K27Ac across the enhancer repertoire. The deposition of H3K27Ac may then act to sequester chromatin remodelers like Brg1 that in turn sequester cohesin to initiate loop extrusion (42). Thus, a common theme is now emerging in which transcription factors, like E2A, sequester cohesin to promote large-scale alterations in chromatin folding, enabling V_k regions to encounter J_k elements with distinct frequencies that are independent of genomic separation.

MATURE B CELL DEVELOPMENT

After successful VDJ rearrangement and receptor editing of the Ig light chain genes, E and Id proteins further instruct the development of pre-B cells. This transition is mediated by upregulation of Id3 and a reduction in E protein abundance triggered by BCR signaling (49). E47 levels therefore decline in transitional B cells followed by a near complete loss of E47 expression in mature B cells. Genetic studies showed that high levels of E2A promote follicular B cell development while high Id3 abundance favors the marginal zone B cell fate (33, 49). E2-2 serves an overlapping role controlling this developmental decision as revealed by the transfer of E2A- and E2-2-deficient fetal liver cells into irradiated Rag-deficient mice (33). E protein activity is also essential for the development of germinal center and plasma cells (83). Likewise, in the absence of Id3 expression germinal center B cell development is severely affected (84). Specifically, when researchers abrogated Id3 expression in germinal center B cells, the expression of genes encoding for components of antigen receptors, cytokine receptors, and chemokine receptors was severely perturbed (83). E2A and E2-2 activity is also essential for the developmental progression of plasma cells (29, 84, 85). E2A and E2-2 promote plasma cell identity by directly activating Blimp1 and Xbp1 expression (84, 85). Together these studies show that HLH proteins play

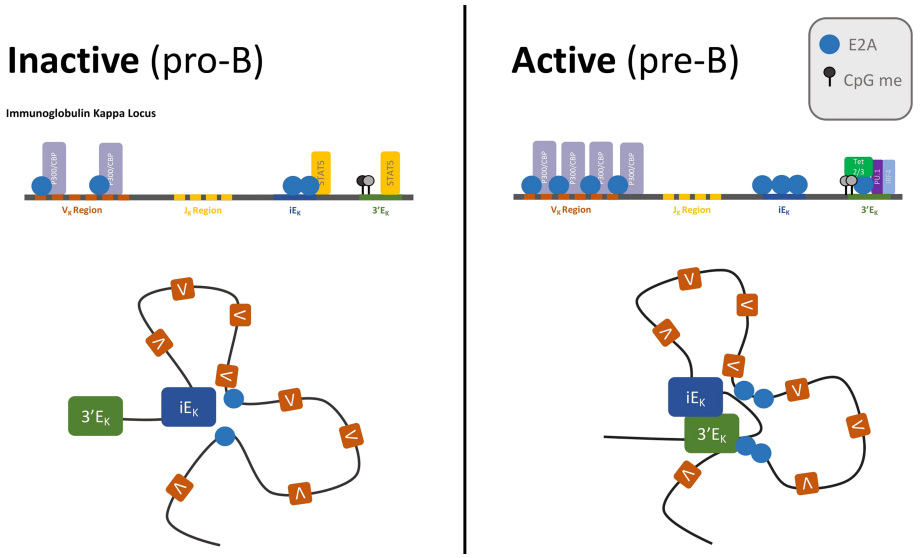

FIGURE 3 | Enhancer activation by E2A drives IgK recombination.The IgK locus is poised for recombination in pro-B cells—when E2A is bound to V_K genes, V_K regions have acetylated histone marks, and the locus is already contracted, with iE_K making extensive contacts with the V_K region. Activation of the locus coincides with E2A activation of the IgK enhancers. Pre-BCR signaling and IL-7/STAT5 attenuation render the iE_K and $3'E_K$ enhancers insensitive to STAT5. E2A occupancy at the enhancers increases, and the Tet proteins are recruited to $3'E_K$ where they demethylate CpG residues. The now accessible $3'E_K$ enhancer forms extensive contacts with the V_K region.

instrumental roles in orchestrating the response of B cells to exposure of infectious agents.

Class Switch Recombination and Somatic Hypermutation

Activation of mature naïve B cells initiates class switch recombination (CSR) and somatic hypermutation (SHM). E proteins regulate both CSR and SHM by 1) transcriptionally regulating key factors involved in these processes, 2) interacting directly with CSR and SHM proteins and targeting them to Ig genes, and 3) by increasing the chromatin accessibility of Ig genes.

The enzyme activation induced cytidine deaminase (AID) is required for both CSR and SHM. AID deaminates cytosine bases to uracils. In CSR, the DNA repair factor UNG then excises these uracil bases and DNA repair factors convert these SSBs to DSBs (86). In SHM, mutations are generated by a variety of error prone DNA repair mechanisms that are employed to repair the mismatched U:G bases (87). E2A and E2-2 directly promote expression of AID by binding to regulatory elements in the *Aicda* locus (the AID gene) and increasing chromatin accessibility of enhancer elements (85, 88, 89). Loss of E protein activity in activated B cells inhibits CSR, due in part to loss of AID expression (85, 90, 91). CSR to IgG1 expression is blocked in in E2A/E2-2 DKO mice due to loss of AID expression (85). Overexpression of Id2 reduces AID expression in activated B cells (92). However, a balance of E protein activity must be maintained for normal CSR, as Id2 also plays an inhibitory role in CSR. Id2 deficient B cells undergo CSR to IgE at a much high frequency than that of wild-type B cells (93).

E2A proteins also bind directly to Ig genes to promote SHM and CSR. E2A forms a complex with AID, Pax5, ETS1 and IRF4 that functions to target AID to sites within the Igh locus (94, 95). E2A and E2-2 promote CSR by opening chromatin at the 3'RR enhancer and activating GLT of switch regions. E2A/E2-2 DKO mice have impaired CSR to IgE, due to loss of activation of the 3'RR enhancer and IgE GLT (85). Further, E-box binding sites within Ig enhancers promote efficient SHM (96–99). E2A may help direct AID to DNA, and genome wide E2A occupancy is associated with AID targeting (99). Finally, we suggest that E2A proteins act to promote CSR and SHM by initiating loop extrusion across the switch regions and V gene segments and note that E2A likely plays an additional role in promoting phase separated droplets to orchestrate CSR and SHM.

REGULATION OF EARLY T CELL DEVELOPMENT BY HLH PROTEINS

A fraction of LMPPs develops into early T progenitors (ETPs) that then home to the thymus (**Figure 4**). E2A and HEB promote homing by modulating chemokine receptor expression, including CXCR4, to direct thymocytes to the cortex (100). Here, ETPs encounter Delta-class Notch ligands. An ensemble of genes involved in Notch signaling are directly activated by E47 (21). Together with E2A, Notch signaling prevents the activation of B-lineage and myeloid factors and promotes T lineage development. These functions are opposed by Id1 and Id2 expression in these early progenitors and in double negative (DN) T cells, promoting an innate lymphoid fate instead. T cell

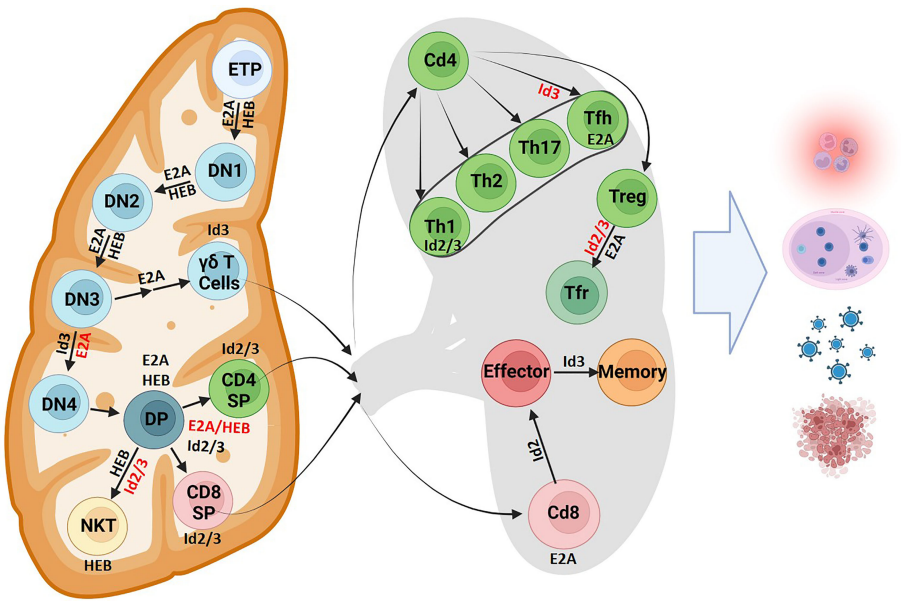

FIGURE 4 | T cell fate and differentiation is directed by E protein activity. The role of E proteins and their antagonists in T cell development is shown in the thymus and lymphoid organs. Protein factors supportive of differentiation or cell maintenance are indicated in black font next to the arrows or adjacent to the cell respectively. Protein factors with repressive functions are indicated in red font (Created with BioRender.com).

progression was blocked in CLPs with disrupted E2A and HEB activity, instead favoring differentiation to alternative lineages (101). Once ETPs migrate to the thymus, E2A and HEB, in coordination with Notch signaling instruct further development (21, 32, 102–104). In developing thymocytes, E proteins modulate the expression of gene programs including those involved in cell cycle progression, pre-TCR signaling and cytokine gene expression (105). Prominent amongst the genes activated by E47 expression are CDK6, Socs1/2, Ets, Foxo1, and GATA3. The E2A proteins may act coordinately with Bcl11b, another key factor known to establish T cell identity, to activate a common set of target genes (106, 107). Researchers have found that E2A binds regulatory elements in a distal long non-coding RNA, ThymoD (108). ThymoD transcription is initiated from within the Bcl11b intergenic region where it acts to promote T cell commitment by repositioning the Bcl11b enhancer from a heterochromatic environment at the lamina to the euchromatic compartment located in the nuclear interior (107, 108). The overlapping gene expression profiles between E2A, Bcl11b, and ThymoD knockout mice combined with the evidence of E2A binding to elements within ThymoD implicates the possibility that E2A could indirectly initiate Bcl11b expression by modulating non-coding transcription. A prominent Bcl11b target in developing thymocytes is Id2. Interestingly, the majority of genes regulated by Bcl11b are also modulated by Id2 (104, 109). HEB also performs multiple, unique functions in thymocyte development. Elegant studies revealed that an alternatively spliced form of HEB, named HEBAlt, increases the development of T cell progenitors (110, 111). Subsequent studies showed that in the absence of HEB T cell progenitors adopt alternative cell fates (112). HEB also directly activates the expression of pre-Tα, a component of the pre-TCR complex (113). Thus, a detailed picture is now emerging in which E2A and HEB act collaboratively to orchestrate the development of early T cell progenitors.

GENERATION OF T CELL DIVERSITY

T lymphocytes rearrange their TCRα, TCRβ, TCRγ and TCRδ loci to generate diverse T cell receptor repertoires. T cell receptor rearrangements initiate at the $CD4^-$ $CD8^-$ double negative (DN) stage of thymocyte development. TCRβ, TCRγ and TCRδ rearrangements all occur simultaneously in DN2 cells. Successful rearrangement of TCRγ and TCRδ loci results in expression of a γδ TCR and potential development into the γδ T cell lineage. Successful rearrangement of a TCRβ chain results in expression of a pre-TCR. Pre-TCR signaling allows progression to the DN4 cell stage where TCRα rearrangements occur, so that cells may become αβ T cells. The E-ID axis plays critical roles in T cell development by regulating rearrangement events at all four TCR loci, orchestrating the αβ versus γδ cell fate decision, and by enforcing key developmental checkpoints.

αβ/γδ T Cell Lineage Decisions

The E-ID axis determines whether cells adopt the αβ or γδ T cell fate. γδ TCR signaling strength is a critical determinant in the choice to become αβ or γδ T cells. Stronger γδTCR signals favor lineage commitment to the γδ T cell fate, while weaker γδ TCR signals favor commitment to the αβ T cell fate (114, 115).

γδTCR signals mediate lineage decisions through activation of the Erk-Egr-Id3 pathway (114, 116). In response to TCR signaling, Egr induces a level of Id3 expression that is proportional to the TCR signaling strength, and Id3 expression levels correlate with commitment to the γδ cell fate. Cells committed to the γδ cell fate display higher levels of Id3 expression (114). Id3 plays a critical role in γδ/αβ lineage fate decision. Loss of Id3 decreases the number of γδTCR+ cells, however Id3 overexpression does not increase the number of γδTCR+ cells or influence their maturation (114). Thus, Id3 is necessary but not sufficient to drive the γδ cell fate. Egr acts upstream of Id3 to promote the γδ cell fate, but likely also effects other pathways besides Id3 to promote γδ T cell development, as Egr1 overexpression is sufficient to increase the frequency of γδ T cell (114, 116). The increase in γδ T cells depends in part, on Id3 activation, as the Egr1 overexpression phenotype is diminished in an Id3 deficient background (116). Id3 regulates the γδ/αβ lineage fate decision by promoting the survival of γδ T cells and repressing the survival of αβ T cells in response to strong TCR signals. In response to a strong γδ TCR signal, Id3 deficiency increases the expression of the anti-apoptotic protein Bcl-XL and the survival of cells committed to the αβ lineage, while reducing expression of the anti-apoptotic protein Bcl-2 in mature γδ T cells (116).

The E-ID Axis Regulates Rag Expression in the T Cell Lineage

There are two waves of Rag expression in developing T cells. The first wave peaks in DN cells at a time when the TCRβ, TCRδ, and TCRγ loci rearrange. The second wave peaks in double positive (DP) T cells during the course of TCRα rearrangements (117). The E-Id protein axis is critical to coordinate these waves of Rag expression. The E proteins, E2A and HEB, positively regulate the expression of Rag1 and Rag2 in DN and DP cells (30, 37, 118). Many studies have sought to characterize the cis-regulatory elements that regulate Rag expression in thymocytes (41, 119, 120). Rag1 and Rag2 share a single genetic locus and their expression in T cells depends on two overlapping cis regulatory elements, the Rag-T cell enhancer (*R-TEn*) and the anti-silencer element (ASE). E2A directly binds to E-boxes in *R-TEn*, which is located within ASE, as well as the Rag1 promoter and upregulates Rag1/2 expression by coordinating or maintaining the assembly of a transcriptionally active chromatin hub at the Rag locus in both DN and DP cells. Deletion of *R-TEn* induces developmental blocks at the DN3 and DP cell stages (41, 120). Following productive TCR rearrangements, Id3 protein expression is upregulated in response to pre-TCR signaling, positive selection and γδ TCR signaling (116, 121, 122). Enforced expression of Id3 in T cell progenitors reduces levels of Rag1 and Rag2 (123). These studies suggest that Id proteins function to promote allelic exclusion by antagonizing E2A binding at the Rag locus, which downregulates Rag1/2 expression and thus prevents further TCR rearrangements (124).

TCRβ Rearrangement

In addition to positively regulating Rag expression, high E protein activity in early thymocyte development promotes TCRβ rearrangements by increasing chromatin accessibility of the locus. The murine TCRβ locus is composed of V_β, D_β, and J_β genes. The locus recombines in a step-wise manner, with D_β to J_β rearrangement occurring before V_β to D_β J_β rearrangement (125). Recombination of the TCRβ locus is dependent on the TCRβ enhancer (Eβ), which drives germline transcription at and promotes chromatin accessibility of the D_β-J_β gene clusters (126–129). In DN thymocytes, E2A binds to conserved E-box binding sites in Eβ, the Dβ2 promoters, and the majority of Vβ promoters and drives germline transcription from Vβ promoters as well as H3 histone acetylation at Vβ, Dβ and Jβ genes in dosage dependent manners, likely by directly binding to and recruiting the histone acetyl transferases CBP and p300 (37, 68, 130, 131).

E2A deficient and null mice have reduced numbers of thymocytes, exhibit a partial block in thymocyte development at the DN1 stage, and display gene dosage dependent deficiencies in both D_β-J_β and V_β-$D_\beta J_\beta$ rearrangements (37, 103, 132). HEB plays a modest role in TCRβ recombination. HEB deficient mice show dosage independent deficiencies in V_β germline transcription, and do not display a partial developmental block until the ISP stage (113, 131). It is possible that the modest defects in rearrangement seen in E2A and HEB deficient mice are caused by loss of a single E protein being compensated for by homodimers of the remaining E-protein (133, 134). Studies designed to address concerns of compensation generated mice with double conditional knockouts of HEB and E2A at an early stage in lymphocyte development ($HEB^{fl/fl}$ $E2A^{fl/fl}$ $Lck^{+/Cre}$), as well as mice that express a dominant negative HEB gene ($HEB^{bm/bm}$), which contains a mutation in the DNA binding region of HEB and forms non-functional heterodimers with E2A (134, 135). Studies with these mice confirmed that E2A and HEB can partially functionally compensate for one another. Both $HEB^{fl/fl}$ $E2A^{fl/fl}$ $Lck^{+/Cre}$ and $HEB^{bm/bm}$ exhibit severe developmental blocks at the DN stage, and $HEB^{bm/bm}$ show severely impaired V_β-$D_\beta J_\beta$ rearrangements (134, 135). Together, these data show that the E-proteins play essential and overlapping roles in controlling TCRβ locus assembly.

β-Selection

Successful rearrangement of a TCRβ chain results in expression of a pre-TCR containing the TCRβ chain and a surrogate light chain TCRα (pre-Tα). Pre-TCR signaling indicates rearrangement of a productive TCRβ allele, ensures allelic exclusion by blocking further TCRβ rearrangement, and allows cells to transition past the β-selection checkpoint and develop into DN4 and DP cells. The E-Id axis acts on many levels to regulate proper development at the β-selection checkpoint. E47 and HEB regulate pre-Tα expression in a dose sensitive manner (135, 136). E2A and HEB double conditional knockout mice exhibit a severe developmental block at the DN3 stage, exhibit normal TCRβ rearrangements, but have reduced pre-Tα expression, suggesting that this block could be due to lack of pre-Tα protein (135).

The E-Id axis also regulates the proliferation of thymocytes before and after β-selection. Prior to β-selection, E2A activity suppresses IL-7 induced proliferation. DN3 cells engaging in TCRβ rearrangement are cell cycle arrested in G1. After

productive TCRβ rearrangement, pre-TCR signaling induces many rounds of proliferation as cells transition to DN4 and DP stages. HEB and E2A are necessary to keep DN3 cells in a low or non-proliferating state prior to pre-TCR signaling (135, 137, 138). Pre-TCR signaling inhibits E protein activity primarily by inducing expression of Id3 and by promoting E2A degradation. This loss of E protein activity then allows proliferative expansion of DP thymocytes. Upregulation of Id3 and silencing of E protein activity functions to ensure allelic exclusion by reducing E2A occupancy at the TCRβ enhancer and Vβ regions, as well as CBP and H3 acetylation at Vβ regions. Inhibition of E2A is essential for allelic exclusion. Enforced expression of E47 in DN thymocytes that already contain a functional TCRβ transgene enables continued rearrangement of the TCRβ loci (37). Further, E2A is essential to enforce the β-selection checkpoint. E2A deficiency allows thymocytes that have not undergone TCRβ rearrangement to bypass selection and develop into DP and even single positive thymocytes (137, 138).

TCRα Rearrangement

The TCRα and TCRδ genes share a single genetic locus, with the TCRδ genes nested within the TCRα genes, such that rearrangement of the TCRα gene results in deletion of the entire TCRδ gene (139). Rearrangements of the TCRα/δ locus are regulated by two enhancers, E_α and E_δ (140). In DP cells, pre-TCR signaling deactivates the E_δ enhancer, activates the E_α enhancer, and promotes the formation of a chromatin hub in which CTCF and cohesin mediate long range chromatin interactions between Eα, the more proximal 3' Vα/δ and the more 5' distal Jα promoters and drives germline transcription (141). The Eα enhancer contains three E-boxes, two of which are occupied by E2A prior to pre-TCR signaling. The third E-box site is bound by E2A only in DP cells and is not occupied in $HEB^{-/-}$ cells, which suggests that this site is bound by a E2A-HEB heterodimer (142). Eα does not drive TCRα expression in mature αβ T cells and is inactivated following positive selection. Following Eα inactivation the TCRα chromatin hub dissolves. There is a loss of long-range enhancer-promoter interactions, activating histone modifications (H4K3me1 and H4K3me3), and E2A and HEB binding to the enhancer (143).

TCRγ Rearrangement

E2A and HEB promote TCRγ rearrangements. E2A and HEB are each sufficient to initiate TCRγ rearrangements in non-lymphoid cells expressing Rag1 and Rag2 (144). The TCRγ locus is composed of 3 functional clusters: Cγ1, Cγ2, and Cγ3. Rearrangement of the Cγ1 cluster has been the most extensively studied. The Cγ1 cluster contains four Vγ genes and one Jγ gene (Jγ1). Vγ genes in the Cγ1 cluster rearrange with Jγ1 in a developmentally ordered manner. The more proximal Vγ3 and Vγ4 rearrange in early fetal thymocytes, while the more distal Vγ2 and Vγ5 rearrange later in development (140).

E2A regulates ordered Vγ rearrangements (145, 146). In fetal thymocytes, both Vγ2 and Vγ3 genes have permissive chromatin states, and the rearrangement preference for Vγ3 depends on its more proximal location to Jγ1 (147–150). In adults thymocytes, selection of Vγ genes for rearrangement depends on the Vγ promoters (151). E2A regulates ordered rearrangement of Vγ genes by increasing chromatin accessibility at Vγ2 and reducing chromatin accessibility at Vγ3 in adult thymocytes. E2A and HEB bind directly to the Vγ2 gene *in vivo* and positively regulate GLTs from and histone acetylation at the Vγ2 gene in a dose dependent manner. E2A deficient mice have reduced Vγ2 rearrangements in both fetal and adult thymocytes. Further, E2A represses Vγ3 GLTs in adult mice, and E2A deficient mice have increased Vγ3 rearrangements in adult thymocytes. These results indicate that while E2A promotes Vγ2 rearrangement in both fetal in adult thymocytes, ordered rearrangement depends on specific repression of the fetal Vγ3 gene in adult thymocytes by E2A (145, 146).

TCRδ Rearrangement

Unlike other antigen receptor loci composed of V, D and J gene segments, V_δ to D_δ rearrangement usually precedes D_δ to J_δ rearrangement (152). E2A has a role in promoting V_δ-D_δ rearrangements, but not in D_δ-J_δ rearrangements (145). Expression of E2A or HEB with Rag in non-lymphoid cells can induce V_δ-D_δ rearrangements (144). Like in TCRγ development, the TCRδ locus rearranges particular V_δ genes at specific stages in development. Recombination of Vδ1 predominates in early fetal development, but Vδ1 rearrangement is rare in the adult thymus. Vδ5 rearrangement begins later in development and predominates in the adult thymus (152, 153). E2A acts to both positively and negatively regulate rearrangement of particular Vδ genes in adult and fetal thymocytes. E2A represses Vδ1 rearrangement in adult thymocytes in a dose dependent manner, and E2A deficient mice exhibit increased rearrangements involving Vδ1. E2A also promotes rearrangement of the predominantly adult gene Vδ5. Vδ5 rearrangements that usually predominate in the adult thymus are not present in E2A deficient mice. In E2A deficient mice, Vδ5 rearrangements are reduced fetal thymocytes in a dose dependent manner and in adult thymocytes in a dose independent manner (145).

MODULATION OF NATURAL KILLER T CELL DEVELOPMENT AND REARRANGEMENT

While Id3 is generally involved in orchestrating γδ cell fate, the Id proteins restrict development of a specific subset of γδ T cells, γδ NKT cells. γδ NKT cells are innate-like γδ T cells that express a semi-invariant receptor (Vγ1.1Vδ6.3), and are associated with many innate like characteristics. Loss of Id3 expression in γδ T cells leads to higher E protein activity, upregulation of Egr2, PLZF, and c-Myc and proliferative expansion of γδ NKT cells (154). Id3 deficient mice also show an expanded population of γδ NKT cells (155–157). Id2 either cooperates with or can compensate for Id3, and γδ NKT cells are expanded even more so in Id3 deficient mice that also have compromised Id2 function (157). Deletion of Id2 promotes a smaller expansion of γδ T cells, although interestingly, this expansion of γδ is not limited to cells expressing Vγ1.1Vδ6.3 (157). Id2 and Id3 restrict development

into the NKT γδ T cell fate by inhibiting E protein activity, and deletion of E proteins in Id deficient mice reverts the expansion of NKT γδ T cells (156, 157).

The mechanism by which inhibition of Id protein activity expands the γδ NKT population is unclear. It is possible that γδ NKT expansion could be a result of increased rearrangement, however there are conflicting findings regarding whether the expansion of Vγ1.1 γδ NKT cells in Id3 deficient mice occurs at the expense of other γδ T cells. It has been reported that $Id3^{-/-}$ mice have reduced numbers of Vγ2 and Vγ3 dendritic epidermal T cell (DETC) subsets (116). Others note that the expanded use of the Vγ1.1 gene is not at the expense of other Vγ genes, and that the number of cells expressing Vγ2 and Vγ5 genes is the same, though the proportion of γδ T cells expressing them is reduced (158). One possible explanation for the expansion of γδ NKT cells is that these cells are normally deleted as a result of excessive γδTCR signaling, but Id3 deficiency allows them to escape deletion and proliferate (159).

A small fraction of DP thymocytes differentiate into invariant NKT (iNKT) cells, driven by heightened E protein activity and modulation of Id2/3 protein expression (160). Upon positive selection, iNKT cells further mature into multiple subsets, including NKT1, NKT2 and NKT17 cells. These developmental transitions are again instructed by E-Id protein activity to indirectly impact CD8+ T cell fate (161–165). iNKT cells express an invariant TCRα chain composed of the distally located $V_{\alpha}14$-$J_{\alpha}18$ gene segments, which recombine in secondary TCRα rearrangements. Several rounds of Vα to Jα recombination occur during TCRα rearrangement. Primary rearrangements of the TCRα locus make use of the most proximal 3' Vα genes and most distal 5' Jα genes. Secondary rearrangements make use of more 5' Vα and 3' Jα segments. Recombination is terminated when cells either pass positive selection or undergo cell death. Prolonged survival at the DP stage allows cells to undergo more sequential arrangements before undergoing cell death. HEB cooperates with TCF-1 to promote the survival of DP thymocytes by positively regulating the anti-apoptotic gene Bcl-XL (118, 166–169). DP thymocytes which lack HEB have an impaired ability to survive, rearrange their distal Jα genes less, and completely lack iNKT cells. This loss of iNKT cells is attributed to the shortened lifespan of DP cells and subsequent deficiencies in secondary TCRα rearrangements, as ectopic expression of Bcl-XL restores secondary TCRα rearrangements and iNKT development (118). Taken together, these data indicated that HEB instructs the generation of a diverse αβ T cell repertoire, enabling usage of all distally located genes and the development of iNKT cells (**Figure 5**).

MODULATION OF MATURE T CELL DEVELOPMENT

E protein activity mediates the development of DP thymocytes into unique developmental fates within the CD4+ or CD8+ T cell compartments. The role of E2A and HEB in supporting the development of an appropriate ratio of CD4+ to CD8+ T cells is now well established (170–172). These E proteins bind to CD4 E-box site to support CD4+ development while antagonizing Id2/3 activity is required for CD8+ development (171–173). E proteins mediate this development by modulating CCR7 and IL7Rα expression (171). Conversely, this activity is suppressed by Id proteins to guide CD8+ development (171). After the successful rearrangement of the TCRβ and TCRα loci, TCR-signaling induces Id3 expression, which is then maintained to enforce a naïve state in peripheral T cells (174). Id2 is then upregulated at a

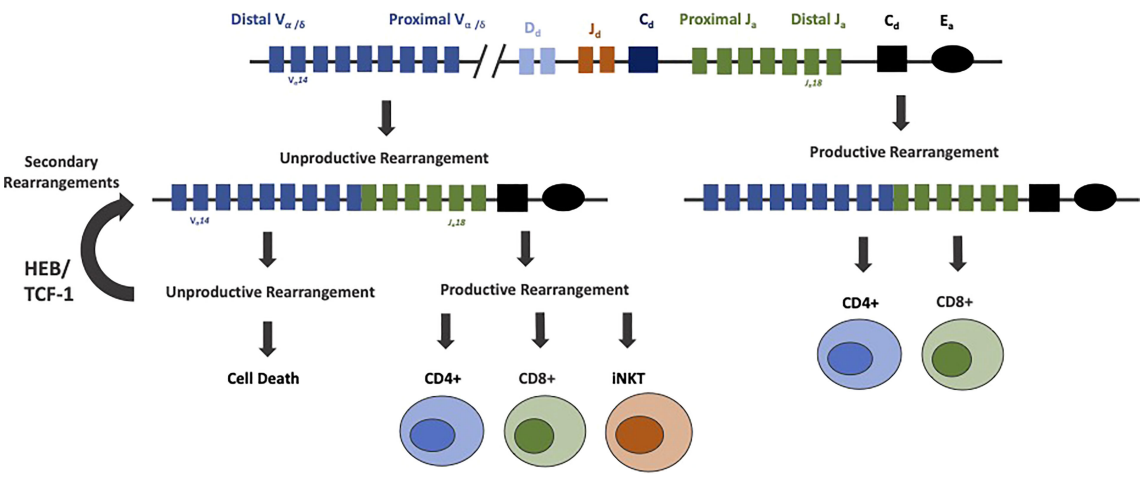

FIGURE 5 | HEB prolongs survival of DP thymocytes and rearrangements of distal V_{α} and J_{α} gene segments. Rearrangement of the TCRα locus proceeds by a deletional mechanism, in which the more proximal gene segments rearrange first. Cells that undergo productive rearrangements that pass positive selection mature into CD4+ and CD8+ mature T cells. Cells with unproductive rearrangements undergo secondary rearrangements, until the cells either produce a productive TCRα allele or undergo cell death. HEB, along with TCF-1, promotes rearrangements of more distal TCRα genes by prolonging cell survival during this process. HEB is crucial for the rearrangement of distal TCRα genes, production of a diverse αβ T cell repertoire, and generation of iNKT cells expressing an invariant TCRα protein composed of the distally located $V_{\alpha}14$ and $J_{\alpha}18$ genes.

later stage through an unknown pathway. Id2 was found to downregulate Id3 while Id3 had no effect on Id2 expression, indicating that some of the Id2-mediated effects on gene expression may be indirect (175). In summary, sustained sequestration of E proteins by Id proteins may maintain thymic single positive T cells in a naïve state.

CD4+ T cells are instructed towards unique developmental fates by the delicate balancing and timing of E protein activity. Unopposed E protein activity readily leads to the development of innate variant follicular helper T cell (T_{FH}) cells (174). In peripheral CD4+ T cells, Id2 and Id3 act to support the Th1 development while restraining T_{FH} lineage differentiation (176). In parallel studies it was shown that Id2 suppresses T_{FH} development and expansion by activating the PI3K–AKT–mTORC1–Hif1a and c-myc/p19Arf pathways (173, 177). An alternative pathway that underpins T_{FH} cell development may involve the induction of Bcl6 expression, which in turn inhibits Id2 expression (176). By permitting T_{FH} development, E proteins also influence the formation of germinal centers, with higher amounts of GC and PC B cells found in both thymi and peripheral lymphoid organs derived from mice that harbor Id2/3 deletions (173, 174). These findings highlight the importance of E protein activity in T cells in coordinating a B cell response and for germinal center adaptive immune cell development. Id proteins also orchestrate developmental progression of Treg cells (178, 179). Upon depletion of Id2 and Id3 expression in Treg cells mice readily develop Th2-cell mediated inflammatory disease (178–180). Collectively these studies revealed that E and Id proteins modulate the development of an ensemble of distinct peripheral CD4+ T cells to combat infection and suppress the development of autoimmune disease.

Id2 and Id3 also regulate E protein activity to instruct CD8+ T cell development. Naïve CD8+ T cells stimulated by the appropriate antigen readily elevate E-protein DNA binding (181). A series of elegant studies revealed that the E-Id protein axis also controls the developmental progression of CD8+ effector and memory T cells (175, 182–186). High levels of Id2 expression are required to instruct CD8+ effector T differentiation while suppressing the development of CD8+ memory cells (182, 183, 185). Conversely, upregulated Id3 expression promoted the development of Cd8+ memory cells (175). Id proteins further perform a key role in orchestrating the development of long-lived resident memory (Trm) cells (187). In summary, E and Id proteins play critical roles in orchestrating the development of an ensemble of immune cell types that act collectively to combat infection.

TRANSCRIPTIONAL BURSTING AND RNA DECAY PATHWAYS DICTATE E2A AND E2-2 mRNA HETEROGENEITY

Very early studies revealed that E47 protein abundance is noisy in naïve B cells. While a small proportion of naïve B cells express detectable levels of E47, E47 abundance is uniformly high in activated B cells (90). Consistent with these observations, more recent studies showed that E47 mRNA abundance varied across the naïve B cell population while heterogeneity in E47 mRNA levels in activated B cells was low (188). These findings raised the question as to how such differences in mRNA abundance and heterogeneity are established. Quantitative studies have addressed this question (188). E2A and E2-2 bursting frequencies and mRNA life-times differ between naïve and activated B cells. In naïve B cells, E2A and E2-2 bursting frequencies are low and mRNA life-times are short. Conversely in activated B cells E2A and E2-2 bursting frequencies are high and mRNA life-times are long (188). These findings bring into question how alterations in E2A and E2-2 mRNA life-times are established. One possible mechanism involves miRNA instructed fine-tuning of E2A and E2-2 mRNA abundance, and it will be important to identify potential miRNAs that target HLH genes. Finally, we would like to consider a role for heterogeneity in E2A and E2-2 mRNA abundance in instructing lymphocyte activation. We suggest that upon interacting of the BCR with invading pathogens, E2A and E2-2 heterogeneity in mRNA abundance permits a swift and clonal response. In such a scenario, the few B naïve cells that are actively bursting across the B cell population are primed to readily undergo CSR or rapidly develop into differentiating plasma cells. Conversely, increased E2A and E2-2 bursting frequencies and lower mRNA decay rates in activated B cells may decrease heterogeneity in E2A and E2-2 abundance to orchestrate B cell maturation. We propose that similar mechanisms instruct the immune response in T cells. Upon viral or tumor encounters the decision to differentiate into effector or memory T cell fate is similarly dictated by the combined alterations in E2A, E2-2 and HEB bursting frequencies and mRNA life-times.

CONCLUSION

Over three decades of research have highlighted critical functions of E- and Id-proteins in instructing adaptive immune development. E-proteins activate B- and T-lineage specific gene programs to specify B and T cell fate. They promote the assembly of antigen receptor loci to generate a diverse antibody and TCR repertoire. In maturing thymocytes, the E- and Id-proteins promote thymocyte selection. In peripheral B and T cells, the rise and fall in E- and Id-proteins orchestrate the development of an array of regulatory, effector and memory cell types. In mechanistic terms, E-proteins sequester histone acetyltransferases across the enhancer landscape to promote the deposition of H3K27Ac. The deposition of H3K27Ac, in turn, initiates loop extrusion to assemble a wide ensemble of loops across antigen receptor loci and down-stream target genes. We suggest that these proteins also assemble loop domains into nuclear condensates to regulate antigen receptor loci rearrangement and lineage specific programs of gene expression. Finally, we propose that alterations in HLH

bursting frequencies and mRNA life-times increase and/or narrow heterogeneity in mRNA abundance to establish B or T cell identity, thereby instructing the developmental progression of peripheral effector and memory lymphocytes in response to invading pathogens.

AUTHOR CONTRIBUTIONS

MA and ZW wrote the manuscript and designed figures with inputs from CM. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, et al. Interactions Between Heterologous Helix-Loop-Helix Proteins Generate Complexes That Bind Specifically to a Common DNA Sequence. *Cell* (1989) 58:537–44. doi: 10.1016/0092-8674(89)90434-0
2. Shivdasanl RA, Mayer EL, Orkin SH. Absence of Blood Formation in Mice Lacking the T-Cell Leukaemia Oncoprotein Tal-1/SCL. *Nature* (1995) 373:432–4. doi: 10.1038/373432a0
3. Semerad CL, Mercer EM, Inlay MA, Weissman IL, Murre C. E2A Proteins Maintain the Hematopoietic Stem Cell Pool and Promote the Maturation of Myelolymphoid and Myeloerythroid Progenitors. *Proc Natl Acad Sci USA* (2009) 106:1930–5. doi: 10.1073/pnas.0808866106
4. Souroullas GP, Salmon JM, Sablitzky F, Curtis DJ, Goodell MA. Adult Hematopoietic Stem and Progenitor Cells Require Either Lyl1 or Scl for Survival. *Cell Stem Cell* (2009) 4(2):180–6. doi: 10.1016/j.stem.2009.01.001
5. Singh SK, Singh S, Gadomski S, Sun L, Valentin M, Chen X, et al. Id1 Ablation Protects Hematopoietic Stem Cells From Stress-Induced Exhaustion and Aging. *Cell Stem Cell* (2018) 23:252–65. doi: 10.1016/j.stem.2018.06.001
6. Dias S, Månsson R, Gurbuxani S, Sigvardsson M, Kee BL. E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* (2008) 29:217–27. doi: 10.1016/j.immuni.2008.05.015
7. Ikawa T, Kawamoto H, Wright LYT, Murre C. Long-Term Cultured E2A-Deficient Hematopoietic Progenitor Cells Are Pluripotent. *Immunity* (2004) 20:349–60. doi: 10.1016/S1074-7613(04)00049-4
8. Nazaraliyev A, Richard E, Sawai CM. *In-Vivo* Differentiation of Adult Hematopoietic Stem Cells From a Single-Cell Point of View. *Curr Opin Hematol* (2020) 27:241–7. doi: 10.1097/MOH.0000000000000587
9. Chapple RH, Tseng YJ, Hu T, Kitano A, Takeichi M, Hoegenauer KA, et al. Lineage Tracing of Murine Adult Hematopoietic Stem Cells Reveals Active Contribution to Steady-State Hematopoiesis. *Blood Adv* (2018) 2(11):1220–8. doi: 10.1182/bloodadvances.2018016295
10. Bouderlique T, Peña-Pérez L, Kharazi S, Hils M, Li X, Krstic A, et al. The Concerted Action of E2-2 and HEB Is Critical for Early Lymphoid Specification. *Front Immunol* (2019) 10:455. doi: 10.3389/fimmu.2019.00455
11. Prabhu S, Ignatova A, Park ST, Sun XH. Regulation of the Expression of Cyclin-Dependent Kinase Inhibitor P21 by E2A and Id Proteins. *Mol Cell Biol* (1997) 17:10. doi: 10.1128/MCB.17.10.5888
12. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High Incidence of T-Cell Tumors in E2A-Null Mice and E2A/Id1 Double-Knockout Mice. *Mol Cell Biol* (1997) 17(12):7317–27. doi: 10.1128/MCB.17.12.7317
13. Ikawa T, Masuda K, Huijskens MJAJ, Satoh R, Kakugawa K, Agata Y, et al. Induced Developmental Arrest of Early Hematopoietic Progenitors Leads to the Generation of Leukocyte Stem Cells. *Stem Cell Rep* (2015) 5:716–27. doi: 10.1016/j.stemcr.2015.09.012
14. Mercer EM, Lin YC, Benner C, Jhunjhunwala S, Dutkowski J, Flores M, et al. Multilineage Priming of Enhancer Repertoires Precedes Commitment to the B and Myeloid Cell Lineages in Hematopoietic Progenitors. *Immunity* (2011) 35:413–25. doi: 10.1016/j.immuni.2011.06.013
15. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A Committed Precursor to Innate Lymphoid Cells. *Nature* (2014) 508:397–401. doi: 10.1038/nature13047
16. Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW. Transcription From the RAG1 Locus Marks the Earliest Lymphocyte Progenitors in Bone Marrow. *Immunity* (2002) 17:117–30. doi: 10.1016/S1074-7613(02)00366-7
17. Mansson R, Zandi S, Welinder E, Tsapogas P, Sakaguchi N, Bryder D, et al. Single-Cell Analysis of the Common Lymphoid Progenitor Compartment Reveals Functional and Molecular Heterogeneity. *Blood* (2010) 115:2601–9. doi: 10.1182/blood-2009-08-236398
18. Inlay MA, Bhattacharya D, Sahoo D, Serwold T, Seita J, Karsunky H, et al. Ly6d Marks the Earliest Stage of B-Cell Specification and Identifies the Branchpoint Between B-Cell and T-Cell Development. *Genes Dev* (2009) 23:2376–81. doi: 10.1101/gad.1836009
19. Jensen CT, Åhsberg J, Sommarin MNE, Strid T, Somasundaram R, Okuyama K, et al. Dissection of Progenitor Compartments Resolves Developmental Trajectories in B-Lymphopoiesis. *J Exp Med* (2018) 215:1947–63. doi: 10.1084/jem.20171384
20. Seet CS, Brumbaugh RL, Kee BL. Early B Cell Factor Promotes B Lymphopoiesis With Reduced Interleukin 7 Responsiveness in the Absence of E2A. *J Exp Med* (2004) 199:1689–700. doi: 10.1084/jem.20032202
21. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E Proteins and Notch Signaling Cooperate to Promote T Cell Lineage Specification and Commitment. *J Exp Med* (2006) 203:1329–42. doi: 10.1084/jem.20060268
22. Lin H, Grosschedl R. Failure of B-Cell Differentiation in Mice Lacking the Transcription Factor EBF. *Nature* (1995) 376(6537):263–7. doi: 10.1038/376263a0
23. Decker T, Pasca di Magliano M, McManus S, Sun Q, Bonifer C, Tagoh H, et al. Stepwise Activation of Enhancer and Promoter Regions of the B Cell Commitment Gene Pax5 in Early Lymphopoiesis. *Immunity* (2009) 30:508–20. doi: 10.1016/j.immuni.2009.01.012
24. Welinder E, Mansson R, Mercer EM, Bryder D, Sigvardsson M, Murre C. The Transcription Factors E2A and HEB Act in Concert to Induce the Expression of FOXO1 in the Common Lymphoid Progenitor. *Proc Natl Acad Sci USA* (2011) 108:17402–7. doi: 10.1073/pnas.1111766108
25. Mansson R, Welinder E, Åhsberg J, Lin YC, Benner C, Glass CK, et al. Positive Intergenic Feedback Circuitry, Involving EBF1 and FOXO1, Orchestrates B-Cell Fate. *Proc Natl Acad Sci USA* (2012) 109:21028–33. doi: 10.1073/pnas.1211427109
26. Jaleco AC, Stegmann APA, Heemskerk MHM, Couwenberg F, Bakker AQ, Weijer K, et al. Genetic Modification of Human B-Cell Development: B-Cell Development Is Inhibited by the Dominant Negative Helix Loop Helix Factor Id3. *Blood* (1999) 94:2637–46. doi: 10.1182/blood.v94.8.2637.420k22_2637_2646
27. Kee BL, Rivera RR, Murre C. Id3 Inhibits B Lymphocyte Progenitor Growth and Survival in Response to TGF-β. *Nat Immunol* (2001) 2:242–7. doi: 10.1038/85303
28. Beck K, Peak MM, Ota T, Nemazee D, Murre C. Distinct Roles for E12 and E47 in B Cell Specification and the Sequential Rearrangement of Immunoglobulin Light Chain Loci. *J Exp Med* (2009) 206:2271–84. doi: 10.1084/jem.20090756

29. Kwon K, Hutter C, Sun Q, Bilic I, Cobaleda C, Malin S, et al. Instructive Role of the Transcription Factor E2A in Early B Lymphopoiesis and Germinal Center B Cell Development. *Immunity* (2008) 28:751–62. doi: 10.1016/j.immuni.2008.04.014
30. Schlissel M, Voronova A, Baltimore D. Helix-Loop-Helix Transcription Factor E47 Activates Germ-Line Immunoglobulin Heavy-Chain Gene Transcription and Rearrangement in a Pre-T-Cell Line. *Genes Dev* (1991) 5:1367–76. doi: 10.1101/gad.5.8.1367
31. Bain G, Maandag ECR, Izon DJ, Amsen D, Kruisbeek AM, Weintraub BC, et al. E2A Proteins Are Required for Proper B Cell Development and Initiation of Immunoglobulin Gene Rearrangements. *Cell* (1994) 79:885–92. doi: 10.1016/0092-8674(94)90077-9
32. Zhuang Y, Cheng P, Weintraub H. B-Lymphocyte Development Is Regulated by the Combined Dosage of Three Basic Helix-Loop-Helix Genes, E2A, E2-2, and HEB. *Mol Cell Biol* (1996) 16:2898–905. doi: 10.1128/mcb.16.6.2898
33. Wikström I, Forssell J, Goncalves M, Colucci F, Holmberg D. E2-2 Regulates the Expansion of Pro-B Cells and Follicular Versus Marginal Zone Decisions. *J Immunol* (2006) 177:6723–9. doi: 10.4049/jimmunol.177.10.6723
34. Shen C-P, Kadesch T. B-Cell-Specific DNA Binding by an E47 Homodimer. *Mol Cell Biol* (1995) 15(8):4518–24. doi: 10.1128/MCB.15.8.4518
35. Chu C, Kohtz DS. Identification of the E2A Gene Products as Regulatory Targets of the G1 Cyclin-Dependent Kinases. *J Biol Chem* (2001) 276:8524–34. doi: 10.1074/jbc.M008371200
36. Bradney C, Hjelmeland M, Komatsu Y, Yoshida M, Yao TP, Zhuang Y. Regulation of E2A Activities by Histone Acetyltransferases in B Lymphocyte Development. *J Biol Chem* (2003) 278:2370–6. doi: 10.1074/jbc.M211464200
37. Agata Y, Tamaki N, Sakamoto S, Ikawa T, Masuda K, Kawamoto H, et al. Regulation of T Cell Receptor β Gene Rearrangements and Allelic Exclusion by the Helix-Loop-Helix Protein, E47. *Immunity* (2007) 27:871–84. doi: 10.1016/j.immuni.2007.11.015
38. Blume J, Ziętara N, Witzlau K, Liu Y, Sanchez OO, Puchałka J, et al. Mir-191 Modulates B-Cell Development and Targets Transcription Factors E2A, Foxp1, and Egr1. *Eur J Immunol* (2019) 49:121–32. doi: 10.1002/eji.201847660
39. Lin YC, Benner C, Mansson R, Heinz S, Miyazaki K, Miyazaki M, et al. Global Changes in the Nuclear Positioning of Genes and Intra-and Interdomain Genomic Interactions That Orchestrate B Cell Fate. *Nat Immunol* (2012) 13:1196–204. doi: 10.1038/ni.2432
40. Lin YC, Jhunjhunwala S, Benner C, Heinz S, Welinder E, Mansson R, et al. A Global Network of Transcription Factors, Involving E2A, EBF1 and Foxo1, That Orchestrates the B Cell Fate. *Nat Immunol* (2011) 11:635–43. doi: 10.1038/ni.1891.A
41. Miyazaki K, Watanabe H, Yoshikawa G, Chen K, Hidaka R, Aitani Y, et al. The Transcription Factor E2A Activates Multiple Enhancers That Drive Rag Expression in Developing T and B Cells. *Sci Immunol* (2020) 5:1–15. doi: 10.1126/sciimmunol.abb1455
42. Zhu Y, Denholtz M, Lu H, Murre C. Calcium Signaling Instructs NIPBL Recruitment at Active Enhancers and Promoters *via* Distinct Mechanisms to Reconstruct Genome Compartmentalization. *Genes Dev* (2021) 35:65–81. doi: 10.1101/GAD.343475.120
43. Denholtz M, Zhu Y, He Z, Lu H, Isoda T, Döhrmann S, et al. Upon Microbial Challenge, Human Neutrophils Undergo Rapid Changes in Nuclear Architecture and Chromatin Folding to Orchestrate an Immediate Inflammatory Gene Program. *Genes Dev* (2019) 5:149–65. doi: 10.1101/808568
44. Zhu Y, Gong K, Denholtz M, Chandra V, Kamps MP, Alber F, et al. Comprehensive Characterization of Neutrophil Genome Topology. *Genes Dev* (2017) 2:141–53. doi: 10.1101/gad.293910.116
45. Lio CW, Zhang J, González-Avalos E, Hogan PG, Chang X, Rao A. Tet2 and Tet3 Cooperate With B-Lineage Transcription Factors to Regulate DNA Modification and Chromatin Accessibility. *Elife* (2016) 5:e18290. doi: 10.7554/eLife.1829
46. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-I1 and RAG-2, Adjacent Genes That Synergistically Activate V(D)J Recombination. *Science* (1990) 248:1517–23. doi: 10.1126/science.2360047
47. Schatz DG, Oettinger MA, Baltimore D. The V(D)J Recombination Activating Gene, Rag-1. *Cell* (1989) 6:1035–48. doi: 10.1016/0092-8674(89)90760-5
48. Grawunder U, Leu TMJ, Schatz DG, Werner A, Rolink AG, Melchers F, et al. Down-Regulation of RAG1 and RAG2 Gene Expression in Preb Cells After Functional Lmmunoglobulin Heavy Chain Rearrangement. *Immunity* (1995) 3:601–8. doi: 10.1016/1074-7613(95)90131-0
49. Quong MW, Martensson A, Langerak AW, Rivera RR, Nemazee D, Murre C. Receptor Editing and Marginal Zone B Cell Development Are Regulated by the Helix-Loop-Helix Protein, E2A. *J Exp Med* (2004) 8:1101–12. doi: 10.1084/jem.20031180
50. Schebesta M, Pfeffer PL, Busslinger M. Control of Pre-BCR Signaling by Pax5-Dependent Activation of the BLNK Gene. *Immunity* (2002) 4:473–85. doi: 10.1016/s1074-7613(02)00418-1
51. Borghesi L, Aites J, Nelson S, Lefterov P, James P, Gerstein R. E47 Is Required for V(D)J Recombinase Activity n Common Lymphoid Progenitors. *J Exp Med* (2005) 12:1669–77. doi: 10.1084/jem.20051190
52. Hsu L-Y, Lauring J, Liang H-E, Greenbaum S, Cado D, Zhuang Y, et al. A Conserved Transcriptional Enhancer Regulates RAG Gene Expression in Developing B Cells. *Immunity* (2003) 1:105–17. doi: 10.1016/s1074-7613(03)00181-x
53. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, et al. Ordered Rearrangement of Immunoglobulin Heavy Chain Variable Region Segments. *EMBO J* (1984) 6:1209–19. doi: 10.1002/j.1460-2075.1984.tb01955.x
54. Choi JK, Shen CP, Radomska HS, Eckhardt LA, Kadesch T. E47 Activates the Ig-Heavy Chain and Tdt Loci in Non-B Cells. *EMBO J* (1996) 18:5014–21. doi: 10.1002/j.1460-2075.1996.tb00881.x
55. Romanow WJ, Langerak AW, Goebel P, Wolvers-Tettero ILM, van Dongen JJM, Feeney AJ, et al. E2A and EBF Act in Synergy With the V(D)J Recombinase to Generate a Diverse Immunoglobulin Repertoire in Nonlymphoid Cells. *Mol Cell* (2000) 5:343–53. doi: 10.1016/S1097-2765(00)80429-3
56. Zhang Z, Espinoza CR, Yu Z, Stephan R, He T, Williams GS, et al. Transcription Factor Pax5 (BSAP) Transactivates the RAG-Mediated VH-to-DJH Rearrangement of Immunoglobulin Genes. *Nat Immunol* (2006) 6:616–24. doi: 10.1038/ni1339
57. Sayegh C, Jhunjhunwala S, Riblet R, Murre C. Visualization of Looping Involving the Immunoglobulin Heavy-Chain Locus in Developing B Cells. *Genes Dev* (2005) 3:322–7. doi: 10.1101/gad.1254305
58. Jhunjhunwala S, van Zelm MC, Peak MM, Cutchin S, Riblet R, van Dongen JJM, et al. The 3d Structure of the Immunoglobulin Heavy-Chain Locus: Implications for Long-Range Genomic Interactions. *Cell* (2008) 2:265–79. doi: 10.1016/j.cell.2008.03.024
59. Kosak ST, Skok JA, Medina KL, Riblet R. Subnuclear Compartmentalization of Immunoglobulin Loci During Lymphocyte Development. *Science* (2002) 5565:158–62. doi: 10.1126/science.1068768
60. Roldán E, Fuxa M, Chong W, Martinez D, Novatchkova M, Busslinger M, et al. Locus 'Decontraction' and Centromeric Recruitment Contribute to Allelic Exclusion of the Immunoglobulin Heavy-Chain Gene. *Nat Immunol* (2005) 1:31–41. doi: 10.1038/ni1150
61. Ebert A, McManus S, Tagoh H, Medvedovic J, Salvagiotto G, Novatchkova M, et al. The Distal VH Gene Cluster of the Igh Locus Contains Distinct Regulatory Elements With Pax5 Transcription Factor-Dependent Activity in Pro-B Cells. *Immunity* (2011) 2:175–87. doi: 10.1016/j.immuni.2011.02.005
62. Verma-Gaur J, Hauser J, Grundström T. Negative Feedback Regulation of Antigen Receptors Through Calmodulin Inhibition of E2A. *J Immunol* (2012) 12:6175–83. doi: 10.4049/jimmunol.1103105
63. Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M. Pax5 Induces V -to- DJ Rearrangements and Locus Contraction of the Immunoglobulin Heavy-Chain Gene. *Genes Dev* (2004) 4:411–22. doi: 10.1101/gad.291504
64. Hauser J, Wallenius A, Sveshnikova N, Saarikettu J, Grundström T. Calmodulin Inhibition of E2A Stops Expression of Surrogate Light Chains of the Pre-B-Cell Receptor and CD19. *Mol Immunol* (2010) 5:1031–8. doi: 10.1016/j.molimm.2009.11.015
65. Hauser J, Verma-Gaur J, Grundström T. Broad Feedback Inhibition of Pre-B-Cell Receptor Signaling Components. *Mol Immunol* (2013) 3-4:247–53. doi: 10.1016/j.molimm.2012.12.002
66. Murre C, Mccaw PS, Baltimore D. A New DNA Binding and Dimerization Motif in Lmmunoglobulin Enhancer Binding, Daughterless, Myod, and Myc Proteins. *Cell* (1989) 5:777–83. doi: 10.1016/0092-8674(89)90682-x

67. Karki S, Kennedy DE, Mclean K, Grzybowski AT, Maienschein-Cline M, Banerjee S, et al. Regulated Capture of Vκ Gene Topologically Associating Domains by Transcription Factories. *Cell Rep* (2018) 9:2443–56. doi: 10.1016/j.celrep.2018.07.091
68. Sakamoto S, Wakae K, Anzai Y, Murai K, Tamaki N, Miyazaki M, et al. E2A and CBP/P300 Act in Synergy to Promote Chromatin Accessibility of the Immunoglobulin κ Locus. *J Immunol* (2012) 11:5547–60. doi: 10.4049/jimmunol.1002346
69. Kleiman E, Loguercio S, Feeney AJ. Epigenetic Enhancer Marks and Transcription Factor Binding Influence Vκ Gene Rearrangement in Pre-B Cells and Pro-B Cells. *Front Immunol* (2018) 9:2074. doi: 10.3389/fimmu.2018.02074
70. Van der Stoep N, Gorman JR, Alt FW. Reevaluation of 3'Ekappa Function in Stage- and Lineage-Specific Rearrangement and Somatic Hypermutation. *Immunity* (1998) 6:743–50. doi: 10.1016/s1074-7613(00)80579-8
71. Xu Y, Davidson L. Deletion of the Ig Light Chain Intronic Enhancer/Matrix Attachment Region Impairs But Does Not Abolish VJ Rearrangement. *Immunity* (1996) 4:377–85. doi: 10.1016/s1074-7613(00)80251-4
72. Gorman JR, Van der Stoep N, Monroe R, Cogne M, Davidson L, Alt FW. The Ig(Kappa) Enhancer Influences the Ratio of Ig(Kappa) Versus Ig (Lambda) B Lymphocytes. *Immunity* (1996) 3:241–52. doi: 10.1016/s1074-7613(00)80319-2
73. Inlay MA, Tian H, Lin T, Xu Y. Important Roles for E Protein Binding Sites Within the Immunoglobulin κ Chain Intronic Enhancer in Activating VKJK Rearrangement. *J Exp Med* (2004) 9:1205–11. doi: 10.1084/jem.20041135
74. Johnson K, Hashimshony T, Sawai CM, Pongubala JMR, Skok JA, Aifantis I, et al. Regulation of Immunoglobulin Light-Chain Recombination by the Transcription Factor IRF-4 and the Attenuation of Interleukin-7 Signaling. *Immunity* (2008) 3:335–45. doi: 10.1016/j.immuni.2007.12.019
75. Mandal M, Powers SE, Ochiai K, Georgopoulos K, Kee BL, Singh H, et al. Ras Orchestrates Exit From the Cell Cycle and Light-Chain Recombination During Early B Cell Development. *Nat Immunol* (2009) 10:1110–7. doi: 10.1038/ni.1785
76. Hodawadekar S, Park K, Farrar MA, Atchison ML. A Developmentally Controlled Competitive STAT5–PU.1 DNA Binding Mechanism Regulates Activity of the Ig κe3′ Enhancer. *J Immunol* (2012) 5:2276–84. doi: 10.4049/jimmunol.1102239
77. Nagulapalli S, Atchison ML. Transcription Factor Pip can Enhance DNA Binding by E47, Leading to Transcriptional Synergy Involving Multiple Protein Domains. *Mol Cell Biol* (1998) 8:4639–50. doi: 10.1128/mcb.18.8.4639
78. Nagulapalli S, Goheer A, Pitt L, McIntosh LP, Atchison ML. Mechanism of E47-Pip Interaction on DNA Resulting in Transcriptional Synergy and Activation of Immunoglobulin Germ Line Sterile Transcripts. *Mol Cell Biol* (2002) 10:7337–50. doi: 10.1128/mcb.22.20.7337-7350.2002
79. Lazorchak AS, Schlissel MS, Zhuang Y. E2A and IRF-4/Pip Promote Chromatin Modification and Transcription of the Immunoglobulin κ Locus in Pre-B Cells. *Mol Cell Biol* (2006) 3:810–21. doi: 10.1128/mcb.26.3.810-821.2006
80. Manoharan A, du RC, AG R, Matthias P. *De Novo* DNA Methyltransferases Dnmt3a and Dnmt3b Regulate the Onset of Igκ Light Chain Rearrangement During Early B-Cell Development. *Eur J Immunol* (2015) 8:2343–55. doi: 10.1002/eji.201445035
81. Bossen C, Murre CS, Chang AN, Mansson R, Rodewald HR, Murre C. The Chromatin Remodeler Brg1 Activates Enhancer Repertoires to Establish B Cell Identity and Modulate Cell Growth. *Nat Immunol* (2015) 7:775–84. doi: 10.1038/ni.3170
82. Stadhouders R, de Bruijn MJW, Rother MB, Yuvaraj S, de Almeida CR, Kolovos P, et al. Pre-B Cell Receptor Signaling Induces Immunoglobulin κ Locus Accessibility by Functional Redistribution of Enhancer-Mediated Chromatin Interactions. *PloS Biol* (2014) (2):e1001791. doi: 10.1371/journal.pbio.1001791
83. Chen S, Miyazaki M, Chandra V, Fisch KM, Chang AN, Murre C. Id3 Orchestrates Germinal Center B Cell Development. *Mol Cell Biol* (2016) 36:2543–52. doi: 10.1128/mcb.00150-16
84. Gloury R, Zotos D, Zuidscherwoude M, Masson F, Liao Y, Hasbold J, et al. Dynamic Changes in Id3 and E-Protein Activity Orchestrate Germinal Center and Plasma Cell Development. *J Exp Med* (2016) 213:1095–111. doi: 10.1084/jem.20152003
85. Wöhner M, Tagoh H, Bilic I, Jaritz M, Poliakova DK, Fischer M, et al. Molecular Functions of the Transcription Factors E2A and E2-2 in Controlling Germinal Center B Cell and Plasma Cell Development. *J Exp Med* (2016) 7:1201–21. doi: 10.1084/jem.20152002
86. Xu Z, Zan H, Pone EJ, Mai T, Casali P. Immunoglobulin Class-Switch DNA Recombination: Induction, Targeting and Beyond. *Nat Rev Immunol* (2012) 7:517–31. doi: 10.1038/nri3216
87. Pilzecker B, Jacobs H. Mutating for Good: DNA Damage Responses During Somatic Hypermutation. *Front Immunol* (2019) 10:438. doi: 10.3389/fimmu.2019.00438
88. Hauser J, Sveshnikova N, Wallenius A, Baradaran S, Saarikettu J. Grundström T. B-Cell Receptor Activation Inhibits AID Expression Through Calmodulin Inhibition of E-Proteins. *Proc Natl Acad Sci USA* (2008) 4:1267–72. doi: 10.1073/pnas.0708220105
89. Sayegh CE, Quong MW, Agata Y, Murre C. E-Proteins Directly Regulate Expression of Activation-Induced Deaminase in Mature B Cells. *Nat Immunol* (2003) 6:586–93. doi: 10.1038/ni923
90. Quong MW, Harris DP, Swain SL, Murre C. E2A Activity Is Induced During B-Cell Activation to Promote Immunoglobulin Class Switch Recombination. *EMBO J* (1999) 22:6307–18. doi: 10.1093/emboj/18.22.6307
91. Goldfarb AN, Flores JP, Lewandowska K. Involvement of the E2A Basic Helix-Loop-Helix Protein in Immunoglobulin Heavy Chain Class Switching. *Mol Immunol* (1996) 11-12:947–56. doi: 10.1016/s0161-5890(96)00047-8
92. Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, et al. The Balance Between Pax5 and Id2 Activities Is the Key to AID Gene Expression. *J Exp Med* (2003) 9:1427–37. doi: 10.1084/jem.20030802
93. Sugai M, Gonda H, Kusunoki T, Katakai T, Yokota Y, Shimizu A. Essential Role of Id2 in Negative Regulation of Ige Class Switching. *Nat Immunol* (2003) 1:25–30. doi: 10.1038/ni874
94. Hauser J, Grundström C, Kumar R, Grundström T. Regulated Localization of an AID Complex With E2A, PAX5 and IRF4 at the Igh Locus. *Mol Immunol* (2016) 80:78–90. doi: 10.1016/j.molimm.2016.10.014
95. Grundström C, Grundström C, Kumar A, Priya A, Negi N, Grundström T. ETS1 and PAX5 Transcription Factors Recruit AID to Igh DNA. *Eur J Immunol* (2018) 10:1687–97. doi: 10.1002/eji.201847625
96. Michael N, Ming Shen H, Longerich S, Kim N, Longacre A, Storb U. The E Box Motif Caggtg Enhances Somatic Hypermutation Without Enhancing Transcription. *Immunity* (2010) 2:235–42. doi: 10.1016/s1074-7613(03)00204-8
97. Tanaka A, Shen HM, Ratnam S, Kodgire P, Storb U. Attracting AID to Targets of Somatic Hypermutation. *J Exp Med* (2010) 2:405–15. doi: 10.1084/jem.20090821
98. Yabuki M, Ordinario EC, Cummings WJ, Fujii MM, Maizels N. E2A Acts in Cis in G1 Phase of Cell Cycle to Promote Ig Gene Diversification. *J Immunol* (2009) 1:408–15. doi: 10.4049/jimmunol.182.1.408
99. Dinesh RK, Barnhill B, Ilanges A, Wu L, Michelson DA, Senigl F, et al. Transcription Factor Binding at Ig Enhancers Is Linked to Somatic Hypermutation Targeting. *Eur J Immunol* (2020) 3:380–95. doi: 10.1002/eji.201948357
100. Kadakia T, Tai X, Kruhlak M, Wisniewski J, Hwang IY, Roy S, et al. E-Protein–Regulated Expression of CXCR4 Adheres Preselection Thymocytes to the Thymic Cortex. *J Exp Med* (2019) 216:1749–61. doi: 10.1084/jem.20182285
101. Miyazaki M, Miyazaki K, Chen K, Jin Y, Turner J, Moore AJ, et al. The E-Id Protein Axis Specifies Adaptive Lymphoid Cell Identity and Suppresses Thymic Innate Lymphoid Cell Development. *Immunity* (2017) 46:818–34. doi: 10.1016/j.immuni.2017.04.022
102. Bain G, Murre C. The Role of E-Proteins in B- and T-Lymphocyte Development. *Semin Immunol* (1998) 10(2):143–53. doi: 10.1006/smim.1998.0116
103. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2A Deficiency Leads to Abnormalities in Alphabeta T-Cell Development and to Rapid Development of T-Cell Lymphomas. *Mol Cell Biol* (1997) 17:4782–91. doi: 10.1128/mcb.17.8.4782
104. Schotte R, Dontje W, Nagasawa M, Yasuda Y, Bakker AQ, Spits H, et al. Synergy Between IL-15 and Id2 Promotes the Expansion of Human NK Progenitor Cells, Which Can be Counteracted by the E Protein HEB Required to Drive T Cell Development. *J Immunol* (2010) 184:6670–9. doi: 10.4049/jimmunol.0901508

105. Schwartz R, Engel I, Fallahi-Sichani M, Petrie HT, Murre C. Gene Expression Patterns Define Novel Roles for E47 in Cell Cycle Progression, Cytokine-Mediated Signaling, and T Lineage Development. *Proc Natl Acad Sci USA* (2006) 103:9976–81. doi: 10.1073/pnas.0603728103
106. Ikawa T, Hirose S, Masuda K, Kakugawa K, Satoh R, Shibano-Satoh A, et al. An Essential Developmental Checkpoint for Production of the T Cell Lineage. *Science* (2010) 329:89–93. doi: 10.1126/science.1188989
107. Longabaugh WJR, Zeng W, Zhang JA, Hosokawa H, Jansen CS, Li L, et al. Bcl11b and Combinatorial Resolution of Cell Fate in the T-Cell Gene Regulatory Network. *Proc Natl Acad Sci USA* (2017) 114:5800–7. doi: 10.1073/pnas.1610617114
108. Isoda T, Moore AJ, He Z, Chandra V, Aida M, Denholtz M, et al. Non-Coding Transcription Instructs Chromatin Folding and Compartmentalization to Dictate Enhancer-Promoter Communication and T Cell Fate. *Cell* (2017) 171:103–119.e18. doi: 10.1016/j.cell.2017.09.001
109. Hosokawa H, Romero-wolf M, Yui MA, Ungerbäck J, Quiloan MLG, Matsumoto M, et al. Bcl11b Sets Pro-T Cell Fate by Site-Specific Cofactor Recruitment and by Repressing Id2 and Zbtb16. *Nat Immunol* (2018) 19:183–93 . doi: 10.1038/s41590-018-0238-4
110. Wang D, Claus CL, Vaccarelli G, Braunstein M, Schmitt TM, Zúñiga-Pflücker JC, et al. The Basic Helix-Loop-Helix Transcription Factor Hebalt is Expressed in Pro-T Cells and Enhances the Generation of T Cell Precursors. *J Immunol* (2006) 177:109–19. doi: 10.4049/jimmunol.177.1.109
111. Braunstein M, Anderson MK. Developmental Progression of Fetal HEB -/- Precursors to the Pre-T-Cell Stage is Restored by Hebalt. *Eur J Immunol* (2010) 40:3173–82. doi: 10.1002/eji.201040360
112. Braunstein M, Anderson MK. HEB-Deficient T-Cell Precursors Lose T-Cell Potential and Adopt an Alternative Pathway of Differentiation. *Mol Cell Biol* (2011) 31:971–82. doi: 10.1128/mcb.01034-10
113. Barndt R, Dai MF, Zhuang Y. A Novel Role for HEB Downstream or Parallel to the Pre-TCR Signaling Pathway During Alpha Beta Thymopoiesis. *J Immunol* (1999) 163(6):3331–43.
114. Haks MC, Lefebvre JM, Lauritsen JPH, Carleton M, Rhodes M, Miyazaki T, et al. Attenuation of γδtcr Signaling Efficiently Diverts Thymocytes to the αβ Lineage. *Immunity* (2005) 5:595–606. doi: 10.1016/j.immuni.2005.04.003
115. Hayes SM, Li LQ, Love PE. TCR Signal Strength Influences αβ/γδ Lineage Fate. *Immunity* (2005) 5:583–93. doi: 10.1016/j.immuni.2005.03.014
116. Lauritsen JPH, Wong GW, Lee SY, Lefebvre JM, Ciofani M, Rhodes M, et al. Marked Induction of the Helix-Loop-Helix Protein Id3 Promotes the Γδ T Cell Fate and Renders Their Functional Maturation Notch Independent. *Immunity* (2009) 4:565–75. doi: 10.1016/j.immuni.2009.07.010
117. Wilson A, Held W, Robson Macdonald H. Two Waves of Recombinase Gene Expression in Developing Thymocytes. *J Exp Med* (1994) 4:1355–60. doi: 10.1084/jem.179.4.1355
118. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An Essential Role for the Transcription Factor HEB in Thymocyte Survival, Tcra Rearrangement and the Development of Natural Killer T Cells. *Nat Immunol* (2010) 3:240–9. doi: 10.1038/ni.1845
119. Yannoutsos N, Barreto V, Misulovin Z, Gazumyan A, Yu W, Rajewsky N, et al. A Cis Element in the Recombination Activating Gene Locus Regulates Gene Expression by Counteracting a Distant Silencer. *Nat Immunol* (2004) 4:443–50. doi: 10.1038/ni1053
120. Naik AK, Byrd AT, Lucander ACK, Krangel MS. Hierarchical Assembly and Disassembly of a Transcriptionally Active RAG Locus in CD4 + CD8 + Thymocytes. *J Exp Med* (2019) 1:231–43. doi: 10.1084/jem.20181402
121. Miyazaki M, Rivera RR, Miyazaki K, Lin YC, Agata Y, Murre C. The Opposing Roles of the Transcription Factor E2A and Its Antagonist Id3 That Orchestrate and Enforce the Naive Fate of T Cells. *Nat Immunol* (2011) 10:992–1001. doi: 10.1038/ni.2086
122. Bain G, Cravatt CB, Loomans C, Alberola-Ila J, Hedrick SM, Murre C. Regulation of the Helix-Loop-Helix Proteins, E2A and Id3, by the Ras-ERK MAPK Cascade. *Nat Immunol* (2001) 2:165–71. doi: 10.1038/84273
123. Blom B, Heemskerk MHM, Verschuren MCM, van Dongen JJM, Stegmann APA, Bakker AQ, et al. Disruption of αβ But Not of Γδ T Cell Development by Overexpression of the Helix-Loop-Helix Protein Id3 in Committed T Cell Progenitors. *EMBO J* (1999) 18:2793–802. doi: 10.1093/emboj/18.10.2793
124. Miyazaki K, Miyazaki M. The Interplay Between Chromatin Architecture and Lineage-Specific Transcription Factors and the Regulation of Rag Gene Expression. *Front Immunol* (2021) 12:659761. doi: 10.3389/fimmu.2021.659761
125. Sleckman BP, Bassing CH, Hughes MM, Okada A, D'auteuil M, Wehrly TD, et al. Mechanisms That Direct Ordered Assembly of T Cell Receptor Locus V, D, and J Gene Segments. *Proc Natl Acad Sci USA* (2000) 14:7975–79780. doi: 10.1073/pnas.130190597
126. Bouvier G, Watrin F, Naspetti M, Verthuy C, Naquet P, Ferrierm P. Deletion of the Mouse T-Cell Receptor B Gene Enhancer Blocks Ab T-Cell Development. *Proc Natl Acad Sci USA* (1996) 15:7877–81. doi: 10.1073/pnas.93.15.7877
127. Bories J-C, Demengeottt J, Davidsont L, Alt FW. Gene-Targeted Deletion and Replacement Mutations of the T-Cell Receptor Beta-Chain Enhancer: The Role of Enhancer Elements in Controlling V(D)J Recombination Accessibility. *Proc Natl Acad Sci USA* (1996) 15:7871–6. doi: 10.1073/pnas.93.15.7871
128. Ferrier P, Krippl B, Blackwell TK, Furley AJW, Suh H, Winoto A, et al. Separate Elements Control DJ and VDJ Rearrangement in a Transgenic Recombination Substrate. *EMBO J* (1990) 1:117–25. doi: 10.1002/j.1460-2075.1990.tb08087.x
129. Capone M, Watrin F, Fernex C, Horvat B, Krippl B, Wu L, et al. Tcrβ and Tcrα Gene Enhancers Confer Tissue- and Stage-Specificity on V(D)J Recombination Events. *EMBO J* (1993) 11:4335–46. doi: 10.1002/j.1460-2075.1993.tb06118.x
130. Tripathi RK, Mathieu N, Spicuglia S, Payet D, Verthuy C, Bouvier G, et al. Definition of a T-Cell Receptor β Gene Core Enhancer of V(D)J Recombination by Transgenic Mapping. *Mol Cell Biol* (2000) 1:42–53. doi: 10.1128/mcb.20.1.42-53.2000
131. Jia J, Dai M, Zhuang Y. E Proteins Are Required to Activate Germline Transcription of the TCR Vβ8.2 Gene. *Eur J Immunol* (2008) 10:2806–20. doi: 10.1002/eji.200838144
132. Kee BL, Bain G, Murre C. IL-7rα and E47: Independent Pathways Required for Development of Multipotent Lymphoid Progenitors. *EMBO J* (2002) 1-2:103–13. doi: 10.1093/emboj/21.1.103
133. Sawada S, Littman DR. A Heterodimer of HEB and an E12-Related Protein Interacts With the CD4 Enhancer and Regulates Its Activity in T-Cell Lines. *Mol Cell Biol* (1993) 9:5620–8. doi: 10.1128/mcb.13.9.5620-5628.1993
134. Barndt RJ, Dai M, Zhuang Y. Functions of E2A-HEB Heterodimers in T-Cell Development Revealed by a Dominant Negative Mutation of HEB. *Mol Cell Biol* (2000) 18:6677–85. doi: 10.1128/mcb.20.18.6677-6685.2000
135. Wojciechowski J, Lai A, Kondo M, Zhuang Y. E2A and HEB Are Required to Block Thymocyte Proliferation Prior to Pre-TCR Expression. *J Immunol* (2007) 9:5717–26. doi: 10.4049/jimmunol.178.9.5717
136. Petersson K, Ivars F, Sigvardsson M. The Ptα Promoter and Enhancer Are Direct Targets for Transactivation by E Box-Binding Proteins. *Eur J Immunol* (2002) 3:911–20. doi: 10.1002/1521-4141(200203)32:3<911::AID-IMMU911>3.0.CO;2-H
137. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early Thymocyte Development Is Regulated by Modulation of E2A Protein Activity. *J Exp Med* (2001) 6:733–45. doi: 10.1084/jem.194.6.733
138. Engel I, Murre C. E2A Proteins Enforce a Proliferation Checkpoint in Developing Thymocytes. *EMBO J* (2004) 1:202–11. doi: 10.1038/sj.emboj.7600017
139. Majumder K, Bassing CH, Oltz EM. Regulation of Tcrb Gene Assembly by Genetic, Epigenetic, and Topological Mechanisms. *Adv Immunol* (2015) 128:273–306. doi: 10.1016/bs.ai.2015.07.001
140. Krangel MS. Mechanics of T Cell Receptor Gene Rearrangement. *Curr Opin Immunol* (2009) 2:133–9. doi: 10.1016/j.coi.2009.03.009
141. Shih HY, Verma-Gaur J, Torkamani A, Feeney AJ, Galjart N, Krangel MS. Tcra Gene Recombination Is Supported by a Tcra Enhancer- and CTCF-Dependent Chromatin Hub. *Proc Natl Acad Sci USA* (2012) 50:E3493–502. doi: 10.1073/pnas.1214131109
142. del Blanco B, García-Mariscal A, Wiest DL, Hernández-Munain C. Tcra Enhancer Activation by Inducible Transcription Factors Downstream of Pre-TCR Signaling. *J Immunol* (2012) 188:3278–93. doi: 10.4049/jimmunol.1100271
143. del Blancoa B, Angulo Ú, Krangel MS, Hernández-Munain C. T-Cell Receptor α Enhancer Is Inactivated in αβ T Lymphocytes. *Proc Natl Acad Sci USA* (2015) 7:E1744–53. doi: 10.1073/pnas.1406551112

144. Langerak AW, Wolvers-Tettero ILM, Van Gastel-Mol EJ, Oud MECM, Van Dongen JJM. Basic Helix-Loop-Helix Proteins E2A and HEB Induce Immature T-Cell Receptor Rearrangements in Nonlymphoid Cells. *Blood* (2001) 8:2456–65. doi: 10.1182/blood.V98.8.2456
145. Bain G, Romanow WJ, Albers K, Havran WL, Murre C. Positive and Negative Regulation of V(D)J Recombination by the E2A Proteins. *J Exp Med* (1999) 2:289–300. doi: 10.1084/jem.189.2.289
146. Nozaki M, Wakae K, Tamaki N, Sakamoto S, Ohnishi K, Uejima T, et al. Regulation of TCR Vγ2 Gene Rearrangement by the Helix-Loop-Helix Protein, E2A. *Int Immunol* (2011) 5:297–305. doi: 10.1093/intimm/dxr005
147. Baker J, Cado D, Raulet D. Developmentally Programmed Rearrangement of T Cell Receptor Vgamma Genes Is Controlled by Sequences Immediately Upstream of the Vgamma Genes. *Immunity* (1998) 2:159–68. doi: 10.1016/s1074-7613(00)80598-1
148. Agata Y, Katakai T, Ye S-K, Sugai M, Gonda H, Honjo T, et al. Brief Definitive Report Histone Acetylation Determines the Developmentally Regulated Accessibility for T Cell Receptor Gene Recombination. *J Exp Med* (2001) 7:873–9. doi: 10.1084/jem.193.7.873
149. Xiong N, Kang C, Raulet DH. Redundant and Unique Roles of Two Enhancer Elements in the Tcrgamma Locus in Gene Regulation and Gammadelta T Cell Development. *Immunity* (2002) 3:453–63. doi: 10.1016/s1074-7613(02)00285-6
150. Xiong N, Zhang L, Kang C, Raulet DH. Gene Placement and Competition Control T Cell Receptor Γ Variable Region Gene Rearrangement. *J Exp Med* (2008) 4:929–38. doi: 10.1084/jem.20071275
151. Xiong N, Baker JE, Kang C, Raulet DH. The Genomic Arrangement of T Cell Receptor Variable Genes Is a Determinant of the Developmental Rearrangement Pattern. *Proc Natl Acad Sci USA* (2004) 1:260–5. doi: 10.1073/pnas.0303738101
152. Chien Y, Iwashima M, Wettstein D, Kaplan KB, Elliott JF, Born W, et al. T-Cell Receptor Delta Gene Rearrangements in Early Thymocytes. *Nature* (1987) 6150:722–6. doi: 10.1038/330722a0
153. Iwashima M, Green A, Davis M, Chien Y-H. Variable Region (V Delta) Gene Segment Most Frequently Utilized in Adult Thymocytes Is 3' of the Constant (C Delta) Region. *Proc Natl Acad Sci USA* (1988) 21:8161–5. doi: 10.1073/pnas.85.21.8161
154. Zhang B, Jiao A, Dai M, Wiest DL, Zhuang Y. Id3 Restricts Γδ NKT Cell Expansion by Controlling Egr2 and C-Myc Activity. *J Immunol* (2018) 5:1452–9. doi: 10.4049/jimmunol.1800106
155. Ueda-Hayakawa I, Mahlios J, Zhuang Y. Id3 Restricts the Developmental Potential of Γδ Lineage During Thymopoiesis. *J Immunol* (2009) 9:5306–16. doi: 10.4049/jimmunol.0804249
156. Verykokakis M, Boos MD, Bendelac A, Kee BL. SAP Protein-Dependent Natural Killer T-Like Cells Regulate the Development of CD8+ T Cells With Innate Lymphocyte Characteristics. *Immunity* (2010) 2:203–15. doi: 10.1016/j.immuni.2010.07.013
157. Zhang B, Lin Y-Y, Dai M, Zhuang Y. Id3 and Id2 Act as a Dual Safety Mechanism in Regulating the Development and Population Size of Innate-Like Γδ T Cells. *J Immunol* (2014) 3:1055–63. doi: 10.4049/jimmunol.1302694
158. Verykokakis M, Boos MD, Bendelac A, Adams EJ, Pereira P, Kee BL. Inhibitor of DNA Binding 3 Limits Development of Murine Slam-Associated Adaptor Protein-Dependent "Innate" Γδ T Cells. *PloS One* (2010) 5:203–15. doi: 10.1371/journal.pone.0009303
159. Fahl SP, Kappes DJ, Wiest DL. TCR Signaling Circuits in αβ/Γδ T Lineage Choice. In: 476 Soboloff J, Kappes, DJ, editor. *Signaling Mechanisms Regulating T Cell Diversity and Function.* Boca Raton, Florida: CRC Press (2017) 85–104.
160. Roy S, Moore AJ, Love C, Reddy A, Rajagopalan D, Dave SS, et al. Id Proteins Suppress E2A-Driven Invariant Natural Killer T Cell Development Prior to TCR Selection. *Front Immunol* (2018) 9:42. doi: 10.3389/fimmu.2018.00042
161. Monticelli LA, Yang Y, Knell J, D'Cruz LM, Cannarile MA, Engel I, et al. Transcriptional Regulator Id2 Controls Survival of Hepatic NKT Cells. *Proc Natl Acad Sci USA* (2009) 106:19461–6. doi: 10.1073/pnas.0908249106
162. Hu T, Wang H, Simmons A, Bajaña' S, Zhao Y, Kovats S, et al. Increased Level of E Protein Activity During Invariant NKT Development Promotes Differentiation of Invariant NKT2 and Invariant NKT17 Subsets. *J Immunol* (2013)191:5065–73 . doi: 10.4049/jimmunol.1301546.Increased
163. Verykokakis M, Krishnamoorthy V, Iavarone A, Lasorella A, Sigvardsson M, Kee BL. Essential Functions for ID Proteins at Multiple Checkpoints in Natural Killer T Cell Development. *J Immunol* (2013) 191:5973–83. doi: 10.4049/jimmunol.1301521
164. D'Cruz LM, Stradner MH, Yang CY, Goldrath AW. E and Id Proteins Influence Invariant Natural Killer T Cell Sublineage Differentiation and Proliferation. *J Immunol* (2014) 192:2227–36. doi: 10.4049/jimmunol.1302904
165. Li J, Wu D, Jiang N, Zhuang Y. Combined Deletion of Id2 and Id3 Genes Reveals Multiple Roles for E Proteins in Invariant NKT Cell Development and Expansion. *J Immunol* (2013) 191:5052–64. doi: 10.4049/jimmunol.1301252
166. Ioannidis V, Beermann F, Clevers H, Held W. The β-Catenin-Tcf-1 Pathway Ensures Cd4 + Cd8 + Thymocyte Survival. *Nat Immunol* (2001) 8:691–7. doi: 10.1038/90623
167. Huang Z, Xie H, Ioannidis V, Held W, Clevers H, Sadim MS, et al. Transcriptional Regulation of CD4 Gene Expression by T Cell Factor-1/β-Catenin Pathway. *J Immunol* (2006) 8:4880–7. doi: 10.4049/jimmunol.176.8.4880
168. Sharma A, Berga-Bolaños R, Sen JM, Alberola-Ila J. T Cell Factor-1 Controls the Lifetime of CD4+ CD8+ Thymocytes *In Vivo* and Distal T Cell Receptor α-Chain Rearrangement Required for NKT Cell Development. *PloS One* (2014) 12:e115803. doi: 10.1371/journal.pone.0115803
169. Emmanuel AO, Arnovitz S, Haghi L, Mathur PS, Mondal S, Quandt J, et al. TCF-1 and HEB Cooperate to Establish the Epigenetic and Transcription Profiles of CD4 + CD8 + Thymocytes. *Nat Immunol* (2018) 12:1366–78. doi: 10.1038/s41590-018-0254-4
170. Bain G, Quong MW, Soloff RS, Hedrick SM, Murre C. Thymocyte Maturation Is Regulated by the Activity of the Helix-Loop- Helix Protein, E47. *J Exp Med* (1999) 190:1605–16. doi: 10.1084/jem.190.11.1605
171. Jones-Mason ME, Zhao X, Kappes D, Lasorella A, Iavarone A, Zhuang Y. E Protein Transcription Factors Are Required for the Development of CD4(+) Lineage T Cells. *Immunity* (2012) 36(3):348–61. doi: 10.1016/j.immuni.2012.02.010
172. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte Selection Is Regulated by the Helix-Loop-Helix Inhibitor Protein, Ld3. *Immunity* (2000) 12:17–26. doi: 10.1016/S1074-7613(00)80155-7
173. Miyazaki M, Miyazaki K, Chen S, Chandra V, Wagatsuma K, Agata Y, et al. The E–Id Protein Axis Modulates the Activities of the PI3K–AKT–Mtorc1–Hif1a and C-Myc/P19arf Pathways to Suppress Innate Variant TFH Cell Development, Thymocyte Expansion, and Lymphomagenesis. *Genes Dev* (2015) 29:409–25. doi: 10.1101/gad.255331.114
174. Miyazaki M, Rivera RR, Miyazaki K, Lin YC, Agata Y and Murre C. The Opposing Roles of E2A and Id3 That Orchestrate and Enforce the Naïve T Cell Fate. *Nat Immunol* (2012) 12:992–1001. doi: 10.1016/j.physbeh.2017.03.040
175. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, et al. The Transcriptional Regulators Id2 and Id3 Control the Formation of Distinct Memory CD8 + T Cell Subsets. *Nat Immunol* (2011) 12:1221–9. doi: 10.1038/ni.2158
176. Shaw LA, Belanger S, Omilusik K, Cho S, Scott-Browne JP, Jp N, et al. Id2 Reinforces Th1 Cell Differentiation and Inhibits E2A to Repress Tfh Cell Differentiation. *Nat Immunol* (2016) 77:834–43. doi: 10.1038/ni.3461
177. Omilusik KD, Shaw LA, Goldrath AW. Remembering One's ID/E-Ntity: E/ID Protein Regulation of T Cell Memory. *Curr Opin Immunol* (2013) 25:660–6. doi: 10.1016/j.coi.2013.09.004
178. Sullivan JM, Höllbacher B, Campbell DJ. Cutting Edge: Dynamic Expression of Id3 Defines the Stepwise Differentiation of Tissue-Resident Regulatory T Cells. *J Immunol* (2019) 202:31–6. doi: 10.4049/jimmunol.1800917
179. Miyazaki M, Miyazaki K, Chen S, Itoi M, Miller M, Lu LF, et al. Id2 and Id3 Maintain the Regulatory T Cell Pool to Suppress Inflammatory Disease. *Nat Immunol* (2014) 15:767–76. doi: 10.1038/ni.2928
180. Cross S, Linker J, Kay E, Leslie FM. The Transcriptional Regulator Id2 Is Critical for Adipose-Resident Regulatory T Cell Differentiation, Survival and Function. *Physiol Behav* (2016) 176:100–6. doi: 10.4049/jimmunol.1900358
181. D'Cruz LM, Lind KC, Wu BB, Fujimoto JK, Goldrath AW. Loss of E Protein Transcription Factors E2A and HEB Delays Memory-Precursor Formation During the CD8 + T-Cell Immune Response. *Eur J Immunol* (2012) 42:2031–41. doi: 10.1002/eji.201242497

182. Cannarile MA, Lind NA, Rivera R, Sheridan AD, Camfield KA, Wu BB, et al. Transcriptional Regulator Id2 Mediates Cd8+ T Cell Immunity. *Nat Immunol* (2006) 7:1317–25. doi: 10.1038/ni1403
183. Knell J, Best JA, Lind NA, Yang E, D'Cruz LM, Goldrath AW. Id2 Influences Differentiation of Killer Cell Lectin-Like Receptor G1 Hi Short-Lived CD8+ Effector T Cells. *J Immunol* (2013) 190:1501–9. doi: 10.4049/jimmunol.1200750
184. Schauder D, Shen J, Kasmani M, Kudek M, Burns R, Cui W. E2a-Regulated Epigenetic Landscape Promotes Memorycd8 T Cell Differentiation. *PNAS* (2021) 118:2022. doi: 10.1073/pnas.2013452118/-/DCSupplemental
185. Masson F, Minnich M, Olshansky M, Bilic I, Mount AM, Kallies A, et al. Id2-Mediated Inhibition of E2A Represses Memory CD8 + T Cell Differentiation. *J Immunol* (2013) 190:4585–94. doi: 10.4049/jimmunol.1300099
186. Omilusik KD, Nadjsombati MS, Shaw LA, Yu B, Justin Milner J, Goldrath AW. Sustained Id2 Regulation of E Proteins Is Required for Terminal Differentiation of Effector CD8 + T Cells. *J Exp Med* (2018) 215:773–83. doi: 10.1084/jem.20171584
187. Milner JJ, Toma C, He Z, Kurd NS, Nguyen QP, McDonald B, et al. Heterogenous Populations of Tissue-Resident CD8+ T Cells Are Generated in Response to Infection and Malignancy. *Immunity* (2020) 52:808–24. doi: 10.1016/j.immuni.2020.04.007
188. Zhou Y, Murre C. Bursty Gene Expression and Mrna Decay Pathways Orchestrate B Cell Activation. *Sci Adv* (2021) 7(49):1–10. doi: 10.1126/sciadv.abm0819

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EDITED BY
Michele Kay Anderson,
University of Toronto, Canada

REVIEWED BY
Cristina Hernández-Munain,
Spanish National Research Council (CSIC), Spain
Christopher S. Seet,
University of California, Los Angeles, United States

*CORRESPONDENCE
Lena Boehme
lena.boehme@ugent.be
Tom Taghon
tom.taghon@ugent.be

†These authors share first authorship

‡These authors share last authorship

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Transcriptional dynamics and epigenetic regulation of E and ID protein encoding genes during human T cell development

Juliette Roels[1,2,3†], Jolien Van Hulle[1†], Marieke Lavaert[1], Anna Kuchmiy[1,3], Steven Strubbe[1], Tom Putteman[1], Bart Vandekerckhove[1,3], Georges Leclercq[1,3], Filip Van Nieuwerburgh[3,4], Lena Boehme[1*‡] and Tom Taghon[1,3*‡]

[1]Department of Diagnostic Sciences, Ghent University, Ghent, Belgium, [2]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [3]Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [4]Laboratory of Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium

T cells are generated from hematopoietic stem cells through a highly organized developmental process, in which stage-specific molecular events drive maturation towards αβ and γδ T cells. Although many of the mechanisms that control αβ- and γδ-lineage differentiation are shared between human and mouse, important differences have also been observed. Here, we studied the regulatory dynamics of the E and ID protein encoding genes during pediatric human T cell development by evaluating changes in chromatin accessibility, histone modifications and bulk and single cell gene expression. We profiled patterns of ID/E protein activity and identified up- and downstream regulators and targets, respectively. In addition, we compared transcription of E and ID protein encoding genes in human versus mouse to predict both shared and unique activities in these species, and in prenatal versus pediatric human T cell differentiation to identify regulatory changes during development. This analysis showed a putative involvement of TCF3/E2A in the development of γδ T cells. In contrast, in αβ T cell precursors a pivotal pre-TCR-driven population with high ID gene expression and low predicted E protein activity was identified. Finally, in prenatal but not postnatal thymocytes, high HEB/TCF12 levels were found to counteract high ID levels to sustain thymic development. In summary, we uncovered novel insights in the regulation of E and ID proteins on a cross-species and cross-developmental level.

KEYWORDS

E proteins, ID proteins, T cell development, human, thymocytes, gene regulation, epigenetics, gene regulatory networks

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1 Introduction

Cellular differentiation is directed by alternating cues for proliferation and differentiation of precursor cells to their final state. In many different cell types E proteins and their inhibitory antagonists ID proteins play an indispensable role in guiding this process. E proteins are basic helix-loop-helix (bHLH) transcription factors that can engage histone modifiers, transcriptional co-activators and DNA binding proteins. As homodimers or heterodimers with other HLH protein family members they bind the six nucleotide CANNTG E box motif in the DNA, which is where their name originates from (1, 2). As such, E proteins can support multiple developmental programs by inducing cell cycle arrest and allowing cellular differentiation (2, 3).

ID proteins, on the other hand, are members of the HLH protein family (4). They can engage with E proteins to inhibit their function by competitive interaction. All ID proteins lack the basic DNA binding domain found in bHLH proteins. Therefore, E-ID dimers cannot bind DNA, which interferes with the E proteins' transcription factor activity. Generally, the inhibitory interaction of ID with E proteins lifts the cell cycle arrest and promotes cell cycle re-entry at the expense of differentiation, hence their name, Inhibitor of Differentiation (3).

There are three E protein encoding genes, *TCF3* (also known as E2A), *TCF4* (also referred to as E2-2) and *TCF12* (also known as HEB). In addition, TCF3 and TCF12 each have two annotated isoforms that are generated by either alternative splicing (TCF3: E12/E47), or alternative transcription initiation (TCF12: HEBalt/HEBcan), respectively (5). On the other hand, four genes code for ID proteins, namely *ID1* to *ID4.* The level of redundancy between different members of the ID and E protein family is not entirely clear. It is thought that the combined expression level of the different E or ID proteins is a major determinant for differentiation (6–9); however, on top of that, each protein likely has its own unique functions, which can be appreciated by single gene murine knockout experiments (10).

During hematopoiesis, E and ID proteins play an indispensable role at numerous differentiation stages from hematopoietic stem cells (HSCs) to functional myeloid and lymphoid cells. The balance between ID (ID1) and E proteins (E47) can guide HSCs in the direction of myeloid or lymphoid precursors, respectively (11). Similarly, during lymphoid development, the lineage decision between natural killer (NK) and T cells is directed by the ratio of ID to E proteins, with high ID (ID2 and ID3) protein activity favoring NK cell fate (12, 13). In contrast, the fate of Dendritic Cells (DCs) is determined by the activity of different TCF4 isoforms regulating the plasmacytoid DC (pDC) versus conventional DC (cDC) lineage entry (14). The short *TCF4* isoform is expressed in both cDCs and pDCs but is actively repressed by ID2 (under influence of BCL11A) during cDC development. pDCs on the other hand, have specific expression of the long *TCF4* isoform, which is needed for their development (14).

T cells develop in the thymus from multipotent lymphoid precursors. During this differentiation process, multiple decision checkpoints exist to generate the wide variety of conventional (αβ) and unconventional (γδ, CD8αα, MAIT, Treg and NK-T) T cells (15, 16). First, bone marrow-derived progenitors gradually differentiate into immature T-lineage specified cells and eventually commit to the T cell fate, excluding potential for other lineages (Figure 1A). From here onwards, committed thymocytes start to rearrange their δ, γ and β T cell receptor (TCR) chains in a process known as V(D)J recombination, mediated by the Recombination-Activating Gene (RAG) proteins (17). Successfully rearranged δ and γ chains pair together to form a γδ TCR, which instructs the developing thymocyte to differentiate further into mature γδ T cells, whereas predecessors of αβ T cells require additional selection steps. *TCRB* rearranging T cells form a pre-TCR by combining the β and the invariant pTα chain during a process called β-selection (15). If the pre-TCR signals with adequate intensity, the rearrangement of the α–chain is initiated, which results in progression to the $CD4^+CD8\beta^+$ double positive (DP) stage of T cell development. DP thymocytes cells undergo negative and subsequently positive selection to ultimately result in mature naïve CD4 or CD8 single positive (SP) αβ T cells. Alternatively, DP thymocytes cells can also give rise to NKT or MAIT cells (18).

During several stages of T cell development E and ID protein driven transcriptional regulation is crucial to ensure proper T cell generation. For instance, the absence of TCF3 partially blocks the earliest stages of thymic differentiation in the mouse (19, 20). TCF3 is an activator of NOTCH1 and of NOTCH1 target genes including *Hes1* and *Dtx1*, thereby priming early thymocyte differentiation (21, 22). Next, TCF3 also activates *Ptcra* and *Rag* expression, which are necessary to initiate TCR rearrangements (22–24). TCF12 is equally essential for T cell development, which is illustrated by *Tcf12*-deficient mice that develop a thymic differentiation arrest, albeit later than *Tcf3*-deficient mice, before the transition to DP thymocytes cells (8). This can in part be explained by TCF12 cooperating with the TCF3 isoform E47 to increase accessibility of the TCR Vβ locus (25). During T cell development, starting from the formation of the pre-TCR, E protein activity is gradually inhibited by ID proteins. This is partially mediated by TCR-mediated induction of *Egr1* expression *via* the RAS–ERK–MAP kinase (MAPK) pathway, which in turn activates *Id3* transcription (26, 27). The γδ TCR is known to give a stronger signal than the pre-TCR (28), which is consistent with high expression of *Id3* in immature γδ T cells (28–31). Moreover, *Id3* expression in γδ T cells remains at higher levels, while a significant decrease in expression can be observed for differentiating αβ thymocytes, further indicating the specificity of ID3 for γδ T cells (32–34). High ID3 activity

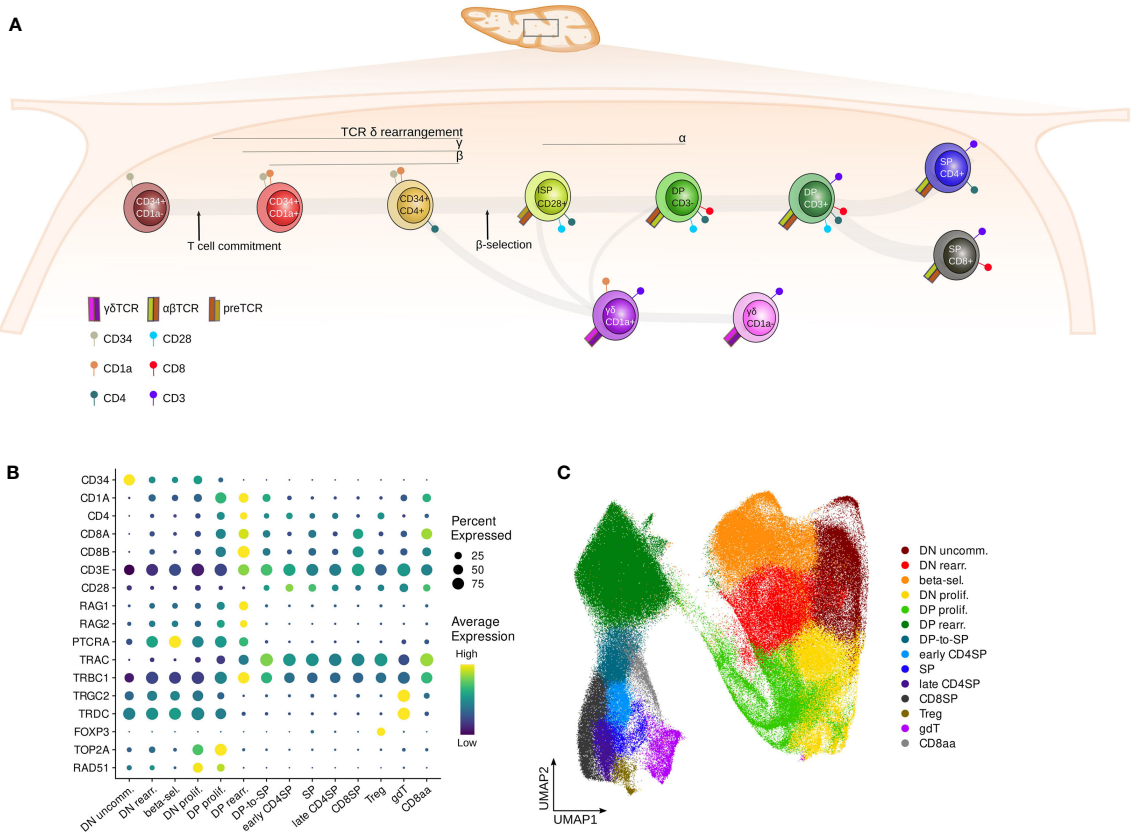

FIGURE 1
Surface markers distinguish subsequent stages of human thymocyte development. **(A)** Schematic depiction of stages of T cell development in the human thymus. **(B)** Dot plot visualizing pseudo-bulk expression of known thymocyte markers per annotated cluster in the pediatric single cell data set. Non-imputed data was log-normalized, averaged and scaled by gene. **(C)** UMAP visualizing the annotated clusters in the pediatric single cell thymus data set.

was recently shown to inhibit *Tcf1* expression in γδ T cells in order to lock in the γδ-lineage fate and effector potential in fetal murine thymocytes (35).

Most of our current knowledge about E and ID protein function during T cell development is based on studies in mice. However, there are some key differences in thymocyte differentiation between mouse and human, especially during the αβ-γδ lineage bifurcation. For instance, both murine and human thymocyte development include an Immature Single Positive (ISP) stage ($CD4^+CD8^-$ in human, $CD8^+CD4^-$ in mice), but while in mice this occurs after β-selection, in human the ISP stage precedes β-selection. Moreover, the order and timing of TCR locus rearrangement differs between the two species, with *TCRD*, *TCRG* and *TCRB* loci rearranging in this chronological order in the human thymus, whereas in mouse V(D)J recombination of the *Tcrb* locus occurs earlier (36). Human thymocytes have also been shown to retain γδ potential throughout a long developmental window since $TCR\gamma\delta^+$ DP thymocytes can be detected, whereas in wildtype mice, γδ potential is usually extinguished by the time they reach the DP stage (37, 38). Further mechanistic differences between mice and human, such as dependance on Notch signaling, have been established (34).

Similarly, fetal T cell development also differs from postnatal development in several aspects. A quantitative imbalance of thymic output of γδ T cell subsets is observed in prenatal compared to postnatal human T cell development (39, 40). The gene expression dynamics of ID proteins, particularly *ID1* and *ID2*, are also different in fetal compared to postnatal thymocytes as shown by analyses in mice (41). However, whether this difference in expression levels is directly linked to the differences in thymic output is currently unknown. In adult thymocytes, TCF3 does block certain *TCRG* V rearrangements that are specifically recombined in a fetal context (19, 42), which may also indicate a role for E and ID proteins in the balance of γδ T cells before and after birth (43–45).

In this study, we employed bulk and single cell sequencing profiling methods to uncover the regulatory roles of E and ID proteins during human T cell development. We compared our extensive human postnatal thymic data to murine postnatal and

human prenatal datasets to gain a better understanding of species-specific and developmental differences, which is of great importance for translational studies. Next, we used gene regulatory network analysis to gain a better understanding of the fine regulatory influence that E and ID protein encoding gene expression has on developing thymocytes. This comprehensive analysis confirmed that many of the findings in mice also hold true in a human context. Nevertheless, we found prominent differences between human and murine expression dynamics of *TCF3*. Furthermore, in human, we found evidence for a regulatory role of TCF3 only after increased accessibility of the *TCRG* locus, which is delayed compared to mouse. Using single cell analysis, we next identified a small cluster of immature γδ T cells that is characterized by *ID3* and *TCF3* expression. In contrast, a cluster of β-selecting cells was identified along the αβ-lineage trajectory that has a very high ID to E protein ratio, likely induced by pre-TCR signaling. Finally, prenatal thymocytes showed an early induction of ID gene expression and stronger TCF12 transcription seems to compensate for this. In conclusion, we here provide a comprehensive analysis of E and ID protein encoding gene activity during thymic differentiation and uncover novel insights into the function of these proteins in different thymic developmental lineages in human.

2 Materials and methods

2.1 Bulk data analysis

Bulk expression profiling by RNA-seq, chromatin accessibility profiling by ATAC-seq and histone modification profiling (H3K4me3, H3K27ac and H3K27me3) by ChIPmentation was previously generated by our group on developing T cells subsets (46). The IGV Genome Browser was used for visualization of all sequencing tracks. RNA expression counts are shown as transcripts per million reads (TPM).

To identify putative transcription factor binding sites, transcription factor footprinting analysis was performed. Transcription factor footprinting combines information from ChIP-seq derived transcription factor motifs with chromatin accessibility information from ATAC-seq. The presence of a TF prevents the cleavage of DNA, leaving a unique footprint in ATAC-seq reads. This method increases the accuracy of predicting transcription factors' presence at their binding sites. For footprinting analysis, Bed files generated from ATAC-seq data were used after peak calling with MACS2, as previously described (46, 47). The footprinting analysis was done with the Regulatory Genomics Toolbox (RGT) functions "rgt-HINT" and "rgt-motif analysis matching" (48) using the JASPAR vertebrate motif database (49).

2.2 Single cell data generation

2.2.1 Antibodies

CD1a-APC (Biolegend), CD4-PE-Cy7 (Biolegend), CD4-PE (Biolegend), CD8a-FITC (Biolegend), CD8a-APC-Cy7 (Biolegend), CD45-BV510 (BD), CD3-APC (Biolegend)

2.2.2 Cell type enrichment on postnatal thymus samples

Pediatric thymus from children undergoing cardiac surgery was obtained according to and used with the approval of the Medical Ethical Commission of Ghent University Hospital, Belgium. Thymus tissue was cut into small pieces and digested with 1.6 mg/ml collagenase (Gibco, 17104-019) in IMDM medium for 30 min at 37°C to generate a single cell suspension. The reaction was quenched with 10% FBS and the thymocyte suspension was passed through a 70 µm filter to remove undigested tissue. Cells were frozen in FBS containing 10% DMSO and stored in liquid nitrogen until needed. Upon thawing, thymocytes were enriched for cell types of interest ($CD34^+$ cells, ISPs, DPs, $TCR\gamma\delta^+$ thymocytes) using bead-based enrichment/depletion and FACS. To obtain DP thymocytes, cells were labelled with antibodies and FACS sorting was used to obtain equal proportions of $CD8\alpha^+CD4^+CD3^+$ and $CD45^+CD8\alpha^+CD4^+CD3^-$ thymocytes. $CD34^+$ cells were obtained through enrichment with CD34 magnetic-activated cell-sorting (MACS) microbeads (Miltenyi, 130-046-703), labelled with anti-CD1a and subsequently FACS sorted to include equal proportions of $CD1a^+$ and $CD1a^-$ cells. To enrich ISPs, thymocytes were labelled with anti-CD3 (clone OKT3, produced in-house) and anti-glycophorin A (clone 10F7MN, produced in-house) and $CD3^+$ and $Glycophorin^+$ cells were subsequently depleted using sheep anti-mouse IgG magnetic Dynabeads (Invitrogen). This was followed by FACS sorting for $CD3^-CD8\alpha^-CD4^+$ thymocytes. To obtain $TCR\gamma\delta^+$ thymocytes, cells were enriched using anti-γδ TCR Hapten antibodies and anti-Hapten MACS microbeads (Miltenyi, 130-050-701) according to the manufacturer's instructions and subsequently FACS sorted for $TCR\gamma\delta^+CD3^+$.

2.2.3 Library preparation and sequencing

The sorted cells were resuspended in PBS containing 0.04% BSA at a concentration of approximately 1200 cells/µl. 16.5µl cell suspension per sample was loaded onto a Next GEM Chip G (10X Genomics) according to the manufacturer's instructions and the Chromium Controller was used to generate GEMs. Reverse transcription, amplification and library preparation were carried out using the Next GEM Single Cell 3' GEM v3.1 kit (10X Genomics) according to the manufacturer's instructions. Libraries were multiplexed and sequenced to a mean depth of 23.000-54.000 reads/cell.

2.3 Single cell data analysis

2.3.1 Preprocessing

Published sequencing data was downloaded from ArrayExpress, GEO and NODE (see Data Availability Statement and Table S1). All fastq files were mapped to the human reference genome GRCh38 using CellRanger version 6.0.1 (10X Genomics). Subsequently, prenatal and pediatric data were analyzed separately. H5 files were loaded into R and analyzed using the Seurat package (50). Cells with over 10% (pediatric data) or 7.5% mitochondrial reads (prenatal data), fewer than 700 reads or expressing fewer than 250 genes were considered to be of low quality and removed from the dataset. The scDblFinder package (51) was used to identify and exclude doublets. In addition, cells with unusually high gene count were removed, with the cutoff varying from >2500 to >6000 genes per cell depending on the sequencing depth of the respective library. Finally, genes expressed in fewer than 10 cells across the entire dataset were removed as non-informative.

Gene expression was log-normalized and the 2000 most variable genes (HVGs) were identified using Seurat. To correct for cell cycle-dependent effects but preserve information about proliferative vs. quiescent cell states, cell cycle scoring was conducted using the G2/M and S phase marker genes provided in the Seurat package and the difference between G2M and S scores was regressed out. Moreover, differences in sequencing depth between samples were regressed out and data was scaled and centered.

2.3.2 Dimensionality reduction, batch correction and clustering

PCA was performed on the scaled HVGs. To reduce batch effects between samples, MNN correction was applied to the PCA matrix *via* the reducedMNN function from the Batchelor package (52). For this step, every library was considered as a separate batch and the merge order was manually specified to guarantee the largest possible overlap in cell types between subsequently merged libraries. The corrected PCA was used to generate an SNN graph (k=50), which was then used to conduct Louvain clustering with an initial resolution of 0.3. Large clusters were further subclustered with a resolution of 0.1-0.8 to identify additional subpopulations of interest. UMAP was used to visualize the results and known marker genes for distinct stages of thymocyte development were used to annotate the clusters (Figure 1B). Clusters with comparable expression of marker genes were merged to obtain the larger annotated clusters used for downstream analyses (Figure 1C). Non-relevant clusters, such as dendritic cells, B cells, stromal cells and NK cells, were removed from the dataset prior to downstream analyses.

2.3.3 DGE, imputation and cell scoring

Differential gene expression analysis for clusters of interest was carried out in a one-vs-all manner on the normalized data *via* the FindMarkers function from the Seurat package using a Wilcoxon Rank Sum test and Bonferroni correction. Prior to visualization, Markov affinity-based graph imputation of cells (MAGIC) (53) was used to denoise the data and impute dropout values.

The UCell package (54) was used to perform cell scoring. To establish the E:ID score, *TCF3*, *TCF4* and *TCF12* were given positive weights while *ID1*, *ID2* and *ID3* carried negative weights. For Notch scoring the following genes were considered indicators of Notch signaling activity: *TCF7*, *HES1*, *HES5*, *HEY1*, *DTX1*, *NOTCH1*, *NOTCH3*, *IL7R*, *CD7*, *PTCRA*, *MYC*, *CCND1*, *NRARP* and *TCF3*.

2.3.4 Pseudotime analysis

The destiny package (55) was used to establish a diffusion map based on the first 20 principal components. Subsequently, the first diffusion component was used as pseudotime measure. Proliferating cells showed inconsistent clustering and were therefore removed from the pseudotime ordering; moreover, only αβ-lineage cells were included in the analysis. The tradeseq package (56) was used to fit a generalized additive model (GAM) on the cell pseudotimes and to determine smoothed gene expression values. Data was scaled and plotted using pheatmap (57).

2.3.5 Gene Regulatory Network (GRN) analysis

GRN analysis was conducted using pySCENIC (58) and SIGNET (59). Due to the compute-intensive nature of the two pipelines the dataset was downsampled to a representative subset of 50.000 and 10.000 cells, respectively. A list of human transcription factors as well as motif ranking databases (mc9nr hg38 500bpUp100Dw and TSS+/-10kbp) were obtained from the online resources provided by the Aerts lab.

In line with the recommended pySCENIC workflow, the GRNBoost2 algorithm (60) was used to determine co-expression modules between transcription factors and potential targets. Subsequently, regulon prediction was carried out using cisTarget based on HGNC motif annotations and motif ranking databases. Finally, the regulon activity per cell was determined *via* enrichment scoring for the regulon target genes using AUCell (61).

For detection of transcription factor-target co-expression modules with SIGNET the same list of transcription factors was supplied as for pySCENIC. RcisTarget (61) was used to prune the modules based on motif rankings and HGNC annotations.

2.3.6 Automated cell type annotation of prenatal data

The singleR package (62) was used to carry out automated annotation of cell types in the prenatal dataset. For this purpose, a pseudobulk gene expression reference was generated from the pediatric single cell dataset. SingleR was then used to infer labels

for individual cells based on similarity to the gene expression signature of the annotated clusters in the pediatric data.

3 Results

3.1 Expression of E and ID protein encoding genes throughout human thymocyte development

To obtain a better understanding of the activity of E and ID proteins during human T cell development, we made use of bulk RNA-seq, ATAC-seq and ChIPmentation data for distinct stages of human thymocyte development as described earlier (46) (Figure 1A). In addition, we compiled a comprehensive scRNA-seq dataset from multiple different sources (63–65) including several new libraries (Figure S1A, Table S1), incorporating approximately 280.000 thymocytes from 13 pediatric donors between the ages of 9 days and 13 years. Sufficient coverage of rare developmental stages was achieved through enrichment for specific cell types prior to library preparation (Figures S1B and Table S1). UMAP-based dimensionality reduction, unsupervised clustering (Figure S1C) and manual annotation of the data based on known cell type markers (Figures 1B and S1D, E) was carried out. In this process, subclusters with comparable marker gene expression were merged to form larger annotated cell populations (Figure 1C). This confirmed that the single cell dataset spans thymocytes across all developmental stages, from the most immature precursors to the fully differentiated naïve T cells (Figure 1C).

To establish gene expression trends along thymocyte differentiation, we evaluated the transcript levels of the genes that encode each of the E and ID proteins in both the bulk samples, and in the continuum of the single cell dataset.

Analysis of *TCF3* RNA levels revealed high expression in immature thymocytes up until the earliest lineage-specific stages of αβ and γδ T cell development (β-selected ISP CD28$^+$ and TCRγδ $^+$CD1a$^+$ cells, respectively), followed by a gradual downregulation in both lineages with ongoing maturation (Figure 2A, top+middle). Even though *TCF3* gene expression was reduced in more mature thymocytes, active promoter marks (H3K4me3 and H3K27ac) and a complete absence of repressive chromatin modifications (H3K27me3) were detected at the *TCF3* locus throughout thymocyte development, supporting sustained *TCF3* transcription (Figure 2A, bottom). Furthermore, we detected the expression of both TCF3 isoforms *E12* and *E47* at comparable levels in the bulk RNA-seq dataset (data not shown).

Similar to *TCF3*, *TCF4* transcript levels were found to follow a downward trend as T cell development progressed (Figure 2B, top+middle). However, *TCF4* transcription already decreased early on, at the T cell commitment stage (CD34$^+$CD1a$^+$), and the immature αβ/γδ-lineage cells showed a substantial reduction in *TCF4* RNA levels compared to preceding stages. This suggests a swift shutdown of *TCF4* transcription in differentiating thymocytes, in contrast to *TCF3* expression, which is maintained throughout a wider developmental window. Downregulation of *TCF4* was associated with a moderate reduction of H3K27ac and H3K4me3 at the gene promoter of the long *TCF4* isoform (Figure 2B, bottom). However, the promoter of the short *TCF4* isoform displayed profound H3K27ac marks in the earliest developmental stages but was completely shut down by the CD34$^+$CD4$^+$ stage. Ultimately, for both isoforms, a decrease in chromatin accessibility after the DP stage was observed, consistent with the drop in transcriptional activity (Figure 2B, bottom).

The third E protein encoding gene, *TCF12*, exhibited a very different expression pattern with low RNA levels in uncommitted thymocytes and an initial peak around the putative αβ/γδ bifurcation point (CD34$^+$CD4$^+$) (Figure 2C, top+middle). In cells of the γδ-lineage, *TCF12* transcription was subsequently reduced, while αβ-lineage cells were found to experience a second window of strong *TCF12* expression at the DP stage, followed by rapid downregulation at the DP-SP transition. TCF12/HEB has two known isoforms, HEBcan and HEBalt, both originating from alternative transcript initiation (5). In our dataset, there was no evidence for a distinctly active alternative start site for *HEBalt* in developing human thymocytes. Indeed, a complete absence of open chromatin or active promoter methylation was observed at this site (Figure 2C, bottom) and expression of the N-terminal HEBalt-specific exon was not detected. Therefore, *HEBalt* transcripts are presumably only very lowly expressed, if at all, in our human thymic dataset.

In contrast to the *HEBalt* promoter, the promoter region of *HEBcan* did exhibit H3K4me3 and K3K27ac histone marks, as well as open chromatin. This was already evident in the most immature thymocytes, thus preceding the higher transcription levels, which suggests that the most immature thymocytes are primed for *TCF12* upregulation (Figure 2C, bottom). Shutdown of *TCF12* expression in the αβ-lineage was accompanied by chromatin closure and loss of H3K27ac in SP thymocytes. In contrast, in γδ-lineage thymocytes permissive chromatin marks were lost in mature CD1a$^-$ cells, but chromatin accessibility was maintained, suggesting a different mechanism of transcriptional downregulation in these cells.

Since E protein activity is crucially controlled through inhibitory dimerization with ID proteins (66), we also assessed ID transcript levels throughout thymocyte development. *ID1* gene expression exhibited a rapid increase, followed by a steep decline, with the highest levels detected in CD3$^-$ DP thymocytes, whereas few *ID1* transcripts were identified in the preceding immature stages or the more mature TCRαβ$^+$ SP and TCRγδ$^+$ thymocytes (Figure 3A, top). This pattern resembles that of *TCF12*, but comparison of expression at the single cell level on UMAP showed that there was surprisingly little overlap in cells

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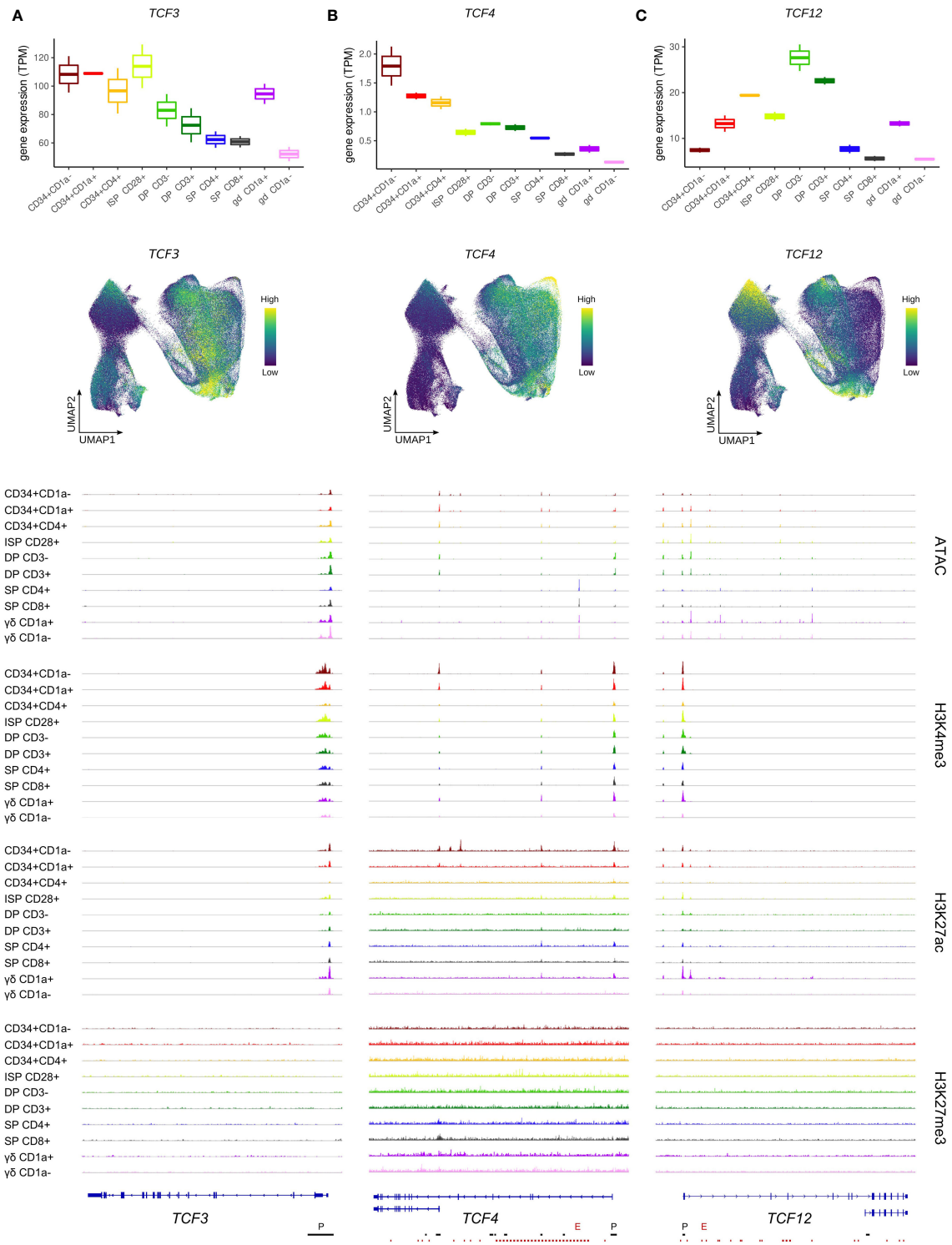

FIGURE 2
Expression of E protein encoding genes throughout human thymocyte development. **(A)** Transcript levels of *TCF3* according to bulk RNA-seq (top) and single cell data (middle), and epigenetic profile at the *TCF3* gene locus. **(B)** Transcript levels of *TCF4* according to bulk RNA-seq (top) and single cell data (middle), and epigenetic profile at the *TCF4* gene locus. Long and short isoform of TCF4 are shown. **(C)** Transcript levels of *TCF12* according to bulk RNA-seq (top) and single cell data (middle), and epigenetic profile at the *TCF12* gene locus. Long (HEBcan) and short isoform (HEBalt) of TCF12 are shown. Locations of promoters (P) and enhancers (E) were retrieved from the Ensembl Regulatory Build and are indicated below the gene structure. UMAP visualizations were generated using imputed data.

expressing high levels of *ID1* or *TCF12* (Figure 3A, middle, and Figure S2A). Indeed, *ID1* expression was found to be rather heterogeneous especially in rearranging and β-selecting thymocytes, with some cells exhibiting strong *ID1* expression whereas other cells at the same developmental stage showed very low *ID1* transcript levels. This suggests that bulk expression profiles indeed do not entirely reflect the fine-grained dynamics of *ID1* expression throughout early differentiation. Remarkably, the *ID1* locus was marked by both repressive and activating histone modifications (H3K27me3 and H3K27ac) (Figure 3A, bottom). This suggests the presence of poised regulatory elements that can rapidly and temporarily switch to active promoters/enhancers over the course of development, which is consistent with the expression dynamics observed in the single cell data.

In the bulk data, *ID2* transcription was not detected in the most immature stages of human T cell development (Figure 3B, top), although the single cell data suggested two small subsets of cells expressing ID2 in the immature DN and β-selection clusters (Figure 3B, middle). Widespread induction of *ID2* was observed in both the αβ- and γδ-lineage committed cells and reached peak levels in the most mature SP and TCRγδ$^+$ cells (Figure 3B, top+middle). The increased expression in later stages of thymic development was accompanied by higher H3K4me3 and H3K27ac levels (Figure 3B, bottom). Combined, this suggests a role for ID2 in the late stages of T cell development or maybe even only in mature cells, with limited function in the early differentiation steps.

ID3 gene expression followed a similar pattern, with low RNA levels throughout the most immature stages and a progressive upregulation during αβ-lineage differentiation in DP and SP thymocytes (Figure 3C, top), although induction seemed to occur slightly earlier than that of *ID2* as visible in the UMAP plots (Figures 3B, C, middle). In contrast, in the γδ-lineage a striking increase in ID3 transcripts was evident in immature TCRγδ$^+$ cells, which is consistent with reports of ID3 being an important regulator of murine γδ T cell development (29, 67). The strong initial upregulation of *ID3* in immature CD1a$^+$ γδ T cells was followed by a reduction during further γδ-lineage maturation to levels comparable with those in SP TCRαβ$^+$ thymocytes (Figure 3C, top). In addition, the single cell data suggested a drop in *ID3* levels in more mature SP thymocytes, which was not discernible from the bulk expression profiles (Figure 3C, middle). In disagreement with its expression pattern, *ID3* was found to exhibit high levels of H3K27ac at the transcription start site and in the gene body in immature thymocytes, which were extinguished by the SP stage (Figure 3C, bottom). H3K4me3 marks were also found in the gene body throughout most developmental stages and therefore cannot explain the transcriptional upregulation of the *ID3* gene in immature TCRγδ$^+$ cells and SP thymocytes. However, in these *ID3*^{high} cell types a prevalent H3K27me3 site immediately upstream of *ID3* was remarkably depleted of this histone modification, while it had persistent methylation from the most immature stages up until the DP-SP transition (Figure 3C, bottom). In addition, chromatin accessibility at the *ID3* transcription start site was increased in cells of the γδ lineage. Thus, the involvement of both H3K27ac and H3K27me3 as well as chromatin opening in cell type-specific regulation of *ID3* expression point again to a very complex regulatory mechanism of *ID* gene expression during thymic development.

Finally, no noticeable *ID4* expression was detected in any of the thymocyte stages, which is consistent with previous reports (68) (Figure S2B).

3.2 E and ID protein encoding gene expression in human and murine thymic development

Thymic expression of E and ID protein encoding genes in the mouse has been studied in detail (7, 25, 69–73) and transcript levels at distinct stages have been mapped by the Immunological Genome Project Consortium (74). We made use of this resource to perform an inter-species comparison of gene expression trends during thymocyte development. Of note, a direct and accurate stage-by-stage comparison between mouse and human is difficult since some developmental stages do not have matching phenotypic markers in both species, especially the most immature thymocyte stages.

In general, highly similar trends were observed for the expression of most E and ID genes in human and mouse thymocytes (Figure S3). *ID2* and *ID3* displayed the same late upregulation in both species, with peak expression in the αβ-lineage SP and the γδ T cell stages, respectively (Figure S3). Likewise, high initial levels of *TCF4* expression and its subsequent downregulation were observed in murine and human cells. The previously described bimodal expression profile of *TCF12* with peaks around the human β-selection checkpoint and in DP thymocytes was also mirrored in the mouse.

TCF3 expression peaked in ISP thymocytes in both species, even though these represent different developmental stages in both species, but in human thymocytes this was preceded by consistently high expression levels, whereas murine thymocytes showed only a gradual *Tcf3* upregulation with low levels at the DN1 stage (Figure S3). Some differences were also observed in the transcription profile of *ID1*, which displayed peak expression in DP thymocytes of both human and mouse but seemed to fluctuate in mouse DN thymocytes (Figure S3). This variability might be caused by transient or heterogeneous upregulation of *Id1* throughout the DN stage, as previously noted for the human single cell dataset (Figure 3A, middle). Alternatively, variable *Id1* expression may be attributed to the overall low levels of *Id1* in mouse thymocytes, especially when compared to those of *Id2*

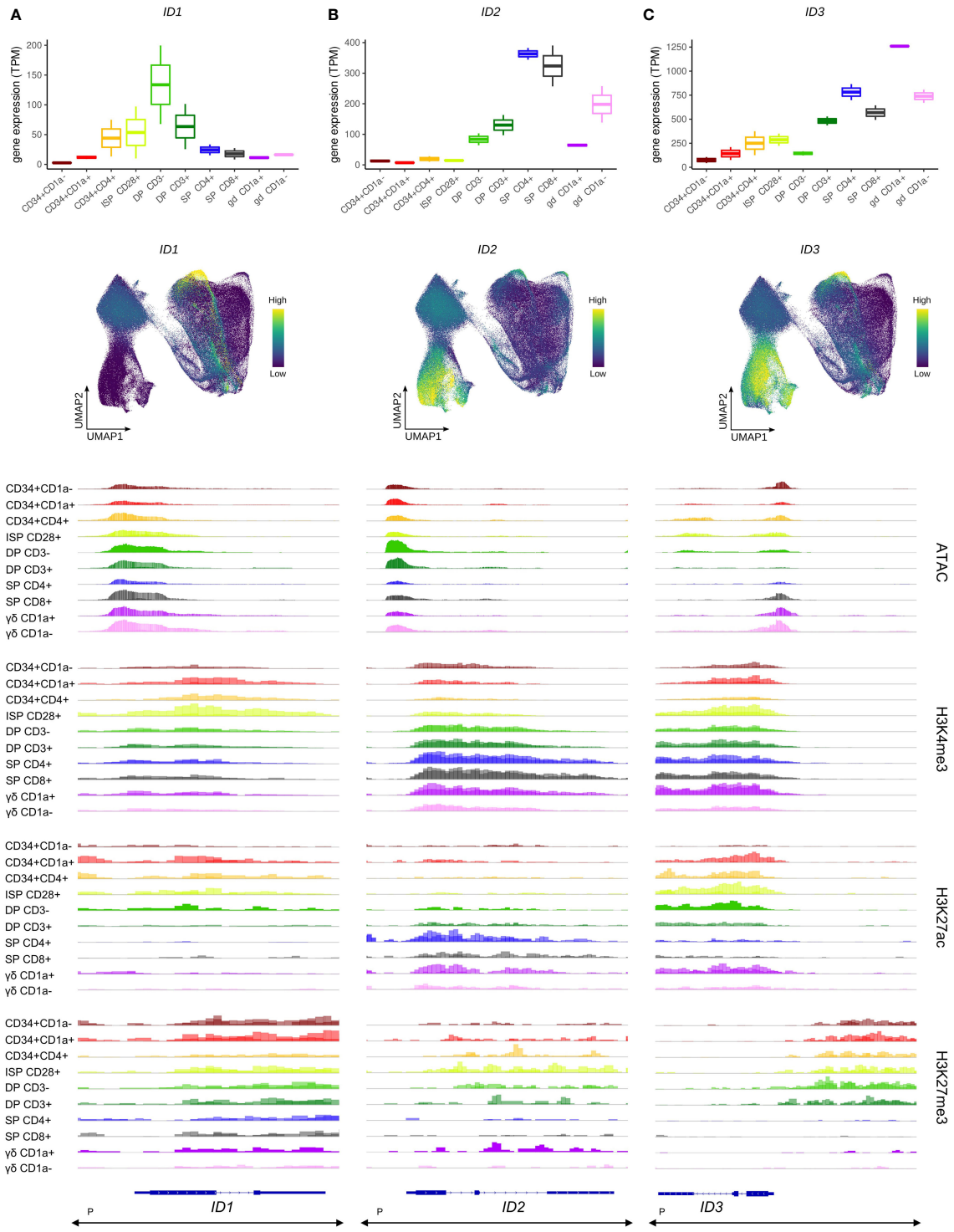

FIGURE 3
Transcription of ID protein encoding genes in developing human thymocytes. **(A)** Transcript levels of *ID1* according to bulk RNA-seq (top) and single cell data (middle), and epigenetic profile at the *ID1* gene locus. **(B)** Transcript levels of *ID2* according to bulk RNA-seq (top) and single cell data (middle), and epigenetic profile at the *ID2* gene locus. **(C)** Transcript levels of *ID3* according to bulk RNA-seq (top) and single cell data (middle), and epigenetic profile at the *ID3* gene locus. Locations of promoters (P) for all three genes was retrieved from the Ensembl Regulatory Build and was found to span the entire locus as indicated below the gene structure. UMAP visualizations were generated using imputed data.

and *Id3*. This raises questions about the biological relevance of *Id1* expression in the mouse thymus, whereas *ID1* levels in human thymocytes are moderately high and may therefore reflect an actual functional role for ID1 in human T cell development.

Despite the overall similarities in the transcriptional dynamics of E and ID protein encoding genes in human and murine thymocytes, the few observed discrepancies should be considered when attempting to model human T cell development in a mouse system. Especially the differences in the *TCF3* expression profiles between the two species suggest that many findings regarding TCF3 functions in early thymocyte differentiation in mouse might need caution when translating to human.

3.3 E and ID protein encoding genes during initial lineage decisions in the thymus

To gain deeper insight into the biological significance of the expression of E and ID protein encoding genes in differentiating thymocytes, we carried out trajectory analysis and gene regulatory network (GRN) prediction on the single cell data, and transcription factor footprinting analysis on the bulk RNA-seq and ATAC-seq datasets.

Given the important role for E and ID proteins during lineage decisions, we assessed E and ID gene regulation at the earliest stages of T cell development, during which cells can still branch off towards other hematopoietic lineages. Indeed, at the most immature stage, which represents a subset of $CD34^{+}CD1a^{-}$ cells, thymocytes have not yet fully committed to the T-lineage and still have the potential to give rise to other non-T cell types, including DCs (63, 75). Our gene expression analysis indicated high levels of *TCF4* RNA in these immature cells, but continuous downregulation in T-committed thymocytes (Figure 2B, top), suggesting a potential role very early on in thymocyte differentiation. GRN analysis on the single cell dataset identified two regulons with exceptionally high activity in the most immature thymocytes, which was quickly extinguished in subsequent stages (Figure 4A). Interestingly, both regulons included *TCF4* as a target gene and were predicted to be driven by *IRF8* and *SPI1* (encoding PU.1). Expression of these two transcriptional regulators was indeed found to be high in immature thymocytes and preceded that of *TCF4* (Figure 4B). Moreover, PU.1 and IRF8 footprints were detected in the open, active chromatin regions at the *TCF4* regulatory elements in $CD34^{+}$ thymocytes (Figure 4C). TCF4, PU.1 and IRF8 are all known to be crucial transcription factors for DC development (76–78) but a previous study seems to place TCF4 upstream of PU.1 and IRF8 (79). In accordance with this, E protein motifs were indeed also detected at the *SPI1* and *IRF8* loci (data not shown). However, our regulon prediction results and temporal order of *TCF4*, *SPI1* and *IRF8* expression in immature thymocytes raise the possibility of TCF4 not (just) as regulator

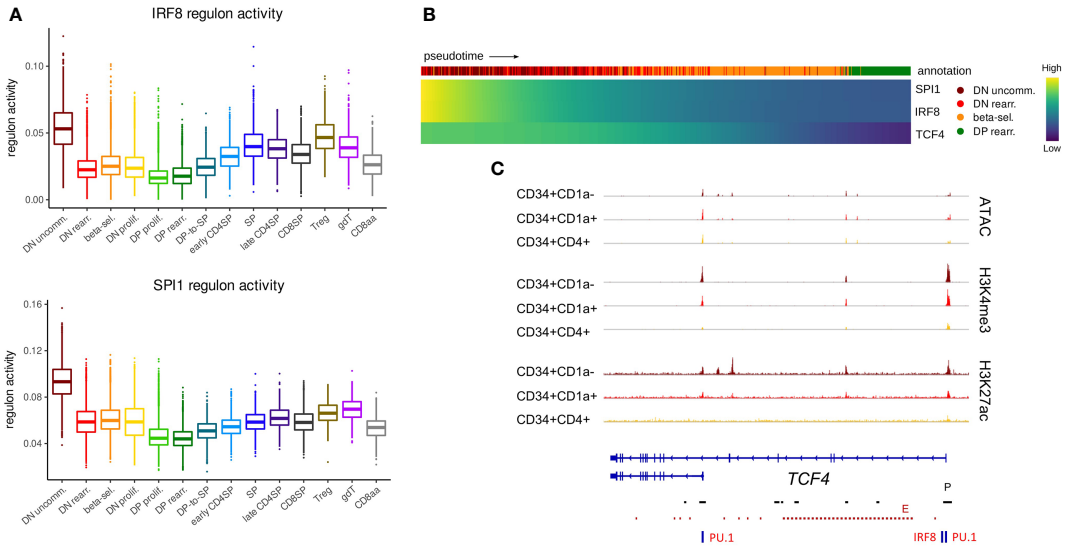

FIGURE 4
TCF4, IRF8 and SPI1 are predicted to form a regulatory network in uncommitted DN thymocytes. **(A)** Activity of IRF8 and PU.1 regulons per cluster as predicted by running pySCENIC on the single cell data set. **(B)** Heatmap showing gene expression along differentiation pseudotime in immature thymocytes. Smoothed gene expression was determined based on a generalized additive model fitted on the cell pseudotimes, cells in pseudotime window of interest were selected and expression was scaled by gene prior to visualization. **(C)** Genome browser view of ATAC, H3K4me3, H3K27ac and PU.1/IRF8 motifs at the *TCF4* locus. Locations of promoters (P) and enhancers (E) were retrieved from the Ensembl Regulatory Build and are indicated below the gene structure.

but also as target of *SPI1*/PU.1 and IRF8. In addition, they strongly suggest that *TCF4* expression in immature thymocytes might reflect a more prominent role in supporting DC compared to T cell development.

The expression of a short *TCF4* isoform has previously been described in cDCs and pDCs as well as other cell types, whereas the long *TCF4* isoform seems to be exclusively expressed in pDCs (14). Therefore, we assessed the footprint analyses of the promoters of the long and short *TCF4* isoform separately. The long *TCF4* isoform, which displayed more stable chromatin accessibility, was shown to be driven by both IRF8 and PU.1 (Figure 4C). In contrast, for the short isoform we only found evidence for binding of PU.1 but not IRF8. Interestingly, previous research identified PU.1 as a repressor of pDC fate within the DC-lineage (80). Therefore, we hypothesize a role for the interplay between PU.1 and the long *TCF4* isoform in guiding immature DCs (or $CD34^+CD1a^-$ unspecified thymocytes) to the pDC fate. Moreover, the differing dynamics of epigenetic changes at the individual promoters suggest divergent expression windows and upstream regulators for both *TCF4* isoforms, although the consequences of this remain to be established.

Once committed to the T-lineage, thymocytes are still bipotent and can adopt either the αβ or γδ T-cell fate, depending on the TCR that they assemble and the signals they receive. The expression patterns of E and ID protein encoding genes suggested particularly high E but low ID encoding transcript levels throughout the first stages of thymocyte development. This indicates potentially strong E protein activity in this phase, which prompted us to investigate the possible consequences. To develop into functional T cells, thymocytes undergo V(D)J recombination to be able to produce a wide range of TCRs with different specificities. For the *TCRD*, *TCRG* and *TCRB* loci, this rearrangement takes place during the immature stages that precede β-selection and the DP stage. Several studies in mice have implicated TCF3 in *Tcrg* locus accessibility and consequently in initiation and regulation of V(D)J recombination of this gene (72, 81) but known differences exist between human and mice in the order and coordination of TCR locus rearrangements (36). Therefore, we explored the possibility of TCF3 involvement in *TRGC* rearrangement in human thymocytes. Expression of *RAG* genes, which mediate V(D)J recombination, was already evident in early $CD34^+$ subsets in our bulk dataset (Figure 5A). In the single cell dataset, *RAG* expression was very low in the immature thymocyte stages and could not be reliably identified. However, transcription of *TRGC* and *TRDC* was clearly detected and can signify not only expression of a mature γ- or δ-chain but also ongoing rearrangement at these loci (Figure 5B). Notably, we observed that cells initially express *TRGC1* and later switch to *TRGC2*, while mature γδ T cells with surface expression of the γδ TCR almost exclusively use *TRGC2* (Figure S4A). This suggests that *TRGC2* is involved in the formation of the functional TCR, whereas *TRGC1* might only be transcribed in the course of rearrangement. We found that, according to pseudotime, *TCF3* and *TCF4* expression reached high levels at the same time as *TRGC1* and slightly before *TRGC2*, suggesting that they could be involved in coordinating chromatin opening and transcription of this region. We did indeed detect multiple TCF3 motifs at the *TCRG* locus, all of which were associated with regions of accessible chromatin and permissive histone marks in $CD34^+$ thymocytes, indicative of an active role of TCF3 at these sites (Figure 5C). Some TCF4 and few TCF12 motifs were also observed, but these did not consistently align with any observable epigenetic features. Analysis of a potential relationship between expression of the E protein encoding genes and transcription of the *TCRG* locus revealed a positive correlation ($r = 0.31$) between *TCF3* and *TRGC2* levels in DN thymocytes, which was not observed to the same extent for *TCF4* or *TCF12* ($r = -0.06$ and $r = 0.11$, respectively) (Figure 5D). Curiously, this correlation was not detected for *TCF3* and *TRGC1* ($r = -0.04$), which seems to be driven by a subpopulation of immature DN thymocytes that express high levels of *TRGC1* but not *TCF3* (Figure 5E). This suggests that, in human, TCF3 may not be required to promote accessibility at the *TCRG* locus but instead might control the expression of the rearranged γ-chain to enable γδ TCR assembly. Despite similar expression windows of *TRDC* and *TRGC2*, no direct correlation was observed between *TCF3* and *TRDC* transcription ($r = -0.03$) (Figure S4B), which indicates that TCF3 is probably not responsible for controlling chromatin accessibility or active transcription at the TCRD locus. However, multiple TCF3 motifs were detected across the TCRD locus (Figure S4C), suggesting that TCF3 might be involved in coordinating V(D)J recombination of the δ-chain, as reported previously for TCF3 knockout mouse model (19, 82). Of note, TCF4 and TCF12 transcription was negatively or not at all correlated with that of TRDC and TRGC1 (Figure S4D) and few motifs were detected at either locus, therefore the two factors are unlikely to be key regulators of V(D)J recombination of TCRD and TCRG.

In human, rearrangement of the *TCRB* locus is thought to occur slightly after the *TCRD* and *TCRG* loci (36) and functionality of the β-chain is assessed by assembly with the surrogate pTα (encoded by *PTCRA*) to form the pre-TCR. TCF3 and TCF12 have both been shown to bind to regulatory sequences at the *Ptcra* locus in mouse thymocytes, but it seems that TCF3 is the main driver of *Ptcra* expression, whereas TCF12 plays a secondary synergistic role but is not able to induce high *Ptcra* transcription by itself (83). Using footprint analysis of our ATAC-seq data, we indeed identified a motif common for all E proteins as well as a TCF12-specific motif at the transcription start site of *PTCRA*, which overlapped with open chromatin and permissive H3K27 acetylation in immature thymocytes (Figure 6A). Gene expression analysis along pseudotime revealed that *PTCRA* transcription coincided

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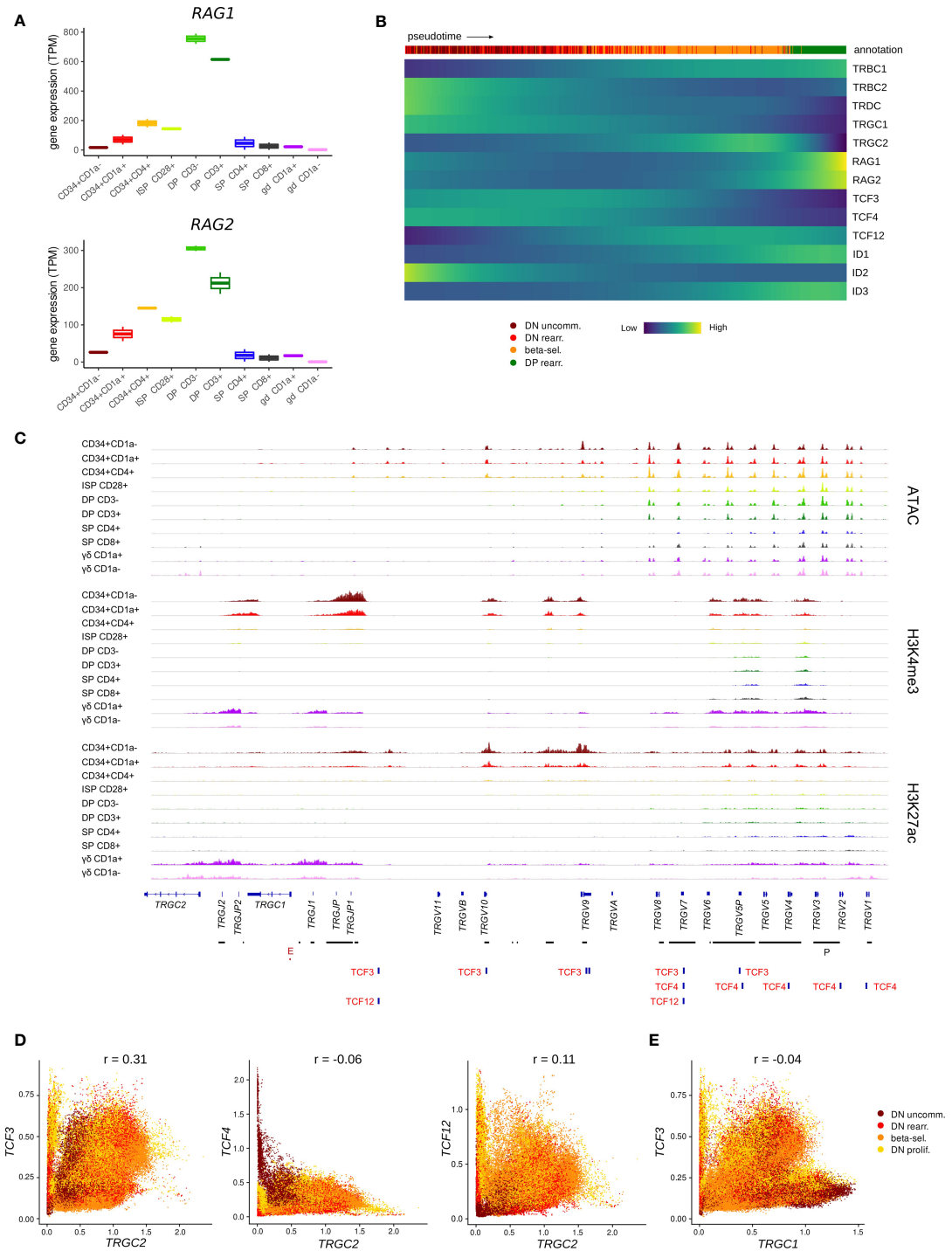

FIGURE 5
TCF3 expression is positively correlated with transcription of *TRGC2*. **(A)** Expression of *RAG1* and *RAG2* at different stages of thymocyte development according to bulk RNA-seq data. **(B)** Heatmap showing gene expression along differentiation pseudotime in immature thymocytes. Smoothed gene expression was determined based on a generalized additive model fitted on the cell pseudotimes, cells in pseudotime window of interest were selected and expression was scaled by gene prior to visualization. **(C)** Genome browser view of ATAC, H3K4me3, H3K27ac and E protein motifs at the *TCRG* locus. Locations of promoters (P) and enhancers (E) were retrieved from the Ensembl Regulatory Build and are indicated below the gene structure. **(D)** Scatter plot for imputed transcript levels of E protein encoding genes and *TRGC2* in immature thymocytes. Cells are colored by cluster and Pearson correlation coefficient is shown. **(E)** Scatter plot for imputed transcript levels of *TCF3* and *TRGC1* in immature thymocytes. Cells are colored by cluster and Pearson correlation coefficient is shown.

with the upregulation of *TCF12* but was preceded by a *TCF3* expression peak (Figure 6B). Regulatory network prediction with SCENIC and SIGNET identified *PTCRA* as a potential target of TCF12, whereas the putative regulatory interaction between TCF3 and PTCRA was found to be weaker (SCENIC) or not detected at all (SIGNET). A possible role of TCF12 in *PTCRA* transcription was also supported by the finding that expression levels of both genes in DN thymocytes exhibit a positive correlation (r = 0.48), whereas no correlation was observed for *TCF3*/*TCF4* vs. *PTCRA* (r = -0.03 and r = -0.28, respectively) (Figure 6C). Together, these observations indicate that, similar to descriptions in mouse, E proteins might be involved in the transcriptional induction of *PTCRA* during human T cell development in the thymus. While the presented analyses seem to favor TCF12 rather than TCF3 as the main transcriptional regulator, *in vitro* validation will be required to assess the true impact of both E proteins on pTα expression and to explore any potential synergism or interdependence.

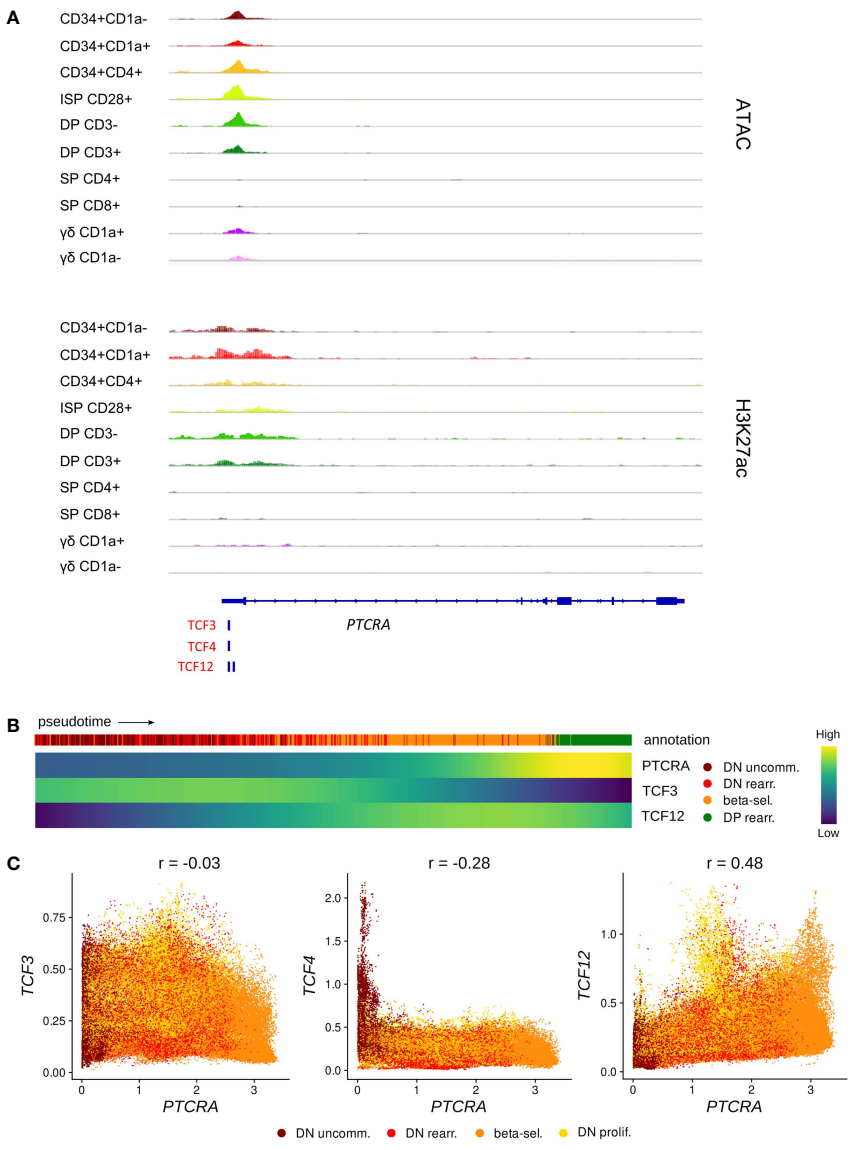

FIGURE 6
TCF12 is predicted to act as positive regulator of *PTCRA* transcription. **(A)** Genome browser view of ATAC, H3K27ac and E protein motifs in the *PTCRA* promoter region. Locations of promoters (P) and enhancers (E) were queried in the Ensembl Regulatory Build but none were found for this locus. **(B)** Heatmap showing gene expression along differentiation pseudotime in immature thymocytes. Smoothed gene expression was determined based on a generalized additive model fitted on the cell pseudotimes, cells in pseudotime window of interest were selected and expression was scaled by gene prior to visualization. **(C)** Scatter plot for imputed transcript levels of E protein encoding genes and *PTCRA* in immature thymocytes. Cells are colored by cluster and Pearson correlation coefficient is shown.

In summary, these observations imply that E proteins play a role in the indispensable processes that allow human thymocytes to develop into either αβ or γδ T cells, prior to the actual fate decision.

3.4 E and ID protein encoding genes in αβ T cell development

A critical test that thymocytes need to pass on their path to become mature αβ T cells is β-selection, which involves assembly of the pre-TCR to assess the successful rearrangement of the TCR β-chain. Analysis of the single cell thymocyte data revealed a small subset of cells (cluster 28, Figure S1C and Figure S5A) within the β-selecting cluster that expressed unusually high levels of ID protein encoding genes (Figure 7A). This was particularly remarkable for *ID2* and *ID3*, for which widespread expression is only induced much later in development, as described above (Figures 3B, C). Since ID proteins are known to inhibit E proteins and high ID levels therefore indicate low E protein activity, we used gene signature scoring to determine an E:ID score based on E (positive weight) and ID (negative weight) transcript levels for each cell. Visualization on the UMAP confirmed an extremely low E:ID score for cluster 28, whereas surrounding cells exhibited a high score (Figure 7B). This indicates a rapid but temporally restricted transcriptional induction of ID protein encoding genes and suggests a high potential for robust E protein inhibition in this subset of cells. It has previously been demonstrated that E protein activity needs to be transiently

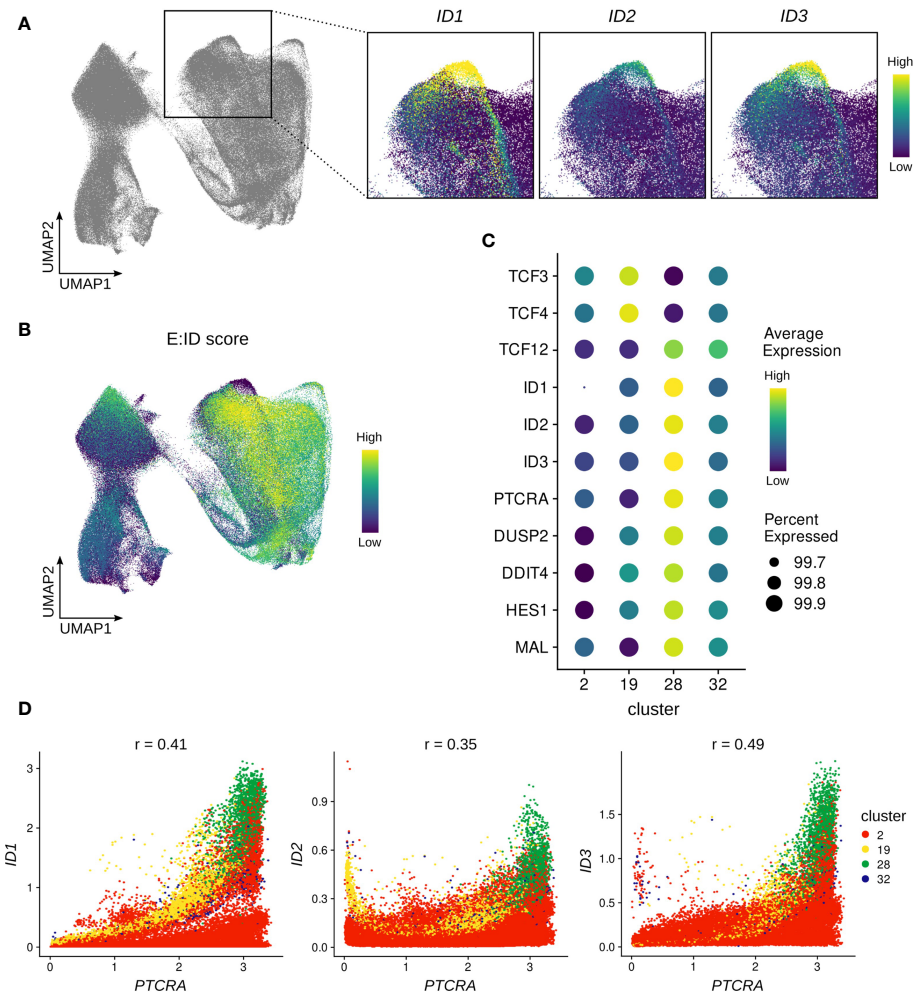

FIGURE 7
ID protein encoding genes are strongly induced in a subset of β-selecting thymocytes. **(A)** UMAP visualization of ID protein encoding gene expression in the β-selecting thymocyte cluster. **(B)** UMAP visualization of the E:ID score calculated on a per-cell basis. **(C)** Dot plot visualizing pseudo-bulk expression of E and ID protein encoding genes and cluster 28 marker genes. Imputed, gene-scaled expression is shown for all subclusters comprising the beta-selecting cluster. **(D)** Scatter plot for imputed transcript levels of ID protein encoding genes and *PTCRA* in β-selecting thymocytes. Cells are colored by β-selecting subcluster and Pearson correlation coefficient is shown.

shut down following β-selection to initiate differentiation of αβ-lineage thymocytes (25). Therefore, it is likely that cluster 28 reflects cells at this specific stage of human T cell development.

To further characterize cluster 28, we conducted differential gene expression analysis which identified *ID1*, *ID3*, and *PTCRA* as the main markers of this cluster. However, we also detected significantly elevated transcript levels for *DUSP2*, *DDIT4*, *HES1* and *MAL* in comparison to the rest of the dataset (Figure 7C). All of these genes have previously been linked to TCR signaling (84–87), and are therefore indicative of strong ongoing pre-TCR activity in cluster 28. It is known that *Id3* expression in thymocytes can be triggered *via* MAPK signaling as a consequence of TCR engagement (27), and it is possible that *ID1* and *ID2* can be similarly induced by pre-TCR signaling. Gene co-expression analysis did indeed reveal a positive correlation between *PTCRA* and *ID1* (r=0.41) or *ID3* (r=0.49) transcript levels in cells of the β-selection cluster, with cluster 28 cells exhibiting the highest expression levels for all 3 genes (Figure 7D). Moreover, GRN analysis predicted a regulatory connection between ID1 and PTCRA, although the nature and direction of the relationship cannot easily be established for non-transcription factors using this approach.

It has been proposed that the relatively weak signal transmitted by the pre-TCR is insufficient to permit further maturation of αβ-lineage cells, and that supplementary Notch signaling is required to achieve transient E protein inhibition and thereby developmental progression (88). NOTCH gene signature scoring, based on expression of known NOTCH target genes (see Material & Methods), did indeed show a high score in cluster 28, providing an explanation for the high levels of the NOTCH target *HES1* in these cells. Nevertheless, the score was equally high in the remaining cells in the β-selection cluster and therefore cannot fully explain the isolated upregulation of ID protein encoding genes (Figure S5B). Finally, to rule out a potential contamination with cells expressing a γδ TCR as a source of strong TCR signaling, we assessed *TRGC2*/*TRDC* transcription in the cells from cluster 28 which confirmed substantially lower levels compared to the γδ T cell subclusters (Figure S5C). This strongly suggests that pre-TCR signaling can induce high levels of ID gene expression in human thymocytes in a subset of β-selecting cells.

Following β-selection, thymocytes progress to the DP stage which encompasses the rearrangement of the *TCRA* locus. Assessment of the rearranging DP cluster in the single cell data indicated a gradual decrease in the transcript levels of all three E proteins with highest levels observed in the most immature rearranging DPs and low levels in cells that started to embark on the transition to the SP stage (Figure 8A). Incidentally, the subgroup of cells with elevated transcription of E protein encoding genes exhibited relatively low ID gene expression. This was also clearly demonstrated by the previously determined E:ID score which indicated that cells undergo a rapid switch from a high to low E-ID ratio as they mature (Figure 7B). Analysis of *RAG* expression levels revealed high *RAG1* and *RAG2* quantities in the $(E:ID)^{high}$ cell group (Figure 8B). In line with this, gene-gene co-expression analysis confirmed a positive correlation between *TCF3*/*TCF12* and *RAG1/2* transcript levels (Figure 8C), whereas *ID1*/*ID3* levels were anticorrelated with those of *RAG1* (Figure 8D). Expression profiles along pseudotime also pointed towards an inverse expression pattern for RAG genes and *ID1* (Figure 8E). Regulon prediction suggested TCF12 and ID1 as putative regulators of *RAG1/2*, but no regulatory relationship with TCF3 was detected. However, a TCF3 binding site was indeed identified at the transcription start site of *RAG2*, in addition to consensus E protein binding sites at a putative upstream enhancer and at the transcription start site of the short *RAG1* isoform, which were all associated with increased accessibility and a permissive epigenetic signature in DP thymocytes (Figure 8F). Together, these findings suggest a possible role for TCF12 and potentially also TCF3 in the upregulation of *RAG* genes in DP thymocytes. Binding of TCF12 and TCF3 to the *Rag* locus has indeed been shown before, and *Tcf3*- or *Tcf3*/*Tcf12*-deficient mice display a moderate or severe impairment in the upregulation of *Rag1* and *Rag2* in DP thymocytes (89, 90). Hence, our observation in human thymocytes is in line with previously published mouse data describing crucial roles of E proteins during *Tcra* rearrangement *via* regulation of Rag expression. Of note, the role of TCF12 in DP thymocytes seems to extend further to regulation of cell viability *via* transcriptional upregulation of *Rorc*. We could also confirm a positive correlation for *TCF12* and *RORC* expression in our data (r=0.73) (Figure S6A) and a regulatory relationship between the two factors was identified *via* GRN analysis.

In contrast, the role of ID1 in rearranging DP thymocytes has not been studied in much detail, but some reports suggest that *Id1* overexpression during murine T and B cell development results in severely reduced *Rag1/2* expression (91, 92). In addition, it is known that *Id3* needs to be downregulated in DP thymocytes to permit *Rag* expression (93) and *Id3* overexpression in thymocytes results in reduced *Rag1/2* levels (94). This indicates that *ID1* and *ID3* expression in DP thymocytes negatively regulates *RAG* transcription and therefore modulates or terminates *TCRA* rearrangement. Given the staggered timepoints of upregulation, it is likely that ID1 only has a moderate effect on *RAG* transcription, whereas *ID3* induction coincides with and might therefore be responsible for the complete shutdown of *RAG* expression (Figure 8B and Figure 3C). Since this takes place around the positive/negative selection stage as indicated by the upregulation of *TRAC* and *CD5* (Figure S6B), initiation of *ID3* expression may represent a response to TCR signaling and

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subsequent downregulation of *RAG1/2* would be required to prohibit further rearrangements in positively selected cells.

In summary, our data support the hypothesis that, like in mouse, TCF3 and TCF12 are involved in the upregulation of *RAG* expression in rearranging DP human thymocytes, whereas ID1 and ID3 seem to exert an inhibitory function towards *RAG* transcription. Whether this is achieved solely through E protein inhibition or involves other regulatory mechanisms remains to be explored.

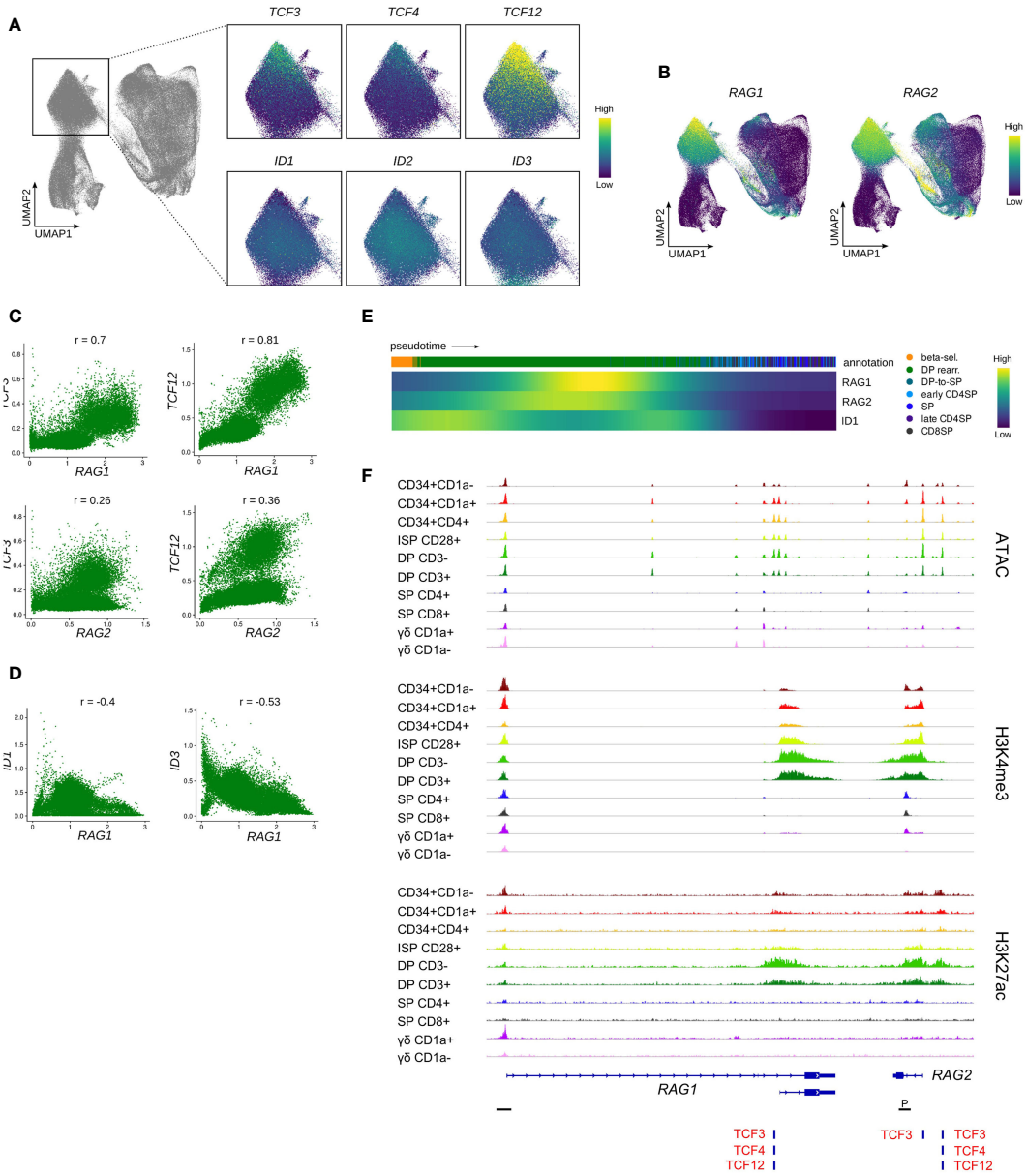

FIGURE 8
Transcription of E protein encoding genes is associated with *RAG* expression in rearranging DP thymocytes. **(A)** UMAP visualization of E and ID protein encoding gene expression in the rearranging DP thymocyte cluster. **(B)** UMAP visualization of *RAG1* and *RAG2* expression according to the single cell thymocyte data set. Transcripts were imputed prior to visualization. **(C)** Scatter plot for imputed transcript levels of *TCF3*, *TCF12*, *RAG1* and *RAG2* in rearranging DP thymocytes. Pearson correlation coefficient is shown. **(D)** Scatter plot for imputed transcript levels of *ID1*, *ID3*, and *RAG1* in rearranging DP thymocytes. Pearson correlation coefficient is shown. **(E)** Heatmap showing gene expression along differentiation pseudotime in DP and SP thymocytes. Smoothed gene expression was determined based on a generalized additive model fitted on the cell pseudotimes, cells in pseudotime window of interest were selected and expression was scaled by gene prior to visualization. **(F)** Genome browser view of ATAC, H3K4me3, H3K27ac and E protein motifs at regulatory regions of the *RAG* gene locus. Locations of promoters (P) were retrieved from the Ensembl Regulatory Build and are indicated below the gene structure.

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3.5 E and ID protein encoding genes in non-conventional T cells

The inclusion of γδ T cells and CD8αα T cells in our established datasets allowed us to assess expression of E and ID protein encoding genes in these non-conventional T cell types. ID3 is a well-known regulator of γδ T cell development and has been shown to be upregulated following strong TCR signaling. Consistent with this and the understanding that strong TCR signals are associated with adoption of γδ fate, *ID3* levels were highest in immature γδ T cells according to bulk RNA-seq analyses (Figure 3C). In the single cell data, we identified a subset of cells with surface γδ TCR expression that displayed a γδ-lineage gene expression signature according to clustering results but that still grouped with DN thymocytes (Figure 9A). Moreover, this subset of cells showed very low expression of maturation markers such as CD73 (encoded by *NT5E*), *CD44*, *CD27*, *CD69* and *IL7R* and γδ effector genes, like *NKG7*, *KLRB1* and *GNLY*, were not yet upregulated (Figure S6C), which identifies them as very immature γδ T cells. Curiously, *ID3* levels were only moderate in this subset (Figure 9B), which suggests that these cells have only just received a TCR signal and are still in the process of upregulating *ID3*.

Regulon prediction in the single cell dataset identified two regulons for the AP-1 family transcription factors FOS and JUND that displayed especially strong activity in γδ T cells (Figure 9C). *ID3* was suggested as a target gene of both regulons and in support of that, we were able to identify an AP-1 family motif downstream of the *ID3* gene at differentially accessible sites in γδ T cells (Figure 9D), indicating that the two factors might indeed confer *ID3* upregulation. Importantly, AP-1 transcription factors are known downstream mediators of TCR

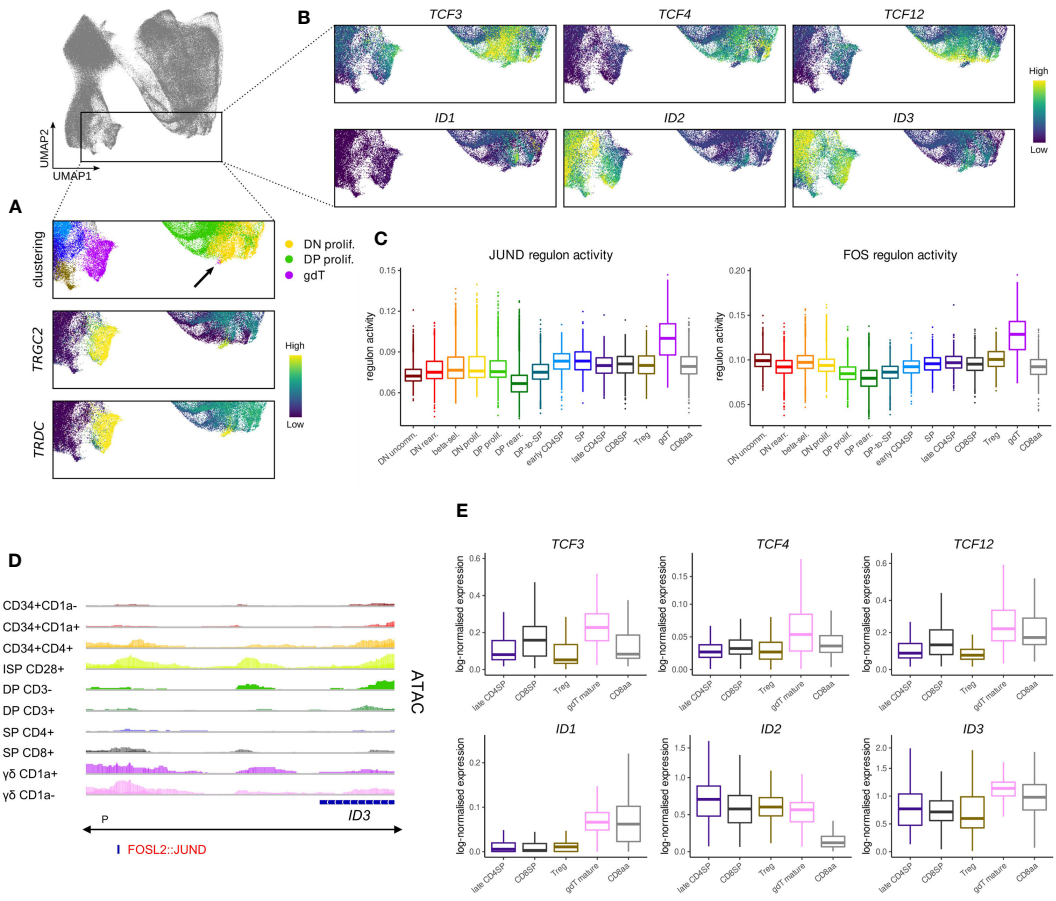

FIGURE 9
Transcription of *TCF4* and *TCF12* but not *TCF3* is shut down in cells committing to the γδ lineage. **(A)** UMAP visualization of *TRGC2* and *TRDC* expression in thymocytes of the γδ lineage. Annotated clusters are depicted in the top panel. **(B)** UMAP visualization of E and ID protein encoding genes in thymocytes of the γδ lineage. **(C)** Activity of JUND and FOS regulons per cluster as predicted by running pySCENIC on the single cell data set. **(D)** Genome browser view of chromatin accessibility and AP-1 family motif at a regulatory region downstream of *ID3*. Location of the ID3 promoter (P), as indicated below the gene structure, was found to span the entire locus. **(E)** Pseudo-bulk expression of E and ID protein encoding genes in mature thymocytes based on the single cell data set. For γδ lineage cells only a subcluster of mature γδTCR$^+$ cells as indicated by low levels of CD1a was included.

signaling, which validates induction of *ID3* transcription as a result of TCR activity.

Further analysis of the immature γδ T populations in bulk and single cell data suggested that these cells do not express notable levels of *TCF4*, *TCF12*, *ID1* or *ID2* at this stage (Figure 9B). However, we noted high levels of *TCF3* in the very immature subset which only dropped gradually as γδ T cells became more mature. While TCF3 is known to play a crucial role in *Tcrg*/*Tcrd* locus gene rearrangement (72, 81), and is therefore indispensable for γδ T cell development, additional roles in γδ-lineage differentiation processes have not been studied in much detail. It is possible that TCF3 protein activity is quickly diminished following *ID3* induction since ID3 has been shown to not only inhibit TCF3 function but also to mediate a reduction in protein levels (88). Nevertheless, it remains unclear why *TCF3* transcripts continue to be expressed following γδ-lineage commitment whereas *TCF12* expression is extinguished much more rapidly.

Comparison of transcript levels for E and ID protein encoding genes in the mature cell types that we identified in the single cell data confirmed generally low expression of *TCF3*, *TCF4* and *TCF12* in conventional αβ-lineage cells as well as in γδ and CD8αα$^+$ T cells, with some minor variability between cell types (Figure 9E). *ID1* levels appeared to be higher in γδ and CD8αα$^+$ T cells, but due to the low total transcript quantities, the biological relevance of this difference may be negligible. In contrast, *ID2* levels were remarkably similar in the γδ and conventional αβ lineage cells, while much lower quantities were detected in CD8αα$^+$ cells (Figure 9E). This contradicts findings in murine CD8αα$^+$ T cells, which appear to exhibit higher *Id2* levels in comparison with CD8αβ$^+$ T cells (95, 96). It is possible that this difference stems from the analysis of thymic vs. peripheral cells. However, it has been proposed that CD8αα$^+$ T cell development is independent of ID2 (97), in which case the biological significance of differential *ID2* transcription is uncertain. *ID3* levels were only slightly higher in mature γδ T cells compared to the other analyzed cell types. This could be explained by the moderate downregulation of *ID3* that is associated with the maturation of γδ T cells and the upregulation of *ID3* in SP thymocytes. ID3 is often described as γδ-specific transcriptional modulator, but these observations suggest that this characteristic only extends to immature cell types, perhaps reflecting TCR signaling events that impact the lineage choice.

Notably, the regulatory T cells that were identified in the single cell dataset expressed similar levels of all E and ID genes as CD4$^+$ and CD8$^+$ SPs, which indicates that mature naïve αβ T cells do not exhibit differential transcription of these factors.

3.6 E and ID protein encoding genes during prenatal T cell development

T cell differentiation in the thymus starts very early during embryonic development and especially cells of the γδ lineage have been shown to exhibit notable differences between prenatal and postnatal origin, although this has been predominantly studied in mice thus far. To investigate potential changes in E and ID gene expression that are associated with human development, we used published single cell data (64, 98, 99) to establish a prenatal dataset consisting of around 112.000 cells from 20 donors, with samples covering a continuous age window from 8 weeks post conception (wpc) to 17 wpc (Figure S7A, Table S1). To visualize the age progression while retaining enough cells from each cell type to avoid donor- or source-specific biases, we further distinguished between cells from embryonic (≤ 10 wpc) and fetal (>10 wpc) donors (Figure S7B, C). We used the pediatric dataset as reference for automated cell type annotation *via* label transfer to identify clusters with similar gene expression profiles (Figures S7C-E).

Comparison of embryonic, fetal and pediatric data revealed substantially higher transcript levels for *ID1* and *TCF12* in prenatal thymocytes (Figure 10A). Elevated *ID1* levels in fetal thymocytes have indeed been described before in mice (41), but the biological significance remains unclear. Strikingly, gene expression profiles for *ID2* and *ID3* differed substantially between prenatal and postnatal thymocytes. Whereas both genes were only upregulated at the DP-SP transition in pediatric T cell development as laid out above, their induction was shifted to much earlier stages in prenatal development (Figure 10A and Figure S7F). This is remarkable because it signifies substantial levels of ID gene expression in DN thymocytes, which we generally determined to be an ID^{low} phase in the pediatric thymus. The anticipated consequence of this is a more pronounced repression of E protein activity in immature prenatal thymocytes, which is also supported by the difference in the E:ID score in pre- and postnatal samples (Figure 10B). This might directly influence thymocyte maturation and differentiation based on the roles of TCF3 and TCF12 described above. Hence, it is possible that elevated *TCF12* levels in embryonic and fetal thymocytes represent a compensatory mechanism to retain some TCF12 activity despite strong ID gene expression.

4 Discussion

In this manuscript, we have established an overview of the regulation of E and ID protein encoding genes during human T cell development, using both bulk and single cell profiling methods to understand gene expression and epigenetic regulation of these genes and their regulatory networks. Comparison with murine thymocytes revealed some potential differences in the stage-specific expression and thus most likely also the activity of these genes. Furthermore, a remarkable shift in the E/ID gene expression ratio was observed in the early stages of human T cell development during the transition from fetal to postnatal thymopoiesis.

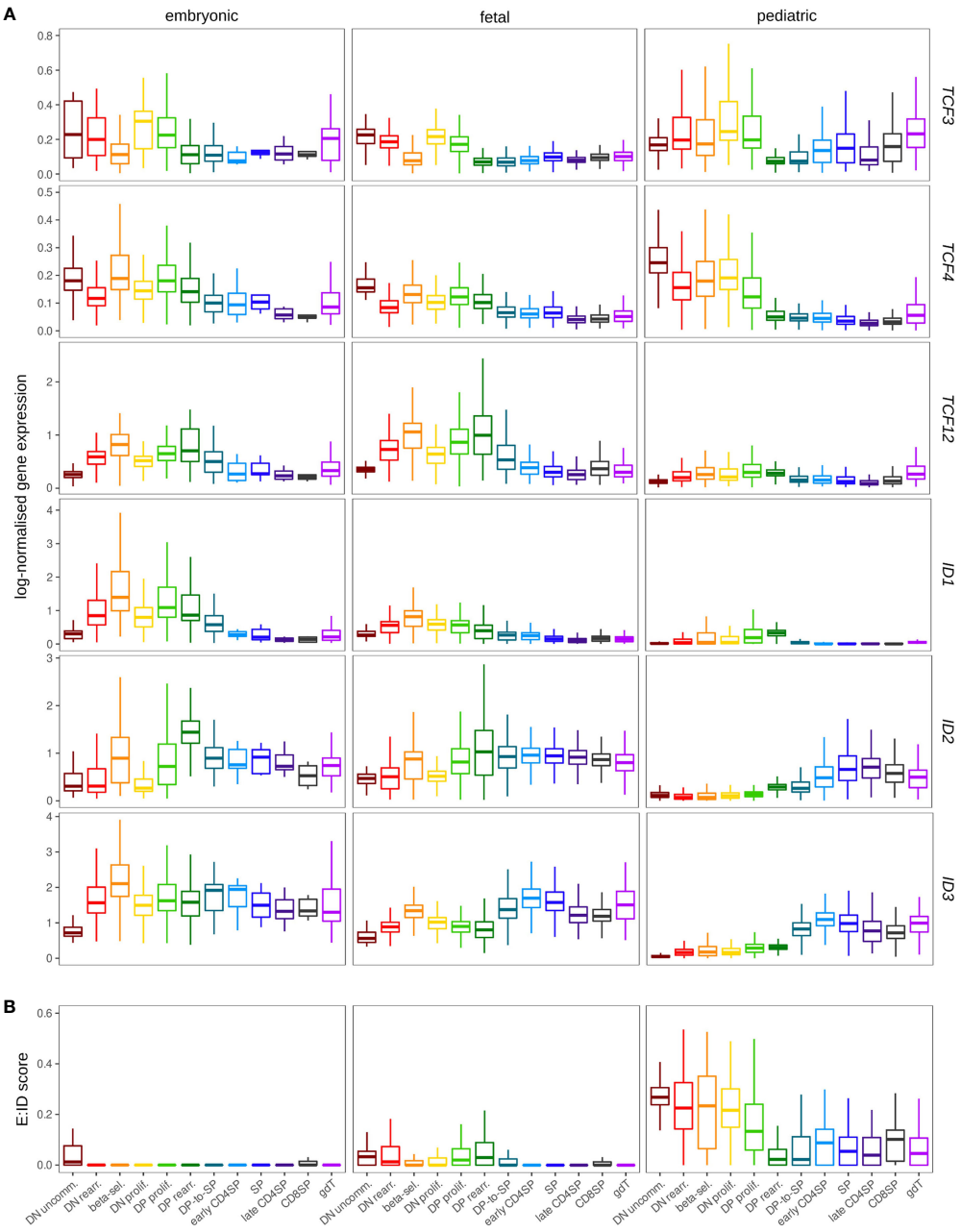

FIGURE 10
Expression of ID protein encoding genes exhibits different patterns in the pre- and postnatal thymocytes. **(A)** Pseudo-bulk expression of E and ID protein encoding genes in thymocytes based on prenatal and pediatric single cell data sets. The prenatal data was divided into embryonic (≤10 wpc) and fetal (>10 wpc) samples. Note that only very few SP, early/late $CD4^+$ and $CD8^+$ SP thymocytes were detected in embryonic samples (see Figure S7C). **(B)** Average E:ID score per cell type in embryonic, fetal, and pediatric thymocytes.

Our analyses revealed several interesting differences and similarities between the gene expression dynamics of E and ID protein encoding genes. Both *ID1* and *TCF12* seem to be largely absent in immature and mature thymocytes and instead reach their expression peak when cells are midway through their developmental progression towards αβ-lineage cells. In contrast, *TCF3* and *TCF4* expression levels are highest in immature stages and extinguished in mature thymocytes, whereas *ID2* and *ID3* display the opposite pattern with upregulation relatively late in the developmental course. However, the expression windows for *TCF3*/*TCF4* and *ID2*/*ID3* are not completely identical, which suggests that their

regulation is controlled by different upstream mechanisms. Importantly, the inverse transcription pattern for E and ID proteins indicates a crucial requirement for E protein expression in the early phase of T cell development but possibly also a need for E protein shutdown *via* degradation or inhibitory dimerization with ID proteins at later stages.

At the epigenetic level, the promoter histone modification H3K4me3 was highly correlated with RNA expression for all E and ID protein encoding genes, as anticipated. Remarkably, however, the corresponding chromatin regions remained largely accessible, and thus permissive for expression, throughout all developmental stages, rendering it feasible to rapidly alter the expression levels in response to new regulatory inputs that can be derived from both environmental and intracellular stimuli. This is important in the case of E and ID proteins given their strong involvement in both TCR generation and signaling, respectively, which are both critical determinators of thymocyte maturation.

Some of the E and ID protein encoding gene expression patterns displayed remarkable features. According to the single cell data, *TCF3* RNA levels were found to be highest in proliferating DN and DP thymocytes, which seems to contradict previous reports of TCF3 acting as inhibitor of proliferation in support of TCR rearrangements, to which E proteins also contribute by regulating *RAG* gene expression. This may point towards differences between RNA and protein levels as well as additional layers of protein activity regulation. E proteins not only form heterodimers with ID proteins that inhibit their activity, they also heterodimerize with other tissue and stage specific factors that thereby can regulate E protein activity (100). In addition, it is established that E protein phosphorylation can induce degradation, for instance following ERK activation downstream of NOTCH and TCR signaling (26, 29).

A surprising characteristic that we observed was the heterogeneous *ID1* expression in β-selecting and rearranging DNs that partially overlapped with *ID3* expression in those early stages. While the *ID1* expression in the rearranging DNs may reflect some early thymocytes that have just successfully rearranged the TCR β-chain and thus are on their way to go through the β-selection process, the difference in *ID1* and *ID3* expression in β-selected cells is intriguing and we hypothesize that this may possibly reflect a differential impact of both ID proteins with respect to their impact on E protein dependent RAG expression or TCR gene locus accessibility. Such differential mechanisms following β-selection may relate to the preferential usage of the distal versus proximal TCRα V gene segments during the development of CD8αα versus the conventional CD8αβ T cells, which has previously been observed (101). Indeed, that biased use of V-J pairs in CD8αα T cells appears to deviate between the pre- and postnatal thymus (64), in line with the developmental differences in *ID1* levels that we detected. In addition, it has been shown in mice that TCF3 is involved in controlling the order of *Tcrg* rearrangements and thereby determines which γδ TCR clonotypes can be generated (19, 42). Clonotypes that are exclusively generated before birth seem to make use of *Tcrg* elements that do not rely on TCF3 presence for their recombination. In contrast, TCF3 activity is required during postnatal γδ T cell development to prevent rearrangement of said fetal-specific region and instead permit a switch to different clonotypes. Although it is unclear if there are preferential ID/E protein dimerization complexes, ID1 and ID3 may have similar differential impacts on *TCRA* V gene segment usage following β-selection. Similarly, we speculate that the high ID levels we observed in the prenatal DN thymocytes may control TCF3 activity in order to ensure correctly timed *TCRG* locus rearrangement, which may lead to the development of fetal γδ T cells with restricted TCR diversity (39). In any case, it has previously been shown in mice that αβ lineage development is not disrupted in absence of ID3 (29). Thus, the simultaneous upregulation of *ID1*, *ID2* and *ID3* observed in our dataset indicates a possible compensation by the other family members in *Id3*-deficient thymocytes. Whether or not ID1 and ID2 might have a specific role following human β-selection remains to be investigated, but our analysis clearly points towards a fast and strong but also highly transient upregulation of ID gene expression in response to pre-TCR signaling, most likely to achieve temporary inhibition of E protein activity to prevent further TCR rearrangements during this proliferative transition.

In the course of investigating a potential role of *TCF3* in the rearrangement of the *TCRG* locus in postnatal development, we noted that mature γδ T cells exhibited strong preferential usage of *TRGC2*, whereas both *TRGC1* and *TRGC2* were actively transcribed in DN thymocytes. While there is evidence that in other mammals *Trgc* usage can differ between thymus and periphery and that circulating γδ T cells vary in their expression of different *Trgc* segments (102, 103), we hypothesize that our observation is instead related to the age-dependent generation of distinct γδ subsets. It has previously been shown that *TRGC1* is predominantly used by Vγ9Vδ2$^+$ cells, while *TRGC2* does not display preferential association with certain *TRGV* segments (104). Vγ9Vδ2$^+$ cells are mainly generated in early fetal development and a switch to Vδ2$^-$ subtypes takes place in mid-gestation (105). As a consequence, Vγ9Vδ2$^+$ cells only make up a small minority of γδ T cells in the postnatal thymus, which provides an explanation for the low expression of *TRGC1* in mature thymic γδ T cells observed in our pediatric data set. In line with this, *TRGC1* expression in DN thymocytes is likely caused by germline transcription at the *TCRG* locus but might not reflect any actual involvement in the assembly of a functional γ-chain. Assessment of *TRGC1* and *TRGC2* transcript levels in γδ T cells identified in the prenatal data set did not reveal a bias

for either segment, which seems to confirm that preferential *TRGC2* usage is an age-specific phenomenon. Due to the limited number of γδ T cells in the prenatal data set and batch effects between samples from different developmental stages, a more detailed investigation of the potential shift from *TRGC1* to *TRGC2* was not possible. Targeted enrichment of γδ T cells from fetal thymi in combination with TCR sequencing will be key to further unravel the use of different TRGC segments in pre- and postnatally developing γδ T cells.

Although the expression patterns for *TCF3* and *TCF12* seem to point towards similar preferential requirements for γδ and αβ T cell development, respectively, as observed in mice (8, 106), both the bulk and single cell RNA-seq expression profiles do reveal stages of overlapping expression which may relate to both redundant and/or unique regulatory roles with respect to TCR rearrangements or other processes that control T cell development. Combined with the largely overlapping *ID1*/*ID3* and *ID2*/*ID3* expression patterns during early and late human T cell development, respectively, and in the absence of any solid information on E-ID dimerization preferences, it is clear that functional studies with genetic approaches will be required to fully understand the specific roles of the E and ID proteins during human T cell development. Given the altered expression ratio of E/ID protein encoding genes during pre- and postnatal human T cell development, this will be required in both developmental windows and should be feasible now using CRISPR-mediated gene-editing tools in combination with the available *in vitro* models that support human T-lineage differentiation from various stem cell and hematopoietic precursor sources (16, 107, 108).

In summary, we here provide an in-depth analysis of the transcriptional dynamics of E and ID protein encoding genes in human postnatal thymocytes and provide insights into how these integrate in the broader molecular mechanisms that control distinct stages of human T cell development, both upstream and downstream of these genes. Our study provides novel insights into the unique regulatory roles of E and ID proteins during human T cell development and encourages additional research to unravel their detailed function in this context.

Data availability statement

The newly generated data presented in the study are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO repository, accession code GSE205439. Previously generated and published pediatric data from our lab are available on GEO with the accession codes GSE151081 (46), GSE144870 (63) and GSE206710 (64) and on ArrayExpress with the accession code E-MTAB-8581 (64). Publicly available single cell datasets were retrieved from GEO with the accession code GSE139042 (65). Normalized gene count tables for mouse bulk RNA-seq data were obtained from the ImmGen dataset repository (http://rstats.immgen.org/DataPage/, GEO accession GSE109125). The publicly available prenatal single cell datasets were retrieved from ArrayExpress (accession code E-MTAB-8581) (64), GEO (accession code GSE133341) (99) and NODE (accession code OEP001185) (98).

Ethics statement

The studies involving human participants were reviewed and approved by Ghent University Hospital ethical committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

Study conception and design: LB, JR, TT. Data collection: AK, LB, ML, SS, FVN. Analysis and interpretation of results: LB, JR, JVH, TP, GL, BV, TT. Draft manuscript preparation: LB, JR, JVH, TT. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.960918/full#supplementary-material

References

1. Jones S. An overview of the basic helix-loop-helix proteins. *Genome Biol* (2004) 5:226. doi: 10.1186/gb-2004-5-6-226

2. Kee BL. E and ID proteins branch out. *Nat Rev Immunol* (2009) 9:175–84. doi: 10.1038/nri2507

3. Engel I, Murre C. The function of e- and id proteins in lymphocyte development. *Nat Rev Immunol* (2001) 1:193–9. doi: 10.1038/35105060

4. Murre C. Helix–loop–helix proteins and the advent of cellular diversity: 30 years of discovery. *Genes Dev* (2019) 33:6–25. doi: 10.1101/gad.320663.118

5. Wang D, Claus CL, Vaccarelli G, Braunstein M, Schmitt TM, Zúñiga-Pflücker JC, et al. The basic helix-Loop-Helix transcription factor HEBAlt is expressed in pro-T cells and enhances the generation of T cell precursors. *J Immunol* (2006) 177:109–19. doi: 10.4049/jimmunol.177.1.109

6. Wikström I, Forssell J, Penha-Goncalves MN, Bergqvist I, Holmberg D. A role for E2-2 at the DN3 stage of early thymopoiesis. *Mol Immunol* (2008) 45:3302–11. doi: 10.1016/j.molimm.2008.02.012

7. Barndt RJ, Dai M, Zhuang Y. Functions of E2A-HEB heterodimers in T-cell development revealed by a dominant negative mutation of HEB. *Mol Cell Biol* (2000) 20:6677–85. doi: 10.1128/mcb.20.18.6677-6685.2000

8. Barndt R, Dai MF, Zhuang Y. A novel role for HEB downstream or parallel to the pre-TCR signaling pathway during αβ thymopoiesis. *J Immunol* (1999) 163:3331–43.

9. Peverali FA, Ramqvist T, Saffrich R, Pepperkok R, Barone MV, Philipson L. Regulation of G1 progression by E2A and id helix-loop-helix proteins. *EMBO J* (1994) 13:4291–301. doi: 10.1002/j.1460-2075.1994.tb06749.x

10. Wang LH, Baker NE. E proteins and ID proteins: Helix-Loop-Helix partners in development and disease. *Dev Cell* (2015) 35:269–80. doi: 10.1016/j.devcel.2015.10.019

11. Cochrane SW, Zhao Y, Welner RS, Sun X-H. Balance between id and e proteins regulates myeloid-versus-lymphoid lineage decisions. *Blood* (2009) 113:1016–26. doi: 10.1182/blood-2008-06-164996.The

12. Boos MD, Yokota Y, Eberl G, Kee BL. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of e protein activity. *J Exp Med* (2007) 204:1119–30. doi: 10.1084/jem.20061959

13. Ikawa T, Fujimoto S, Kawamoto H, Katsura Y, Yokota Y. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. *Proc Natl Acad Sci* (2001) 98:5164–9. doi: 10.1073/pnas.091537598

14. Grajkowska LT, Ceribelli M, Lau CM, Warren ME, Tiniakou I, Nakandakari Higa S, et al. Isoform-specific expression and feedback regulation of e protein TCF4 control dendritic cell lineage specification. *Immunity* (2017) 46:65–77. doi: 10.1016/j.immuni.2016.11.006

15. Taghon T, Waegemans E, Van de Walle I. Notch signaling during human T cell development. In: F Radtke, editor. *Notch regulation of the immune system*. Springer (2012). 75–97 p. doi: 10.3109/08820137709055812

16. Strubbe S, Taghon T. Modeling of human T cell development *in vitro* as a read-out for hematopoietic stem cell multipotency. *Biochem Soc Trans* (2021) 49:2113–22. doi: 10.1042/BST20210144

17. Krangel MS. Mechanics of T cell receptor gene rearrangement. *Curr Opin Immunol* (2009) 21:133–9. doi: 10.1016/j.coi.2009.03.009

18. Pellicci DG, Koay HF, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and γδ T cells emerge. *Nat Rev Immunol* (2020) 20:756–70. doi: 10.1038/s41577-020-0345-y

19. Bain G, Romanow WJ, Albers K, Havran WL, Murre C. Positive and negative regulation of V(D)J recombination by the E2A proteins. *J Exp Med* (1999) 189:289–300. doi: 10.1084/jem.189.2.289

20. Bain G, Engel I, Robanus Maandag EC, Te Riele HP, Voland JR, Sharp LL, et al. E2A deficiency leads to abnormalities in αβ T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol* (1997) 17:4782–91. doi: 10.1128/mcb.17.8.4782

21. Dias S, Månsson R, Gurbuxani S, Sigvardsson M, Kee BL. E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity* (2008) 29:217–27. doi: 10.1016/j.immuni.2008.05.015

22. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E proteins and notch signaling cooperate to promote T cell lineage specification and commitment. *J Exp Med* (2006) 203:1329–42. doi: 10.1084/jem.20060268

23. Tremblay M, Herblot S, Lécuyer E, Hoang T. Regulation of pTα gene expression by a dosage of E2A, HEB, and SCL. *J Biol Chem* (2003) 278:12680–7. doi: 10.1074/jbc.M209870200

24. Braunstein M, Anderson MK. HEB in the spotlight: Transcriptional regulation of T-cell specification, commitment, and developmental plasticity. *Clin Dev Immunol* (2012) 2012:1–15. doi: 10.1155/2012/678705

25. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early thymocyte development is regulated by modulation of E2A protein activity. *J Exp Med* (2001) 194:733–45. doi: 10.1084/jem.194.6.733

26. Nie L, Xu M, Vladimirova A, Sun X-H. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J* (2003) 22:5780–92. doi: 10.1093/emboj/cdg567

27. Bain G, Cravatt CB, Loomans C, Alberola-Ila J, Hedrick SM, Murre C. Regulation of the helix-loop-helix proteins, E2A and Id3, by the ras-ERK MAPK cascade. *Nat Immunol* (2001) 2:165–71. doi: 10.1038/84273

28. Zarin P, Chen ELY, In TSH, Anderson MK, Zúñiga-Pflücker JC. Gamma delta T-cell differentiation and effector function programming, TCR signal strength, when and how much? *Cell Immunol* (2015) 296:70–5. doi: 10.1016/j.cellimm.2015.03.007

29. Lauritsen JPH, Wong GW, Lee SY, Lefebvre JM, Ciofani M, Rhodes M, et al. Marked induction of the helix-Loop-Helix protein Id3 promotes the γδ T cell fate and renders their functional maturation notch independent. *Immunity* (2009) 31:565–75. doi: 10.1016/j.immuni.2009.07.010

30. Hayes SM, Love PE. Distinct structure and signaling potential of the gammadeltaTCR complex. *Immunity* (2002) 16:827–38. doi: 10.1016/S1074-7613(02)00320-5

31. Hayes SM, Li LQ, Love PE. TCR signal strength influences αβ/γδ lineage fate. *Immunity* (2005) 22:583–93. doi: 10.1016/j.immuni.2005.03.014

32. Taghon T, Yui MA, Pant R, Diamond RA, Rothenberg EV. Developmental and molecular characterization of emerging β- and γδ-selected pre-T cells in the adult mouse thymus. *Immunity* (2006) 24:53–64. doi: 10.1016/j.immuni.2005.11.012

33. Xi H, Schwartz R, Engel I, Murre C, Kersh GJ. Interplay between RORγt, Egr3, and e proteins controls proliferation in response to pre-TCR signals. *Immunity* (2006) 24:813–26. doi: 10.1016/j.immuni.2006.03.023

34. Taghon T, Rothenberg EV. Molecular mechanisms that control mouse and human TCR-αβ and TCR-γδ T cell development. *Semin Immunopathol* (2008) 30:383–98. doi: 10.1007/s00281-008-0134-3

35. Fahl SP, Contreras AV, Verma A, Qiu X, Harly C, Radtke F, et al. The e protein-TCF1 axis controls γδ T cell development and effector fate. *Cell Rep* (2021) 34:108716. doi: 10.1016/j.celrep.2021.108716

36. Joachims ML, Chain JL, Hooker SW, Knott-Craig CJ, Thompson LF. Human αβ and γδ thymocyte development: TCR gene rearrangements, intracellular TCRβ expression, and γδ developmental potential–differences between men and mice. *J Immunol* (2006) 176:1543–52. doi: 10.4049/jimmunol.176.3.1543

37. Van Coppernolle S, Vanhee S, Verstichel G, Snauwaert S, van der Spek A, Velghe I, et al. Notch induces human T-cell receptor γ δ+ thymocytes to differentiate along a parallel, highly proliferative and bipotent CD4 CD8 double-positive pathway. *Leukemia* (2012) 26:127–38. doi: 10.1038/leu.2011.324

38. Offner F, Van Beneden K, Debacker V, Vanhecke D, Vandekerckhove B, Plum J, et al. Phenotypic and functional maturation of TCR gammadelta cells in the human thymus. *J Immunol* (1997) 158:4634–41. doi: 10.1016/s0165-2478(97)87771-1

39. Tan L, Fichtner AS, Bruni E, Odak I, Sandrock I, Bubke A, et al. A fetal wave of human type 3 effector γδ cells with restricted TCR diversity persists into adulthood. *Sci Immunol* (2021) 6:1–15. doi: 10.1126/sciimmunol.abf0125

40. Dimova T, Brouwer M, Gosselin F, Tassignon J, Leo O, Donner C, et al. Effector vγ9vδ2 t cells dominate the human fetal γδ t-cell repertoire. *Proc Natl Acad Sci USA* (2015) 112:E556–65. doi: 10.1073/pnas.1412058112

41. David-Fung E-S, Yui MA, Morales M, Wang H, Taghon T, Diamond RA, et al. Progression of regulatory gene expression states in fetal and adult pro-t-cell development. *Immunol Rev* (2006) 209:212–36. doi: 10.1111/j.0105-2896.2006.00355.x

42. Nozaki M, Wakae K, Tamaki N, Sakamoto S, Ohnishi K, Uejima T, et al. Regulation of TCR Vγ2 gene rearrangement by the helix-loop-helix protein, E2A. *Int Immunol* (2011) 23:297–305. doi: 10.1093/intimm/dxr005

43. Iwashima M, Green A, Davis MM, Chien Y-H. Variable region (Vδ) gene segment most frequently utilized in adult thymocytes is 3' of the constant (Cδ) region. *Proc Natl Acad Sci* (1988) 85:8161–5. doi: 10.1073/pnas.85.21.8161

44. Papadopoulou M, Tieppo P, McGovern N, Gosselin F, Chan JKY, Goetgeluk G, et al. TCR sequencing reveals the distinct development of fetal and adult human Vγ9Vδ2 T cells. *J Immunol* (2019) 203:1468–79. doi: 10.4049/jimmunol.1900592

45. Krangel MS, Yssel H, Brocklehurst C, Spits H. A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: Evidence for controlled gene rearrangement and cytokine production. *J Exp Med* (1990) 172:847–59. doi: 10.1084/jem.172.3.847

46. Roels J, Kuchmiy A, De Decker M, Strubbe S, Lavaert M, Liang KL, et al. Distinct and temporary-restricted epigenetic mechanisms regulate human αβ and γδ T cell development. *Nat Immunol* (2020) 21:1280–92. doi: 10.1038/s41590-020-0747-9

47. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-seq (MACS). *Genome Biol* (2008) 9: R137.1–R.9. doi: 10.1186/gb-2008-9-9-r137

48. Li Z, Schulz MH, Look T, Begemann M, Zenke M, Costa IG. Identification of transcription factor binding sites using ATAC-seq. *Genome Biol* (2019) 20:1–21. doi: 10.1109/ISDA.2006.171

49. Castro-Mondragon JA, Riudavets-Puig R, Rauluseviciute I, Berhanu Lemma R, Turchi L, Blanc-Mathieu R, et al. JASPAR 2022: The 9th release of the open-access database of transcription factor binding profiles. *Nucleic Acids Res* (2022) 50: D165–73. doi: 10.1093/nar/gkab1113

50. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, Zheng S, Butler A, et al. Integrated analysis of multimodal single-cell data. *Cell* (2021) 184:3573–87. doi: 10.1016/j.cell.2021.04.048

51. Germain P-L, Lun A, Macnair W, Robinson MD, Gartner ZJ, McGinnis C, et al. Doublet identification in single-cell sequencing data using scDblFinder. *F1000Research* (2022) 10:1–25. doi: 10.12688/f1000research.73600.1

52. Haghverdi L, Lun ATL, Morgan MD, Marioni JC. Batch effects in single-cell RNA-sequencing data are corrected by matching mutual nearest neighbors. *Nat Biotechnol* (2018) 36:421–7. doi: 10.1038/nbt.4091

53. van Dijk D, Sharma R, Nainys J, Yim K, Kathail P, Carr AJ, et al. Recovering gene interactions from single-cell data using data diffusion. *Cell* (2018) 174:716–729.e27. doi: 10.1016/j.cell.2018.05.061

54. Andreatta M, Carmona SJ. UCell: Robust and scalable single-cell gene signature scoring. *Comput Struct Biotechnol J* (2021) 19:3796–8. doi: 10.1016/j.csbj.2021.06.043

55. Angerer P, Haghverdi L, Büttner M, Theis FJ, Marr C, Buettner F. Destiny: Diffusion maps for large-scale single-cell data in r. *Bioinformatics* (2016) 32:1241–3. doi: 10.1093/bioinformatics/btv715

56. Van den Berge K, Roux de Bézieux H, Street K, Saelens W, Cannoodt R, Saeys Y, et al. Trajectory-based differential expression analysis for single-cell sequencing data. *Nat Commun* (2020) 11:1–13. doi: 10.1038/s41467-020-14766-3

57. Kolde R. *Pheatmap: pretty heatmaps*, R Package Version 1.0.12. (2012). Available from: https://CRAN.R-project.org/package=pheatmap.

58. Van de Sande B, Flerin C, Davie K, De Waegeneer M, Hulselmans G, Aibar S, et al. A scalable SCENIC workflow for single-cell gene regulatory network analysis. *Nat Protoc* (2020) 15:2247–76. doi: 10.1038/s41596-020-0336-2

59. Luo Q, Yu Y, Lan X. SIGNET: single-cell RNA-seq-based gene regulatory network prediction using multiple-layer perceptron bagging. *Brief Bioinform* (2021) 23(1): 1–14. doi: 10.1093/bib/bbab547

60. Moerman T, Aibar Santos S, Bravo González-Blas C, Simm J, Moreau Y, Aerts J, et al. GRNBoost2 and arboreto: Efficient and scalable inference of gene regulatory networks. *Bioinformatics* (2019) 35:2159–61. doi: 10.1093/bioinformatics/bty916

61. Aibar S, González-Blas CB, Moerman T, Huynh-Thu VA, Imrichova H, Hulselmans G, et al. SCENIC: Single-cell regulatory network inference and clustering. *Nat Methods* (2017) 14:1083–6. doi: 10.1038/nmeth.4463

62. Aran D, Looney AP, Liu L, Wu E, Fong V, Hsu A, et al. Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat Immunol* (2019) 20:163–72. doi: 10.1038/s41590-018-0276-y

63. Lavaert M, Liang KL, Vandamme N, Park JE, Roels J, Kowalczyk MS, et al. Integrated scRNA-seq identifies human postnatal thymus seeding progenitors and regulatory dynamics of differentiating immature thymocytes. *Immunity* (2020) 52:1088–1104.e6. doi: 10.1016/j.immuni.2020.03.019

64. Park JE, Botting RA, Conde CD, Popescu DM, Lavaert M, Kunz DJ, et al. A cell atlas of human thymic development defines T cell repertoire formation. *Science* (2020) 367:1–11. doi: 10.1126/science.aay3224

65. Le J, Park JE, Ha VL, Luong A, Branciamore S, Rodin AS, et al. Single-cell RNA-seq mapping of human thymopoiesis reveals lineage specification trajectories and a commitment spectrum in T cell development. *Immunity* (2020) 52:1105–1118.e9. doi: 10.1016/j.immuni.2020.05.010

66. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* (1990) 61:49–59. doi: 10.1016/0092-8674(90)90214-Y

67. Haks MC, Lefebvre JM, Lauritsen JPH, Carleton M, Rhodes M, Miyazaki T, et al. Attenuation of γδTCR signaling efficiently diverts thymocytes to the αβ lineage. *Immunity* (2005) 22:595–606. doi: 10.1016/j.immuni.2005.04.003

68. Riechmann V, Van Crüchten I, Sablitzky F. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, 1d2 and Id3. *Nucleic Acids Res* (1994) 22:749–55. doi: 10.1093/nar/22.5.749

69. Engel I, Murre C. E2A proteins enforce a proliferation checkpoint in developing thymocytes. *EMBO J* (2004) 23:202–11. doi: 10.1038/sj.emboj.7600017

70. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, ld3. *Immunity* (2000) 12:17–26. doi: 10.1016/S1074-7613(00)80155-7

71. Wojciechowski J, Lai A, Kondo M, Zhuang Y. E2A and HEB are required to block thymocyte proliferation prior to pre-TCR expression. *J Immunol* (2007) 178:5717–26. doi: 10.4049/jimmunol.178.9.5717

72. Ghosh JK, Romanow WJ, Murre C. Induction of a diverse T cell receptor γ/δ repertoire by the helix-loop-helix proteins E2A and HEB in nonlymphoid cells. *J Exp Med* (2001) 193:769–75. doi: 10.1084/jem.193.6.769

73. Xu W, Carr T, Ramirez K, McGregor S, Sigvardsson M, Kee BL. E2A transcription factors limit expression of Gata3 to facilitate T lymphocyte lineage commitment. *Blood* (2013) 121:1534–42. doi: 10.1182/blood-2012-08-449447

74. Yoshida H, Lareau CA, Ramirez RN, Rose SA, Maier B, Wroblewska A, et al. The cis-regulatory atlas of the mouse immune system. *Cell* (2019) 176:897–912.e20. doi: 10.1016/j.cell.2018.12.036

75. Porritt HE, Rumfelt LL, Tabrizifard S, Schmitt TM, Zúñiga-Pflücker JC, Petrie HT. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* (2004) 20:735–45. doi: 10.1016/j.immuni.2004.05.004

76. Schiavoni G, Mattei F, Sestili P, Borghi P, Venditti M, Morse HC, et al. ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8α+ dendritic cells. *J Exp Med* (2002) 196:1415–25. doi: 10.1084/jem.20021263

77. Carotta S, Dakic A, D'Amico A, Pang SHM, Greig KT, Nutt SL, et al. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity* (2010) 32:628–41. doi: 10.1016/j.immuni.2010.05.005

78. Esashi E, Liu Y-J. E-box protein E2-2 is a crucial regulator of plasmacytoid DC development. *Eur J Immunol* (2008) 38:2386–8. doi: 10.1002/eji.200838763

79. Cisse B, Caton ML, Lehner M, Maeda T, Scheu S, Locksley R, et al. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* (2008) 135:37–48. doi: 10.1016/j.cell.2008.09.016

80. Chopin M, Lun AT, Zhan Y, Schreuder J, Coughlan H, D'Amico A, et al. Transcription factor PU.1 promotes conventional dendritic cell identity and

function *via* induction of transcriptional regulator DC-SCRIPT. *Immunity* (2019) 50:77–90.e5. doi: 10.1016/j.immuni.2018.11.010

81. Langerak AW, Wolvers-Tettero ILM, Van Gastel-Mol EJ, Oud MECM, Van Dongen JJM. Basic helix-loop-helix proteins E2A and HEB induce immature T-cell receptor rearrangements in nonlymphoid cells. *Blood* (2001) 98:2456–65. doi: 10.1182/blood.V98.8.2456

82. Dauphars DJ, Mihai A, Wang L, Zhuang Y, Krangel MS. Trav15-dv6 family tcrd rearrangements diversify the tcra repertoire. *J Exp Med* (2021) 219:1–11. doi: 10.1084/jem.20211581

83. Takeuchi A, Yamasaki S, Takase K, Nakatsu F, Arase H, Onodera M, et al. E2A and HEB activate the pre-TCRα promoter during immature T cell development. *J Immunol* (2001) 167:2157–63. doi: 10.4049/jimmunol.167.4.2157

84. Antón O, Batista A, Millán J, Andrés-Delgado L, Puertollano R, Correas I, et al. An essential role for the MAL protein in targeting lck to the plasma membrane of human T lymphocytes. *J Exp Med* (2008) 205:3201–13. doi: 10.1084/jem.20080552

85. Wong GW, Knowles GC, Mak TW, Ferrando AA, Zúñiga-Pflücker JC. HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRβ-selected mouse thymocytes. *Blood* (2012) 120:1439–48. doi: 10.1182/blood-2011-12-395319

86. Lang R, Raffi FAM. Dual-specificity phosphatases in immunity and infection: An update. *Int J Mol Sci* (2019) 20:1–26. doi: 10.3390/ijms20112710

87. Reuschel EL, Wang JF, Shivers DK, Muthumani K, Weiner DB, Ma Z, et al. REDD1 is essential for optimal T cell proliferation and survival. *PloS One* (2015) 10:1–13. doi: 10.1371/journal.pone.0136323

88. Lauritsen JPH, Wong WG, Lee S-Y, Lefebvre JM, Ciofani M, Rhodes M, et al. Differential induction of Id3 signals lineage divergence, notch independent differentiation, and functional maturation of γδ T cells. *Immunity* (2009) 31:565–75. doi: 10.1016/j.immuni.2009.07.010.Differential

89. Naik AK, Byrd AT, Lucander ACK, Krangel MS. Hierarchical assembly and disassembly of a transcriptionally active RAG locus in CD4 + CD8 + thymocytes. *J Exp Med* (2019) 216:231–43. doi: 10.1084/jem.20181402

90. Miyazaki K, Watanabe H, Yoshikawa G, Chen K, Hidaka R, Aitani Y, et al. The transcription factor E2A activates multiple enhancers that drive rag expression in developing T and b cells. *Sci Immunol* (2020) 5:1–15. doi: 10.1126/sciimmunol.abb1455

91. Kim D, Peng X-C, Sun X-H. Massive apoptosis of thymocytes in T-Cell-Deficient Id1 transgenic mice. *Mol Cell Biol* (1999) 19:8240–53. doi: 10.1128/mcb.19.12.8240

92. Sun X-H. Constitutive expression of the Id1 gene impairs mouse b cell development. *Cell* (1994) 79:893–900. doi: 10.1016/0092-8674(94)90078-7

93. Anderson MK. At The crossroads: Diverse roles of early thymocyte transcriptional regulators. *Immunol Rev* (2006) 209:191–211. doi: 10.1111/j.0105-2896.2006.00352.x

94. Blom B, Heemskerk MHM, Verschuren MCM, Van Dongen JJM, Stegmann APA, Bakker AQ, et al. Disruption of alpha beta but not of gamma delta T cell development by overexpression of the helix-loop-helix protein Id3 in committed T cell progenitors. *EMBO J* (1999) 18:2793. doi: 10.1093/EMBOJ/18.10.2793

95. Sheng H, Marrero I, Maricic I, Fanchiang SS, Zhang S, Sant'Angelo DB, et al. Distinct PLZF+ CD8αα+ unconventional T cells enriched in liver use a cytotoxic mechanism to limit autoimmunity. *J Immunol* (2019) 203:2150–62. doi: 10.4049/jimmunol.1900832

96. Yamagata T, Benoist C, Mathis D. A shared gene-expression signature in innate-like lymphocytes. *Immunol Rev* (2006) 210:52–66. doi: 10.1111/j.0105-2896.2006.00371.x

97. Van Kaer L, Scott Algood HM, Singh K, Parekh VV, Greer MJ, Piazuelo MB, et al. CD8αα+ innate-type lymphocytes in the intestinal epithelium mediate mucosal immunity. *Immunity* (2014) 41:451–64. doi: 10.1016/j.immuni.2014.08.010

98. Li Y, Zeng W, Li T, Guo Y, Zheng G, He X, et al. Integrative single-cell transcriptomic analysis of human fetal thymocyte development. *Front Genet* (2021) 12:679616. doi: 10.3389/fgene.2021.679616

99. Zeng Y, Liu C, Gong Y, Bai Z, Hou S, He J, et al. Single-cell RNA sequencing resolves spatiotemporal development of pre-thymic lymphoid progenitors and thymus organogenesis in human embryos. *Immunity* (2019) 51:930–948.e6. doi: 10.1016/j.immuni.2019.09.008

100. Jones ME, Zhuang Y. Stage-specific functions of e-proteins at the β-selection and T-cell receptor checkpoints during thymocyte development. *Immunol Res* (2011) 49:202–15. doi: 10.1007/s12026-010-8182-x

101. Verstichel G, Vermijlen D, Martens L, Goetgeluk G, Brouwer M, Thiault N, et al. The checkpoint for agonist selection precedes conventional selection in human thymus. *Sci Immunol* (2017) 2:1–12. doi: 10.1126/sciimmunol.aah4232

102. Herzig C, Blumerman S, Lefranc MP, Baldwin C. Bovine T cell receptor gamma variable and constant genes: Combinatorial usage by circulating γδ T cells. *Immunogenetics* (2006) 58:138–51. doi: 10.1007/s00251-006-0097-2

103. Heng TSP, Painter MWThe Immunological Genome Project Consortium. The immunological genome project: networks of gene expression in immune cells. *Nat Immunol* (2008) 9:1091–4. doi: 10.1038/ni1008-1091

104. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. *Immunol Today* (1989) 10:186–8. doi: 10.1016/0167-5699(89)90321-6

105. Fichtner AS, Ravens S, Prinz I. Human γδ TCR repertoires in health and disease. *Cells* (2020) 9:1–14. doi: 10.3390/cells9040800

106. Bain G, Quong MW, Soloff RS, Hedrick SM, Murre C. Thymocyte maturation is regulated by the activity of the helix-loop-helix protein, E47. *J Exp Med* (1999) 190:1605–16. doi: 10.1084/jem.190.11.1605

107. Montel-Hagen A, Seet CS, Li S, Chick B, Zhu Y, Chang P, et al. Organoid-induced differentiation of conventional T cells from human pluripotent stem cells. *Cell Stem Cell* (2019) 24:376–89. doi: 10.1016/j.stem.2018.12.011

108. Seet CS, He C, Bethune MT, Li S, Chick B, Gschweng EH, et al. Generation of mature T cells from human hematopoietic stem and progenitor cells in artificial thymic organoids. *Nat Methods* (2017) 14:521–30. doi: 10.1038/nmeth.4237

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EDITED BY
Michele Kay Anderson,
University of Toronto, Canada

REVIEWED BY
Xiao-Hong Sun,
Oklahoma Medical Research Foundation, United States
Kamalakannan Rajasekaran,
Genentech, Inc., United States

*CORRESPONDENCE
Sin-Hyeog Im
iimsh@postech.ac.kr
Dipayan Rudra
drudra@shanghaitech.edu.cn

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Signaling networks controlling ID and E protein activity in T cell differentiation and function

Sung-Min Hwang[1], Sin-Hyeog Im[2,3,4]* and Dipayan Rudra[5]*

[1]Department of Obstetrics and Gynecology, Weill Cornell Medicine, New York, NY, United States, [2]Department of Life Sciences, Pohang University of Science and Technology, Pohang, South Korea, [3]Institute for Convergence Research and Education, Yonsei University, Seoul, South Korea, [4]ImmunoBiome Inc., Bio Open Innovation Center, Pohang, South Korea, [5]School of Life Science and Technology, ShanghaiTech University, Shanghai, China

E and inhibitor of DNA binding (ID) proteins are involved in various cellular developmental processes and effector activities in T cells. Recent findings indicate that E and ID proteins are not only responsible for regulating thymic T cell development but also modulate the differentiation, function, and fate of peripheral T cells in multiple immune compartments. Based on the well-established E and ID protein axis (E-ID axis), it has been recognized that ID proteins interfere with the dimerization of E proteins, thus restricting their transcriptional activities. Given this close molecular relationship, the extent of expression or stability of these two protein families can dynamically affect the expression of specific target genes involved in multiple aspects of T cell biology. Therefore, it is essential to understand the endogenous proteins or extrinsic signaling pathways that can influence the dynamics of the E-ID axis in a cell-specific and context-dependent manner. Here, we provide an overview of E and ID proteins and the functional outcomes of the E-ID axis in the activation and function of multiple peripheral T cell subsets, including effector and memory T cell populations. Further, we review the mechanisms by which endogenous proteins and signaling pathways alter the E-ID axis in various T cell subsets influencing T cell function and fate at steady-state and in pathological settings. A comprehensive understanding of the functions of E and ID proteins in T cell biology can be instrumental in T cell-specific targeting of the E-ID axis to develop novel therapeutic modalities in the context of autoimmunity and cancer.

KEYWORDS

E proteins, ID proteins, E-ID axis, T cell differentiation, T cell function, regulatory T (Treg) cells, signaling pathways

Introduction

A diverse network of transcription factors (TFs) and modulators regulate the expression of relevant genes involved in lymphocyte generation and function (1, 2). E and ID proteins are well-characterized transcriptional regulators that belong to the helix-loop-helix (HLH) family of proteins (3). They are widely recognized to play a significant role in developing lymphocytes, particularly B and T cells (4–9). E and ID proteins are crucially involved in various stages of thymic T cell development. For instance, E2A and HEB, which represents the major E proteins, have been demonstrated to play crucial roles in the early stages of thymocytes differentiation (10–13).

Several types of E proteins are identified, forming active homo- and heterodimers within the HLH proteins, binding to DNA, and regulating the transcription of multiple target genes in T cells (7, 8). Considerable evidence in mice shows that active E proteins are primarily engaged in the generation, differentiation, and effector function of different peripheral CD4 T cell subsets and CD8 T cell populations. On the other hand, ID proteins, encoded by four different genes (Id1-Id4), lack DNA binding activity and, through E-ID heterodimerization, modulate gene expression primarily by interfering with the DNA binding and transcription-related activities of E proteins (5). While various transcription factors regulate the expression of E proteins, the ID proteins are the only regulators that inhibit the transcription factor activity of E proteins through a mechanism that interferes with the formation of dimers within E proteins. Unlike E proteins, which are ubiquitously expressed in many tissues and cells, ID proteins are found to be expressed in a tissue- and cell-specific manner (14). For instance, Id2 and Id3 are predominantly expressed in T cells compared with Id1 and Id4. Indeed, Id2 and Id3 are recognized for their crucial roles in multiple discrete steps of T cell development and the differentiation and effector function of various CD4 and CD8 T cells. They have also been shown to suppress the generation of innate-like γδ (15) and invariant NKT (iNKT) cells (16), reinforcing αβ CD4 and CD8 T cell development in the thymus.

As E and ID proteins play critical roles in T cells through well-established molecular dynamics; the balance between these proteins has the potential to alter the E-ID axis-mediated global transcriptional program, affecting T cell phenotypes, and contributing to many aspects of autoimmune diseases, inflammation, and cancer progression (17). It is becoming clear that various TFs and extrinsic signaling pathways can affect the expression and stability of E and ID proteins by influencing the interactions between them. This review focuses on a brief overview of E and ID proteins and their molecular relationship, the interplay between E and ID proteins in the regulation of peripheral T cell activation, differentiation, and function, and the mechanisms by which TFs and extrinsic signaling pathways act on altering the E-ID axis in a context-dependent manner to dictate T cell function and fate.

Molecular dissection of E and ID proteins

For decades, it has been well known that E and ID proteins interact closely with each other as transcription regulators, and their structure and mode of interaction are established. This section will describe the overview of E and ID proteins, highlighting the protein structure/domains and molecular features of their interaction in the context of transcription regulation.

E proteins

E proteins are a family of TFs that recognize a consensus DNA sequence (CANNTG) known as an enhancer box (E-box). E proteins are encoded from three genes, E2A, HEB, and E2-2, which encode multiple proteins through alternative splicing. The E2A gene encodes E12 and E47 proteins, while the HEB and E2-2 genes encode both canonical (HEBcan and E2-2can) and alternative splice variants (HEBalt and E2-2alt) proteins (5). These E proteins are ubiquitously expressed and function in many tissues and cell types. E proteins contain several conserved domains, including the basic HLH (bHLH) domain and transcriptional activation domains (AD) (Figure 1A) (18). A C-terminal bHLH domain consists of approximately 60 amino acids and has two functionally distinct regions: the basic and HLH regions. The basic part is essential for initiating or repressing gene transcription by binding to the E box present downstream of specific target genes. On the other hand, the HLH region contains two amphipathic α-helices with a linking loop and is required for protein-protein interaction with other HLH proteins. Since the E proteins bind to the E-box of genomic DNA by forming homo- or heterodimers to initiate the transcription of target genes, the bHLH domain is an essential part of these two distinct processes governing the transcriptional machinery of the E proteins. In addition, E proteins also contain two transcriptional activation AD domains, AD1 and AD2. These two domains have been shown to recruit co-transcriptional activators, such as CBP/p300 and Spt/Ada/Gcn5 acetyltransferase (SAGA) complex (Figure 1A), to promote the transcriptional activity of a target gene (19). Contrary to acting on transcriptional activation, it has been demonstrated that corepressors, such as ETO family proteins and leukemogenic AML1-ETO fusion protein, can interact with AD1 (Figure 1A) (20, 21). These interactions contribute to transcriptional repression mechanisms of E proteins by inhibiting the recruitment of coactivators on target genes

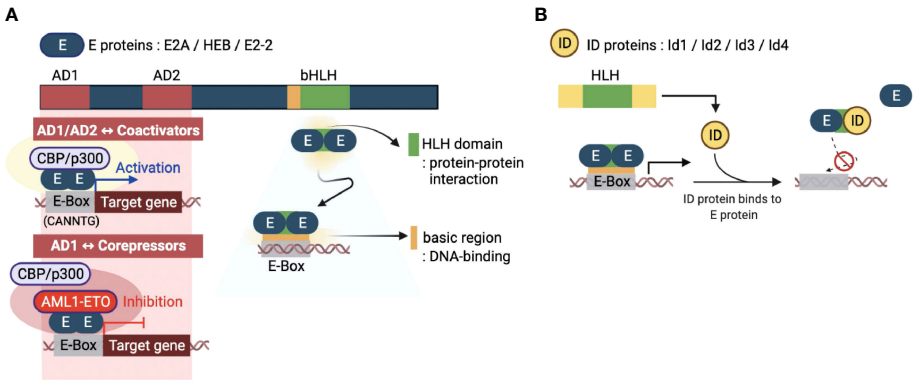

FIGURE 1
Molecular basis of E and ID protein functions. **(A)** An overview of the key domains of E proteins (E2A, HEB, and E2-2) - transcriptional activation domains (AD1 and AD2) and basic helix-loop-helix domain (bHLH). The AD1 and AD2 domains enable the E-box sequence (CANNTG) bound E protein homodimerization. E proteins function as transcriptional activators or repressors through recruitment of coactivators (CBP/p300) or corepressors (AML1-ETO, acute myeloid leukemia1-eight-twenty one oncoprotein), respectively. The bHLH domain consists of two parts: the HLH domain for protein-protein interactions and the basic region for DNA binding. **(B)** The four inhibitors of DNA binding (ID) proteins have one HLH domain in common. The ID protein interferes with the homo- or heterodimerized E proteins through this domain, inhibiting their DNA binding and transcription-related activities. This figure was created with BioRender.com.

ID proteins

The inhibitor of DNA binding (ID) proteins, Id1-Id4, are also members of the HLH protein family. Regarding protein structure/domain and function, all four ID proteins have highly conserved common domains and similar molecular functions. Although ID proteins contain the same HLH domain as E proteins, homodimers or heterodimers between ID proteins can occur in rare circumstances (22). Interestingly, however, they can heterodimerize with bHLH proteins, primarily by inhibiting the formation of DNA-bound bHLH dimers (5). The lack of a basic region of the HLH domain distinguishes the ID proteins from the E proteins. Therefore, ID proteins cannot bind to the promoter regions and directly mediate the transcriptional regulation of genes. Thus, ID proteins operate as dominant-negative regulators of bHLH TFs and indirectly repress transcription of E protein target genes (Figure 1B). Interestingly, there is also evidence that ID proteins have functions unrelated to E proteins (23). The exact mechanism of such 'non-canonical' E-protein independent functions of ID proteins in transcriptional regulation is unknown and requires further investigation.

ID proteins are expressed in various tissues and cell types, including neuronal and immune compartments tumors (24–26). In particular, Id2 and Id3 are dominantly expressed in immune cells and have been demonstrated to control the expression of different genes that play critical roles in the development, differentiation, and function of T cells in steady-state and pathological conditions. In the following section, therefore, we will discuss the effects of ID proteins on the activation and function of T cells through their interaction with E proteins.

The E-ID axis orchestrates peripheral T cell differentiation and function

T cells are one of the key immune cell types that comprise the adaptive immune system, offering cellular protection against pathogenic assaults and capable of eliciting a long-lasting memory response. T cells begin their life cycle as T cell precursors generated in the bone marrow and migrate to the thymus, where they undergo successive stages of development and maturation. During this process, thymic T cell precursors initiate genetically programmed transcriptional cascades that rely on precise functional networks of multiple TFs in response to thymus-related environmental signals (27). Following that, naive T cells matured in the thymus undergo further proliferation and activation as they migrate to secondary lymphoid organs. They are activated upon engagement with peptide-MHC on APC through TCR-CD3 complex molecules and CD28-mediated co-stimulation. In addition, the cytokine milieu act as an essential tertiary factor that determines the differentiation and effector function of specific CD4 T helper cell (Th) subsets, like Th1, Th2, Th17, induced regulatory T cells (iTreg), and T follicular helper T cells (Tfh) as well as promotes the function of cytotoxic CD8 T cells (28). During this process, T cells that receive these signals activate a network of multiple TFs. Numerous studies have revealed different molecular pathways with varied implications on T cell activation, differentiation, function, proliferation, survival, and memory formation (29). The E-ID axis is believed to be responsible for regulating the transcription of several genes involved in the development and

function of T cells. In this section, we will discuss the role of the E-ID axis in determining the differentiation and function in different peripheral T cell subsets in steady-state and pathological conditions.

Th1 and Tfh cells

When a viral infection occurs, the immune system induces naive CD4 T cells to actively differentiate into two lineages: Th1 cells and Tfh cells (30, 31). Th1 cell differentiation drives inflammatory responses and pathogen clearance, whereas Tfh cells enhance germinal center (GC) responses for forming high-affinity antibodies and immunological memory against the virus. While Th1 and Tfh differentiation occur concurrently, these T cell identities are mutually exclusive and are governed by T-bet and Bcl6, which are the master regulators for the differentiation of Th1 and Tfh cells, respectively (32, 33). In the case of Th1 cells, it has been reported that both Id2 and Id3 promote Th1 differentiation in the context of influenza viruses by promoting the expression of T-bet, which is negatively regulated by the E proteins (34). In line with this finding, another study demonstrated that enhanced Id2 expression promotes Th1 differentiation while suppressing E protein-mediated CXCR5 expression, which is essential for Tfh cell differentiation and maturation upon lymphocytic choriomeningitis virus (LCMV) infection (Figure 2A) (35). Id2, therefore reciprocally modulates Th1/Tfh cell differentiation in the course of viral infection and promotes cell-mediated immunity, which does not rely on Tfh cell-mediated humoral response mechanisms to respond to virus infection.

Th17 cells

Th17 cells are one of the CD4 T cell subsets that play a prominent role in maintaining mucosal barrier homeostasis by contributing to pathogen clearance at mucosal surfaces (36, 37). Loss of Th17 populations in the gut mucosal sites is directly associated with increased microbial translocation into the normal sterile tissues, leading to systemic immune activation and inflammation. However, excessive or uncontrolled Th17 activation has been linked to several autoimmune diseases, including multiple sclerosis (MS), arthritis, psoriasis, and lupus (38, 39). Therefore, it is important to understand the mechanisms involved in the differentiation and function of Th17 cells in both homeostatic and pathological settings. Not surprisingly, E and ID proteins are also involved in Th17 differentiation and function. For example, E2A and HEB were found to directly induce RORγt and interleukin-17 (IL-17) (Figure 2B), which are important for the differentiation and function of Th17 cells, respectively (40). Interestingly and

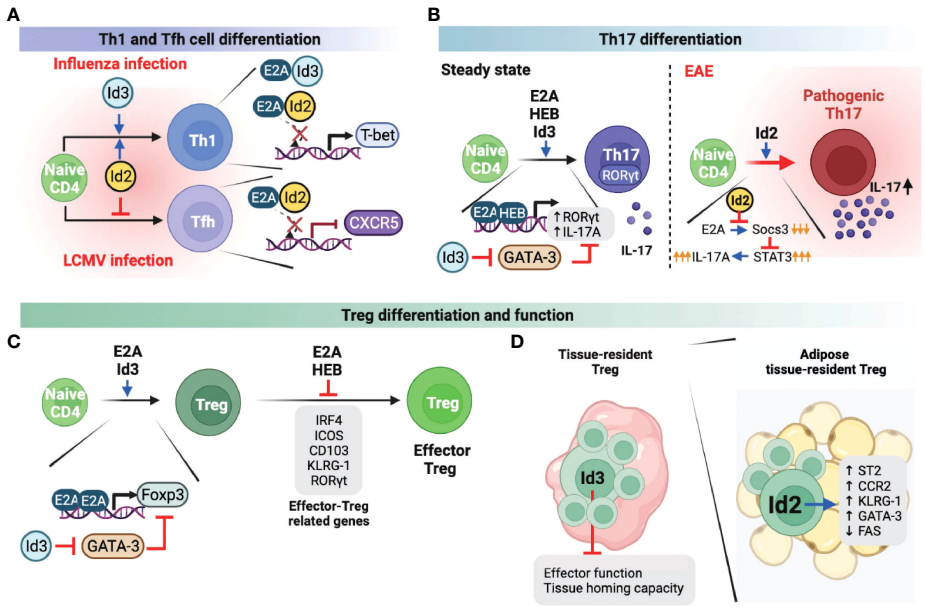

FIGURE 2
Role of E and ID proteins during peripheral CD4 T cell differentiation and function. A-C. E and ID proteins influence Th1 and Tfh cell differentiation **(A)**, Th17 cell differentiation and function **(B)**, and Treg and effector Treg differentiation and function **(C)** under steady-state as well as infectious and autoimmune conditions. **(D)** Id3 negatively influences the tissue-resident Treg effector function and tissue homing capacity. In adipose tissue, tissue-resident Treg cells enhanced Id2 expression, which can positively regulate the Treg effector function and survival. This figure was created with BioRender.com.

somewhat counterintuitively, Id3-deficient naïve CD4 T cells exhibit decreased Th17 differentiation relative to wild-type T cells *in vitro*. Id3 deficiency leads to increased GATA-3 expression, which suppresses RORγt expression (40). In another study, Id2 was associated with increased activation phenotype of CD4 T cells under steady-state conditions, as well as IL17 production upon experimental autoimmune encephalomyelitis (EAE), an animal model of MS, induction, ultimately contributing to severe EAE pathogenesis (Figure 2B) (41). Mechanistically, increased Id2 in activated T cells suppresses E protein-mediated expression of Socs3. Socs3 is a negative regulator of cytokine production through the JAK/STAT pathway (42). Thus enhanced expression of Id2 results in restoration of IL-17A production that is otherwise suppressed by Socs3 (Figure 2B). Taken together, E and ID proteins promote Th17 cell differentiation and function through different regulatory mechanisms, respectively.

Treg cells

The functions of E and ID proteins in Treg cells have also been extensively studied and led to complicated conclusions. Unlike other T cell subsets, Treg cells are a unique subset of CD4 T cells, indispensable for peripheral tolerance (43). It was reported that E2A directly promotes the expression of Foxp3, a well-defined Treg lineage specificity factor, by binding at the *Foxp3* promoter (Figure 2C) (44). In addition, Id3 suppressed GATA-3 expression, which represses the transcription of *Foxp3*, suggesting that E and ID proteins contribute independently of each other towards optimal Foxp3 expression (Figure 2C). In another study, however, E2A and HEB were found to negatively regulate Foxp3 and other effector-related factors in Treg cells such as IRF4, ICOS, CD103, KLRG-1, and RORγt, and consequently inhibited effector Treg differentiation and function (Figure 2C) (45). Based on the conflicting results of E proteins regulating *Foxp3* transcription, the mechanisms by which E proteins regulate *Foxp3* appear to differ, depending on the effector stages of Treg cells, and therefore require further investigation. On the other hand, recent studies discovered that ID proteins play an important role in the differentiation, function, and survival of tissue-resident Treg cells (Figure 2D). For instance, Id2 is highly expressed in adipose-resident Treg cells and is associated with increased expression of the adipose Treg-related genes *Il1rl1* (codes for the IL33 receptor ST2), *Ccr2*, *Klrg1*, and *Gata3*, but suppresses the apoptosis-related gene, *Fas* (46). Another group demonstrated that Id3 is directly associated with decreased effector function and tissue homing capacity of tissue-resident Treg cells (47). However, establishing a direct role of Id3 downregulation in the functional differentiation of tissue-resident Treg cells requires further investigation. ID protein-mediated Treg differentiation and function also contribute to the pathogenicity of autoimmune diseases. In systemic lupus erythematosus (SLE) patients, Id3 expression levels were positively correlated with Treg cell frequencies, and subsequently, mice in which Id proteins were overexpressed showed favorable autoimmune responses (48). In contrast, elevated Id3 expression was found to promote Treg differentiation in hepatitis B virus infection, thereby reducing viral clearance and developing a chronic state of infection (49). Thus, these two independent studies demonstrated that disease prognosis might differ based on Treg differentiation and function, suggesting the importance of targeting Id3 expression in Treg cells according to its context.

CD8 T cells

CD8 cytotoxic T cells are well known for anti-viral immune responses and anti-tumor immunity (50). Currently, there are two studies on the role of the E proteins in association with the formation of memory CD8 T cells responding to infection. One study demonstrated that both E2A and HEB transcriptionally upregulate the effector-associated genes such as *Eomes*, *Id2*, and *Fyb* and increase the generation of memory precursor T cells (51). The other study showed that E2A epigenetically regulates the accessibility of enhancers of memory-related genes such as *Id3*, *Ccr7*, and *Sell*, increasing the frequency of memory precursor effector cells and accelerating memory cell formation (Figure 3A) (52). As expected, Id2 expression suppressed E proteins-mediated gene expression, thereby suppressing the differentiation of memory CD8 T cells (Figure 3A) (53–55). Memory T cells are classified into two types according to the classification of Killer Cell Lectin Like Receptor G1 (KLRG1) and CD127. First, KLRG1^{+}CD127^{low} cells are classified as short-lived effector memory cells, and most of them show rapid effector function and die. Conversely, KLRG1^{-}CD127^{hi} long-lived memory T cells present a vital protective role during acute rechallenge with pathogens such as viruses or bacteria. Interestingly, in the context of LCMV infection, Id2 expression promoted the differentiation of short-lived effector-memory CD8 T cells, while Id3 expression demonstrated functional capability to induce differentiation of long-lived memory progenitors (Figure 3A) (56). However, the exact underlying mechanisms by which Id2 and Id3 are involved in the differentiation process of each memory cell types are yet to be elucidated. Therefore, it may be beneficial to investigate further how the expression of Id2 and Id3 is regulated in association with the signaling pathways that determine each memory T cell subset.

A recent study found that Id3 inhibits the exhaustion of CD8 T cells in the tumor microenvironment (TME) (Figure 3B) (57). Further, an elevated population of exhausted CD8 T cells was correlated with reduced anti-tumor immune response in TME. In fact, these exhausted CD8 T cells display high levels of Tcf1 and Tox, which are known representative markers of CD8 T cell

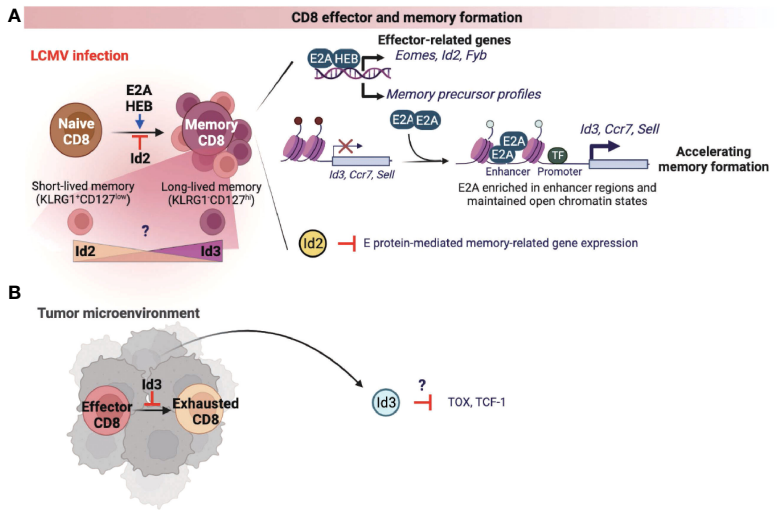

FIGURE 3
E-ID axis in the formation of effector and memory CD8 T cells. E and ID proteins regulate effector and memory CD8 T cell differentiation and function in infection **(A)** and cancer **(B)**. This figure was created with BioRender.com.

exhaustion, and overexpression of these two factors also directly induces T cell dysfunction. These data warrants further investigations to understand whether Id3 can regulate Tcf1 or Tox expression (Figure 3B), which is implicated in the differentiation and function of exhausted CD8 T cells in the TME.

Endogenous factors and cell-extrinsic signaling pathways impact the E-ID axis in T cell fate and function

Recent studies have demonstrated that several endogenous proteins and extrinsic signaling pathways affect development, differentiation, function, and memory formation by altering the E-ID axis under steady-state and pathological conditions. In this section, we will emphasize some of the major endogenous proteins and extrinsic signaling pathways that influence the balance of the E-ID axis by controlling the expression of each of the E and ID proteins and their protein-protein interactions, leading to phenotypic changes and functional reprogramming of T cells in a context-dependent manner (Figure 4).

Endogenous proteins associated with the E-ID axis

The zinc-finger transcription factor Bcl11b is a critical regulator of differentiation and survival during T cell development in the thymus (58–60). Hosokawa et al. discovered that Bcl11b, highly expressed at a specific pro-T cell lineage commitment stage (DN2-DN3), inhibits Id2 expression (61). In addition, the effect of depleting Bcl11b on gene expression in pro-T cells remarkably overlaps the impact of depleting E2A, suggesting that Bcl11b and E2A interact very closely during the pro-T cell development. In agreement with this finding, another study demonstrated that Bcl11b-dependent target genes are parallelly regulated by E2A during T cell development (62), indicating that Bcl11b plays a critical role in remodeling the E-ID axis through active suppression of Id2 expression. Given the importance of the E-ID axis in T cell development, further studies are needed to identify other transcription factors that contribute to the functionalities of the E-ID axis by regulating E and ID protein expressions.

Two other zinc finger transcription factors, Egr2 and Egr3, mediate self-tolerance by T lymphocytes and NKT cell development (63, 64). Miao et al. reported that Egr2 and Egr3 regulate clonal expansion and differentiation of virus-responsive T cells by directly promoting Id3 expression and other effector genes (65). Nevertheless, the exact mechanism remains unclear.

T cell factor 1 (Tcf1) is the key transcription factor of the canonical Wnt signaling pathway (66). Tcf1 plays an essential role in controlling T cell development, the differentiation of specific CD4 T helper (Th) subsets, and the formation of memory and stem-cell-like CD8 T cells following various types of viral infections (67). Tcf1 closely interacts with the E-protein HEB to establish epigenetic and transcriptional profiles of double-positive thymocytes (68). Mechanistically, TCF-1 inhibits Notch signaling, which protects HEB from Notch-induced proteasomal degradation, suggesting that Tcf1 is

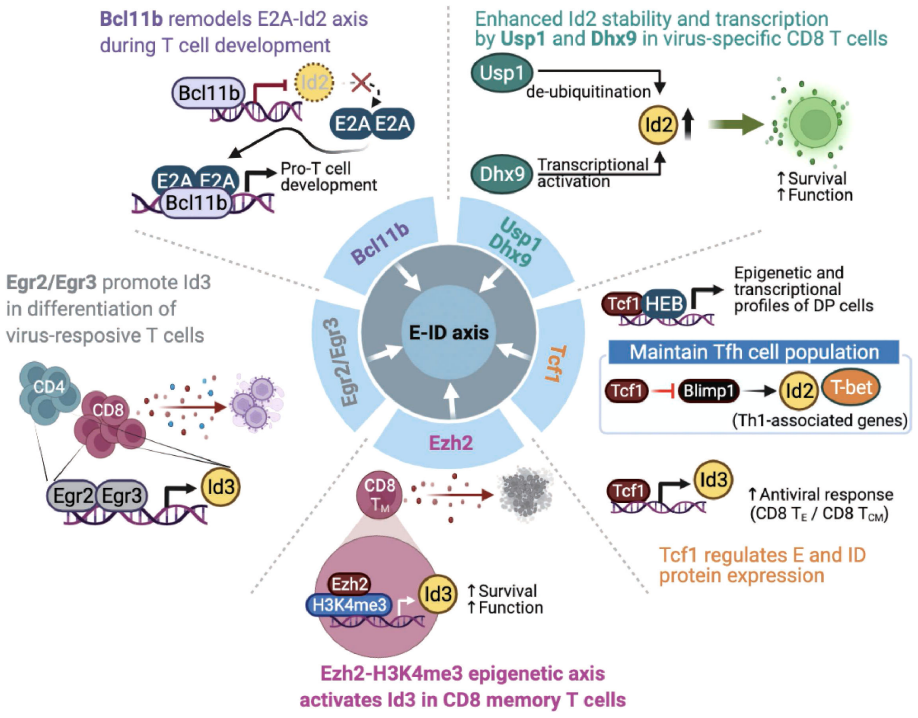

FIGURE 4
Endogenous factors influence the E-ID axis in T cell fate and function. The roles and mode of action of endogenous proteins that control E and ID protein expression and affect T cell development, differentiation, and function (Bcl11b, B-cell lymphoma/leukemia 11B; Egr2/Egr3, early growth response 2/early growth response 3; Ezh2, Enhancer of zeste homolog 2; H3K4me3, histone H3 lysine 4 trimethylation; Tcf1, T cell factor 1; Blimp1, B-lymphocyte-induced maturation protein 1; Usp1, ubiquitin-specific protease 1; Dhx9, DExH-Box Helicase 9; T_E, Effector T cells; T_{CM}, Central Memory T cells). This figure was created with BioRender.com.

involved in the stability of E proteins. In response to acute viral infection, another study found that Tcf1 maintains T follicular T helper (Tfh) cell population by suppressing Blimp1, which promotes Th1-associated effector genes such as T-bet and Id2 expression in Tfh cells (69). Since E protein induces CXCR5, associated with Tfh cell migration into B cell follicles and subsequent further differentiation of Tfh cells (70), Tcf1-mediated Blimp1 repression may serve as a unique mechanism for maintaining the Tfh population in the context of viral infection. For CD8 T cells, a recent study discovered that Tcf1 directly modulates the expression of Id3, which is important for both effector and central memory function of CD8 T cells, thereby affecting optimal CD8 T cell activity in the context of viral infection (71). Further, ectopic expression of Tcf1 was associated with increased expression of Id3 and several key effector components known to counteract CD8 T cell exhaustion upon LCMV infection, eventually leading to reinforced CD8 T cells mediated antiviral response (72).

The histone methyltransferase Ezh2 is involved in forming CD8 T cell memory precursors and contributes to the antitumor activity of CD8 memory T cells in the tumor microenvironment. Ezh2 was found to promote the expression of Id3 for maintaining the function of effector and memory CD8 T cells. Interestingly, Ezh2 was found to promote the expression of Id3 by enhancing H3K4me3 modification on its gene locus, which is distinct from the well-known repressive H3K27me3 promoting activity of Ezh2 (73). Considering these exciting observations, further studies are required to clarify the precise roles of various epigenetic modifying enzymes related to the E-ID axis in CD8 memory T cell formation and function in tumor microenvironments.

Interestingly, recent studies have demonstrated that the E-ID axis involved in T cell function is also regulated by factors whose functions extend beyond transcription regulation. For example, the de-ubiquitinase Usp1, which is known to stabilize Id1-Id3, contributes to maintaining stem cell properties in osteosarcoma and mesenchymal stem cells (74, 75). In activated T cells, Usp1 interacts with Id2 and Id3, protects the stability of Id2 protein in the context of viral infection, and maintains the proliferative potential and memory phenotype differentiation of virus-specific CD8 effector T cells (76). Similarly, Jiao et al. demonstrated that DExD/H-box helicase 9 (Dhx9) is required for a proper CD8 T cell response against acute viral infection. Interestingly, contrary to the well-established role of Dhx9 as a cytosolic DNA-sensor, Dhx9 was

found to directly increase the transcription level of Id2 expression, thereby affecting the survival and function of viral-specific CD8 T cells (77). Mechanistically, the authors discovered that two domains of Dhx9, double-stranded RNA binding motif (DSRM) and oligonucleotide/oligosaccharide binding fold (OB_Fold) domain, play an essential role in directly binding to the *Id2* promoter and consequently regulating the level of Id2 transcription.

Extrinsic signaling pathways associated with the E-ID axis

It is becoming clear that extracellular cytokine signaling can further modulate the E-ID axis dependent transcriptional reprograms in a T cell lineage-specific and context-dependent manner, consequently governing T cell phenotypes implicated in several disease outcomes (Figure 5). Interleukin-7 (IL-7) appears to be an important determinant in this context. A recent study discovered that Interleukin-7 (IL-7) signaling promotes Foxo1-Tcf1-Id3 pathways to maintain memory CD8 T cell differentiation, survival, and function (78). Although IL-7 signaling is well-established to play an important role in T cell survival and proliferation (79, 80), before this finding, it was primarily believed to function by enhancing the expression of anti-apoptotic Bcl-2 family of proteins, especially Mcl1 and Bcl-2 (81). In another study, Han et al. demonstrated that when mice were administered with the *Mycobacterium tuberculosis* (*M. tuberculosis*) subunit vaccine and adeno-associated virus-mediated IL-7, Id3 expression was directly upregulated, contributing to long-term memory CD4 and CD8 T cells response against *M. tuberculosis* infection (82). The underlying mechanism, however, is yet to be elucidated.

Interleukin-21 (IL-21) is an IL-2 family cytokine produced by activated T cells, mainly by natural killer T (NKT) cells, Th17 cells, and Tfh cells, to regulate immune responses (83, 84). Elevated amounts of IL-21 have been reported in several autoimmune diseases (85) such as inflammatory bowel disease (86), rheumatoid arthritis (87), type 1 diabetes (88), and systemic lupus erythematosus (89). Interestingly, a recent study demonstrated that IL-21 signaling directly inhibits Id3 *via* STAT3, promoting differentiation of hyper-activating Tfh cells, exacerbating the pathogenesis of Sjogren's syndrome (90). Hence, it is evident that IL-21 affects the E-ID axis under specific inflammatory conditions, thereby influencing the differentiation and function of disease-related target cells. In addition to IL-21, Interleukin-2 (IL-2) and Interleukin-15 (IL-15) are also major cytokines that regulate T cell differentiation, proliferation, effector function, and memory formation (91, 92). Given that the E-ID axis is critical for effector T cell function and memory T cell formation, further investigation is required to determine

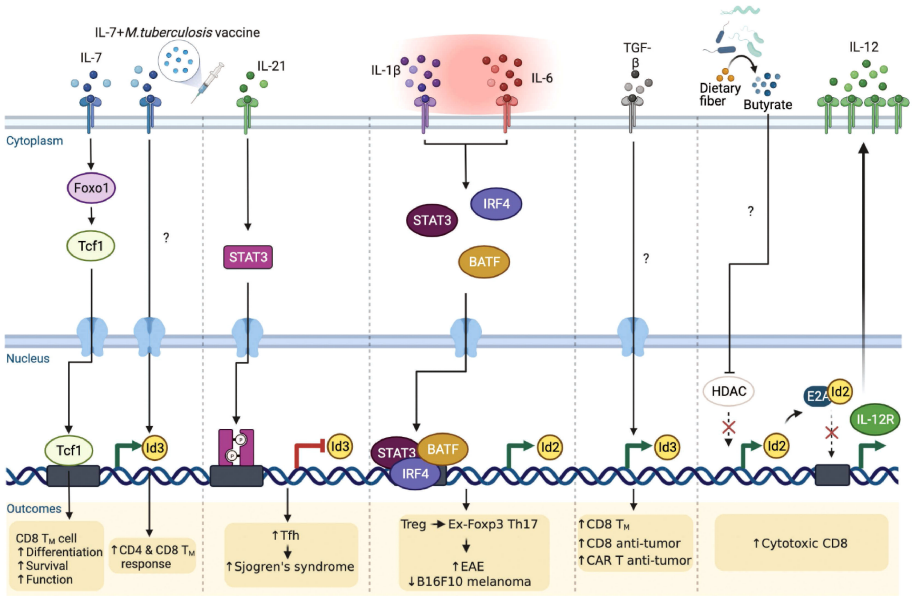

FIGURE 5
Extrinsic signaling pathways affect E-ID axis-mediated T cell function in infection, autoimmunity, and cancer. External signaling pathways affect the expression of E and ID proteins as well as the E-ID axis in various T cell subsets, resulting in changes in the phenotype of T cells and alleviating or exacerbating diseases (Foxo1, Forkhead Box O1; STAT3, Signal Transducer And Activator Of Transcription 3; IRF4, Interferon Regulatory Factor 4; BATF, Basic Leucine Zipper ATF-Like Transcription Factor; TGF-β, Transforming growth factor β; HDAC, Histone deacetylase; T_M, Memory T cells; CAR, Chimeric Antigen Receptor). This figure was created with BioRender.com.

how IL-2 and IL-15 downstream signaling affect the E-ID axis in this context.

Under the inflammatory milieu in the context of autoimmune diseases, Treg cells are known to convert into IL-17 producing cells (93–95). These Treg cells that have lost Foxp3 and become Th17 cells are called "ex-Foxp3 Th17" cells. Previously, our group demonstrated a unique mechanism by which the pro-inflammatory cytokines, IL-1β and IL-6, by altering the E-ID axis, contribute to Treg cell plasticity (96). In this context, we found that Id2 expression is intrinsically repressed during Treg cell differentiation, which is likely one of the reasons why Treg cells stably maintain the expression of Foxp3 in an E2A-dependent manner. In an inflammatory setting, particularly in experimental autoimmune encephalomyelitis (EAE), elevated IL-1β and IL-6 mediated STAT3/IRF4/BATF signaling cascades promote Id2 activation. Enhanced Id2 blocks the binding of E2A to the *Foxp3* locus and renders Treg cells into pathogenic ex-Foxp3 Th17 cells, resulting in exacerbated EAE pathogenesis. Interestingly, in mice bearing B16-F10 melanoma, artificially promoting the plasticity of Treg cells upon transient ectopic expression of Id2 effectively inhibited tumor growth (96). Thus, re-balancing the E-ID axis in a context-dependent manner may be beneficial in treating autoimmunity and cancer.

In terms of promoting the anti-tumor activity of tumor-infiltrating T cells in association with the E-ID axis, a recent study showed that *ex-vivo* stimulation of human T cells with exogenous transforming growth factor beta (TGFβ) leads to the accumulation of central memory T cells that exhibit relatively superior antitumor function than effector T cells (97). This is achieved by upregulating the memory-associated regulatory factor Id3 and improving the anti-tumor activity of T cells and chimeric antigen receptor-expressing T cells. However, the role played by TGFβ leading to the upregulation of Id3 and the target genes regulated downstream of the E-ID axis in this context requires further investigation.

Moreover, it is worth mentioning that metabolites derived from intestinal microbes can directly regulate the E-ID axis, affecting anti-tumor immunity. A recent study demonstrated butyrate, one of the short-chain fatty acids (SCFAs), to be directly involved in the upregulation of Id2 expression by inhibiting histone deacetylase (HDAC) activity (98). Elevated Id2, interfering with the activity of E2A, restores the expression of E2A-repressed IL-12R and consequently activates IL-12 signaling, which is important for the cytotoxic activity of CD8 T cells. Curiously enough, this Id2-expression promoting activity of butyrate appears to be a cell type and microenvironment-specific phenomenon since, contrary to CD8 T cells, butyrate is known to promote iTreg induction from TCR-stimulated CD4 T cells in the presence of TGFβ (99, 100), which according to our finding is negatively affected by enhanced expression of Id2. Thus, it will be interesting to determine further how specific metabolites of gut microbiota associated with cancer and inflammatory diseases affect T cell differentiation and function within the E-ID axis.

Concluding remarks

In the past few years, several studies have clarified the functions and mechanisms of the E-ID axis determining T cell phenotypes in inflammation and cancer; however, further research is necessary to understand the role of E and ID proteins in the metabolic reprogramming of T cells in health and disease. In particular, an integrated mechanistic understanding of the E-ID axis regulating metabolic pathways or rate-limiting enzymes in glycolysis or mitochondrial fatty acid oxidation could provide opportunities for developing effective therapeutic interventions to promote the anti-cancer function of tumor-infiltrating T cells. Moreover, interesting areas focused on improving tumor immunotherapy are the reversal of T cell exhaustion and the maintenance of stem-like memory T cells, which have been demonstrated as long-lived, self-renewing T cell populations important for sustained antitumor immunity in the TME (101). The ID proteins not only inhibit the differentiation of exhausted CD8 T cells in the TME but are also involved in generating diverse memory CD8 T cell subsets under viral infection conditions. Therefore, understanding the E-ID axis in this context could contribute to developing new approaches to improving the better outcomes of cancer immunotherapy.

Besides T cells, several studies have suggested that ID proteins in cancer cells play an important role in promoting tumor progression and metastasis (14, 102). There is increasing evidence that the functional inhibition of ID proteins by pharmacological drugs in cancer cells suppresses cancer cell proliferation under physiological conditions. For example, a recent study showed that the chemical compound, AK-778-XXMU, is a potent Id2 antagonist that can be used to treat gliomas (14, 103). On the contrary, ID protein expression improves the function of tumor-infiltrating T cells and virus-reactive T cells. In line with this finding, further preclinical studies need to be conducted utilizing humanized immunocompetent mouse models to determine durable responses of the pharmacological agonists in promoting and stabilizing ID protein expression in T cells that may prevent disease progression and/or recurrence in patients.

In this review, we discussed the critical roles of the E-ID axis in controlling T cell homeostasis and function under steady-state conditions and various pathological settings. Understanding the complexities of the multiple factors and extrinsic signaling pathways associated with the E-ID axis and further defining the pros and cons of targeting the E-ID axis to affect T cell responses is likely to emerge as an area of enormous therapeutic relevance in the future for immune-mediated diseases and cancer.

Author contributions

S-MH: conceptualization, investigation, writing, and visualization of the manuscript. S-HI and DR: conceptualization, investigation, review, manuscript editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

S-HI is the CEO of the company ImmunoBiome Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Spinner CA, Lazarevic V. Transcriptional regulation of adaptive and innate lymphoid lineage specification. *Immunol Rev* (2021) 300(1):65–81. doi: 10.1111/imr.12935

2. Hosokawa H, Rothenberg EV. How transcription factors drive choice of the T cell fate. *Nat Rev Immunol* (2021) 21(3):162–76. doi: 10.1038/s41577-020-00426-6

3. Murre C. Helix-Loop-Helix proteins and the advent of cellular diversity: 30 years of discovery. *Genes Dev* (2019) 33(1-2):6–25. doi: 10.1101/gad.320663.118

4. Murre C. Helix-Loop-Helix proteins and lymphocyte development. *Nat Immunol* (2005) 6(11):1079–86. doi: 10.1038/ni1260

5. Kee BL. E And id proteins branch out. *Nat Rev Immunol* (2009) 9(3):175–84. doi: 10.1038/nri2507

6. de Pooter RF, Kee BL. E proteins and the regulation of early lymphocyte development. *Immunol Rev* (2010) 238(1):93–109. doi: 10.1111/j.1600-065X.2010.00957.x

7. Miyazaki K, Watanabe H, Yoshikawa G, Chen K, Hidaka R, Aitani Y, et al. The transcription factor E2a activates multiple enhancers that drive rag expression in developing T and b cells. *Sci Immunol* (2020) 5(51):1–15. doi: 10.1126/sciimmunol.abb1455

8. Jones-Mason ME, Zhao X, Kappes D, Lasorella A, Iavarone A, Zhuang Y. E protein transcription factors are required for the development of Cd4(+) lineage T cells. *Immunity* (2012) 36(3):348–61. doi: 10.1016/j.immuni.2012.02.010

9. Kadakia T, Tai X, Kruhlak M, Wisniewski J, Hwang IY, Roy S, et al. E-Protein-Regulated expression of Cxcr4 adheres preselection thymocytes to the thymic cortex. *J Exp Med* (2019) 216(8):1749–61. doi: 10.1084/jem.20182285

10. Xu W, Carr T, Ramirez K, McGregor S, Sigvardsson M, Kee BL. E2a transcription factors limit expression of Gata3 to facilitate T lymphocyte lineage commitment. *Blood* (2013) 121(9):1534–42. doi: 10.1182/blood-2012-08-449447

11. Braunstein M, Anderson MK. Heb-deficient T-cell precursors lose T-cell potential and adopt an alternative pathway of differentiation. *Mol Cell Biol* (2011) 31(5):971–82. doi: 10.1128/MCB.01034-10

12. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor heb in thymocyte survival, tcra rearrangement and the development of natural killer T cells. *Nat Immunol* (2010) 11(3):240–9. doi: 10.1038/ni.1845

13. Miyazaki M, Rivera RR, Miyazaki K, Lin YC, Agata Y, Murre C. The opposing roles of the transcription factor E2a and its antagonist Id3 that orchestrate and enforce the naive fate of T cells. *Nat Immunol* (2011) 12(10):992–1001. doi: 10.1038/ni.2086

14. Roschger C, Cabrele C. The id-protein family in developmental and cancer-associated pathways. *Cell Commun Signal* (2017) 15(1):7. doi: 10.1186/s12964-016-0161-y

15. Zhang B, Lin YY, Dai M, Zhuang Y. Id3 and Id2 act as a dual safety mechanism in regulating the development and population size of innate-like gammadelta T cells. *J Immunol* (2014) 192(3):1055–63. doi: 10.4049/jimmunol.1302694

16. Verykokakis M, Krishnamoorthy V, Iavarone A, Lasorella A, Sigvardsson M, Kee BL. Essential functions for id proteins at multiple checkpoints in invariant nkt cell development. *J Immunol* (2013) 191(12):5973–83. doi: 10.4049/jimmunol.1301521

17. Belle I, Zhuang Y. E proteins in lymphocyte development and lymphoid diseases. *Curr Top Dev Biol* (2014) 110:153–87. doi: 10.1016/B978-0-12-405943-6.00004-X

18. Cano A, Portillo F. An emerging role for class I bhlh E2-2 proteins in emt regulation and tumor progression. *Cell Adh Migr* (2010) 4(1):56–60. doi: 10.4161/cam.4.1.9995

19. Denis CM, Chitayat S, Plevin MJ, Wang F, Thompson P, Liu S, et al. Structural basis of Cbp/P300 recruitment in leukemia induction by E2a-Pbx1. *Blood* (2012) 120(19):3968–77. doi: 10.1182/blood-2012-02-411397

20. Plevin MJ, Zhang J, Guo C, Roeder RG, Ikura M. The acute myeloid leukemia fusion protein Aml1-eto targets e proteins *Via* a paired amphipathic helix-like tbp-associated factor homology domain. *Proc Natl Acad Sci U.S.A.* (2006) 103(27):10242–7. doi: 10.1073/pnas.0603463103

21. Gow CH, Guo C, Wang D, Hu Q, Zhang J. Differential involvement of E2a-corepressor interactions in distinct leukemogenic pathways. *Nucleic Acids Res* (2014) 42(1):137–52. doi: 10.1093/nar/gkt855

22. Sun XH, Copeland NG, Jenkins NA, Baltimore D. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-Loop-Helix proteins. *Mol Cell Biol* (1991) 11(11):5603–11. doi: 10.1128/mcb.11.11.5603-5611.1991s

23. Ling F, Kang B, Sun XH. Id proteins: Small molecules, mighty regulators. *Curr Top Dev Biol* (2014) 110:189–216. doi: 10.1016/B978-0-12-405943-6.00005-1

24. Chu YH, Lin JD, Nath S, Schachtrup C. Id proteins: Emerging roles in cns disease and targets for modifying neural stemcell behavior. *Cell Tissue Res* (2022) 387(3):433–49. doi: 10.1007/s00441-021-03490-z

25. Wu H, Shao Q. The role of inhibitor of binding or differentiation 2 in the development and differentiation of immune cells. *Immunobiology* (2019) 224(1):142–6. doi: 10.1016/j.imbio.2018.09.006

26. Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. Id proteins in cell growth and tumorigenesis. *Cancer Cell* (2003) 3(6):525–30. doi: 10.1016/s1535-6108(03)00141-7

27. Irla M. Instructive cues of thymic T cell selection. *Annu Rev Immunol* (2022) 40:95–119. doi: 10.1146/annurev-immunol-101320-022432

28. Bonelli M, Shih HY, Hirahara K, Singelton K, Laurence A, Poholek A, et al. Helper T cell plasticity: Impact of extrinsic and intrinsic signals on transcriptomes and epigenomes. *Curr Top Microbiol Immunol* (2014) 381:279–326. doi: 10.1007/82_2014_371

29. Ruterbusch M, Pruner KB, Shehata L, Pepper M. *In vivo* Cd4(+) T cell differentiation and function: Revisiting the Th1/Th2 paradigm. *Annu Rev Immunol* (2020) 38:705–25. doi: 10.1146/annurev-immunol-103019-085803

30. Vella LA, Herati RS, Wherry EJ. Cd4(+) T cell differentiation in chronic viral infections: The tfh perspective. *Trends Mol Med* (2017) 23(12):1072–87. doi: 10.1016/j.molmed.2017.10.001

31. Swain SL, McKinstry KK, Strutt TM. Expanding roles for Cd4(+) T cells in immunity to viruses. *Nat Rev Immunol* (2012) 12(2):136–48. doi: 10.1038/nri3152

32. Vacchio MS, Ciucci T, Gao Y, Watanabe M, Balmaceno-Criss M, McGinty MT, et al. A thpok-directed transcriptional circuitry promotes Bcl6 and maf expression to orchestrate T follicular helper differentiation. *Immunity* (2019) 51 (3):465–78 e6. doi: 10.1016/j.immuni.2019.06.023

33. Sheikh AA, Groom JR. Transcription tipping points for T follicular helper cell and T-helper 1 cell fate commitment. *Cell Mol Immunol* (2021) 18(3):528–38. doi: 10.1038/s41423-020-00554-y

34. Han X, Liu H, Huang H, Liu X, Jia B, Gao GF, et al. Id2 and Id3 are indispensable for Th1 cell differentiation during influenza virus infection in mice. *Eur J Immunol* (2019) 49(3):476–89. doi: 10.1002/eji.201847822

35. Shaw LA, Belanger S, Omilusik KD, Cho S, Scott-Browne JP, Nance JP, et al. Id2 reinforces Th1 differentiation and inhibits E2a to repress tfh differentiation. *Nat Immunol* (2016) 17(7):834–43. doi: 10.1038/ni.3461

36. Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. *Nat Rev Immunol* (2017) 17(9):535–44. doi: 10.1038/nri.2017.50

37. Korn T, Bettelli E, Oukka M, Kuchroo VK. Il-17 and Th17 cells. *Annu Rev Immunol* (2009) 27:485–517. doi: 10.1146/annurev.immunol.021908.132710

38. Burkett PR, Meyer zu Horste G, Kuchroo VK. Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. *J Clin Invest* (2015) 125(6):2211–9. doi: 10.1172/JCI78085

39. Dong C. Th17 cells in development: An updated view of their molecular identity and genetic programming. *Nat Rev Immunol* (2008) 8(5):337–48. doi: 10.1038/nri2295

40. Zhang F, Fuss IJ, Yang Z, Strober W. Transcription of rorgammat in developing Th17 cells is regulated by e-proteins. *Mucosal Immunol* (2014) 7 (3):521–32. doi: 10.1038/mi.2013.69

41. Lin YY, Jones-Mason ME, Inoue M, Lasorella A, Iavarone A, Li QJ, et al. Transcriptional regulator Id2 is required for the Cd4 T cell immune response in the development of experimental autoimmune encephalomyelitis. *J Immunol* (2012) 189(3):1400–5. doi: 10.4049/jimmunol.1200491

42. Kleinsteuber K, Heesch K, Schattling S, Sander-Juelch C, Mock U, Riecken K, et al. Socs3 promotes interleukin-17 expression of human T cells. *Blood* (2012) 120(22):4374–82. doi: 10.1182/blood-2011-11-392738

43. Sharma A, Rudra D. Emerging functions of regulatory T cells in tissue homeostasis. *Front Immunol* (2018) 9:883. doi: 10.3389/fimmu.2018.00883

44. Maruyama T, Li J, Vaque JP, Konkel JE, Wang W, Zhang B, et al. Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. *Nat Immunol* (2011) 12(1):86–95. doi: 10.1038/ni.1965

45. Han X, Huang H, Gao P, Zhang Q, Liu X, Jia B, et al. E-protein regulatory network links tcr signaling to effector treg cell differentiation. *Proc Natl Acad Sci U.S.A.* (2019) 116(10):4471–80. doi: 10.1073/pnas.1800494116

46. Frias ABJr., Hyzny EJ, Buechel HM, Beppu LY, Xie B, Jurczak MJ, et al. The transcriptional regulator Id2 is critical for adipose-resident regulatory T cell differentiation, survival, and function. *J Immunol* (2019) 203(3):658–64. doi: 10.4049/jimmunol.1900358

47. Sullivan JM, Hollbacher B, Campbell DJ. Cutting edge: Dynamic expression of Id3 defines the stepwise differentiation of tissue-resident regulatory T cells. *J Immunol* (2019) 202(1):31–6. doi: 10.4049/jimmunol.1800917

48. Liu C, Yu S, Jin R, Long Y, Lu S, Song Y, et al. Correlation of the levels of DNA-binding inhibitor Id3 and regulatory T cells with sle disease severity. *J Autoimmun* (2020) 113:102498. doi: 10.1016/j.jaut.2020.102498

49. Liu C, Zeng X, Yu S, Ren L, Sun X, Long Y, et al. Up-regulated DNA-binding inhibitor Id3 promotes differentiation of regulatory T cell to influence antiviral immunity in chronic hepatitis b virus infection. *Life Sci* (2021) 285:119991. doi: 10.1016/j.lfs.2021.119991

50. Collier JL, Weiss SA, Pauken KE, Sen DR, Sharpe AH. Not-So-Opposite ends of the spectrum: Cd8(+) T cell dysfunction across chronic infection, cancer and autoimmunity. *Nat Immunol* (2021) 22(7):809–19. doi: 10.1038/s41590-021-00949-7

51. D'Cruz LM, Lind KC, Wu BB, Fujimoto JK, Goldrath AW. Loss of e protein transcription factors E2a and heb delays memory-precursor formation during the Cd8+ T-cell immune response. *Eur J Immunol* (2012) 42(8):2031–41. doi: 10.1002/eji.201242497

52. Schauder DM, Shen J, Chen Y, Kasmani MY, Kudek MR, Burns R, et al. E2a-regulated epigenetic landscape promotes memory Cd8 T cell differentiation. *Proc Natl Acad Sci USA* (2021) 118(16):1–12. doi: 10.1073/pnas.2013452118

53. Masson F, Minnich M, Olshansky M, Bilic I, Mount AM, Kallies A, et al. Id2-mediated inhibition of E2a represses memory Cd8+ T cell differentiation. *J Immunol* (2013) 190(9):4585–94. doi: 10.4049/jimmunol.1300099

54. Knell J, Best JA, Lind NA, Yang E, D'Cruz LM, Goldrath AW. Id2 influences differentiation of killer cell lectin-like receptor G1(Hi) short-lived Cd8+ effector T cells. *J Immunol* (2013) 190(4):1501–9. doi: 10.4049/jimmunol.1200750

55. Omilusik KD, Nadjsombati MS, Shaw LA, Yu B, Milner JJ, Goldrath AW. Sustained Id2 regulation of e proteins is required for terminal differentiation of effector Cd8(+) T cells. *J Exp Med* (2018) 215(3):773–83. doi: 10.1084/jem.20171584

56. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory Cd8+ T cell subsets. *Nat Immunol* (2011) 12(12):1221–9. doi: 10.1038/ni.2158

57. Jin Y, Hu P, Sun H, Yang C, Zhai J, Wang Y, et al. Expression of Id3 represses exhaustion of anti-tumor Cd8 T cells in liver cancer. *Mol Immunol* (2022) 144:117–26. doi: 10.1016/j.molimm.2022.02.005

58. Wakabayashi Y, Watanabe H, Inoue J, Takeda N, Sakata J, Mishima Y, et al. Bcl11b is required for differentiation and survival of alphabeta T lymphocytes. *Nat Immunol* (2003) 4(6):533–9. doi: 10.1038/ni927

59. Okazuka K, Wakabayashi Y, Kashihara M, Inoue J, Sato T, Yokoyama M, et al. P53 prevents maturation of T cell development to the immature Cd4-Cd8+ stage in Bcl11b-/- mice. *Biochem Biophys Res Commun* (2005) 328(2):545–9. doi: 10.1016/j.bbrc.2005.01.013

60. Inoue J, Kanefuji T, Okazuka K, Watanabe H, Mishima Y, Kominami R. Expression of tcr alpha beta partly rescues developmental arrest and apoptosis of alpha beta T cells in Bcl11b-/- mice. *J Immunol* (2006) 176(10):5871–9. doi: 10.4049/jimmunol.176.10.5871

61. Hosokawa H, Romero-Wolf M, Yui MA, Ungerback J, Quiloan MLG, Matsumoto M, et al. Bcl11b sets pro-T cell fate by site-specific cofactor recruitment and by repressing Id2 and Zbtb16. *Nat Immunol* (2018) 19 (12):1427–40. doi: 10.1038/s41590-018-0238-4

62. Longabaugh WJR, Zeng W, Zhang JA, Hosokawa H, Jansen CS, Li L, et al. Bcl11b and combinatorial resolution of cell fate in the T-cell gene regulatory network. *Proc Natl Acad Sci USA* (2017) 114(23):5800–7. doi: 10.1073/pnas.1610617114

63. Harris JE, Bishop KD, Phillips NE, Mordes JP, Greiner DL, Rossini AA, et al. Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in Cd4+ T cells. *J Immunol* (2004) 173(12):7331–8. doi: 10.4049/jimmunol.173.12.7331

64. Li S, Miao T, Sebastian M, Bhullar P, Ghaffari E, Liu M, et al. The transcription factors Egr2 and Egr3 are essential for the control of inflammation and antigen-induced proliferation of b and T cells. *Immunity* (2012) 37(4):685–96. doi: 10.1016/j.immuni.2012.08.001

65. Miao T, Symonds ALJ, Singh R, Symonds JD, Ogbe A, Omodho B, et al. Egr2 and 3 control adaptive immune responses by temporally uncoupling expansion from T cell differentiation. *J Exp Med* (2017) 214(6):1787–808. doi: 10.1084/jem.20160553

66. Li X, Xiang Y, Li F, Yin C, Li B, Ke X. Wnt/Beta-catenin signaling pathway regulating T cell-inflammation in the tumor microenvironment. *Front Immunol* (2019) 10:2293. doi: 10.3389/fimmu.2019.02293

67. Escobar G, Mangani D, Anderson AC. T Cell factor 1: A master regulator of the T cell response in disease. *Sci Immunol* (2020) 5(53):1–28. doi: 10.1126/sciimmunol.abb9726

68. Emmanuel AO, Arnovitz S, Haghi L, Mathur PS, Mondal S, Quandt J, et al. Tcf-1 and heb cooperate to establish the epigenetic and transcription profiles of Cd4(+)Cd8(+) thymocytes. *Nat Immunol* (2018) 19(12):1366–78. doi: 10.1038/s41590-018-0254-4

69. Shao P, Li F, Wang J, Chen X, Liu C, Xue HH. Cutting edge: Tcf1 instructs T follicular helper cell differentiation by repressing Blimp1 in response to acute viral infection. *J Immunol* (2019) 203(4):801–6. doi: 10.4049/jimmunol.1900581

70. Crotty S. T Follicular helper cell differentiation, function, and roles in disease. *Immunity* (2014) 41(4):529–42. doi: 10.1016/j.immuni.2014.10.004

71. Wang Y, Hu J, Li Y, Xiao M, Wang H, Tian Q, et al. The transcription factor Tcf1 preserves the effector function of exhausted Cd8 T cells during chronic viral infection. *Front Immunol* (2019) 10:169. doi: 10.3389/fimmu.2019.00169

72. Shan Q, Hu SS, Zhu S, Chen X, Badovinac VP, Peng W, et al. Tcf1 preprograms the mobilization of glycolysis in central memory Cd8(+) T cells during recall responses. *Nat Immunol* (2022) 23(3):386–98. doi: 10.1038/s41590-022-01131-3

73. He S, Liu Y, Meng L, Sun H, Wang Y, Ji Y, et al. Ezh2 phosphorylation state determines its capacity to maintain Cd8(+) T memory precursors for antitumor immunity. *Nat Commun* (2017) 8(1):2125. doi: 10.1038/s41467-017-02187-8

74. Williams SA, Maecker HL, French DM, Liu J, Gregg A, Silverstein LB, et al. Usp1 deubiquitinates id proteins to preserve a mesenchymal stem cell program in osteosarcoma. *Cell* (2011) 146(6):918–30. doi: 10.1016/j.cell.2011.07.040

75. Wrighton KH. Mechanisms of disease: Usp1 keeps id proteins stable. *Nat Rev Mol Cell Biol* (2011) 12(11):691. doi: 10.1038/nrm3210

76. Omilusik KD, Nadjsombati MS, Yoshida TM, Shaw LA, Goulding J, Goldrath AW. Ubiquitin specific protease 1 expression and function in T cell immunity. *J Immunol* (2021) 207(5):1377–87. doi: 10.4049/jimmunol.2100303

77. Jiao A, Sun C, Wang X, Lei L, Liu H, Li W, et al. Dexd/H-box helicase 9 intrinsically controls Cd8(+) T cell-mediated antiviral response through noncanonical mechanisms. *Sci Adv* (2022) 8(6):eabk2691. doi: 10.1126/sciadv.abk2691

78. Xu A, Leary SC, Islam MF, Wu Z, Bhanumathy KK, Ara A, et al. Prosurvival il-7-Stimulated weak strength of Mtorc1-S6k controls T cell memory *Via* transcriptional Foxo1-Tcf1-Id3 and metabolic Ampkalpha1-Ulk1-Atg7 pathways. *J Immunol* (2022) 208(1):155–68. doi: 10.4049/jimmunol.2100452

79. Boudil A, Matei IR, Shih HY, Bogdanoski G, Yuan JS, Chang SG, et al. Il-7 coordinates proliferation, differentiation and tcra recombination during thymocyte beta-selection. *Nat Immunol* (2015) 16(4):397–405. doi: 10.1038/ni.3122

80. Chen D, Tang TX, Deng H, Yang XP, Tang ZH. Interleukin-7 biology and its effects on immune cells: Mediator of generation, differentiation, survival, and homeostasis. *Front Immunol* (2021) 12:747324. doi: 10.3389/fimmu.2021.747324

81. Koenen P, Heinzel S, Carrington EM, Happo L, Alexander WS, Zhang JG, et al. Mutually exclusive regulation of T cell survival by il-7r and antigen receptor-induced signals. *Nat Commun* (2013) 4:1735. doi: 10.1038/ncomms2719

82. Han J, Ma Y, Ma L, Tan D, Niu H, Bai C, et al. Id3 and Bcl6 promote the development of long-term immune memory induced by tuberculosis subunit vaccine. *Vaccines (Basel)* (2021) 9(2):1–14. doi: 10.3390/vaccines9020126

83. Spolski R, Leonard WJ. Interleukin-21: A double-edged sword with therapeutic potential. *Nat Rev Drug Discov* (2014) 13(5):379–95. doi: 10.1038/nrd4296

84. Tangye SG, Ma CS. Regulation of the germinal center and humoral immunity by interleukin-21. *J Exp Med* (2020) 217(1):1–13. doi: 10.1084/jem.20191638

85. Long D, Chen Y, Wu H, Zhao M, Lu Q. Clinical significance and immunobiology of il-21 in autoimmunity. *J Autoimmun* (2019) 99:1–14. doi: 10.1016/j.jaut.2019.01.013

86. Jiang W, Su J, Zhang X, Cheng X, Zhou J, Shi R, et al. Elevated levels of Th17 cells and Th17-related cytokines are associated with disease activity in patients with inflammatory bowel disease. *Inflammation Res* (2014) 63(11):943–50. doi: 10.1007/s00011-014-0768-7

87. Yuan FL, Hu W, Lu WG, Li X, Li JP, Xu RS, et al. Targeting interleukin-21 in rheumatoid arthritis. *Mol Biol Rep* (2011) 38(3):1717–21. doi: 10.1007/s11033-010-0285-x

88. Seyfarth J, Mutze N, Antony Cruz J, Kummer S, Reinauer C, Mayatepek E, et al. Cd4(+) T-cells with high common gamma chain expression and disturbed cytokine production are enriched in children with type-1 diabetes. *Front Immunol* (2019) 10:820. doi: 10.3389/fimmu.2019.00820

89. Deng XM, Yan SX, Wei W. Il-21 acts as a promising therapeutic target in systemic lupus erythematosus by regulating plasma cell differentiation. *Cell Mol Immunol* (2015) 12(1):31–9. doi: 10.1038/cmi.2014.58

90. Park JS, Kim SM, Choi J, Jung KA, Hwang SH, Yang S, et al. Interleukin-21-Mediated suppression of the Pax3-Id3 pathway exacerbates the development of sjogren's syndrome *Via* follicular helper T cells. *Cytokine* (2020) 125:154834. doi: 10.1016/j.cyto.2019.154834

91. Ross SH, Cantrell DA. Signaling and function of interleukin-2 in T lymphocytes. *Annu Rev Immunol* (2018) 36:411–33. doi: 10.1146/annurev-immunol-042617-053352

92. Richer MJ, Pewe LL, Hancox LS, Hartwig SM, Varga SM, Harty JT. Inflammatory il-15 is required for optimal memory T cell responses. *J Clin Invest* (2015) 125(9):3477–90. doi: 10.1172/JCI81261

93. Kleinewietfeld M, Hafler DA. The plasticity of human treg and Th17 cells and its role in autoimmunity. *Semin Immunol* (2013) 25(4):305–12. doi: 10.1016/j.smim.2013.10.009

94. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, et al. Pathogenic conversion of Foxp3+ T cells into Th17 cells in autoimmune arthritis. *Nat Med* (2014) 20(1):62–8. doi: 10.1038/nm.3432

95. Massoud AH, Charbonnier LM, Lopez D, Pellegrini M, Phipatanakul W, Chatila TA. An asthma-associated Il4r variant exacerbates airway inflammation by promoting conversion of regulatory T cells to Th17-like cells. *Nat Med* (2016) 22(9):1013–22. doi: 10.1038/nm.4147

96. Hwang SM, Sharma G, Verma R, Byun S, Rudra D, Im SH. Inflammation-induced Id2 promotes plasticity in regulatory T cells. *Nat Commun* (2018) 9(1):4736. doi: 10.1038/s41467-018-07254-2

97. Dahmani A, Janelle V, Carli C, Richaud M, Lamarche C, Khalili M, et al. Tgfbeta programs central memory differentiation in *ex vivo*-stimulated human T cells. *Cancer Immunol Res* (2019) 7(9):1426–39. doi: 10.1158/2326-6066.CIR-18-0691

98. He Y, Fu L, Li Y, Wang W, Gong M, Zhang J, et al. Gut microbial metabolites facilitate anticancer therapy efficacy by modulating cytotoxic Cd8(+) T cell immunity. *Cell Metab* (2021) 33(5):988–1000 e7. doi: 10.1016/j.cmet.2021.03.002

99. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013) 504(7480):451–5. doi: 10.1038/nature12726

100. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013) 504(7480):446–50. doi: 10.1038/nature12721

101. Liu Q, Sun Z, Chen L. Memory T cells: Strategies for optimizing tumor immunotherapy. *Protein Cell* (2020) 11(8):549–64. doi: 10.1007/s13238-020-00707-9

102. Teo WS, Holliday H, Karthikeyan N, Cazet AS, Roden DL, Harvey K, et al. Id proteins promote a cancer stem cell phenotype in mouse models of triple negative breast cancer *Via* negative regulation of Robo1. *Front Cell Dev Biol* (2020) 8:552. doi: 10.3389/fcell.2020.00552

103. Zhong G, Wang Y, Wang Q, Wu M, Liu Y, Sun S, et al. Discovery of novel Id2 antagonists from pharmacophore-based virtual screening as potential therapeutics for glioma. *Bioorg Med Chem* (2021) 49:116427. doi: 10.1016/j.bmc.2021.116427

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Regulation of the Signal-Dependent E Protein HEBAlt Through a YYY Motif Is Required for Progression Through T Cell Development

Kogulan Yoganathan[1,2], *Anqi Yan*[1,2], *Juliana Rocha*[1], *Ashton Trotman-Grant*[1,2], *Mahmood Mohtashami*[1], *Lisa Wells*[1], *Juan Carlos Zúñiga-Pflücker*[1,2] *and Michele K. Anderson*[1,2]*

[1] Biological Sciences, Sunnybrook Research Institute, Toronto, ON, Canada, [2] Department of Immunology, University of Toronto, Toronto, ON, Canada

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***Correspondence:**
Michele K. Anderson
manderso@sri.utoronto.ca

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The E protein transcription factors E2A and HEB are critical for many developmental processes, including T cell development. We have shown that the *Tcf12* locus gives rise to two distinct HEB proteins, with alternative (HEBAlt) and canonical (HEBCan) N-terminal domains, which are co-expressed during early T cell development. While the functional domains of HEBCan have been well studied, the nature of the HEBAlt-specific (Alt) domain has been obscure. Here we provide compelling evidence that the Alt domain provides a site for the molecular integration of cytokine signaling and E protein activity. Our results indicate that phosphorylation of a unique YYY motif in the Alt domain increases HEBAlt activity by 10-fold, and that this increase is dependent on Janus kinase activity. To enable *in vivo* studies of HEBAlt in the T cell context, we generated ALT-Tg mice, which can be induced to express a HA-tagged HEBAlt coding cassette in the presence of Cre recombinases. Analysis of ALT-Tg mice on the Vav-iCre background revealed a minor change in the ratio of ISP cells to CD8+ SP cells, and a mild shift in the ratio of T cells to B cells in the spleen, but otherwise the thymus, spleen, and bone marrow lymphocyte subsets were comparable at steady state. However, kinetic analysis of T cell development in OP9-DL4 co-cultures revealed a delay in early T cell development and a partial block at the DN to DP transition when HEBAlt levels or activity were increased. We also observed that HEBCan and HEBAlt displayed significant differences in protein stability that were resolved in the thymocyte context. Finally, a proteomic screen identified STAT1 and Xpo1 as potential members of HEBAlt-containing complexes in thymocytes, consistent with JAK-induced activation of HEBAlt accompanied by translocation to the nucleus. Thus, our results show that the Alt domain confers access to multiple layers of post-translational control to HEBAlt that are not available to HEBCan, and thus may serve as a rheostat to tune E protein activity levels as cells move through different thymic signaling environments during T cell development.

Keywords: T cell development, HEB, TCF12, gene expression, signal transduction

INTRODUCTION

T cells act as a central organizing hub for adaptive immune responses and provide a first line of innate defense in barrier tissues. These roles are distributed among distinct T cell subsets, which acquire their core functions during T cell development in the thymus. T cell development occurs in the thymus through a series of intermediates that give rise to αβ and γδ T cells. These cells arise from T-lineage committed progenitors known as double negative (DN; CD4-CD8-) cells. DN cells can be further subdivided into successive developmental stages using the markers CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$) (1). Successful rearrangement of TCRβ, expression of TCR signaling components, and assembly of a pre-T cell receptor (pre-TCR) complex allows passage through "β-selection" into the αβ-T lineage (2). β-selected cells downregulate CD25 to become DN4 cells. DN4 cells undergo rapid proliferation and upregulate $CD8^+$ to become immature single positive (ISP) cells. This is followed by upregulation of CD4 to generate double positive (DP; $CD4^+CD8^+$) thymocytes (3). DP cells become quiescent as they commence TCRα rearrangement. Upon αβ TCR signaling, DP cells can differentiate into either conventional $CD4^+$ or $CD8^+$ single positive (SP) cells through a series of intermediate stages that can be followed by expression of CD69 and CD24.

The E protein transcription factors encoded by the *Tcf12* (HEB) and the *Tcf3* (E2A) loci are essential regulators of T cell development. Each major developmental transition that occurs during thymic T cell development is dependent on interactions between E protein transcription factors and their antagonist Id3 (4–8). Prior to β-selection, E proteins directly upregulate the expression of proteins involved in pre-TCR signaling, including pTα, TCRβ, CD3, Lck, LAT, and RAG1/2 (9–12). Pre-TCR, γδ TCR, and αβ TCR signaling lead to the transient upregulation of Id3, which halts E protein activity (13, 14). After T lineage commitment and passage through β-selection, E proteins can regulate gene products that are not expressed at the DN stage, including CD4, TCRα, and Rorγt (7, 15–17). Thus, regulation of different suites of T cell genes at different stages of development is a key feature of E protein activity during T cell development, as has also been observed for GATA3 and Runx factors (18, 19).

HEB and E2A knockout models have provided considerable insight into the roles of these factors at different stages of thymocyte development. HEB deficiency leads to defects in fetal γδ and αβ T cell development (20, 21). HEB disruption also results in a partial block at the DN3 to DN4 transition, an accumulation of ISP cells, and a decrease in CD4 T cells (22, 23). Deletion of E2A results in a partial block at the DN1 to DN2 transition, breakthrough to the DP stage in the absence of pre-TCR or γδTCR signals, an increase in SPs, and leukemic transformation of T cell progenitors (24, 25). Conditional deletion of both HEB and E2A with Lck-Cre in DN thymocytes resulted in the emergence of a rapidly cycling population of IL-7R dependent DN2-like cells (26). These studies and others led to the concept of E proteins as "gatekeepers" that prevent inappropriate differentiation and proliferation prior to receiving pre-TCR, γδTCR, or αβTCR signals (27).

HEB and E2A function as homodimers and heterodimers, but how dimer composition affects target gene expression is poorly understood. In DN thymocytes, two variants of HEB are expressed: HEBAlt (alternative) and HEBCan (canonical) (28). HEBAlt mRNA is downregulated at the DN3 to DN4 transition, leaving HEBCan and E2A as the main E proteins in DP and SP cells (28, 29). HEBCan and E2A share a conserved domain structure, including three activation domains (AD1, AD2, AD3), which interact with other transcriptional regulators, and a basic helix-loop-helix (bHLH) DNA binding and dimerization domain (30–32) (**Figure 1A**; **Figure S1**). HEBAlt includes AD2 and the bHLH domain and lacks AD1. The N-terminus of HEBAlt encodes a 23 amino acid "Alt" domain that is excluded from HEBCan. This configuration arises from the location of the Alt exon between exons 8 and 9 of the *Tcf12* gene locus (**Figure 1B**). Additionally, the AD3 domain spans exons 8 and 9, resulting in the absence of the first half of AD3 in HEBAlt.

HEBAlt and HEBCan are expressed from distinct transcriptional start sites, allowing differential regulation of mRNA expression (**Figure 1B**). HEBCan is expressed throughout T cell development, peaking at the DP stage, whereas HEBAlt mRNA is restricted to DN2 and DN3 cells (28). Thus, differences between HEBAlt and HEBCan could factor into the switching of E protein target genes at the DN to DP transition. Our previous work using retroviral vectors and *in vitro* differentiation systems showed that HEBAlt could enhance entry of uncommitted progenitors into the T-lineage, whereas HEBCan did not (28, 33). HEBAlt could also uniquely restrict B and myeloid cell development, and recruit committed myeloid precursors into the T cell lineage (21, 33, 34). By contrast, overexpression of HEBCan inhibited T cell development, consistent with its role as a gatekeeper. Whether HEBAlt participates in the gatekeeping process has not been resolved, and the function of the Alt domain remains unclear.

In this study, we generated HEBAlt mutant constructs and evaluated their ability to induce transcriptional activation using promoter-reporter luciferase assays. Our results identified a unique triple tyrosine (YYY) motif within the Alt domain that plays a role in the magnitude of HEBAlt-mediated transcriptional activation. We also showed that YYY-mediated elevation of HEBAlt activity is dependent on JAK (Janus tyrosine kinase) activity, and that the YYY motif can be phosphorylated. Furthermore, using an HEBAlt transgenic mouse model, we observed that uncontrolled HEBAlt activity inhibited progress through T cell development, and that HEBAlt protein stability is dependent on cell context. Our results indicate that HEBAlt activity is tightly regulated at the post-translational level, and that disruption of this regulation interferes with T cell development.

METHODS

Mice

The generation of Rosa26-loxP-stop-loxP-HEBAlt-HA (ALT-Tg) mice was performed by Ingenious Targeting Laboratories (Ronkonkoma, NY). Hybrid (129/SvEv x C57BL/6) embryonic stem cells (ESCs) were targeted and microinjected into C57BL/6 blastocysts. Resulting chimeras were mated to WT C57BL/6N mice to generate F1 heterozygous offspring, and those with germline integration were backcrossed for six generations with C57BL/6 mice to fix them on the C57BL/6 background. To induce expression in all hematopoietic cells, ALT-Tg mice were crossed to Vav-iCre mice (Jax; 008610)91. All experiments were conducted using 6-8 wk old littermate controls. Mice were maintained at the Sunnybrook Research Institute and all protocols were approved by the Animal Care Committee.

Construct Generation

HEBAlt (NM_001253864.1) and HEBCan (NM_011544.3) cDNAs were cloned into pCMV-HA or pCMV-myc expression vectors using the KpnI and SalI cloning sites. Site-directed mutagenesis performed on the pCMV-HEBAlt-HA plasmid to generate the mutant constructs using the Agilent QuikChange Site-Directed Mutagenesis Kit or Q5® Site-Directed Mutagenesis Kit. Mutations were confirmed by cloning the inserts into Top10 Competent E. coli cells, followed by plasmid DNA extraction and Sanger Sequencing (TCAG; SickKids, Toronto, Canada).

Cell Culture

Cells lines were cultured under standard conditions at 37°C with 5% CO2. Adherent cells were trypsinized (0.25% Trypsin) for passaging. Both adherent and suspension cells were centrifuged at 550 g for 5 minutes before re-plating in fresh media. HeLa cells, HEK293T cells, and Baby Mouse Kidney (BMK) cells (deficient for *Bax* and *Bak*, kind gift from David Andrews, Sunnybrook) were cultured in DMEM supplemented with 10% FBS and antibiotics (100 mg/mL penicillin and 100 U/mL streptomycin). Jurkat cells were cultured in RPMI supplemented with 10% FBS and antibiotics. SCID.adh cells were cultured in RPMI supplemented with 10% FBS, antibiotics, 1% non-essential amino acids, 1% sodium pyruvate, and 50mM β-mercaptoethanol. OP9-DL4 co-cultures were seeded with LSK (Lin-Sca1+ckit+) cells sorted from bone marrow of WT or ALT-Tg mice, or with GFP+ LSK cells sorted from retrovirally transduced bone marrow, as previously described (35).

Transfections

Cells were seeded 18 h before transfection, and equal amounts of DNA were transfected into each well using Lipofectamine 3000 Transfection Reagent (Invitrogen; Cat. L3000008) or FuGene HD Reagent (Promega; E2311) in OPTI-MEM. Analysis was performed 24 h post-transfection.

Electroporation

For immunoprecipitation experiments, Jurkat cells were transfected with 5 μg of vector DNA by Neon Transfection System based on manufacturer's protocols. After washed with PBS, cells were resuspended in R Buffer to reach a concentration of 2 $\times 10^7$ cells/ml. Using 100 μl Neon tips, cells were electroporated with the parameter of 1350V, 10 ms, three times, in a Neon tube containing E_2 Buffer. After the pulse, cells were quickly transferred into a 6-well-plate with 3 ml RPMI supplemented with 10% FBS and antibiotics and cultured for 2 days.

Dual Luciferase Reporter Assays

Transcriptional activity was assessed by performing Dual Luciferase Reporter (DLR) Assays (Promega; Cat. E1910). The following constructs were co-transfected into cells in 24-well plates using FuGene HD transfection reagent (Promega; Cat. E2311): Renilla Luciferase, 8X-E-box Firefly luciferase reporter construct (pGL3/4 vector) (33), and pCMV-HA HEB expression constructs. Cells were lysed 24 h post-transfection using lysis buffer provided by the DLR assay kit, and luminescence was measured using the BioTek Synergy H1 Hybrid Reader. Firefly Luciferase Units were normalized to Renilla Luciferase Units to calculate Relative Luciferase Units (RLU).

Retroviral Transduction

GFP (empty vector negative control), HEBAlt (ALT), FFF, EEE, and HEBCan (CAN) retrovirus- producing GP+E cell lines were generated using pMIG-IRES-GFP, pMIG-HEBALT-HA-(WT/FFF/EEE)-IRES-GFP, and pMIG-HEBCAN-HA-IRES-GFP vectors, as previously described (36). BMK cells were co-cultured with the retrovirus-producing cell lines overnight (18h) in media containing Polybrene (10mg/μL). GFP+ cells were sorted by flow cytometry and expanded in culture to create stably expressing cell lines.

Immunoprecipitation

SCID.adh cells and HeLa cells, or HEK293T cells transfected with expression constructs using Lipofectamine 2000 one day earlier, were harvested using Pierce lysis buffer supplemented with protease (Thermo Scientific, 78425) and phosphatase inhibitors (EMD Millipore, 4906845001). Lysates were incubated on ice for 30 min, with vigorous vortexing every 10 min, and then pre-washed with Pierce Protein A/G agarose slurry for 1 h at 4°C to block non-specific binding. HA-tagged proteins were subjected to IP at 4°C overnight using antibody-agarose beads (Clontech, 631207) followed by three washes with Pierce lysis buffer or were collected anti-HA-conjugated magnetic beads followed by TBST washes (Thermo Scientific, 88836).

Western Blotting

Cells were lysed using RIPA buffer with protease inhibitors (Halt Protease Inhibitor Cocktail (100X); Thermo Scientific; Cat. 78430). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific; 23225) and equal amounts were used in SDS-PAGE analysis. Lysates were denatured in SDS loading buffer (containing DTT), heated at 100°C for 5 min, and loaded onto acrylamide gels. Sizes were determined using the PageRuler Plus Prestained Protein ladder

(Thermo Scientific, 10 to 250kDa). Samples were transferred from the gel onto a PVDF membrane (Biorad TransBlot Turbo) by the semi-dry transfer method using the TransBlot Turbo Machine (Biorad), and the membrane was blocked overnight at 4°C on a shaker in 1X TBST (Tris-buffered saline with Tween 20 at 0.1% v/v) with 5% skim milk to eliminate non-specific binding. The next day, the blots were probed with primary antibodies in 1X TBST with 5% BSA, washed, and probed with HRP-conjugated secondary antibodies. After washing in TBST, the blot was visualized using the Clarity ECL kit (Biorad) and the Fusion Fx Chemiluminescence and Fluorescence Imager (Vilber Lourmat). Image quantification was performed using ImageJ software. The primary antibodies used in these studies were the HA-Tag polyclonal antibody (Clontech, 631207), pan anti-HEB antibody (Santa Cruz Biotechnology, A-20, sc-357), anti-GAPDH (mouse, EMD Millipore, MAB374), anti-Tubulin (mouse, SCBT; sc-69970), and anti-Alt (in-house). The secondary antibodies were HRP-conjugated Goat anti-Rabbit (Invitrogen, 626120) and Goat anti-Mouse (BioRad, 1706516). The anti-phosphotyrosine antibody (4G10 Platinum) was directly conjugated to HRP (Sigma-Aldrich 16-316).

Protein Stability Assays

For the cyclohexamide experiments, cells were treated with 300 µg/mL cyclohexamide (CHX) to block translation elongation 24 h post-transfection. The cells were lysed at 0 h, 0.5 h, 1 h, and 2 h post-CHX-treatment, and lysates were analyzed by Western blotting. Band densities were quantified using ImageJ.

JAK Inhibition

Jurkat cells were co-transfected with ALT, FFF, EEE or pCMV-HA (negative control) and the 8X E-box luciferase reporter and treated with DMSO (no Ruxolitinib) or 1 mM of the pan-JAK inhibitor Ruxolitinib (Invitrogen, tlrl-rux) for 4 h. Dual luciferase assays were conducted, and the data was depicted as relative luciferase units (RLU) normalized to untreated ALT.

Mass Spectrometry

HEK293 cells transfected with ALT, CAN, EEE, FFF, TR, or empty vector, or whole thymocytes from littermate ALT-Tg mice with or without Vav-Cre, were lysed using RIPA lysis buffer and subjected to IP using the Pierce™ HA-Tag Magnetic IP/Co-IP Kit (ThermoFisher Scientific; 88838) and anti-HA antibodies. Samples were trypsin-digested and analyzed by mass spectrometry at the SPARC BioCentre facility using a Thermo Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (SickKids, Toronto). Scaffold (version Scaffold_5.0.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide Probabilities from Sequest (XCorr Only) and MS-Amanda Proteome Discoverer were assigned by the Scaffold Local FDR algorithm. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm (37) with Scaffold delta-mass correction. Protein probabilities were assigned by the Protein Prophet algorithm (38). A filter of 95% protein identity, 95% peptide identity, and a minimum of 3 spectra were applied to all samples, and then proteins present in the HA negative control were removed.

Post Translational Modification Site Localization

Scaffold PTM (Proteome Software, Portland, OR) was used to annotate PTM sites derived from MS/MS sequencing results using the site localization algorithm developed by Beausoliel et al. (39). MS/MS spectra identified with modified peptides were identified and AScore values and site localization probabilities were calculated to assess the level of confidence in each PTM localization.

Generation of Anti-ALT Antibodies

Anti-ALT antibodies were made by Abcam (Cambridge, U.K). A peptide corresponding to the Alt domain coding region was synthesized, conjugated to KHL, and injected into rabbits. After several rounds of boosting, the rabbits were exsanguinated to provide polyclonal stocks. Specificity was confirmed by Western blotting of HEK293T cells transfected with either HEBAlt- or HEBCan- expressing constructs (**Figure S2A**), and Rag2-/- thymocytes, which express abundant HEBAlt protein (**Figure S2B**).

Statistical Analysis

Data was analyzed using Prism software (GraphPad). Statistical significance was determined by comparing two sets of data with the unpaired two-tailed Student's t test, where a p value of less than 0.05 fulfilled the criteria of statistical significance. The variation between biological replicates of the same group was depicted by graphing the standard error of the mean as error bars. All data shown are reflective of at least two independent experiments. Results of multiple experiments were pooled, normalized, graphed, and shown as individual data points.

RESULTS

Optimal HEBAlt Transcriptional Activity Requires the Last Third of the Alt Domain

We have previously shown that the activity of HEBAlt on an E-box promoter-reporter luciferase construct was lower than HEBCan-induced transcriptional activation (33). This could have been due to the absence of the HEBCan-specific N-terminus, or due to unique properties of the Alt domain. To distinguish between these possibilities, we generated HA-tagged HEBAlt mutant truncation expression constructs using naturally occurring methionines as start codons (**Figures 1C, D**). We removed the first third of the Alt domain (MET1), the first two-thirds, (MET2), the entire Alt domain (TR), or the Alt domain and part of exon 9, which is shared between HEBAlt and HEBCan (MET3) and tested their ability to transactivate the reporter construct as compared with WT (ALT) HEBAlt in HEK293T cells. All constructs were expressed at comparable protein levels (**Figure 1E**). ALT induced luciferase activity was

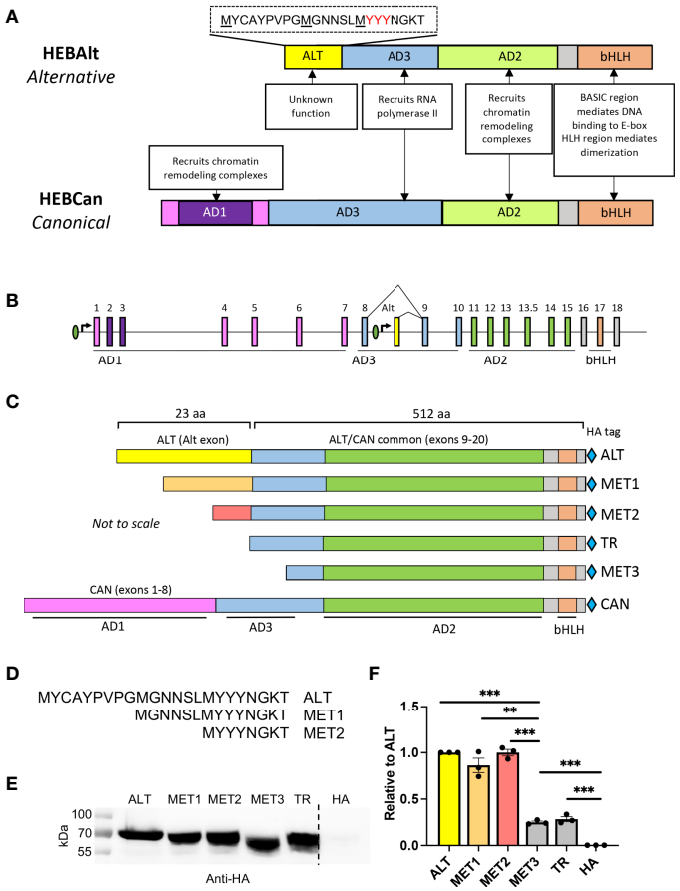


FIGURE 1 | The Alt domain is required for HEBAlt transcriptional activity. **(A)** Diagram of HEBAlt (alternative) and HEBCan (canonical) variant domain structures and functions. AD=activation domain, bHLH = basic DNA binding and helix-loop-helix dimerization. ALT = domain specific to HEBAlt, with amino acid sequence shown above it. **(B)** Structure of the *TCF12* gene locus that encodes both HEBCan and HEBAlt by alternative transcriptional initiation and splicing. Introns = straight line, exons = boxes, promoters = ovals. Exons encoding the functional domains are indicated under the locus and in colors corresponding to **(A)**. **(C)** Domain structures of HEBAlt WT (ALT), MET1, MET2, TR, MET3, and HEBCan (CAN). Domains are color coded to match **(A, B)**. Light blue diamonds = HA epitope tag. **(D)** Amino acid sequence of the wildtype (ALT) and mutated Alt (MET1, MET2) domains. MET3 and TR lack the ALT domain entirely. **(E)** Western blot showing protein expression of wildtype and mutant HEBAlt construct expression as detected by anti-HA. HA indicates HA vector-only control **(F)**. Dual luciferase assay on cells co-transfected with HA-tagged ALT, MET1, MET2, MET3, or TR, plus 8X E box Firefly luciferase and Renilla luciferase constructs. The Y-axis depicts relative luciferase units (RLU) of Firefly to Renilla values, normalized to ALT. ***P ≤ 0.0001, **P ≤ 0.001, ns = non-significant. Note that in **(E)**, some lanes from the same gel are depicted separately, as they were cut and pasted back together from the original image to exclude irrelevant data. None of the domain or construct diagrams are to scale.

above background levels, in agreement with previous studies (33) (**Figure 1F**). The MET1 and MET2 constructs displayed comparable activity to full length ALT, but the TR and MET3 constructs, which lacked the Alt domain completely, had a ~3-fold decrease in activity. These results indicated that the last third of the Alt domain is required for effective transcriptional activation from a multimerized E box site by HEBAlt.

The YYY Residues in the Alt Domain Regulate HEBAlt Activity

Closer inspection of the amino acid residues in the Alt domain revealed an abundance of tyrosine (Y) residues, including a triple tyrosine (YYY) motif. To evaluate whether the Alt domain could respond to signaling through tyrosine kinase-mediated pathways, we generated mutant HEBAlt constructs in which the YYY motif was replaced by EEE, imparting a negative charge and mimicking phosphorylation, or FFF, which cannot be phosphorylated (**Figure 2A**). We transfected these constructs into HEK293T cells and confirmed that EEE and FFF were expressed at similar protein levels to ALT (**Figure 2B**). To evaluate the ability of these constructs to activate transcription from the 8X E box reporter construct, we co-transfected them into BMK (Baby mouse kidney) cells. Consistent with our previous findings, luciferase assays showed that ALT had lower levels of activity than HEBCan (CAN) and E2A (E47) (**Figure 2C**). Strikingly, EEE exhibited a ~10-fold increase in activity over ALT, to levels that surpassed CAN. By contrast, FFF activity was ~2-fold lower than ALT. We also tested the activity of these factors in HEK293T cells on the *Ptcra* promoter, which drives expression of the gene encoding pTα in DN thymocytes

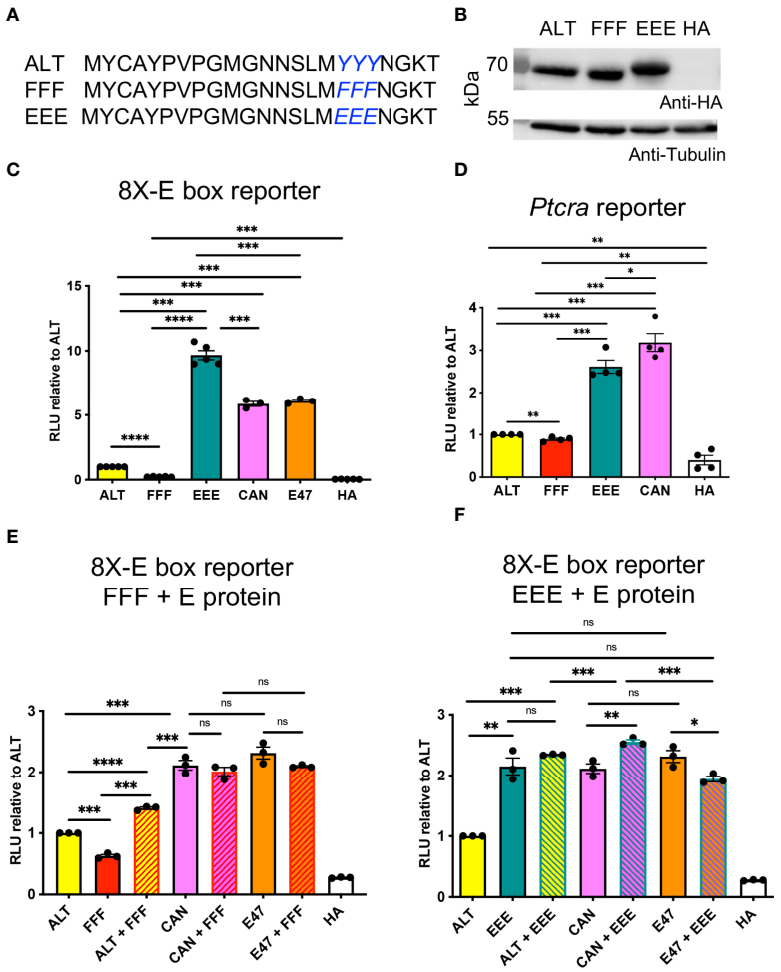


FIGURE 2 | A YYY motif in the Alt domain modulates HEBAlt transcriptional activity. **(A)** Amino acid sequences of the Alt domain of the EEE and FFF constructs. **(B)** Western blot of protein expression for the HA-tagged ALT, FFF, and EEE constructs in HEK293T cells, probed by anti-HA, with anti-tubulin as a loading control. **(C)** Dual luciferase assays on BMK cells transfected with expression constructs for ALT, FFF, EEE, HEBCan (CAN), E2A (E47), or HA empty vector control, along with 8X E box Firefly luciferase and Renilla luciferase constructs. The Y-axis depicts relative luciferase units (RLU) of Firefly to Renilla luciferase values, normalized to ALT. **(D)** Dual luciferase assay on HEK293T cells co-transfected with HA-tagged expression constructs for ALT, FFF, EEE, CAN, or HA empty vector control with a construct in which Firefly luciferase was driven by the Ptcra promoter, and a Renilla luciferase construct. The Y-axis depicts relative luciferase units (RLU) of Firefly to Renilla luciferase values normalized to ALT. **(E)** Constructs were mixed 1:1 to allow the formation of homodimers of ALT, CAN, E2A (E47), or FFF, or heterodimers of ALT, CAN, or E2A with FFF, and activity was measured by luciferase assays using the 8X E box construct. **(F)** Constructs were mixed 1:1 to allow the formation of homodimers of ALT, CAN, E2A (E47), or EEE, or heterodimers of ALT, CAN, or E2A with EEE, and activity was measured in HEK293T cells by luciferase assays using the 8X E box construct. Note that the assays shown in **(E, F)** were conducted at the same time, and the ALT, FFF, EEE, CAN, E47, and HA samples were graphed next to either the FFF or EEE samples for clarity. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.0001, ns, non-significant.

(40) (**Figure 2D**). Under these conditions, ALT and FFF activity were both low, but EEE approximated HEBCan activity. These results suggest that phosphorylation of the YYY motif in the Alt domain might enable HEBAlt to induce transcription more strongly than HEBCan.

FFF Does Not Act as a Dominant Negative Form of ALT

To evaluate whether the FFF acted as a dominant negative form of HEB, we assessed whether mixing FFF with ALT, CAN, or E2A would decrease the overall activity when co-transfected into HEK293T cells with the reporter construct. We found that FFF did not interfere with CAN or E2A transcriptional activity but induced a small increase in ALT activity (**Figure 2E**). Next, we asked whether mixing the EEE mutant with each of the wildtype E proteins would impact their activity. No major impact on CAN or E47 activity was observed with the addition or either EEE, suggesting that a negative charge on one E protein within an E protein dimer is sufficient to drive enhanced transcriptional activation (**Figure 2F**).

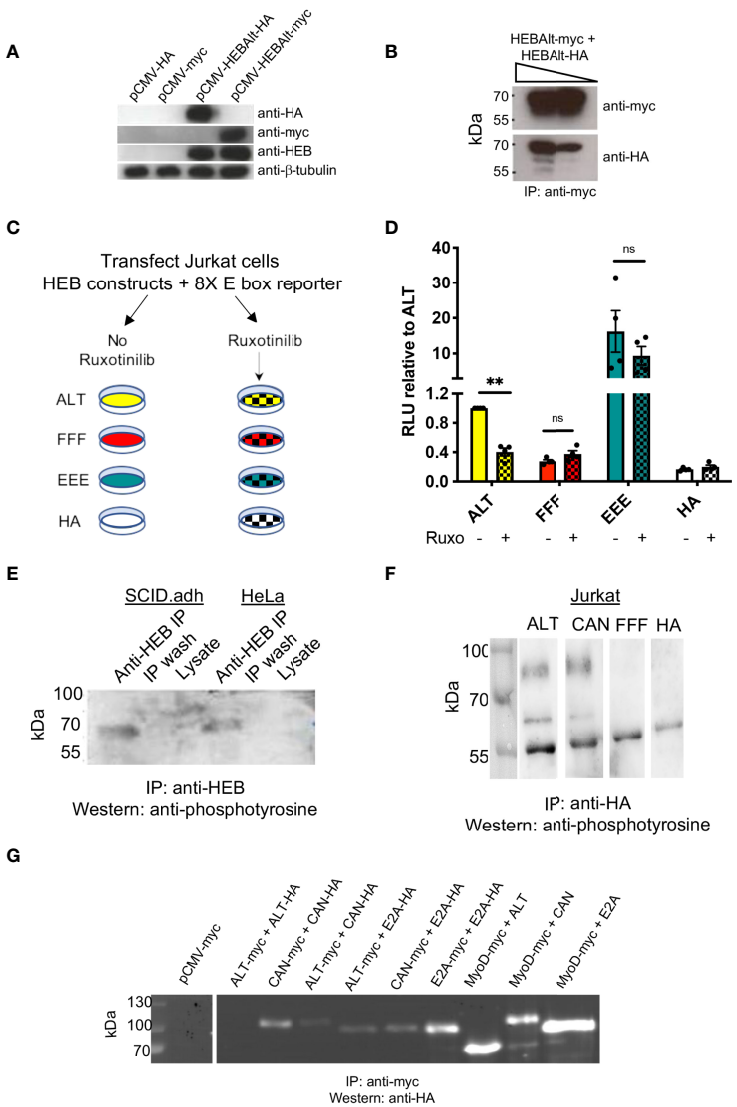


FIGURE 3 | HEBAlt transcriptional activity is decreased by JAK inhibition. **(A)** Western blot of lysates from HEK293T cells transfected with HEBAlt-HA, HA empty vector (pCMV-HA), HEBAlt-myc, or myc empty vector (pCMV-myc) expression constructs probed with anti-HA, anti-myc, anti-HEB (A20) or β-tubulin as a loading control. **(B)** Western blots of HEK293T lysates from co-IPs of HEBAlt-HA and HEBAlt-myc, using anti-myc antibodies for co-IP and anti-HA antibodies for Western protein detection, at a higher (left) or lower (right) concentration of lysate (triangle). **(C)** Diagram of JAK inhibition experimental design. Jurkat cells were co-transfected with HEBAlt (ALT; yellow), FFF (red), EEE (green) or HA empty vector (white), and 8X E box Firefly luciferase and Renilla luciferase constructs. Cells were cultured with (checked) or without (filled) the pan-JAK inhibitor Ruxotinilib (Ruxo) for two days at 1 mM, followed by dual luciferase assay. **(D)** Activation of the reporter construct as measured by dual luciferase assay. The Y-axis depicts relative luciferase units (RLU) of Firefly to Renilla luciferase values normalized to ALT. **P < 0.001, ns, non-significant. **(E)** Detection of endogenous tyrosine phosphorylated protein in the mouse pro-T cell line SCID.adh and human HeLa cells after immunoprecipitation with anti-HEB antibodies, assessed by probing of Western blot with anti-phosphotyrosine antibodies. **(F)** Western blot of lysates extracted from Jurkat cells transfected with ALT or CAN, IP'd with anti-HA, and probed with anti-phosphotyrosine antibodies. **(G)** Expression proteins epitope tagged with myc or HA were contransfected into HEK293T cells and co-immunoprecipitated with anti-myc. Lysates were analyzed by Western blots probed with anti-HA. The discrepancy between ALT-ALT dimerization seen in **(B)** versus **(G)** is likely due to the exposure times on the different blots, since a far longer detection time was necessary to observe ALT-ALT dimers, and the blot in **(G)** was optimized to visualize the range of intensity for the other dimers.

HEBAlt Activity Is Reduced by JAK Inhibitors

To formally assess whether ALT proteins can form homodimers, we generated a myc-tagged ALT construct (**Figure 3A**). ALT-HA and ALT-myc were co-transfected into HEK293T cells, and antibodies were used to immunoprecipitate (IP) ALT-myc. Western blot analysis of HA-tagged constructs clearly showed that ALT-myc and ALT-HA can form a complex (**Figure 3B**), consistent with the existence of HEBAlt homodimers. Next, we assessed whether JAK tyrosine kinases could enhance HEBAlt

activity. ALT or mutant constructs and 8X-E box reporter constructs were co-transfected into Jurkat T cells, which provide a background of low constitutive JAK signaling (41). ALT, FFF, EEE, or control empty vector (HA) transfected Jurkat cells were cultured with or without the JAK inhibitor Ruxolitinib for two days, followed by luciferase assays (**Figure 3C**). As before, FFF displayed lower activity than ALT. In contrast, EEE exhibited a ~20-fold higher activity than ALT (**Figure 3D**). Importantly, JAK inhibition decreased ALT activity but did not significantly affect the activity of FFF or EEE. These results indicate that modulation of JAK function can regulate HEBAlt activity in a YYY-dependent manner.

Phosphorylation of the YYY Motif in the Alt Domain

To assess whether HEBAlt could be phosphorylated, we used anti-HEB (A20) to IP endogenous HEB factors from HeLa and SCID.adh cells (42) (**Figure 3E**). Anti-HEB was used to IP HEB factors, and the Western blot was probed using anti-phosphotyrosine antibodies (4G10). One band around 60 kDa was detected, consistent with phosphorylation of HEBAlt tyrosine residues. To verify the specificity of the endogenous bands, we transfected HA-tagged ALT, CAN, or FFF constructs into Jurkat cells and cultured them for two days. The transfected Jurkat cells were subjected to IP with anti-HA antibodies (**Figure 3F**) and Western blots were probed with anti-phosphotyrosine antibodies. In the ALT and CAN transfected Jurkat cells, we observed two bands, one at the size of HEBCan (upper band; ~100 kD) and another at the size of HEBAlt (lower band; ~60 kD), whereas the FFF sample lacked bands indicating phosphorylated tyrosine. To assess whether the presence of two bands in the each of the ALT and CAN samples might be due to enrichment of complexes containing both HEBAlt and HEBCan, we performed co-IPs in HEK293T cells with myc-tagged and HA-tagged HEBAlt, HEBCan, E2A, and MyoD (**Figure 3G**). MyoD is a Class II bHLH factor that binds more strongly to E proteins than they do with each other. Our results showed that HEBAlt can form heterodimers with HEBCan, E2A, and MyoD. Therefore, the presence of a phosphorylated CAN band in the ALT sample and a phosphorylated ALT band in the CAN sample suggests that both proteins were precipitated as components of complexes containing HA-tagged proteins. Moreover, these bands suggest that both HEBCan and HEBAlt, whether exogenous or endogenous, can be tyrosine phosphorylated, whereas FFF cannot. Moreover, the lack of bands in the FFF lane suggest that it may not be able to form stable dimers with either HEBAlt or HEBCan, providing a partial explanation for its decreased function.

Detection of a Phosphorylated YYY Motif Within the E2-2 Alt Domain

To directly identify phosphorylated residues on HEBAlt, we transfected HEK293T cells with HA-tagged ALT, FFF, EEE, CAN, and TR. After 24 h, protein lysates were generated in the presence of phosphatase inhibitors and subjected to IP with anti-HA. The precipitated proteins were subjected to mass spectrometry sequencing, and ScaffoldPTM was used to identify phosphorylated residues on ALT, FFF, EEE, CAN, and TR (**Figure 4A**). Five unique peptides were recovered from this set of samples, all of which were located downstream of the ALT/CAN junction (**Figures 4B, C**). Spectra consistent with phosphorylation were detected on two serines, two threonines, and one tyrosine. These were sparsely present among the peptides and were differentially represented in each sample (**Figure 4C**). However, we also detected a paralog of HEBAlt, E2-2Alt (43), likely due to co-immunoprecipitation with HEBAlt. The Alt domains of E2-2 and HEB differ by two amino acids (**Figure 4D**), allowing unequivocal identification. This spectrum showed a +80 shift on the last tyrosine of the YYY motif indicating the presence of a phosphate group (**Figure 4E**). It should be noted that E2-2Alt spectra represented an endogenous protein that is not overexpressed, further supporting the capacity of the YYY motif in E protein ALT variants to be directly phosphorylated on at least one tyrosine residue.

A Transgenic Mouse Model for Studying HEBAlt *In Vivo*

Given the lack of reagents available for studying HEBAlt, we generated several new tools to move our studies into the context of T cell development. The first was an antibody that detects the Alt domain (**Figure S2**). These antibodies (anti-ALT) worked well for detection of HEBAlt using Western blots but not for immunoprecipitations. Therefore, we designed a new mouse model with inducible expression of HEBAlt. To enhance the versatility of these mice, we inserted an HA-tagged HEBAlt construct into a loxP-stop-loxP cassette driven by the Rosa26 constitutively expressed promoter to generate ALT-Tg mice (**Figure 5A**). In these mice, the HEBAlt-HA transgene is silent until the stop cassette is removed by Cre-mediated excision, enabling induction of HEBAlt-HA expression in a stage- and lineage-specific manner. We bred these mice to Vav-iCre mice to induce deletion in all hematopoietic cells, and generated ALT-Tg$^{Vav\text{-}iCre}$ (ALT-Tg) mice, which were compared with ALT-Tg (WT) littermates containing the transgene in the absence of Cre.

Phenotyping of Major Thymocyte Subsets in ALT-Tg Mice at Steady State

The ALT-Tg mice had no obvious defects at the level of gross morphology. The adult thymus had normal cellularity (**Figure 5B**) and undisturbed proportions of DN, DP, and SP thymocytes at steady state (**Figures 5C, G**), as well as a normal distribution of DN subsets (**Figures 5D, H**). We also evaluated the percentages of γδ T cells (**Figures 5E, I**), and the distribution of immature versus mature cells within the γδ T cell subset according to expression of CD24 (**Figures 5F, I**). No significant differences were observed. We next undertook a more in-depth analysis of thymocyte subsets that normally do not express HEBAlt at the mRNA level (**Figure 6**). Gating on the CD8 +CD4- subset (**Figure 6A**) revealed that the proportion of immature (CD24+ TCRβ-) cells to mature (CD24-low TCRβ+)

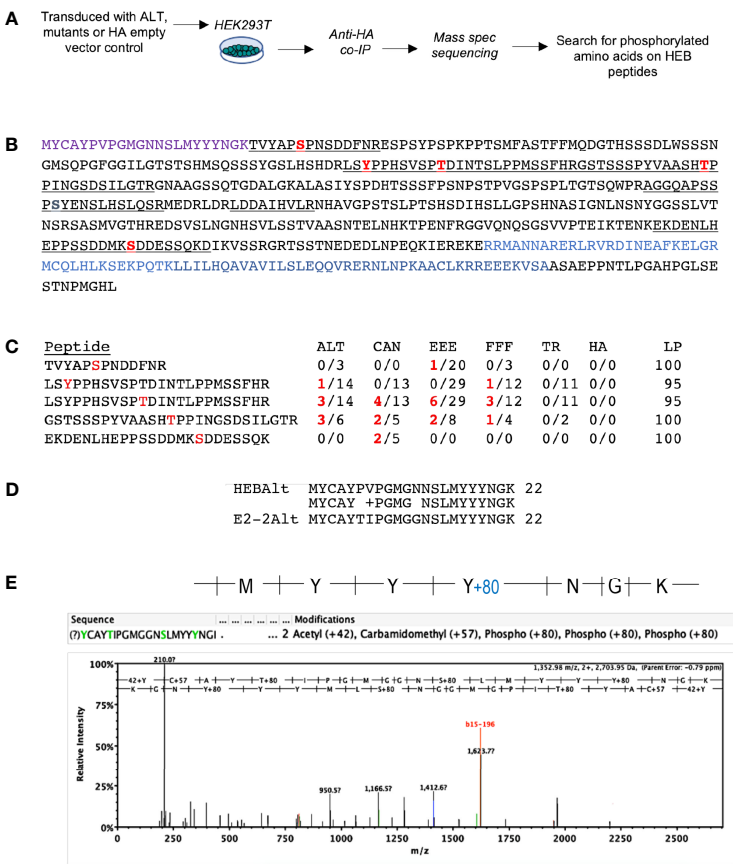


FIGURE 4 | Detection of post-translational modifications in HEBCan, HEBAlt, and HEBAlt mutant proteins transfected into HEK293T cells. **(A)** Diagram of experimental design. HEK293T cells were transfected with HA-tagged constructs and subjected to immunoprecipitation 24 hrs later using anti-HA antibodies. Lysates were subjected to mass spectrometry sequencing, and sequences were analyzed to detect post-translational modifications (PMTs). **(B)** Annotated amino acid sequence of HEBAlt. Purple = Alt domain. Underlined = peptides detected. Red = residues with S/T/Y phosphorylation detected at least once. Blue = bHLH domain. No peptides were detected representing the Alt domain. **(C)** Table showing the numbers and distribution of specific PMTs in each peptide among samples bearing different constructs. LP = probability that the site of phosphorylation has been correctly annotated, as calculated by the Scaffold-PTM software. **(D)** Alignment of the Alt domains of HEB and its paralog E2-2 showing the two amino acid difference between them. **(E)** Spectrum of a peptide identified as E2-2Alt in the FFF sample with a mass shift of +80 at the last tyrosine of the YYY motif, consistent with phosphorylation.

cells was higher in ALT-Tg mice than in WT mice, with a corresponding decrease in the percentage of mature CD8+ SPs in the ALT-Tg mice (**Figures 6B, G**). This was not accompanied by a decrease in the percentage of DP cells, suggesting the increase in CD8+ ISP did not indicate a developmental block at the DN to DP transition. To assess the ratio of mature CD4 to CD8 cells, we first gated on TCRβ+ CD3+ cells (**Figures 6C, H**), and then on CD24- cells within that population (**Figure 6H**). These two populations were present at similar frequencies in WT and ALT-Tg. We next evaluated the frequencies of CD4 and CD8 cells within the mature (TCRβ+ CD3+ CD24-) population and found no differences between WT and ALT-Tg mice (**Figures 6D, I**). To assess whether positive selection was impaired in ALT-Tg mice, we examined CD24 and CD69 expression within the TCRβ+ CD8+ (**Figures 6E, J**) and the TCRβ+ CD4+ (**Figures 6F, K**) populations. Thymocytes from ALT-Tg mice exhibited transient upregulation of CD69 accompanied by CD24 downregulation comparable to WT thymocytes in both the CD4+ and CD8+ SP subsets. Therefore, we found no evidence for alterations in positive selection in the ALT-Tg mice on the C57Bl/6 background with a polyclonal repertoire.

HEBAlt Protein Persists in Thymocytes After mRNA Expression Ceases

To confirm expression of the HEBAlt transgene in ALT-Tg mice, we measured HEBAlt mRNA and protein levels (**Figure 7**). Although HEBAlt mRNA decreased at the DN3 to DN4 transition in the WT mice, as expected, it was sustained up to the DP stage in ALT-Tg thymocytes, as detected by anti-HA (**Figure 7A**). Western blots of sorted thymocyte subsets showed that the transgenic HEBAlt protein was also expressed up to the DP stage, as detected by anti-HA (**Figure 7B**). Surprisingly, we also found that endogenous HEBAlt protein, as detected by the anti-ALT antibody, was present in WT thymocytes subsets that had downregulated HEBAlt mRNA (**Figure 7C**). These

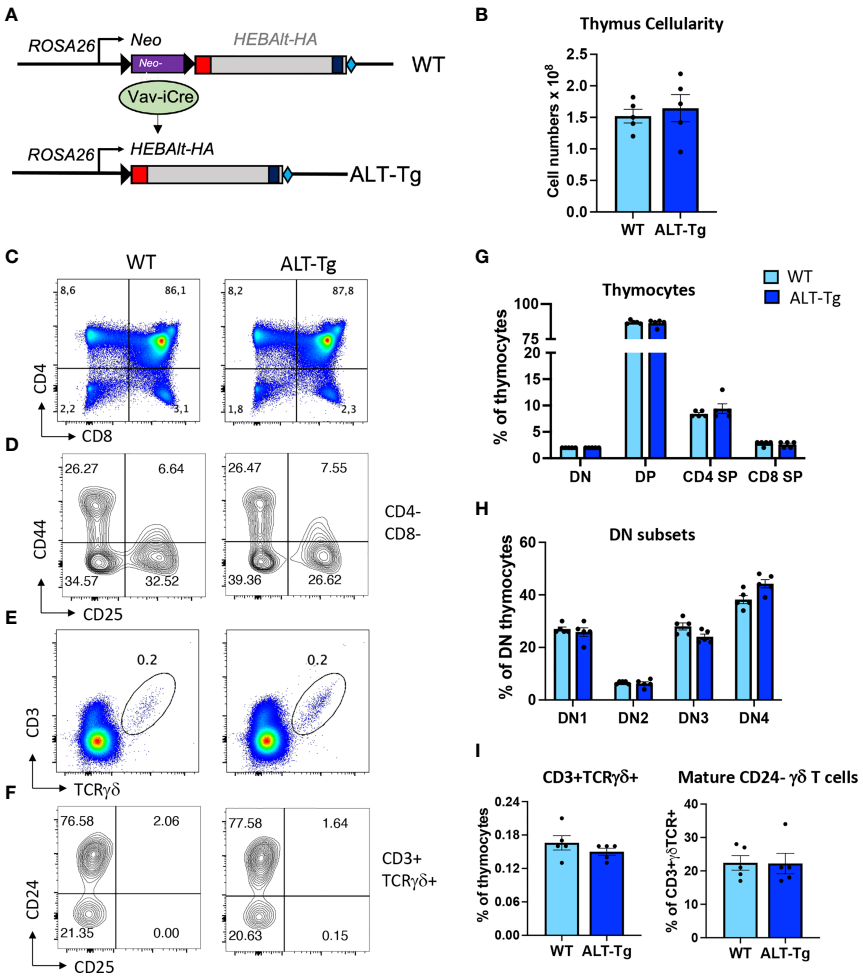


FIGURE 5 | Generation and characterization of conditional HEBAlt-HA transgenic (ALT-Tg)_ mice. **(A)**. An HA-tagged HEBAlt coding cassette was inserted into the ROSA26 locus downstream of a loxP-Neo-stop-loxP cassette in ES cells, giving rise to mice carrying an inducible HEBAlt-HA transgene. These mice were bred to Vav-iCre mice to generate WT (Vav-Cre-) and ALT-Tg (Vav-Cre+) littermates. **(B–E)**. Phenotype of thymic developmental subsets in WT and ALT-Tg mice by flow cytometry within total thymocytes **(B, D)** and and within DN cells **(C, E)**. **(F–H)**. Percentages of γδ T cells within total thymocytes **(F, H)**, and percentages of immature (CD24+) and mature (CD24-) cells within the CD3+TCRγδ+ populations **(G, H)**.

observations suggest that HEBAlt protein stability may be enhanced across the β-selection checkpoint.

The Thymocyte Environment Enhances HEBAlt Protein Stability

Given the discordance between HEBAlt mRNA and HEBAlt protein expression, we evaluated HEBAlt protein stability by performing cycloheximide chase experiments. BMK cells were stably transduced with HA-tagged HEBAlt or HEBCan to provide a non-T cell context for protein stability studies, and these were compared with HA-tagged ALT-Tg thymocytes. Cycloheximide was added to the cells to stop *de novo* protein synthesis, and then washed out (chase). Samples were taken at 0, 0.5, 1, and 2 h after chase, and protein levels were evaluated using Western blots probed with anti-HA and anti-GAPDH. In BMK cells, HEBAlt and HEBCan were expressed at comparable amounts at t=0, but HEBAlt protein levels dropped more precipitously than HEBCan levels, indicative of decreased stability (**Figure 7D**). By contrast, HEBAlt protein appeared more stable in thymocytes than in BMK cells (**Figures 7F, G**), and the levels were statistically indistinguishable from HEBCan in BMK cells after 2 hrs (**Figure 7H**). EEE showed only a minor improvement in HEBAlt stability in BMK cells (**Figures 7E, H**), suggesting that factors other than YYY phosphorylation were at least partially responsible for this feature of HEBAlt biology.

Transgenic HEBAlt Protein is Reduced in Non-Thymic Hematopoietic Cells

We have shown in previous studies that forced expression of HEBAlt was inhibitory to the development of non-T cell lineages *in vitro* (33). We therefore analyzed the proportions of myeloid cells ($CD11b^+$), B cells ($CD19^+$), and the ratio of immature ($B220^{int}$ $CD19^{int}$) to mature B cells ($B220^{hi}$ $CD19^{hi}$) in the bone marrow (**Figure 8A**). We found no significant

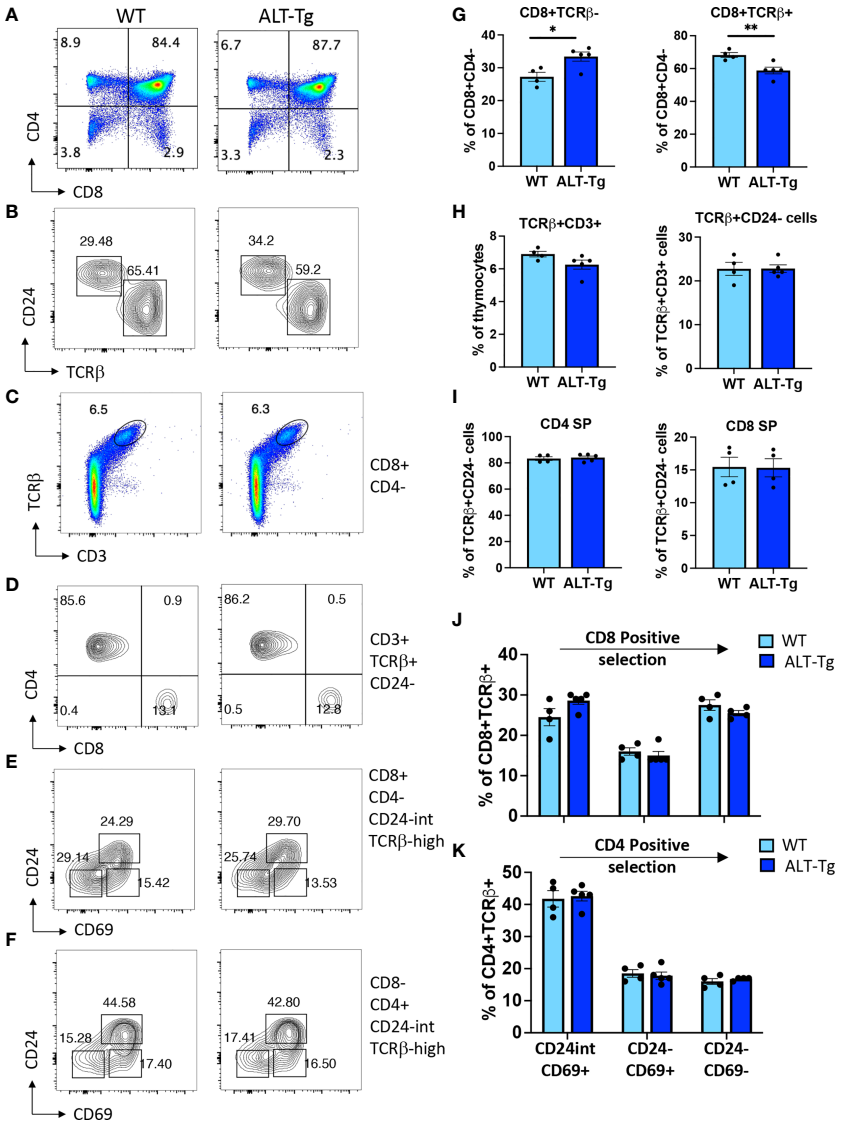


FIGURE 6 | Perturbation of the ISP to CD8 SP thymocyte ratio in ALT-Tg mice. **(A)** Flow cytometry plot showing the gates for CD4+ CD8- and CD4- CD8+ cells. **(B)** Flow cytometry plots showing immature (CD24-hi TCRβ-) and mature (CD24-low TCRβ+) subsets in the CD8+ subset gated as in **(A)**. **(C)** Flow cytometry plots showing the percentages of TCRβ+ CD3+ cells within the total thymocyte population. **(D)** Flow cytometry plot of the percentages of CD4 and CD8 cells within the TCRβ+ CD3+ CD24- population. **(E)** Flow cytometry plot of CD8+ TCRβ+ CD24- cells passing through each stage of positive selection as assessed by sequential downregulation of CD24 and CD69. **(F)** Flow cytometry plot of CD4+ TCRβ+ CD24- cells passing through each stage of positive selection as assessed by sequential downregulation of CD24 and CD69. **(G)** Quantification for replicates of the plots shown in **(B)**. **(H)** Quantification for replicates of the plots shown in **(C)** and for the percentages of CD24- cells within the TCRβ+ CD3+ population, which are not shown as FACS plots. **(I)** Quantification for replicates of the plots shown in **(D)**. **(J)** Quantification for replicates of the plots shown in **(E)**. **(K)** Quantification for replicates of the plots shown in **(F)**. *P < 0.05, **P < 0.001.

differences between the WT and ALT-Tg mice. Absolute numbers of bone marrow cells and splenocytes were also indistinguishable (**Figure 8B**). We also analyzed the percentages of B cells, the ratios of T to B cells, and the ratios of CD4 to CD8 T cells within the $CD3^{+}TCR\beta^{+}$ population in the spleen. There was a slight decrease in the ratio of T cells to B cells in the ALT-Tg spleen, but otherwise no major perturbations were observed (**Figures 8A, C**). Given our observation of differential HEBAlt protein stability in transduced BMK cells versus ALT-Tg thymocytes, we next analyzed the expression of the HEBAlt transgene in spleen and bone marrow subsets from ALT-Tg mice. Thymocytes, RBC-depleted bone marrow cells and splenocytes that had been sorted to obtain T (CD3+), B (CD19+), and myeloid CD11b+ (myeloid)-enriched samples, and these samples were subjected to Western blot analysis with anti-HA. Transgenic HA-tagged

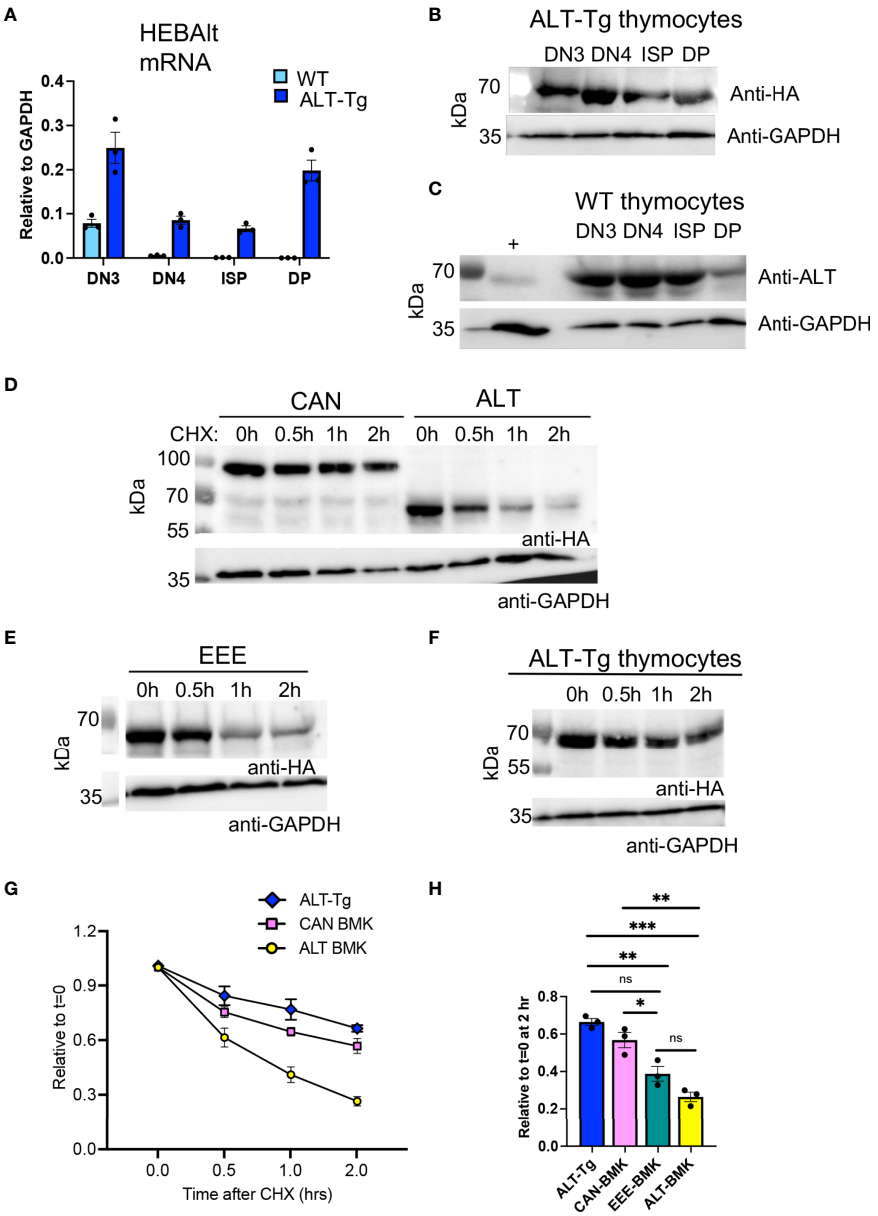


FIGURE 7 | Differential protein stability of HEBAlt in T cell and non-T cell contexts. **(A)** Levels of HEBAlt mRNA in sorted thymocyte subsets from WT and ALT-Tg mice as assessed by qRT-PCR. **(B)** Levels of HEBAlt-HA transgene-derived protein in sorted thymocyte subsets from ALT-Tg mice as determined by Western blots probed with anti-HA or anti-GAPDH as a loading control. **(C)** Levels of endogenous HEBAlt in sorted thymocyte subsets from WT mice as determined by Western blot probed with anti-ALT specific antibodies or anti-GAPDH as a loading control. **(D–F)**. BMK cells were transduced with constructs expressing HA-tagged **(D)** HEBCan (CAN), HEBAlt (ALT) or **(E)** EEE expression vectors and examined for protein stability in parallel with ALT-Tg thymocytes **(F)**. Cells were treated with DMSO or 300 mg/mL cycloheximide (CHX) for 0, 0.5, 1, or 2 h. C, and protein was measured by Western blot detecting using anti-HA or anti-GAPDH as a loading control. **(G)** Time course of quantified band densities (n = 3 independent experiments), relative to GAPDH at t=0 and normalized to untreated sample (t = 0). EEE is not shown to enhance clarity of the plot. **(H)** Comparison of all samples at t = 2 hr of CHX treatment, including EEE. *P < 0.05, **P < 0.001, ***P < 0.0001, ns, non-significant.

HEBAlt protein was expressed strongly in ALT-Tg thymocytes but was undetectable in ALT-Tg bone marrow (**Figure 8D**). Splenic B and T cells had similar levels of HEBAlt-HA protein expression, whereas none was apparent in the myeloid fraction. However, qRT-PCR showed that ALT-Tg cells had higher overall levels of HEBAlt mRNA than their WT littermates in all three tissues, confirming expression of the transgene at the RNA level (**Figure 8E**). Interestingly, HEBAlt-HA protein levels were much stronger in thymocytes than they were in the splenic T or B cells. These results suggest that the thymus

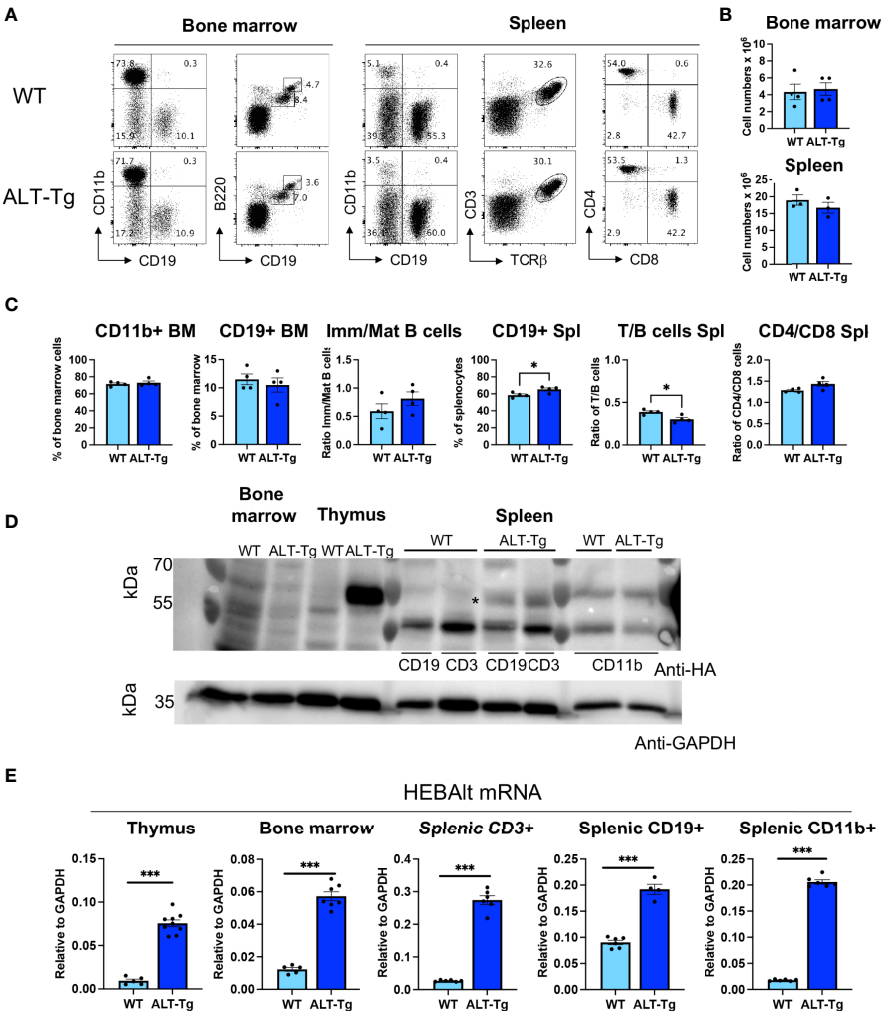


FIGURE 8 | Loss of HEBAlt protein stability in immune cell subsets outside the thymus. A-C. **(A)** Phenotype, **(B)** cell numbers, and **(C)** frequencies or ratios of T cell, B cell, and myeloid subsets in bone marrow and spleen of WT (light blue) and ALT-Tg (dark blue) mice. The bone marrow B220/CD19 plot is gated on CD19+ cells, and the spleen CD4/CD8 plot is gated on CD3+ cells. **(D)** Western blot analysis of transgene-derived HEBAlt-HA protein as detected by anti-HA in WT or ALT-Tg bone marrow, thymocytes, or sorted splenic T (CD3), B (CD19), and myeloid (CD11b) cells. GAPDH is shown as a loading control. The star pinpoints a band that is present in the ALT-Tg splenic T and B cells but not in the WT cells or myeloid cells. **(E)** Expression of total HEBAlt levels in thymus, bone marrow, and splenic T, B, and myeloid cells from WT and ALT-Tg mice, as analyzed by qRT-PCR and normalized to GAPDH, showing that there are higher levels of HEBAlt mRNA in these populations than in WT mice, despite the paucity of transgene-derived HEBAlt protein. *P < 0.05, ***P < 0.0001.

provides a protective environment for HEBAlt stability, that this protection fades in mature T cells, and that it is absent in myeloid cells.

ALT-Tg Thymocytes Exhibit a Delay in T Cell Development *In Vitro*

Considering these observations, we reasoned that the dynamics of steady state T cell development might mask changes caused by elevated levels of HEBAlt in thymocytes in the ALT-Tg mice. We therefore sorted bone marrow Lin-Sca1+Kit+ (LSK) progenitors from WT and ALT-Tg mice and placed them in OP9-DL4 co-cultures (**Figure 9A**), which allowed us follow T cell differentiation over time (**Figures 9B, C**). We found that ALT-Tg cells were subject to developmental delays compared to their WT counterparts, both at early and at later timepoints (**Figure 9C**). No major changes in cellularity were noted (**Figure 9D**), suggesting that these changes were not due to defects in cell growth or survival. Instead, these results indicate that disrupting the normal balance of HEBAlt in T cell precursors partially inhibits T cell development.

Forced Expression of EEE Inhibits T Cell Development at Multiple Stages

We next evaluated the impact of forced expression of the EEE mutant on T cell development. WT bone marrow precursors were transduced with MIGR1-based retroviral vectors encoding HEBAlt (ALT), EEE, or empty vector. GFP+ LSK cells were sorted and placed in OP9-DL4 cell co-cultures for varying

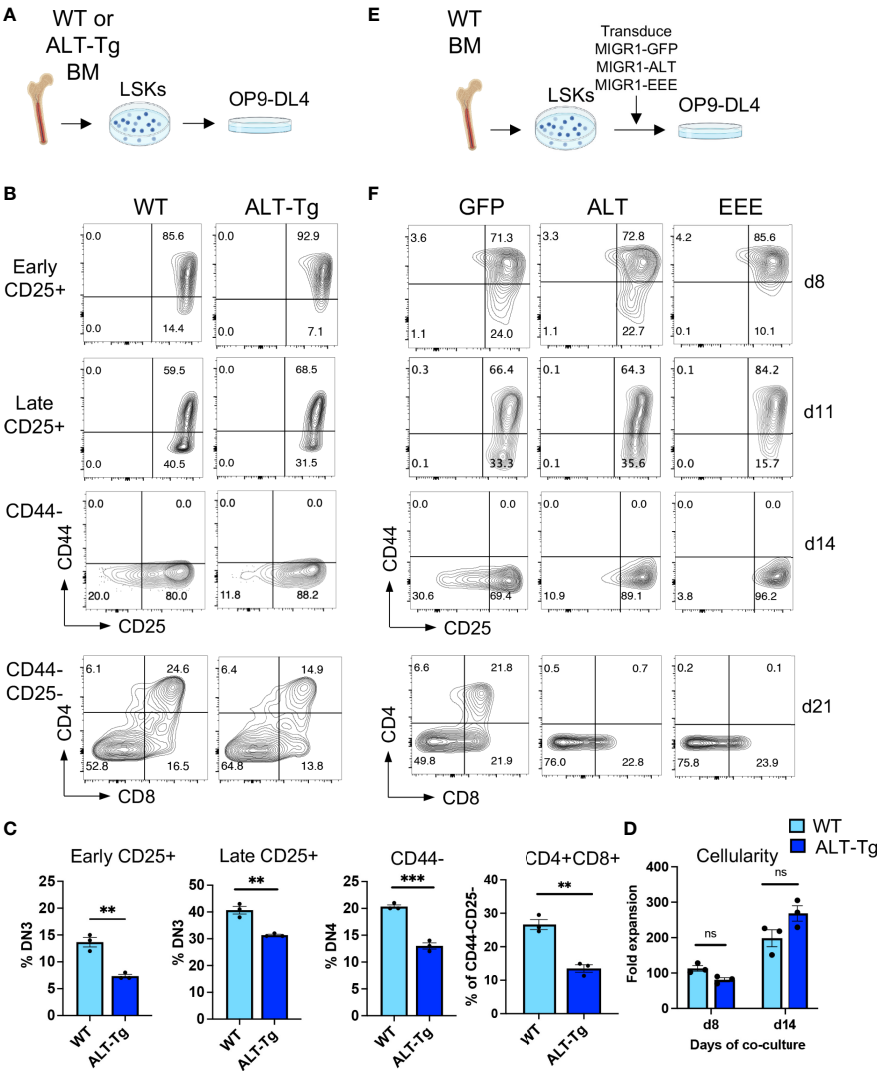
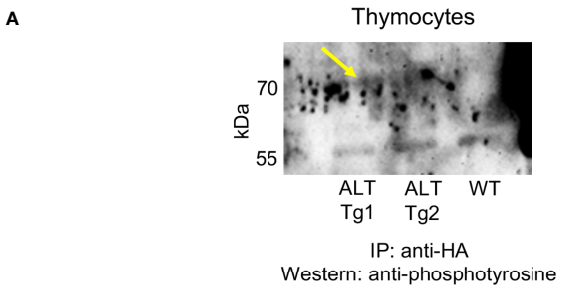


FIGURE 9 | Unrestrained activity of HEBAlt inhibits progress through T cell development. **(A)** Diagram of experimental procedure using precursors from ALT-Tg mice. **(B)** Flow cytometry analysis of progression through T cell development by bone marrow-derived LSKs from ALT-Tg mice co-cultured on OP9-DL4 cells. Gates are shown to the left of each plot. **(C)** Quantification of the populations defined by flow cytometry in **(C)**. **(D)** Fold expansion of cells after 8 or 14 days of OP9-DL4 co-culture. **(E)** Diagram of experimental procedure using precursors from WT mice transduced with retroviral vectors encoding GFP (vector only), ALT, or EEE. **(F)** Flow cytometry analysis of T cell development. Gates are the same as shown in **(B)**. **P < 0.001, ***P < 0.0001, ns, not significant.

amounts of time (**Figure 9E**). Interestingly, progression of ALT-transduced cells from the DN2 to DN3 stages was similar to that seen in WT cultures, whereas the EEE cells exhibited a strong block at this transition (**Figure 9F**). However, both ALT and EEE cultures exhibited a partial block in the DN3 to DN4 transition and failed to progress to the DP stage. Therefore, dysregulation of HEBAlt expression and/or activity can interfere with T cell development.

HEBAlt Is Present in Complexes Containing STAT1 in ALT-Tg Thymocytes

We next set out to determine whether the HEBAlt-HA protein was tyrosine phosphorylated in ALT-Tg thymocytes (**Figure 10**). Unfractionated thymocytes from ALT-Tg or WT littermates were isolated and subjected to co-IP with anti-HA antibodies, followed by Western blot analysis of tyrosine phosphorylation (**Figure 10A**). These results clearly showed that transgene-derived HEBAlt can be tyrosine phosphorylated in ex vivo thymocytes from ALT-Tg mice. To identify putative protein partners of HEBAlt in this context, we subjected WT and ALT-Tg thymocytes to IP using anti-HA antibodies. Two independent experiments were performed, each of which contained biological duplicates (**Figure 10B**). The IP fractions were subjected to trypsin-mediated digestion, followed by mass spectrometry sequencing. The data was analyzed using Scaffold5, with a cut-



C

Identified Proteins	Accession #	Alternate ID	MW	ALT-Tg 1	ALT-Tg 2	ALT-Tg 3	ALT-Tg 4	Total
Signal transducer and activator of transcription OS=Mus musculus OX=10090 GN=Stat1	A0A087WSP5	Stat1	88 kDa	4	2	4	1	11
Exportin-1 OS=Mus musculus OX=10090 GN=Xpo1	XPO1	Xpo1	123 kDa	2	3	3	3	11
rRNA 2'-O-methyltransferase fibrillarin OS=Mus musculus OX=10090 GN=Fbl	FBRL	Fbl	34 kDa	2	3	2	4	11
Transcription factor 12 (Fragment) OS=Mus musculus OX=10090 GN=Tcf12	Q3UXQ3	Tcf12	51 kDa	2	4	1	3	10
Carabin OS=Mus musculus OX=10090 GN=Tbc1d10c	TB10C	Tbc1d10c	50 kDa	3	2	3	1	9
Predicted gene 7324 OS=Mus musculus OX=10090 GN=Gm7324	A0A23BRL8	Gm7324	42 kDa	2	3	1	3	9
PBK1 OS=Mus musculus domesticus OX=10092 GN=Rsl1d1	Q1PG84	Rsl1d1	52 kDa	3	1	2	1	7
Coatomer subunit alpha OS=Mus musculus OX=10090 GN=Copa	COPA	Copa	138 kDa	2	2	2	2	8
Uncharacterized protein OS=Mus musculus OX=10090 GN=Trmt112	Q5M8M3	Trmt112	14 kDa	1	3	1	3	8
Transcription factor A, mitochondrial OS=Mus musculus OX=10090 GN=Tfam	A0A0R4J1N9	Tfam	23 kDa	1	2	2	2	7
KH domain-containing, RNA-binding, signal transduction-associated protein 1 OS=Mus musculus OX=10090 GN=Khdrbs1	KHDF1	Khdrbs1	48 kDa	1	2	1	3	7
Uncharacterized protein OS=Mus musculus OX=10090 GN=Nsun2	A0A156GWH4	Nsun2	88 kDa	2	1	2	1	6
tRNA-splicing ligase RtcB homolog OS=Mus musculus OX=10090 GN=Rtcb	Q3TJ01	Rtcb	55 kDa	1	2	1	2	6
ATP-binding cassette sub-family F member 2 OS=Mus musculus OX=10090 GN=Abcf2	ABCF2	Abcf2	72 kDa	1	1	2	1	5
Uncharacterized protein (Fragment) OS=Mus musculus OX=10090 GN=Capn1	Q3TB79	Capn1	80 kDa	1	1	1	2	5
FACT complex subunit SSRP1 (Fragment) OS=Mus musculus OX=10090 GN=Ssrp1	A2AVI05	Ssrp1	72 kDa	1	1	1	2	5
Protein THEMIS OS=Mus musculus OX=10090 GN=Themis	THMI1	Themis	73 kDa	1	1	1	1	4
Tubulin alpha chain (Fragment) OS=Mus musculus OX=10090 GN=Tuba4a	A0A0A0MQA5	Tuba4a	53 kDa	1	1	1	1	4
Protein DEK OS=Mus musculus OX=10090 GN=Dek	DEK	Dek	43 kDa	1	1	1	1	4
Processing of 1, ribonuclease P/MRP family, (S. cerevisiae) OS=Mus musculus OX=10090 GN=Pop1	Q8K2J5	Pop1	117 kDa	1	1	1	1	4
ATP-dependent RNA helicase DDX1 OS=Mus musculus OX=10090 GN=Ddx1	DDX1	Ddx1	83 kDa	1	1	1	1	4

FIGURE 10 | Identification of potential proteins in HEB-containing complexes in ALT-Tg thymocytes. **(A)** Western blot of thymocyte lysates from two ALT-tg mice and one WT littermate immunoprecipitated with anti-HA and probed with anti-phosphotyrosine. The yellow arrow indicates the bands of ~70 kD, corresponding to the size of HEBAlt, that are present in both ALT-Tg samples but not in the WT sample. **(B)** Diagram of experimental design and protocol. **(C)** Identities, molecular weights, and numbers of spectra detected across four replicates for all proteins identified as potential HEBAlt complex partners by the criteria shown in **(B)**.

off of 95% protein identity and 95% peptide identity. Spectra represented in the littermate controls were used to screen out background hits. Twenty-two proteins represented by at least one spectrum all four replicates were identified (**Figure 10C**). HEBAlt was represented in all four samples for a total of 10 spectra but was not found at all in the negative control samples, consistent with IP-mediated enrichment of HEBAlt-HA. The two most enriched proteins were STAT1, a transcription factor that is phosphorylated and activated by JAK, and Xpo1, which is involved in nuclear export. These results suggest that HEBAlt, like STAT1, can be shuttled between the nucleus and the cytoplasm by Xpo1 (44) in response to cytokine signaling, and that HEBAlt and STAT1 may be coordinately regulated.

DISCUSSION

E proteins are essential regulators of T cell development, but the relative roles of HEB and E2A are not well understood. Here, our studies revealed that HEBAlt functions as a hub for integrating cytokine signaling and E protein activity in a restricted cellular context. This restriction occurs at multiple levels, including mRNA expression, protein stability, and post-translational modifications. Breeching these controls by extending HEBAlt expression past the point at which it is normally turned off, or by bypassing the need for signal-dependent phosphorylation, impeded T cell development, exposing a need for strict control of HEBAlt function. Moreover, our results revealed that the unique YYY motif in the Alt domain provides a mechanism for sensing changes in extracellular cues and may provide an axis for the coordinated regulation of E proteins with other JAK-responsive factors, such as STATs. Thus, the downregulation of HEBAlt at the DN to DP transition may be necessary to remove a mode of E protein activity that is beneficial to early T cell precursors but harmful to later stages of thymocyte development.

HEB proteins are essential to inhibit the development of ILCs even in the presence of Notch ligands (10, 45). Since ILCs require IL-7R signaling, it may be that elevating HEBAlt activity would aid in restricting IL-7R expressing precursors from adopting an ILC fate. This would be especially important at the DN2 stage, when most cells express cell surface IL-7R. By contrast, DN3 cells

are more heterogeneous with respect to levels of IL-7R expression (46). In the adult thymus, a gradient of IL-7 exists that is highest near the corticomedullary junction and lowest near the subcapsular zone (SCZ), where β-selection takes place (47, 48). Therefore, decreasing HEBAlt activity may function to allow β-selection once DN3 cells as they move into the outer cortex where concentrations of IL-7 are low. This would be consistent with the observation that HEB protein is degraded in thymocytes undergoing leukemic transformation, resulting in a decrease in Cdkn1a expression and dysregulated proliferation, in both mice and humans (49).

A connection between E proteins, JAK, and Suppressor of Cytokine Signaling (SOCS) has been previously proposed, in which JAK activate STATs to provide a positive input into E2A activity (50). E2A then upregulates SOCS to inhibit cytokine receptor signaling. We propose that HEBAlt provides the missing link between JAK and E protein activity in early T cell precursors. It remains to be seen whether HEBAlt is directly involved in the regulation of SOCS function. Loss of IL-7R signaling may also be required to silence expression of TCRγ regulatory elements that are activated by STATs (51, 52). Therefore, residual HEBAlt function after b-selection may help to enforce the αβ T cell fate.

Our complimentary approaches using co-cultures and mouse models allowed us to analyze a single wave of T cell development *in vitro* and to monitor T cell development at steady state *in vivo*. Progenitors from ALT-Tg mice in OP9-DL4 co-cultures showed a much stronger defect in T cell development than we observed *in vivo*, akin to what has been previously observed for HEB-deficient and TCF1-deficient mice (21, 53). Given our findings, this may be due in part to the movement of progenitors through different niches in the intact thymus, resulting in modulation of IL-7 availability and regulation of signaling environments that contribute to HEB protein stability (49). In ALT-Tg mice, the mild differences observed in the ISP and CD8 SP subsets were not reflected in the subsets representing progress through positive selection in a background with a polyclonal TCR repertoire. Additional studies of ALT-Tg on backgrounds with fixed TCR transgenes would be helpful in further examining the impact of inappropriate HEBAlt expression on thymocyte maturation.

The modulation of HEBAlt protein stability in different cellular contexts was remarkable. First, we observed that HEBAlt protein persists across the β-selection checkpoint into the ISP stage after its mRNA expression was extinguished. Many studies have described signal-dependent inhibition of E protein activity or stability. These signaling pathways include AKT, casein kinase II, Notch1, MAPK, and calmodulin (54–56, 57). However, it has also been shown that TCF1 protects HEB from degradation in DP thymocytes (58). Therefore, interaction with TCF1 may be one mechanism by which HEBAlt protein could be stabilized after its mRNA expression ceases. This would be consistent with our observation that HEBAlt expressed in the thymocytes of ALT-Tg mice are more stable than those in BMK cells.

Secondly, our results showed that HEBAlt protein was undetectable in non-lymphoid cells in the bone marrow and spleens of ALT-Tg mice, despite strong expression of HEBAlt mRNA. This could be due to fact that myeloid cells express high Id2 levels. Id2 undergoes proteasome-mediated degradation (59), and therefore could destabilize associated E proteins. Thus, our transgenic approach revealed another layer of regulation that could inhibit the induction of E protein target genes outside the lymphoid context. We also observed that thymocytes from ALT-Tg mice had much stronger expression of HEBAlt protein than peripheral T and B cells. Therefore, it is likely that a combination of positive and negative inputs regulates HEB stability in different cellular contexts. Moreover, our results indicate that HEBAlt can provide a unique input to the signals that converge on E protein function to control protein stability and activity.

Taken together, we have defined a new mode of E protein control that links cytokine signaling to E protein activity, and which may be essential for the gatekeeping function exhibited by E2A and HEB prior to β-selection. Additional studies will be needed to determine whether YYY phosphorylated HEBAlt binds to the same gatekeeping target genes as HEBCan and E2A, or whether it imposes another type of control by interaction with distinct protein partners downstream of IL-7R such as STATs or PI3K-responsive transcription factors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**. The mass spectrometry datasets generated for this study can be found in the MassIVE Repository, identifier MSV000088631 (HEK293T cells transfected with HA-tagged HEB constructs) and MSV000089563 (HEBAlt-transgenic thymocytes). Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Sunnybrook Research Institute Animal Care Committee.

AUTHOR CONTRIBUTIONS

KY, MA, and JZ-P conceived of and designed the study. KY, AY, JR, AT, LW, and MM generated the data. KY, AT, AY and MA analyzed the data and wrote the manuscript. MA and JZ-P provided funding for the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.848577/full#supplementary-material

REFERENCES

1. Godfrey DI, Kennedy J, Suda T, Zlotnik A. A Developmental Pathway Involving Four Phenotypically and Functionally Distinct Subsets of CD3-CD4-CD8- Triple-Negative Adult Mouse Thymocytes Defined by CD44 and CD25 Expression. *J Immunol* (1993) 150(10):4244–52.
2. Michie AM, Zuniga-Pflucker JC. Regulation of Thymocyte Differentiation: Pre-TCR Signals and Beta-Selection. *Semin Immunol* (2002) 14(5):311–23. doi: 10.1016/S1044-5323(02)00064-7
3. Dutta A, Zhao B, Love PE. New Insights Into TCR Beta-Selection. *Trends Immunol* (2021) 42(8):735–50. doi: 10.1016/j.it.2021.06.005
4. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early Thymocyte Development is Regulated by Modulation of E2A Protein Activity. *J Exp Med* (2001) 194 (6):733–45. doi: 10.1084/jem.194.6.733
5. Jones ME, Zhuang Y. Stage-Specific Functions of E-Proteins at the Beta-Selection and T-Cell Receptor Checkpoints During Thymocyte Development. *Immunol Res* (2011) 49(1-3):202–15. doi: 10.1007/s12026-010-8182-x
6. Lauritsen JP, Wong GW, Lee SY, Lefebvre JM, Ciofani M, Rhodes M, et al. Marked Induction of the Helix-Loop-Helix Protein Id3 Promotes the Gammadelta T Cell Fate and Renders Their Functional Maturation Notch Independent. *Immunity* (2009) 31(4):565–75. doi: 10.1016/j.immuni.2009.07.010
7. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An Essential Role for the Transcription Factor HEB in Thymocyte Survival, Tcra Rearrangement and the Development of Natural Killer T Cells. *Nat Immunol* (2010) 11(3):240–9. doi: 10.1038/ni.1845
8. Rauch KS, Hils M, Lupar E, Minguet S, Sigvardsson M, Rottenberg ME, et al. Id3 Maintains Foxp3 Expression in Regulatory T Cells by Controlling a Transcriptional Network of E47, Spi-B, and SOCS3. *Cell Rep* (2016) 17 (11):2827–36. doi: 10.1016/j.celrep.2016.11.045
9. Miyazaki K, Watanabe H, Yoshikawa G, Chen K, Hidaka R, Aitani Y, et al. The Transcription Factor E2A Activates Multiple Enhancers That Drive Rag Expression in Developing T and B Cells. *Sci Immunol* (2020) 5(51). doi: 10.1126/sciimmunol.abb1455
10. Miyazaki M, Miyazaki K, Chen K, Jin Y, Turner J, Moore AJ, et al. The E-Id Protein Axis Specifies Adaptive Lymphoid Cell Identity and Suppresses Thymic Innate Lymphoid Cell Development. *Immunity* (2017) 46(5):818–34 e4. doi: 10.1016/j.immuni.2017.04.022
11. Ikawa T, Kawamoto H, Goldrath AW, Murre C. E Proteins and Notch Signaling Cooperate to Promote T Cell Lineage Specification and Commitment. *J Exp Med* (2006) 203(5):1329–42. doi: 10.1084/jem.20060268
12. Takeuchi A, Yamasaki S, Takase K, Nakatsu F, Arase H, Onodera M, et al. E2A and HEB Activate the Pre-TCR Alpha Promoter During Immature T Cell Development. *J Immunol* (2001) 167(4):2157–63. doi: 10.4049/jimmunol.167.4.2157
13. Yashiro-Ohtani Y, He Y, Ohtani T, Jones ME, Shestova O, Xu L, et al. Pre-TCR Signaling Inactivates Notch1 Transcription by Antagonizing E2A. *Genes Dev* (2009) 23(14):1665–76. doi: 10.1101/gad.1793709
14. Dutta A, Venkataganesh H, Love PE. Epigenetic Regulation of T Cell Development. *Int Rev Immunol* (2021), 1–9. doi: 10.1080/08830185.2021.2022661
15. Williams CJ, Naito T, Arco PG, Seavitt JR, Cashman SM, De Souza B, et al. The Chromatin Remodeler Mi-2beta Is Required for CD4 Expression and T Cell Development. *Immunity* (2004) 20(6):719–33. doi: 10.1016/j.immuni.2004.05.005
16. O'Neil J, Shank J, Cusson N, Murre C, Kelliher M. TAL1/SCL Induces Leukemia by Inhibiting the Transcriptional Activity of E47/HEB. *Cancer Cell* (2004) 5(6):587–96. doi: 10.1016/j.ccr.2004.05.023
17. Zhang F, Fuss IJ, Yang Z, Strober W. Transcription of RORgammat in Developing Th17 Cells is Regulated by E-Proteins. *Mucosal Immunol* (2014) 7(3):521–32. doi: 10.1038/mi.2013.69
18. Zhang JA, Mortazavi A, Williams BA, Wold BJ, Rothenberg EV. Dynamic Transformations of Genome-Wide Epigenetic Marking and Transcriptional Control Establish T Cell Identity. *Cell* (2012) 149(2):467–82. doi: 10.1016/j.cell.2012.01.056
19. Hosokawa H, Koizumi M, Masuhara K, Romero-Wolf M, Tanaka T, Nakayama T, et al. Stage-Specific Action of Runx1 and GATA3 Controls Silencing of PU.1 Expression in Mouse Pro-T Cells. *J Exp Med* (2021) 218(8): e20202648. doi: 10.1084/jem.20202648
20. In TSH, Trotman-Grant A, Fahl S, Chen ELY, Zarin P, Moore AJ, et al. HEB is Required for the Specification of Fetal IL-17-Producing Gammadelta T Cells. *Nat Commun* (2017) 8(1):2004. doi: 10.1038/s41467-017-02225-5
21. Braunstein M, Anderson MK. Developmental Progression of Fetal HEB(-/-) Precursors to the Pre-T-Cell Stage Is Restored by HEBAlt. *Eur J Immunol* (2010) 40(11):3173–82. doi: 10.1002/eji.201040360
22. Barndt R, Dai MF, Zhuang Y. A Novel Role for HEB Downstream or Parallel to the Pre-TCR Signaling Pathway During Alpha Beta Thymopoiesis. *J Immunol* (1999) 163(6):3331–43.
23. Braunstein M, Anderson MK. HEB in the Spotlight: Transcriptional Regulation of T-Cell Specification, Commitment, and Developmental Plasticity. *Clin Dev Immunol* (2012) 2012:678705. doi: 10.1155/2012/678705
24. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2A Deficiency Leads to Abnormalities in Alphabeta T-Cell Development and to Rapid Development of T-Cell Lymphomas. *Mol Cell Biol* (1997) 17 (8):4782–91. doi: 10.1128/MCB.17.8.4782
25. Bain G, Quong MW, Soloff RS, Hedrick SM, Murre C. Thymocyte Maturation is Regulated by the Activity of the Helix-Loop-Helix Protein, E47. *J Exp Med* (1999) 190(11):1605–16. doi: 10.1084/jem.190.11.1605
26. Wojciechowski J, Lai A, Kondo M, Zhuang Y. E2A and HEB are Required to Block Thymocyte Proliferation Prior to Pre-TCR Expression. *J Immunol* (2007) 178(9):5717–26. doi: 10.4049/jimmunol.178.9.5717
27. Jones ME, Zhuang Y. Acquisition of a Functional T Cell Receptor During T Lymphocyte Development is Enforced by HEB and E2A Transcription Factors. *Immunity* (2007) 27(6):860–70. doi: 10.1016/j.immuni.2007.10.014
28. Wang D, Claus CL, Vaccarelli G, Braunstein M, Schmitt TM, Zuniga-Pflucker JC, et al. The Basic Helix-Loop-Helix Transcription Factor HEBAlt is Expressed in Pro-T Cells and Enhances the Generation of T Cell Precursors. *J Immunol* (2006) 177(1):109–19. doi: 10.4049/jimmunol.177.1.109
29. Rothenberg EV, Moore JE, Yui MA. Launching the T-Cell-Lineage Developmental Programme. *Nat Rev Immunol* (2008) 8(1):9–21. doi: 10.1038/nri2232
30. Bhalla S, Spaulding C, Brumbaugh RL, Zagort DE, Massari ME, Murre C, et al. Differential Roles for the E2A Activation Domains in B Lymphocytes and Macrophages. *J Immunol* (2008) 180(3):1694–703. doi: 10.4049/jimmunol.180.3.1694
31. Massari ME, Jennings PA, Murre C. The AD1 Transactivation Domain of E2A Contains a Highly Conserved Helix Which Is Required for Its Activity in Both Saccharomyces Cerevisiae and Mammalian Cells. *Mol Cell Biol* (1996) 16 (1):121–9. doi: 10.1128/MCB.16.1.121

32. Chen WY, Zhang J, Geng H, Du Z, Nakadai T, Roeder RG. A TAF4 Coactivator Function for E Proteins That Involves Enhanced TFIID Binding. *Genes Dev* (2013) 27(14):1596–609. doi: 10.1101/gad.216192.113
33. Wang D, Claus CL, Rajkumar P, Braunstein M, Moore AJ, Sigvardsson M, et al. Context-Dependent Regulation of Hematopoietic Lineage Choice by HEBAlt. *J Immunol* (2010) 185(7):4109–17. doi: 10.4049/jimmunol.0901783
34. Braunstein M, Rajkumar P, Claus CL, Vaccarelli G, Moore AJ, Wang D, et al. HEBAlt Enhances the T-Cell Potential of Fetal Myeloid-Biased Precursors. *Int Immunol* (2010) 22(12):963–72. doi: 10.1093/intimm/dxq450
35. Moore AJ, Sarmiento J, Mohtashami M, Braunstein M, Zuniga-Pflucker JC, Anderson MK. Transcriptional Priming of Intrathymic Precursors for Dendritic Cell Development. *Development* (2012) 139(2):373–84. doi: 10.1242/dev.069344
36. Mohtashami M, Shah DK, Kianizad K, Awong G, Zuniga-Pflucker JC. Induction of T-Cell Development by Delta-Like 4-Expressing Fibroblasts. *Int Immunol* (2013) 25(10):601–11. doi: 10.1093/intimm/dxt027
37. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical Statistical Model to Estimate the Accuracy of Peptide Identifications Made by MS/MS and Database Search. *Anal Chem* (2002) 74(20):5383–92. doi: 10.1021/ac025747h
38. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A Statistical Model for Identifying Proteins by Tandem Mass Spectrometry. *Anal Chem* (2003) 75 (17):4646–58. doi: 10.1021/ac0341261
39. Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. A Probability-Based Approach for High-Throughput Protein Phosphorylation Analysis and Site Localization. *Nat Biotechnol* (2006) 24(10):1285–92. doi: 10.1038/nbt1240
40. Petersson K, Ivars F, Sigvardsson M. The pT Alpha Promoter and Enhancer are Direct Targets for Transactivation by E Box-Binding Proteins. *Eur J Immunol* (2002) 32(3):911–20. doi: 10.1002/1521-4141(200203)32:3<911::AID-IMMU911>3.0.CO;2-H
41. So EY, Oh J, Jang JY, Kim JH, Lee CE. Ras/Erk Pathway Positively Regulates Jak1/STAT6 Activity and IL-4 Gene Expression in Jurkat T Cells. *Mol Immunol* (2007) 44(13):3416–26. doi: 10.1016/j.molimm.2007.02.022
42. Carleton M, Ruetsch NR, Berger MA, Rhodes M, Kaptik S, Wiest DL. Signals Transduced by CD3epsilon, But Not by Surface Pre-TCR Complexes, are Able to Induce Maturation of an Early Thymic Lymphoma In Vitro. *J Immunol* (1999) 163(5):2576–85.
43. Grajkowska LT, Ceribelli M, Lau CM, Warren ME, Tiniakou I, Nakandakari Higa S, et al. Isoform-Specific Expression and Feedback Regulation of E Protein TCF4 Control Dendritic Cell Lineage Specification. *Immunity* (2017) 46(1):65–77. doi: 10.1016/j.immuni.2016.11.006
44. Xu D, Farmer A, Collett G, Grishin NV, Chook YM. Sequence and Structural Analyses of Nuclear Export Signals in the NESdb Database. *Mol Biol Cell* (2012) 23(18):3677–93. doi: 10.1091/mbc.e12-01-0046
45. Braunstein M, Anderson MK. HEB-Deficient T-Cell Precursors Lose T-Cell Potential and Adopt an Alternative Pathway of Differentiation. *Mol Cell Biol* (2011) 31(5):971–82. doi: 10.1128/MCB.01034-10
46. Boudil A, Matei IR, Shih HY, Bogdanoski G, Yuan JS, Chang SG, et al. IL-7 Coordinates Proliferation, Differentiation and Tcra Recombination During Thymocyte Beta-Selection. *Nat Immunol* (2015) 16(4):397–405. doi: 10.1038/ni.3122
47. Alves NL, Richard-Le Goff O, Huntington ND, Sousa AP, Ribeiro VS, Bordack A, et al. Characterization of the Thymic IL-7 Niche *In Vivo*. *Proc Natl Acad Sci U.S.A.* (2009) 106(5):1512–7. doi: 10.1073/pnas.0809559106
48. Ribeiro AR, Rodrigues PM, Meireles C, Di Santo JP, Alves NL. Thymocyte Selection Regulates the Homeostasis of IL-7-Expressing Thymic Cortical Epithelial Cells *In Vivo*. *J Immunol* (2013) 191(3):1200–9. doi: 10.4049/jimmunol.1203042
49. Veiga DFT, Tremblay M, Gerby B, Herblot S, Haman A, Gendron P, et al. Monoallelic Heb/Tcf12 Deletion Reduces the Requirement for NOTCH1 Hyperactivation in T-Cell Acute Lymphoblastic Leukemia. *Front Immunol* (2022) 13:867443. doi: 10.3389/fimmu.2022.867443
50. Schwartz R, Engel I, Fallahi-Sichani M, Petrie HT, Murre C. Gene Expression Patterns Define Novel Roles for E47 in Cell Cycle Progression, Cytokine-Mediated Signaling, and T Lineage Development. *Proc Natl Acad Sci U S A* (2006) 103(26):9976–81. doi: 10.1073/pnas.0603728103
51. Masui N, Tani-ichi S, Maki K, Ikuta K. Transcriptional Activation of Mouse TCR Jgamma4 Germline Promoter by STAT5. *Mol Immunol* (2008) 45 (3):849–55. doi: 10.1016/j.molimm.2007.06.157
52. Tani-ichi S, Satake M, Ikuta K. Activation of the Mouse TCRgamma Enhancers by STAT5. *Int Immunol* (2009) 21(9):1079–88. doi: 10.1093/intimm/dxp073
53. Weber BN, Chi AW, Chavez A, Yashiro-Ohtani Y, Yang Q, Shestova O, et al. A Critical Role for TCF-1 in T-Lineage Specification and Differentiation. *Nature* (2011) 476(7358):63–8. doi: 10.1038/nature10279
54. Teachenor R, Beck K, Wright LY, Shen Z, Briggs SP, Murre C. Biochemical and Phosphoproteomic Analysis of the Helix-Loop-Helix Protein E47. *Mol Cell Biol* (2012) 32(9):1671–82. doi: 10.1128/MCB.06452-11
55. Johnson SE, Wang X, Hardy S, Taparowsky EJ, Konieczny SF. Casein Kinase II Increases the Transcriptional Activities of MRF4 and MyoD Independently of Their Direct Phosphorylation. *Mol Cell Biol* (1996) 16(4):1604–13. doi: 10.1128/MCB.16.4.1604
56. Nie L, Xu M, Vladimirova A, Sun XH. Notch-Induced E2A Ubiquitination and Degradation are Controlled by MAP Kinase Activities. *EMBO J* (2003) 22 (21):5780–92. doi: 10.1093/emboj/cdg567
57. Saarikettu J, Sveshnikova N, Grundstrom T. Calcium/calmodulin Inhibition of Transcriptional Activity of E-Proteins by Prevention of Their Binding to DNA. *J Biol Chem* (2004) 279(39):41004–11. doi: 10.1074/jbc.M408120200
58. Emmanuel AO, Arnovitz S, Haghi L, Mathur PS, Mondal S, Quandt J, et al. TCF-1 and HEB Cooperate to Establish the Epigenetic and Transcription Profiles of CD4(+)CD8(+) Thymocytes. *Nat Immunol* (2018) 19(12):1366–78. doi: 10.1038/s41590-018-0254-4
59. Sullivan JM, Havrda MC, Kettenbach AN, Paolella BR, Zhang Z, Gerber SA, et al. Phosphorylation Regulates Id2 Degradation and Mediates the Proliferation of Neural Precursor Cells. *Stem Cells* (2016) 34(5):1321–31. doi: 10.1002/stem.2291

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Barbara L. Kee,
The University of Chicago,
United States

REVIEWED BY
Ivan Maillard,
University of Pennsylvania,
United States
Ana Cumano,
Institut Pasteur, France

*CORRESPONDENCE
Jonathan R. Keller
Kellerjo@mail.nih.gov

†These authors have contributed equally to this work

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Inhibitor of DNA binding proteins revealed as orchestrators of steady state, stress and malignant hematopoiesis

Shweta Singh[1†], Tanmoy Sarkar[1†], Brad Jakubison[1,2], Stephen Gadomski[1], Andrew Spradlin[1], Kristbjorn O. Gudmundsson[1,2] and Jonathan R. Keller[1,2*]

[1]Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute (NCI)-Frederick, Frederick, MD, United States, [2]Basic Science Program, Frederick National Laboratory for Cancer Research, Frederick, MD, United States

Adult mammalian hematopoiesis is a dynamic cellular process that provides a continuous supply of myeloid, lymphoid, erythroid/megakaryocyte cells for host survival. This process is sustained by regulating hematopoietic stem cells (HSCs) quiescence, proliferation and activation under homeostasis and stress, and regulating the proliferation and differentiation of downstream multipotent progenitor (MPP) and more committed progenitor cells. Inhibitor of DNA binding (ID) proteins are small helix-loop-helix (HLH) proteins that lack a basic (b) DNA binding domain present in other family members, and function as dominant-negative regulators of other bHLH proteins (E proteins) by inhibiting their transcriptional activity. ID proteins are required for normal T cell, B cell, NK and innate lymphoid cells, dendritic cell, and myeloid cell differentiation and development. However, recent evidence suggests that ID proteins are important regulators of normal and leukemic hematopoietic stem and progenitor cells (HSPCs). This chapter will review our current understanding of the function of ID proteins in HSPC development and highlight future areas of scientific investigation.

KEYWORDS

ID proteins, hematopoiesis, stem cells, quiescence, stress

Introduction

Hematopoiesis is sustained by a limited number of hematopoietic stem cells (HSCs) that reside in a quiescent state protected from proliferation-induced damage and exhaustion (1, 2). HSCs proliferate and differentiate to give rise to multipotent progenitor (MPP) cells with limited self-renewal potential that give rise to more

restricted progenitor cells to maintain normal numbers of differentiated blood cells (3, 4). Recent evidence suggests that HSCs have a limited number of self-renewing divisions indicating that HSC quiescence and proliferation is a tightly regulated process that protects the host from HSC exhaustion and hematopoietic failure (5, 6). While HSCs are one of the best characterized vertebrate stem cells, a significant effort is still focused on understanding the molecular and cellular mechanisms that regulate HSC quiescence, self-renewal and differentiation to 1) improve methods to expand adult HSCs and their differentiated progeny for bone marrow transplantation (BMT) and cell therapies, 2) identify molecular pathways that direct adult HSC development and expansion from pluripotential stem cells, 3) improve gene editing technology in hematopoietic stem and progenitor cells (HSPCs), and 4) develop new methods to detect, prevent and treat hematologic malignancies.

Inhibitor of DNA binding proteins (ID1-4) are members of the helix-loop-helix (HLH) family of proteins that regulate cell proliferation and differentiation (7–10). ID proteins function as dominant negative regulators of other HLH proteins (E proteins) by inhibiting their DNA binding and transcriptional activity, which is essential for the differentiation and proliferation of normal tissues including muscle, nerve, lymphoid, and embryonic stem cells (11–14). ID proteins also inhibit the transcriptional activity of erythroblast transformation specific (ETS), retinoblastoma (RB), and paired box proteins (PAX) proteins, which affect cell growth and differentiation (15–17). ID proteins are critical transcriptional regulators of hematopoietic cell differentiation, and are required for the proper development of T cells, B cells, dendritic and NK and innate lymphoid cells (13, 14, 18–23). ID proteins have emerged as critical regulators of HSPC quiescence, proliferation and differentiation under homeostasis, inflammatory and genotoxic stress, and aging. These findings suggest that ID proteins could have therapeutic potential to treat myeloid proliferative neoplasia's (MPN), myelodysplastic syndromes (MDS), and clonal hematopoiesis. Therefore, this prospective will review data related to the function of *Id* genes in regulating normal HSPC quiescence and differentiation, and their roles in hematopoietic stress and hematologic malignancies.

Id gene expression in hematopoietic cells

Id gene expression in hematopoietic cells was first reported in the murine erythroleukemia cell line, MEL cells, and its expression was shown to decrease during erythroid differentiation (11, 24–26). Subsequently, *Id1* was detected in an interleukin-3 (IL-3) dependent myeloid progenitor cell line, 32Dcl3 cells, which can be induced to differentiate into neutrophils when cultured in media containing granulocyte-colony stimulating factor (G-CSF) (27). The expression of *Id1* decreased during G-CSF-induced differentiation of 32Dcl3 cells, while the binding of bHLH proteins to a canonical E-box motif increased. Furthermore, enforced expression of *Id1* blocked G-CSF-induced differentiation of 32Dcl3 cells. Taken together, these results suggested that ID1 may function during myeloid cell differentiation by disrupting bHLH protein function, the canonical target of ID proteins. Subsequent studies demonstrated that IL-3 and other myeloid growth factors (G-CSF and granulocyte/macrophage CSF, GM-CSF) induce *Id1* expression and cell proliferation in other IL-3-dependent progenitor cell lines including NFS-60 and FDC-P1 cells, while Id2 expression increases upon withdrawal of IL-3 and cell cycle arrest or differentiation (28–30). The HSC/MPP-like stem cell factor (SCF) -dependent progenitor cell line, EML cells, express ID2 but not ID1, while more committed IL-3-dependent progenitors cell lines derived from EML cells express ID1 but not ID2, suggesting ID2 may function in more primitive hematopoietic progenitor cells. Taken together, these studies demonstrated that ID1 is correlated with increased proliferation/growth and decreased differentiation of hematopoietic progenitor cell (HPC) lines, which is consistent with results from previous studies in other tissues suggesting that ID1 promotes cell proliferation and inhibits cellular differentiation (7, 9, 10, 31, 32). In addition, these studies suggest that ID2 may have functions distinct from ID1 in HSPCs.

Id1 gene expression was increased in normal murine progenitor cells during the early proliferative phase of colony formation in soft agar assays stimulated by myeloid growth factors (IL-3/SCF/IL-1/erythropoietin, EPO), while *Id2* was induced to a lesser extent and no effect on *Id3 or Id4* expression was observed in these assays (29). In comparison, *Id2* levels were increased and *Id1* levels decreased in cells from colonies that contained differentiated progeny after 7-10 days. Thus, *Id1* is expressed in normal proliferating HPCs, while *Id2* expression increases and *Id1* levels decrease as cells exit the cell cycle and differentiate into myeloid cells. Subsequent studies confirmed that myeloid hematopoietic growth factors (HGFs) (SCF, IL-3 and GM-CSF) but not lymphoid HGFs (IL-7) and erythroid/megakaryocyte HGFs (EPO/thrombopoietin, TPO) induce *Id1* expression in purified lineage-negative, Lin-/Sca-1+/c-Kit+ (LSK) BMCs that are enriched for HSCs/MPPs, suggesting that *Id1* may be required for myeloid development, but not lymphoid or erythroid development (33, 34). Analysis of *Id* gene expression in purified HSPC populations showed low levels of *Id1* expression in LSK cells and clonogenic lymphoid progenitors (CLPs), while *Id1* expression was increased in

common myeloid progenitors (CMP), and further increased in more differentiated granulocyte/macrophage progenitors (GMPs), but not in megakaryocyte/erythroid progenitors (MEP), indicating a potential role for *Id1* in myeloid cell development (Figure 1) (33–35). Subsequent analysis of purified HSCs demonstrated that roughly 5-10% of normal HSCs defined as LSK/Flk2-/CD150+/CD48- express *Id1* during steady state hematopoiesis, and that myeloid HGFs induce *Id1* in HSCs (36, 37). While the expression of *Id1* increases in committed myeloid progenitor cells (CMP and GMP), the expression of *Id1* decreases during the final stages of neutrophil maturation. Specifically, FACS purified normal neutrophils express low levels of ID1 protein compared to Lin- cells that are comprised of LSK cells and more committed Lin-/cKit+/Sca-1- (LK) progenitor cells that include CMPs/GMPs (33). ID1 expression decreases during G-CSF- and GM-CSF-induced neutrophil differentiation of myeloid progenitor cell lines, 32Dcl3 and MPRO, respectively (27, 33). In contrast, mature neutrophils in $Id1^{EGFP}$ reporter mice show high levels of ID1/EGFP expression compared to CMP's and GMP's, suggesting that neutrophils express high levels of ID1 (34, 36). However, EGFP might not accurately reflect ID1 protein levels in neutrophils since ID proteins are rapidly degraded and the stability of EGFP and Id1 may differ significantly in these terminally differentiated cells. Future studies are needed to define the precise expression and function of ID1 and other ID genes in maturing neutrophils and macrophages.

ID2 expression is 3-fold higher than *ID1* and 6.5-fold higher than *ID3* in purified human cord blood (CB) HSCs suggesting that human HSCs express high levels of ID2 (38). Little or no expression of ID1 was detected in purified CD34+38- human bone marrow HSPCs, but was induced by myeloid HGFs including GM-CSF/IL-3, but not SCF/EPO (erythroid) and SCF/TPO (megakaryocytes) HGFs (39). Thus, ID1 is expressed at low levels in human HSPCs and is induced by myeloid HGFs, and is consistent with results obtained with murine bone marrow cells (BMCs). Similarly, ID1 protein expression is induced during the early proliferative phase of normal human neutrophil/eosinophil differentiation in culture; after which, ID1 expression rapidly declines, while ID2 steadily increases (40). Furthermore, gain and loss of function studies showed that ID2 expression promotes eosinophil/neutrophil differentiation, and ID1 expression promotes neutrophil differentiation of human CD34+ progenitor cells *in vitro* and *in vivo* (40). Collectively, these results demonstrate that ID1 functions during the early proliferative stages of myeloid development, while ID2 may be required for the final stages of myeloid cell development. Further studies are needed to more precisely define, 1) the cell-specific and temporal expression of *Id1* and *Id2*, 2) how *Id1* and *Id2* gene expression is regulated, and 3) their molecular mechanism(s) of

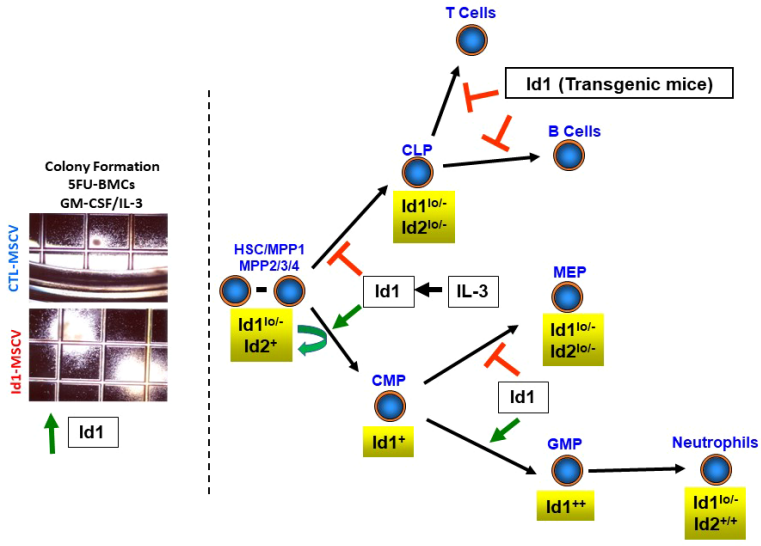

FIGURE 1
Summary of Id gene expression in hematopoietic stem and progenitor cells. Levels of *Id* gene expression are shown for hematopoietic stem cells (HSCs), multipotent progenitor (MPP) cells, clonogenic lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitor cells. Photomicrographs of BMCs harvested from mice three days after the administration of 5-FU, transduced with control and *Id1* expressing retroviral vectors and cultured in methycellulose for 7-10 days (colony formation assay).

action during myeloid cell differentiation in normal human and murine cells.

Insights into *Id* gene function in hematopoietic stem and progenitor cells through the lens of enforced expression

Initial studies to define the physiological function of *Id* genes in hematopoietic cells *in vivo* demonstrated that transgenic mice overexpressing *Id1* or *Id2* during T cell differentiation manifest a severe block in T cell development (Figure 1) (41, 42). Similarly, transgenic mice that overexpress *Id1* during B cell differentiation show impaired B cell development (43). These studies are consistent with the strict requirement for E2A homodimers in B cell development and E2A homo and heterodimers in T cell development, and the dominant negative regulation of E protein function by ID proteins (20, 44).

To study *Id* gene function in HSPCs, *Id* genes were overexpressed in normal murine HSPCs using retroviral vectors and analyzed for growth and differentiation *in vitro* (33). Enforced expression of *Id1* in HSPCs resulted in a significant increase in the size and number of colonies in soft agar in the presence of SCF, IL-3 and GM-CSF (Figure 1) (39). Furthermore, these colonies contained increased numbers of primitive and differentiating myeloid cells, suggesting that *Id1* promotes myeloid cell hyperplasia without blocking differentiation *in vitro*. However, immortalized hematopoietic progenitor cell (HPC) lines that escape normal senescence and differentiation could be readily derived from BMCs overexpressing *Id1* in liquid culture containing SCF (39). The HPC lines resembled CMP-like progenitors by immunophenotype and lineage specific gene expression, and continued to divide in SCF; however, these cells were not arrested in differentiation and retained the ability to differentiate in response to G-CSF and GM-CSF *in vitro*. In other studies, overexpression of *Id1* in purified LSK cells inhibited B cell development and promoted myeloid/dendritic cell development in cultures supplemented with SCF/FLT3/IL-7 (34). Furthermore, LSK/Flk2- and lymphoid-primed multipotent progenitor (LMPP) cells isolated from mice that overexpress a dominant form of E protein (ET2) that prevents ID protein binding, showed increased B cell, and reduced myeloid cell development when cultured *in vitro*. Collectively these studies demonstrate that *Id1* promotes the proliferation of murine HSPCs and myeloid development, while inhibiting lymphoid development *in vitro*.

Id1-overexpressing HSPCs showed increased myeloid and decreased B cell repopulation potential when transplanted into γ-irradiated (IR) recipients *in vivo* (Figure 1) (33, 39). In addition, thymocyte repopulation was inhibited during the early stages of T cell development after transplantation; however, no effect on T cell repopulation was observed in PBCs 4-6 months after BMT. A comparison of Id1/EGFP expression in repopulating T cells and myeloid cells in the same mouse by flow cytometry showed a significant reduction in Id1/EGFP expression in T cells compared to myeloid cells. Thus, it was reasoned that T cells expressing high levels of *Id1* do not repopulate, while low levels of *Id1* expression are permissive for T cell development *in vivo*, and account for the recovered T cell development over time. Historically, murine BMCs cultured in HGFs cocktails that included IL-3 to promote HSC cycling and increase retroviral vector transduction, show significantly reduced lymphoid repopulation when transplanted *in vivo*. It is tempting to speculate that inhibition of lymphoid cell repopulation in these BMT experiments was due to the induction of *Id1* by IL-3. In this regard, HGFs that promote *Id1* expression and inhibit lymphoid development of HSCs should be avoided in HSC expansion media. Finally, mice transplanted with *Id1*-overexpressing HSPCs become moribund within a year after transplantation and develop a myeloid proliferative neoplasia (MPN) including 1) increased myeloid blasts in BM, 2) splenomegaly and extramedullary hematopoiesis (myeloid and erythroid), and 3) myeloid cell infiltration in liver, which does not progress to acute myeloid leukemia (AML) (39). Collectively, enforced expression of *Id1* regulates HSPC fate by promoting myeloid and inhibiting lymphoid development *in vitro* and *in vivo*, and *Id1* can promote HSPC proliferation and immortalize HSPCs *in vitro*, and promote a myeloproliferative disease *in vivo*.

HPC lines were also derived from murine BMCs transduced with lentiviral vectors expressing inducible *Id2* (Id2-HPCs), which were established in conditions that support primitive B cell growth including SCF, FLT-3 and IL-7 on S17 stromal cells (45). The Id2-HPC lines express c-Kit, CD43 and low levels of B220, but lack the expression of other cell surface markers expressed on more mature myeloid and lymphoid lineage cells. Id2-HPC lines are multipotential and differentiate into myeloid cells in the presence of IL-3, GM-CSF, and M-CSF *in vitro* and *in vivo*, under conditions that maintain *Id2* expression. Id2-HPCs differentiate into lymphoid cells (B cell and T cell) upon withdrawal of ID2 *in vitro* and *in vivo* consistent with the ability of ID2 to inhibit E protein function. The lymphoid potential of the Id2-HPC lines was lost with cell passaging suggesting that the Id2-HPC lines may undergo further myeloid differentiation and commitment *in vitro*. It is possible that the Id2-HPC lines that have lost lymphoid potential may resemble the SCF-dependent CMP-like HPC lines that overexpress *Id1* (39). The Id2-HPC line transcriptome resembles the transcriptome of the previously described *E2a*$^{-/-}$ multipotential cell lines (pre-pro-B cells) that were established in SCF, FLT-3, IL-7 and stromal cells (45), which is consistent with the ability of ID2 to inhibit the function of E2A and prevent lymphoid development. In comparison, over expression of *Id2* in

human cord blood CD34+ HSPCs resulted in expansion of CD34+/CD38-/CD90+/CD45RA- HSCs, and a skewing toward myeloid (CMP/MEP) versus lymphoid (B/NK/GMP) by immunophenotype analysis *in vitro*, and reduced lymphoid development when transplanted into NSG mice (38). HSPCs isolated from NSG mice transplanted with ID2-overexpressing CB-HSPCs show increased expression of stem cell genes and genes expressed in primitive myeloid differentiation programs, and decreased expression of B cell factors, *EBF1* and *FOXO1*, which are E2A target genes (46). ID2-mediated expansion of HSCs was rescued by overexpressing E2A *in vitro*, and overexpression of E2A promoted lymphoid development *in vivo*. Knock down of the *E2A* target gene, *EBF1*, which is required for lymphoid development, resulted in an increase in HSC numbers. These results suggested that ID2 expands or maintains HSC numbers by inhibiting lymphoid lineage priming, which leads to a reduction in HSC numbers (38). Taken together, overexpression of *ID2* expands HSCs and restrains B cell development in human HSPCs. These studies suggest that ID2 may function to regulate human and mouse HSCs function *in vivo*.

Hematopoietic progenitor cell lines were also established by overexpressing *Id3* in fetal liver cells and BMC cultures containing SCF, FLT-3, IL-7, and stromal cells, and resemble pre-pro B cells (Id3-HPC lines) (47). Id3-HPC lines are multipotential and retain myeloid, B cell and T cell potential *in vitro* and *in vivo*. The lymphoid potential of Id3-HPC lines was induced by down regulation of *Id3* expression in developing B cells *in vivo*, supporting previous studies that high levels of *Id3* impair B and T cell development (48–51). Secondary transplantation of BMCs from primary BMT recipients that received Id3-HPCs showed residual myeloid reconstitution, but no lymphoid reconstitution suggesting that Id3-HPCs are not HSC/MPP-like cells (47). Id3-HPC lines could not be established from human CB HSPCs; however, *Id3* overexpressing CB HSPCs cells showed enhanced proliferation *in vitro* and limited repopulation potential in NSG mice. Additional experiments are required to further understand the mechanism(s) of *Id3*-mediated immortalization in murine and human HSPCs compared to *Id1* and *Id2* immortalization.

Hematopoietic phenotypes in mouse models of *Id* gene loss of function

Conventional *Id1*$^{-/-}$ mice

The first *Id1*- deficient (*Id1*$^{-/-}$) mouse model was generated by gene targeting, which replaced the first exon and part of the promoter of *Id1* with a neomycin resistance gene cassette. Conventional *Id1*$^{-/-}$ mice were born at normal mendelian frequencies, were fertile and showed normal lifespan with no overt abnormalities (52). No significant difference in the number of mature hematopoietic cells was reported in PBCs, BMCs and spleen cells of *Id1*$^{-/-}$ mice, suggesting that *Id1* is not required for normal hematopoietic development. However, a more detailed analysis of these mice revealed that *Id1*$^{-/-}$ mice have impaired hematopoietic development including 1) increased myeloid cells and decreased lymphoid cells in PBCs, 2) decreased BM cellularity, 3) decreased numbers of LSK cells, 4) decreased numbers of LSK/CD34-/Flk2- cells enriched for HSCs, 5) increased cycling of LSK cells, and 6) increased proliferation and differentiation of LSK cells *in vitro* (35). No difference in the repopulation potential of *Id1*$^{-/-}$ and *Id1*$^{+/+}$ BMCs was observed in primary BMT recipients; however, *Id1*$^{-/-}$ BMCs showed impaired secondary repopulation ability albeit with low statistical significance, suggesting that *Id1*$^{-/-}$ HSCs have a defect in self-renewal. Collectively, these investigators concluded that *Id1*$^{-/-}$ HSCs show increased cycling and increased myeloid commitment, which resulted in decreased HSC self-renewal in secondary BMT recipients, suggesting that *Id1* is required to maintain HSCs.

A second *Id1*$^{-/-}$ mouse model was generated by inserting the EGFP coding sequence downstream of the Id1 transcriptional start site, which results in an *Id1* null allele (*Id1*$^{EGFP/EGFP}$) (37). In comparison to the Jankovic et al. *Id1*$^{-/-}$ mouse model described above, the hematopoietic phenotypes observed in *Id1*$^{EGFP/EGFP}$ mice included decreased numbers of HSCs (LSK/CD150+/CD48-); however, this report showed no effect on the cycling of HSC enriched populations, no difference in the development of mature lymphoid and myeloid cell populations in BMCs or PBCs. *Id1*$^{EGFP/EGFP}$ mice showed no difference in repopulation in primary BMT recipients, but reduced secondary repopulation potential suggesting that *Id1* was required for HSC development and maintenance. However, it should be noted that the secondary BMT was performed 16 days after primary BMT, which is a significant deviation from the typical secondary BMT protocol, which is usually performed 10-16 weeks after primary BMT, when hematopoiesis resembles more steady-state conditions (37). Therefore, it is difficult to conclude from these studies if *Id1* is required for HSC self-renewal.

A third study also analyzed hematopoietic development in the *Id1*$^{-/-}$ mouse model used in the Jankovic et al. study discussed above (35) and confirmed that 1) myeloid cells were increased and lymphoid cells decreased in BMCs and PBCs, 2) BM cellularity was decreased, and 3) LSK cycling was increased (53). This study showed no difference in the number of HSC-enriched cells (LSK/CD34- cells) and HSC function *in vivo*; however, the two studies used different cell surface antigens to immunophenotype the HSCs, which could explain the differences in HSC numbers. Finally, the two studies agreed and showed no difference in HSC repopulation potential in primary BMT recipient mice and differed in serial repopulation

potential leaving open the question whether *Id1* functions in HSC self-renewal.

Based on the known function of *Id1* in other cellular contexts, it might be predicted that loss of *Id1* would lead to reduced cell cycling and increased B cell development in the hematopoietic compartment of $Id1^{-/-}$ mice. However, $Id1^{-/-}$ mice show the opposite hematopoietic phenotypes including increased LSK cycling and proliferation, and decreased B cell development (35, 53). Since *Id1* expression is ablated in all cells in conventional $Id1^{-/-}$ mice, and *Id* genes are widely expressed in other tissues including endothelial cells (ECs) and skeletal stem cells (SSCs) and their progeny, which are cellular constituents of the hematopoietic microenvironment (HME) (8, 54–57), it is possible that loss of *Id1* function in the HME could contribute to the hematopoietic phenotypes observed in conventional $Id1^{-/-}$. Therefore, in the third study, $Id1^{+/+}$ and $Id1^{-/-}$ BMCs were transplanted into γ-IR $Id1^{-/-}$ or $Id1^{+/+}$ recipient mice and monitored for hematopoietic development (53). $Id1^{+/+}$ BMCs transplanted into $Id1^{-/-}$ recipients showed impaired hematopoietic development similar to the hematopoietic phenotypes observed in the conventional $Id1^{-/-}$ mice including decreased BM cellularity, increased myeloid and decreased erythroid development. Importantly, $Id1^{-/-}$ BMCs showed normal hematopoietic development when transplanted into $Id1^{+/+}$ recipient mice. These results were confirmed in a recent study using a mouse model that lacked *Id1* and *Id3* expression in the HME, since HME phenotype was less severe in mice on a pure C57BL/6 background and *Id3* can compensate for loss of *Id1* in some models (58). Transplantation of normal BMCs into γ-IR $Id1^{-/-}$;$Id3^{-/-}$ recipient mice showed a significant decrease in BM cellularity, decreased B cell development, increased HSC cycling and decreased HSC numbers (56). Finally, $Id1^{-/-}$ stromal cells show altered cytokine production *in vitro*, and cytokine levels were deregulated in conventional $Id1^{-/-}$ mice *in vivo*. Collectively, these results demonstrated that *Id1* is required for the proper function of the HME, and that the hematopoietic phenotypes observed in conventional $Id1^{-/-}$ mice could, in part, be explained by the loss of *Id1* function in the HME.

Conditional loss of *Id1* in endothelial cells

Endothelial cells are critical cellular components of the HME and are required to maintain steady state hematopoiesis (59–63). SECs are critical cellular components of the HME and are required to maintain steady state hematopoiesis, in part, through angiocrine signaling (64, 65). Lineage tracing studies using vascular endothelial cadherin (VE-Cad) transgenic mice *Cdh5(PAC)-CreERT2* bred to *Rosa26-mT/mG* mice showed that transition endothelial vessels (H vessels), proliferate to regenerate diaphyseal sinusoidal ECs (SECs) within forty days under homeostasis (66). Recent evidence suggest that *Id* genes promote EC proliferation and vessel regeneration under stress (67–69); thus, it was hypothesized that *Id* genes may be required for proper HME function by maintaining ECs under steady state conditions and stress (56). Therefore, *Cdh5(PAC)-CreERT2* mice were bred to $Id1^{F/F}$;$Id3^{-/-}$ mice to specifically ablate *Id1* and *Id3* expression in ECs, since *Id1* and *Id3* are required for proper HME in mice on a pure C57/BL6 background. Loss of sinusoidal integrity was observed in $Id1^{-/-}$; $Id3^{-/-}$ mice characterized by dilated, leaky, and apoptotic BM SECs that increased in severity over time (56). The proliferation of $Id1^{-/-}$; $Id3^{-/-}$ SECs, and transition endothelial vessels was significantly reduced *in vitro* and *in vivo*, leading to impaired vascular integrity under steady state conditions, which was more severe following acute stress. The disruption in sinusoidal integrity and neovascularization in $Id1^{-/-}$;$Id3^{-/-}$ mice led to a progressive decline in hematopoiesis, marked by increased HSC activation, proliferation, differentiation, migration, and exhaustion. Thus, *Id1* and *Id3* are required for the survival and steady state regeneration of BM SECs, which provide a supportive niche for HSC quiescence and survival. Future studies are needed to examine if *Id* genes regulate other cells in the HME including subtypes of SSCs (Leptin-cre+, Nestin-ER-cre+, and NG2-cre+), which functionally support hematopoiesis, and their downstream progeny including osteoblasts and chondrocytes (60, 63, 70). Future studies are needed to examine Id gene function in the neural niche, since nerve fibers such as adrenergic and cholinergic nerves are instructive for hematopoietic mobilization and quiescence respectively.

Conventional $Id3^{-/-}$ mice

$Id3^{-/-}$ mice are born at normal mendelian frequencies, are fertile, and young mice show no overt phenotypes. $Id3^{-/-}$ mice have normal numbers of developing B cells, but show impaired humoral immunity, B-cell proliferation, and develop a unique autoimmune disease, Sjogren's syndrome, and γδ-T cell hyperplasia with age (49, 50, 71, 72). In addition, $Id3^{-/-}$ mice show severely impaired positive and negative thymocyte selection (50). Transplantation of $Id3^{-/-}$ BMCs into γ-IR mice show normal myeloid and B cell repopulation, but impaired T cell repopulation in secondary recipient mice, suggesting that $Id3^{-/-}$ is not required for HSC maintenance (37). However, further studies are needed to examine if *Id3* is required for HSC self-renewal in serial BMT assays separated by 10-12 weeks (37). In other studies, no difference in donor B cells, T cells, neutrophils, HSCs and MPP repopulation were observed in mice 12 weeks after competitive BMT of $Id1^{-/-}Id3^{-/-}$ BMCs compared to controls, confirming that *Id3* is not required for HSC repopulation of primary BMT recipient mice; however, HSC self-renewal was not evaluated in these assays (56). Therefore, additional studies are needed to examine the requirement of *Id3* in HSC development.

Conventional *Id2*$^{-/-}$ mice

The majority of conventional *Id2*$^{-/-}$ mice show perinatal lethality and are born at less than ten percent of the normal mendelian frequencies. The surviving mice lack Langerhans cells, splenic dendritic cells, and NK cells, and show absence of lymph nodes and Peyer's patches, which demonstrates the requirement for ID2 in the development of these cells (18, 22). Surviving *Id2*$^{-/-}$ mice also show increased B cell development, decreased erythroid cells and no effect on myeloid cell development (73). Gain and loss of *Id2* function studies in normal HSPCs confirmed that *Id2* intrinsically inhibits B cell development *in vivo* by negatively regulating E2A. In addition, these studies showed that ID2 binds to PU.1 and interferes with PU.1's ability to inhibit GATA-1 transcriptional activity, suggesting a potential mechanism of action for how ID2 promotes erythroid development (73). Additional experiments are needed to further explore this mechanism of action, and more precisely define the progenitors that express *Id2*, and where cell fate is determined and how the expression of *Id2* is regulated. In this regard, *Gfi-1* has been identified as a direct transcriptional repressor of *Id2*, and high levels of *Id2* are expressed in *Gfi-1*$^{-/-}$ BMCs, and these mice show defects in B cell, T cell, and neutrophil development (74, 75). In addition, these mice have impaired short term reconstituting cell (STRC) activity or ability to radio-protect lethally irradiated recipient mice, and have significantly reduced numbers of HSCs (74–76). Reducing *Id2* levels in *Gfi-1*$^{-/-}$ mice (*Gfi-1*$^{-/-}$;*Id2*$^{+/-}$ mice) partially restores B cell development by overcoming the block in B cell development at the pro-B cell stage, and reduces myeloid hyperplasia, but does not rescue T cell development. These results provide evidence for a direct link between *Gfi-1* and the B cell transcriptional network *via Id2*, which inhibits E2A function required for B cell development (76). While reducing the levels of *Id2* in *Gfi-1*$^{-/-}$ mice rescued the myeloid hyperplasia in the spleen, it did not rescue neutrophil differentiation. Additional studies are needed to uncover how *Id2* promotes myeloid expansion in *Gfi-1*$^{-/-}$ mice and why normal myeloid development is not restored. Finally, reducing *Id2* levels in *Gfi-1*$^{-/-}$ mice partially restores the number of STRCs, CMP and MEP progenitors and differentiating erythroid cells in the BM bone marrow, which is sufficient to radio-protect lethally irradiated BMT recipient mice (77). Increased red cell production in *Gfi-1*$^{-/-}$;*Id2*$^{+/-}$ mice was correlated with increased expression of *Gata1*, *Eklf* and *EpoR*, which are required for erythroid development. It was proposed that ID2 inhibits E2A/Scl complexes that regulate erythroid gene expression *via* a multiprotein transcription factor complex that binds paired E-box/GATA sites in the promoters of *Gata1, Eklf and EpoR*. However; the precise molecular mechanism(s) of *Id2* action that rescue the erythroid lineage in *Gfi-1*$^{-/-}$ mice remain to be defined. Finally, reducing *Id2* levels in *Gfi-1*$^{-/-}$ mice does not increase HSC numbers and rescue the defect in HSC function in competitive repopulation assays indicating that other genes mediate HSC loss in *Gfi-1*$^{-/-}$ mice. Collectively, these observations suggest that *Id2* regulates HSPC fate at multiple cellular levels, but leaves open the question whether *Id2* regulates HSC development.

Conditional *Id2*$^{-/-}$ mice

Few conventional *Id2*$^{-/-}$ mice survive beyond birth suggesting that the surviving *Id2*$^{-/-}$ mice may have compensated in some way to promote the survival of *Id2*$^{-/-}$ mice. Therefore, the intrinsic requirement of *Id2* in HSCs was evaluated in conditional *Id2*$^{F/F}$ mice. Specifically, BMCs from *Mx1-cre;Id2*$^{F/F}$ mice (78) were transplanted in γ-IR recipient mice (chimeric mice) to reconstitute the host hematopoietic system and eliminate any contribution that loss of *Id2* function might have in the HME. Chimeric mice were treated with *pIpC* to induce interferon production and ablate *Id2* expression in hematopoietic cells six weeks after BMT, and then examined for hematopoietic development after ten weeks. Chimeric mice showed a significant reduction in the total number immunophenotypic HSCs, and decreased donor reconstitution of competitively transplanted primary recipient mice confirming that *Id2* is required to maintain HSCs in chimeric mice (Figure 2). Furthermore, *Id2*$^{-/-}$ chimeric mice showed reduced overall survival due to anemia and BM failure compared to *Id2*$^{+/+}$ chimeric mice indicating that ID2 is intrinsically required for HSC maintenance. Mechanistically, *Id2*$^{-/-}$ HSCs showed increased proliferation and cycling, mitochondrial activation, reactive oxygen species (ROS) production and differentiation *in vitro* and *in vivo*. Pathway analysis of differentially expressed genes in *Id2*$^{+/+}$ and *Id2*$^{-/-}$ HSCs revealed increased expression of genes that regulate cellular proliferation and genes that regulate oxidative phosphorylation in *Id2*$^{-/-}$ HSCs compared to *Id2*$^{+/+}$ HSCs. In addition, gene set expression analysis (GSEA) and pathway analysis revealed that HIF-1α target genes were decreased *Id2*$^{-/-}$ HSCs suggesting that ID2 might affect the levels or function of HIF-1α. In this regard, *HIF-1α*$^{-/-}$ mice show similar hematopoietic phenotypes with *Id2*$^{-/-}$ mice including increased cycling, decreased quiescence, and increased susceptibility to 5-FU treatment (79). HIF-1α protein levels were reduced in purified *Id2*$^{-/-}$ HSCs, and loss of HSC function in *Id2*$^{-/-}$ mice could be restored by chemically stabilizing HIF-1α and overexpression of stabilized HIF-1α *in vitro* and *in vivo*. Mechanistically, ID2 stabilizes HIF-1α by binding to VHL and interfering with HIF-1α ubiquitination and proteasomal degradation (Figure 2). Collectively, ID2 is required to maintain HSC quiescence, a function that is distinct from ID1,

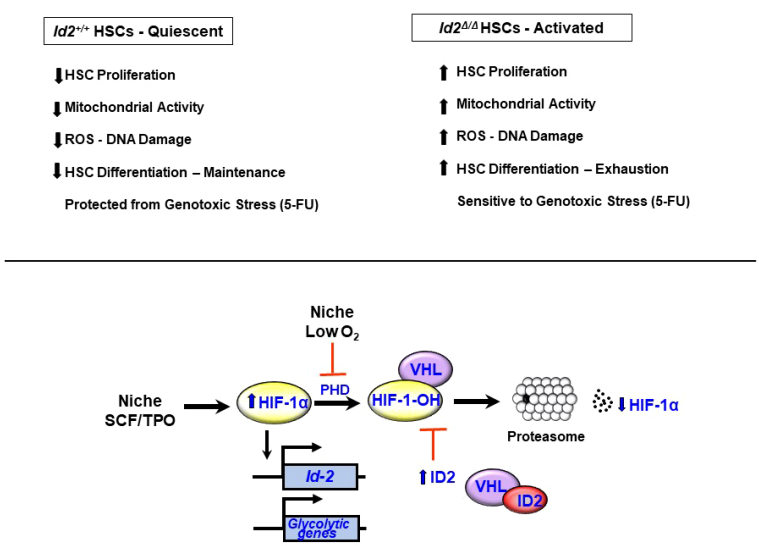

FIGURE 2
Id2 is required for HSC quiescence and maintenance. Summary of hematopoietic phenotypes in *Id2* ablated mice. *Hif-1α* expression is induced in HSCs by stem cell factor (SCF) and thombopoietin (TPO). HIF-1α protein levels are maintained at low levels under normoxic conditions *via* the action of proline hydroxylases (PHDs), which hydroxylate HIF-1α and promotes its association with the VHL complex, ubiquitination and proteasomal degradation. Under low O_2 conditions, PHD is inhibited resulting in reduced levels of hydroxylated HIF-1α, and reduced ubiquitination and stabilization of HIF-1α. ID2 also acts to stabilize HIF-1α by binding to VHL, which prevents ubiquination and proteosomal degradation of HIF-1α and promotes HSC quiescence.

which promotes HSC proliferation. *Id2* and *Id1* expression are inversely correlated during hematopoietic stress, where *Id2* expression decreases in HSCs as they exit quiescence and *Id1* expression increases; after which, *Id1* decreases and *Id2* increases when HSCs resume quiescence (Figure 3). Future experiments are needed to determine if *Id2* regulates quiescence of leukemic stem cells (LSCs), and if there are additional mechanism(s) of *Id2* action in normal and LSCs.

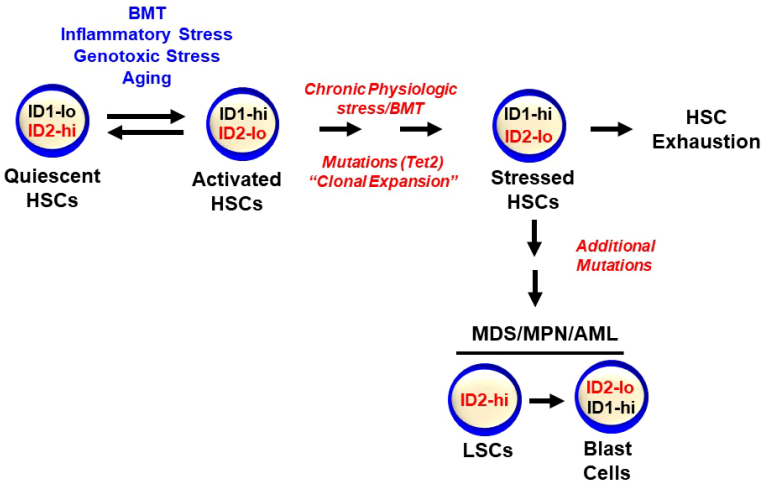

FIGURE 3
Id1 and *Id2* are differentially expressed during HSC activation and quiescence. HSCs express low levels of *Id1* that are induced during stress including BMT, genotoxic and inflammatory stress, and aging. *Id2* is required to maintain HSC quiescence and *Id2* decreases during HSC proliferation and activation, after which, *Id1* levels decrease and *Id2* levels increase as HSCs return to quiescence. Chronic stress can lead to HSC exhaustion and clonal hematopoiesis and hematopoietic malignancies.

ID1 and stress hematopoiesis

Id genes are early response genes that are induced in NIH-3T3 fibroblasts by serum and growth factors, and are required for re-entry of serum-starved cells into cell cycle, demonstrating the requirement for *Id* genes in HGF-induced proliferation (stress) (80). *Id* genes are expressed at low levels in most adult tissues, and are induced by a wide array of extracellular signals in response to stress or injury to promote tissue repair and regeneration (10, 67, 68). HSCs express low levels of *Id1* under steady state conditions, and *Id1* is induced by HGFs that promote myeloid proliferation and differentiation including IL-3 (33, 34). Furthermore, enforced expression of *Id1* in HSPCs promotes myeloid cell proliferation at the expense of lymphoid development, suggesting that ID1 may function during hematopoietic stress (33, 39). Therefore, to evaluate the role of *Id1* in hematopoietic stress, $Id1^{-/-}$ BMCs were serially transplanted in γ-IR recipient mice. No significant difference in the repopulation potential of $Id1^{-/-}$ BMCs compared to $Id1^{-/-}$ BMCs was observed in primary BMT recipients. However, $Id1^{-/-}$ BMCs showed enhanced self-renewal potential and promoted the survival of serially transplanted mice significantly beyond the potential of $Id1^{+/+}$ BMCs (Figure 4) (36). Increased numbers of HSCs were detected in mice serially transplanted with $Id1^{-/-}$ BMCs compared to $Id1^{+/+}$ BMCs, demonstrating that $Id1^{-/-}$ HSCs are maintained and protected from exhaustion during chronic stress. Furthermore, $Id1^{-/-}$ HSCs in serially transplanted mice showed reduced cycling, DNA damage (γ-H2AX phosphorylation), mitochondrial biogenesis and activation, and ROS levels compared to $Id1^{+/+}$ HSCs, indicating that $Id1^{-/-}$ HSCs show increased quiescence during stress (36). Comparative transcriptome analysis of purified donor $Id1^{-/-}$ and $Id1^{+/+}$ HSCs confirmed that $Id1^{-/-}$ HSCs have an increased quiescent molecular signature including reduced expression of genes involved in cell cycle, oxidative phosphorylation, ribosomal biogenesis, and protein synthesis. Taken together, $Id1^{-/-}$ HSCs show increased quiescence and reduced proliferation and activation during hematopoietic stress compared to $Id1^{+/+}$ HSCs.

BMT conditioning regimens including γ-IR damages the HME resulting in acute and chronic inflammation due to the production of alarmins, recruitment of inflammatory cells and the production of pro-inflammatory cytokines (cytokine storm) (81–85). Initial reports demonstrated that myeloid HGFs induce *Id1* expression and proliferation of HSPCs *in vitro* (33, 34). Subsequent studies showed that *Id1* can be induced in HSCs by a variety of proinflammatory cytokines *in vitro*, and HSCs that express *Id1* are actively proliferating (36). In addition, these studies found that *Id1* is induced in HSCs by proinflammatory cytokines *in vivo*, and in HSCs after 6 Gy γ-IR. Thus, during chronic stress $Id1^{-/-}$ HSCs fail to properly respond to cytokine-induced proliferation and differentiation and are protected from exhaustion *in vitro and vivo* during serial BMT.

It is difficult to predict which cytokines in the cytokine storm induce *Id1* in HSCs after BMT *in vivo*; however, many cytokines

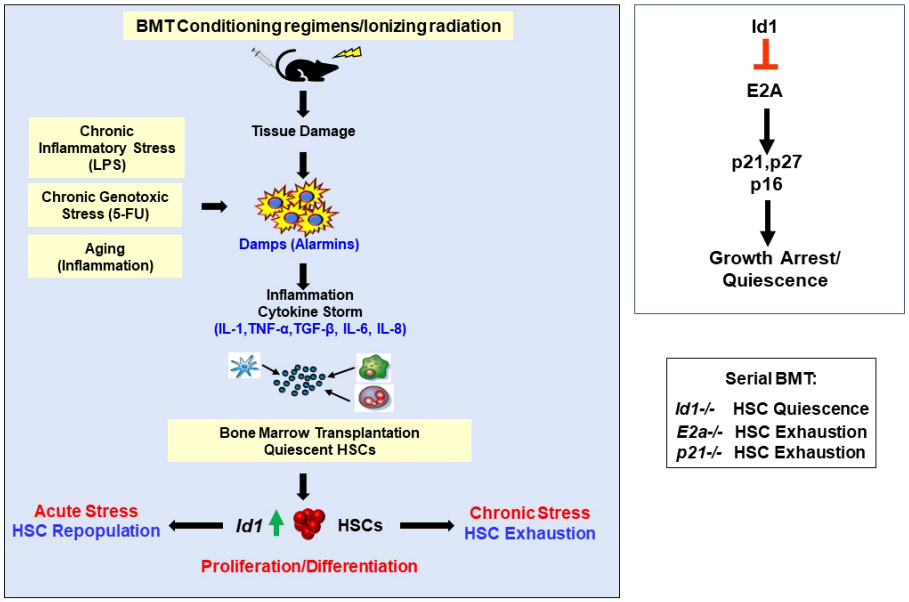

FIGURE 4
Ablation of Id1 Protects HSCs from chronic proliferative stress including BMT, chronic genotoxic and inflammatory stress, and aging. Summary of mechanism of ID1 action in HSCs, and gene knockout mouse models in the molecular pathway.

signal *via* the Jak/Stat pathway suggesting that JAK inhibitors could protect HSCs from chronic proliferative stress. In this regard, JAK1 is an intracellular tyrosine kinase signaling molecule required for HSCs to respond to stress cytokines including IFN-α/β/γ and IL-3 (86). *Jak1* deficient HSCs exhibited increased quiescence, inability to enter cell cycle, reduced response to type I interferons and IL-3, and impaired ability to reconstitute hematopoiesis during BMT and stress (86). Since ID1 proteins are induced by IL-3 in normal HSPCs and cell lines (33), the increased quiescence of *Jak1*$^{-/-}$ HSCs during stress may be mediated, in part, by reduced expression of *Id1*. Therefore, mice were treated with small molecule inhibitors of the JAK/STAT pathway after BMT to inhibit *Id1* induction in HSCs. JAK/STAT inhibitors partially inhibited the induction of *Id1* in HSCs after BMT in γ-IR recipient mice demonstrating that pro-inflammatory cytokines produced after γ-IR induce *Id1* in HSCs *in vivo* (36). Future studies could target other relevant ligands (cytokines), receptors, and downstream signaling pathways involved in proinflammatory cytokine signaling during BMT including IL-6, IL-1, tumor necrosis factor (TNF), IL-8 and others to prevent HSC exhaustion and improve BMT.

It was hypothesized that since *Id1*$^{-/-}$ HSCs are protected from chronic proliferative stress and exhaustion during serial BMT, ablation of *Id1* expression in HSPCs during other conditions of chronic stress including inflammatory and genotoxic stress, and aging could prevent HSC exhaustion (Figure 4). Indeed, studies found that *Id1*$^{-/-}$ HSCs were also preserved in models of chronic proliferative stress including chronic inflammatory stress (LPS) and genotoxic stress (5-FU) (36, 87). In addition, HSCs from aged (2 years old) *Id1*$^{-/-}$ mice resemble more youthful HSCs including, 1) an immunophenotype of young HSCs, 2) increased HSC function in serial BMT assays, 3) increased HSCs in G0, 4) decreased HSC DNA damage, 4) reduced HSC ROS levels, and 5) reduced HSC mitochondrial stress compared to *Id1*$^{+/+}$ HSCs. Future experiments are needed to further define the molecular mechanism(s) that protect *Id1*$^{-/-}$ HSCs from aging. Collectively, these results demonstrate that *Id1*$^{-/-}$ HSCs are more quiescent and resistant to chronic proliferative stress including inflammatory and genotoxic stress and aging. Clonal hematopoiesis, MPN and MDS are associated with increased inflammation (84, 88–90), which could induce *Id1*, promote HSPC proliferation during clonal hematopoiesis, and increase mutational load and genomic instability over time.

Mechanism(s) of Id1 action in hematopoietic stem and progenitor cell growth and quiescence

Id1$^{-/-}$ HSCs fail to properly respond to cytokine-induced proliferation and differentiation and show decreased BrdU incorporation and cell cycling after stress *in vitro* and *in vivo* (36). Since E proteins inhibit cell proliferation, in part, by promoting the expression of cyclin dependent kinase inhibitors (Cdki's) (91–94), and ID proteins inhibit E-proteins resulting in increased proliferation, it is reasonable to hypothesize that the reduced cycling of *Id1*$^{-/-}$ HSCs is due to unrestrained E protein transcriptional activity and increased Cdki expression (Id1-E2a-Cdki pathway) (Figure 4). Increased *p21* expression was observed in *Id1*$^{-/-}$ LSK cells compared to *Id1*$^{+/+}$ HSCs (35). Subsequent reports demonstrated that *Id1*$^{-/-}$ HSCs show increased *p27* and *p16* expression and reduced proliferation in expansion cultures compared to *Id1*$^{+/+}$ HSCs, and *Id1*$^{-/-}$ HSCs show increased expression of *p21* and *p27* after 14 weeks in competitive BMT assays compared to *Id1*$^{+/+}$ HSCs (36). Furthermore, knock down of *E2a* and *p16* expression in *Id1*$^{-/-}$ HSPC expansion cultures increased HSC proliferation suggesting that *Id1* increases HSC proliferation, in part, by restraining E protein function and reducing *p16* expression (36). Importantly, *E2a*$^{-/-}$ HSCs show increased cycling, decreased serial repopulation potential, and decreased expression of *p21* and *p27* expression (95–97), and *p21*$^{-/-}$ HSCs have decreased serial repopulation populations potential (98). Thus, *Id1*$^{-/-}$ HSCs demonstrate opposite hematopoietic phenotypes to *E2a*$^{-/-}$ and *p21*$^{-/-}$ HSCs, whereby *Id1*$^{-/-}$ HSCs show increased quiescence and are protected from hematopoietic stress and exhaustion, and *E2a*$^{-/-}$ and *p21*$^{-/-}$ HSCs show increased proliferation and activation and are sensitive to hematopoietic stress and premature exhaustion. Thus, the Id-E-Cdki pathway critically regulates HSC cycling during stress. In other studies, the E protein(E47) – Cdki (p21) pathway was shown to be important in preventing HSC exhaustion during BMT and 5-FU mediated stress (99). Specifically, genetic experiments examining the requirement for E47- p21 pathway in maintaining HSCs under stress showed that *E47*^{het}*p21*^{het} HSCs exhibit decreased serial BMT repopulation when compared to *E47*^{het}*p21*^{WT}, which have increased p21 levels. The E-Cdki pathway is conserved in endothelial cells (ECs), where *p21* and *p27* RNA expression is increased in ECs that lack *Id* gene expression, and growth inhibition of *Id1*$^{-/-}$*Id3*$^{-/-}$ ECs *in vitro* was rescued by knocking down *E2-2* expression (56). In comparison, *E2a* and *p16* shRNAs partially restore the proliferation of *Id1*$^{-/-}$ HSCs *in vitro*, suggesting that other E and Cdki proteins may regulate quiescence in *Id1* ablated HSCs, or *Id1* regulates HSC functions through other target genes and pathways. Therefore, it will be important to evaluate if knock down of other E proteins, *E2-2* and *Heb*, and other Cdki's rescue *Id1*$^{-/-}$ HSC proliferation *in vitro*. In this regard, the role of *E2-2* and *Heb* in HSC development in conditional mouse models has not been evaluated. Furthermore, it would be important to know if reducing the expression of *p21* and other Cdki's *via* genetic experiments can rescue the hematopoietic phenotypes in *Id1*$^{-/-}$ mice *in vivo*. Finally, additional transcriptomic, proteomic, and

single cell analysis of normal, stressed, or aged *Id1*$^{-/-}$ HSCs might uncover additional molecular pathways that regulate HSC quiescence.

Id Genes and hematopoietic malignancies

Id gene expression has been correlated with the initiation, progression, and metastasis of cancer in many tissues (7, 10, 100). *Id* genes are frequently overexpressed in advanced stage chronic myeloid leukemia (CML), AML and MDS patient samples while low or no levels of ID1 were detected in normal CD34+ HSPCs (39, 101–110). Knock down of *ID1* expression in the AML cell line, MO-7e, resulted in decreased growth and suggested that some AML cells may require ID1 for growth. Furthermore, analysis of *ID* gene expression in an AML patient cohort showed that high levels of *ID* gene expression were correlated with *FLT3-ITD, RAS, EVI-1* and *C/EBPA* mutations, suggesting *ID* gene expression may be induced downstream of oncogene activated signal transduction pathways (111). *ID1* was identified as a common downstream target of oncogenic tyrosine kinases including FLT3-ITD and BCR-ABL (112). Stable KD of *ID1* in K562 (BCR-ABL) and Molm14 (FLT3-ITD) AML cell lines inhibited their growth *in vitro* confirming that *ID1* is required for the growth of some AML cell lines. Analysis of *Id1* expression in a 237 AML patient cohort demonstrated that AML patients with high levels of *ID1* gene expression were less likely to achieve complete remission and were correlated with shorter disease-free survival and overall survival (113). Thus, it was suggested that *ID1* expression levels may provide a molecular tool for refining risk classification of AML. In addition, these studies suggest that Id1 may represent a candidate for targeted therapy to treat AMLs (106).

To explore the intrinsic role of *Id* genes in hematopoietic malignancies, *Id1*-overexpressing HSPCs were transplanted into lethally irradiated recipient mice and monitored for survival. *Id1*-overexpressing mice died roughly one year after BMT compared to control mice, and sick mice showed 1) myeloid/erythroid cell hyperplasia and increased numbers of immature cells in the BM, 2) splenomegaly and extramedullary hematopoiesis, and 3) peripheral blood monocytosis, indicating that these mice succumbed to a MPN that did not progress to AML (39). *ID1* expression is increased in leukemic cells from AML patients with t (8, 21) translocations, and AML1-ETO regulates the *ID1* promoter, suggesting a role for *ID1* in AML1-ETO leukemia (114). Ablation of *Id1* in AML1-ETO transduced murine fetal liver cells delays the onset and development of leukemia by roughly 130 days after transplantation *in vivo* (115). Furthermore, conditional loss of *Id1* in established AML-ETO-leukemia's slows the development of leukemia and promotes animal survival, suggesting that *Id1* is also required for the maintenance of leukemia. Mechanistically, these studies provided evidence that *Id1* promotes AML-ETO leukemic cell growth by interacting with Akt and increasing its activity. In other studies, the progression of leukemia was significantly delayed after transplantation of MLL-AF9-transduced *Id1*$^{-/-}$ FL cells compared to MLL-AF9-transduced *Id1*$^{+/+}$ fetal liver cells (116). The delay in *Id1*$^{-/-}$ MLL-AF9 leukemia was reversed in FL cells that lack *p21*, which is consistent with previous studies demonstrating that ID proteins promote HSPC proliferation by inhibiting the E-Cdki pathway. Interestingly, loss of *Id1* accelerated the progression of MLL-AF9-induced leukemia of BMCs, suggesting that *Id1* is not required for MLL-AF9-induced leukemia of BMCs, and that leukemogenesis may differ significantly for FL and BM HSPCs. The cellular and molecular mechanism(s) that account for the differences in the progression of leukemia in these models is not currently known. Taken together, these studies provide evidence that *ID1* is required for the initiation and progression of oncogene driven leukemias. Future studies are needed to determine if *ID1* is required for AML cell line growth in xenotransplantation and human AML PDX models, and if the recently identified inhibitors of ID1 including cannabidiol and AGX51 show any therapeutic benefit in these models (117–119). Since MPN and MDS and myeloid malignancies that develop with age are strongly correlated with inflammation and *Id1* is induced in HSPCs downstream of pro-inflammatory signals, future studies are needed to better understand the role of *ID1* in these diseases and the molecular mechanism(s) of ID1 action (84, 89, 120).

Ablation of *Id2* in HSCs results in increased proliferation and HSC activation suggesting that ID2 may function as a tumor suppressor. Interestingly, Ko et al. showed that mice transplanted with *Id2*$^{-/-}$ fetal liver cells develop leukocytosis after 6 months that resembles a myeloproliferative disorder, and that over expression of ID2 delays the onset *BCR-ABL*-induced CML *in vivo* (121). In addition, loss of *Id2* expression is associated with increased MLL-AF9-induced leukemia in mice, and over expression of *Id2* inhibits the growth of MV4-11 and MOLM-13 AML cell lines that express MLL-AF9, and Kasumi AML cells that express AML-ETO (122). Together, these results suggest that ID2 may function as a tumor suppressor in hematopoietic malignancies. In addition, mice that lack *Id2* develop intestinal adenomas, and show a hyperproliferation of colon stem cells during embryonic development due to increased Wnt/B-catenin signaling, suggesting that ID2 may function as a tumor suppressor in other cell types (123, 124). Analysis of ID2 expression in 145 AML patient BMCs showed that AML patient cells with high levels of ID2 expression were correlated with lower complete remission and shorter overall survival, and was a predictor of poor chemotherapy response (103). Analysis of *ID2* expression in a subset of MLL-rearranged AML patient cells indicated that MLL patients (35 patients) with high levels of *ID2* expression had a significantly better overall and event free survival than patients with low levels of *ID2* (122). Further

studies are needed to examine if levels of *ID2* expression are prognostic for AML patient subsets, and to determine if ID2 is expressed in LSCs and functions to regulate their quiescence and survival (Figure 3).

Conclusion and perspectives

ID proteins have emerged as important regulators of HSPC quiescence (ID2) and proliferation (ID1). Specifically, low levels of ID1 are expressed in primitive HSPCs, but are induced in HSPC after acute stress including BMT, inflammatory and genotoxic stress, and promote HSPC proliferation and myeloid development, while inhibiting lymphoid development (Figure 3). Upon resolution of an acute stress, HSCs return to quiescence with low levels of ID1 and hematopoiesis resumes under steady state conditions. However, under chronic proliferative stress ID1 levels remain high and HSCs undergo excessive proliferation, exhaustion and bone marrow failure. Reducing ID1 levels during serial BMT, chronic inflammatory and genotoxic stress and aging may be therapeutic to protect HSCs from exhaustion. In addition, since hematopoietic malignancies and bone marrow failure syndromes are often accompanied by inflammation and increased ID1 expression, reducing ID1 levels could be therapeutic by reducing preleukemic proliferation and clonal expansion, which could delay the onset and reduce the incidence of hematopoietic malignancies and bone marrow failure syndromes. ID2 has emerged as a critical regulator of normal HSC quiescence and shows opposite expression to ID1, where ID2 levels decrease as HSCs exit quiescence and ID1 levels increase during cell proliferation. Maintaining high levels of ID2 *in vitro* and *in vivo* could be exploited to expand HSCs for gene and cell therapies including BMT. Opposing expression of ID1 and ID2 is also observed during the final stages of myeloid development, where ID1 expression is increased in myeloid progenitors (CMP/GMP), and then decreases as cells exit the cell cycle and differentiate, while ID2 expression is increased in mature neutrophils. Furthermore, current evidence suggests that ID1 and ID2 may function during neutrophil and eosinophil development, however, gain and loss of function studies are needed to reveal if these genes are required for the differentiation and function of these cells. Since ID2 regulates normal HSC quiescence, the potential role of ID2 in LSC quiescence and resistance to current therapies remains to be explored.

Author contributions

SS, TS, BJ, SG, AS, KG, and JK contributed to writing and editing the review and figures. All authors contributed to the article and approved the submitted version.

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References

1. Trumpp A, Essers M, Wilson A. Awakening dormant haematopoietic stem cells. *Nat Rev Immunol* (2010) 10(3):201–9. doi: 10.1038/nri2726

2. Pinho S, Frenette PS. Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol* (2019) 20(5):303–20. doi: 10.1038/s41580-019-0103-9

3. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* (2010) 2(6):640–53. doi: 10.1002/wsbm.86

4. Ito K, Frenette PS. HSC contribution in making steady-state blood. *Immunity* (2016) 45(3):464–6. doi: 10.1016/j.immuni.2016.09.002

5. Bernitz JM, Kim HS, MacArthur B, Sieburg H, Moore K. Hematopoietic stem cells count and remember self-renewal divisions. *Cell* (2016) 167(5):1296–309.e10. doi: 10.1016/j.cell.2016.10.022

6. Qiu J, Papatsenko D, Niu X, Schaniel C, Moore K. Divisional history and hematopoietic stem cell function during homeostasis. *Stem Cell Rep* (2014) 2 (4):473–90. doi: 10.1016/j.stemcr.2014.01.016

7. Roschger C, Cabrele C. The id-protein family in developmental and cancer-associated pathways. *Cell Commun Signal* (2017) 15(1):7. doi: 10.1186/s12964-016-0161-y

8. Wang LH, Baker NE. E proteins and id proteins: helix-loop-helix partners in development and disease. *Dev Cell* (2015) 35(3):269–80. doi: 10.1016/j.devcel.2015.10.019

9. Norton JD. ID Helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* (2000) 113(Pt 22):3897–905. doi: 10.1242/jcs.113.22.3897

10. Lasorella A, Benezra R, Iavarone A. The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. *Nat Rev Cancer* (2014) 14(2):77–91. doi: 10.1038/nrc3638

11. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* (1990) 61 (1):49–59. doi: 10.1016/0092-8674(90)90214-Y

12. Murre C. Helix-loop-helix proteins and lymphocyte development. *Nat Immunol* (2005) 6(11):1079–86. doi: 10.1038/ni1260

13. de Pooter RF, Kee BL. E proteins and the regulation of early lymphocyte development. *Immunol Rev* (2010) 238(1):93–109. doi: 10.1111/j.1600-065X.2010.00957.x

14. Engel I, Murre C. The function of e- and id proteins in lymphocyte development. *Nat Rev Immunol* (2001) 1(3):193–9. doi: 10.1038/35105060

15. Ohtani N, Zebedee Z, Huot TJ, Stinson JA, Sugimoto M, Ohashi Y, et al. Opposing effects of ets and id proteins on p16INK4a expression during cellular senescence. *Nature* (2001) 409(6823):1067–70. doi: 10.1038/35059131

16. Lasorella A, Noseda M, Beyna M, Yokota Y, Iavarone A. Id2 is a retinoblastoma protein target and mediates signalling by myc oncoproteins. *Nature* (2000) 407(6804):592–8. doi: 10.1038/35036504

17. Roberts EC, Deed RW, Inoue T, Norton JD, Sharrocks AD. Id helix-loop-helix proteins antagonize pax transcription factor activity by inhibiting DNA binding. *Mol Cell Biol* (2001) 21(2):524–33. doi: 10.1128/MCB.21.2.524-533.2001

18. Hacker C, Kirsch RD, Ju XS, Hieronymus T, Gust TC, Kuhl C, et al. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat Immunol* (2003) 4(4):380–6. doi: 10.1038/ni903

19. Jackson JT, Hu Y, Liu R, Masson F, D'Amico A, Carotta S, et al. Id2 expression delineates differential checkpoints in the genetic program of CD8alpha+ and CD103+ dendritic cell lineages. *EMBO J* (2011) 30(13):2690–704. doi: 10.1038/emboj.2011.163

20. Kee BL E. And ID proteins branch out. *Nat Rev Immunol* (2009) 9(3):175–84. doi: 10.1038/nri2507

21. Kee BL, Quong MW, Murre C. E2A proteins: essential regulators at multiple stages of b-cell development. *Immunol Rev* (2000) 175:138–49. doi: 10.1111/j.1600-065X.2000.imr017514.x

22. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* (1999) 397(6721):702–6. doi: 10.1038/17812

23. Boos MD, Yokota Y, Eberl G, Kee BL. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of e protein activity. *J Exp Med* (2007) 204(5):1119–30. doi: 10.1084/jem.20061959

24. Sun XH, Copeland NG, Jenkins NA, Baltimore D. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol Cell Biol* (1991) 11(11):5603–11.

25. Lister J, Forrester WC, Baron MH. Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. *J Biol Chem* (1995) 270(30):17939–46. doi: 10.1074/jbc.270.30.17939

26. Shoji W, Yamamoto T, Obinata M. The helix-loop-helix protein id inhibits differentiation of murine erythroleukemia cells. *J Biol Chem* (1994) 269(7):5078–84. doi: 10.1016/S0021-9258(17)37657-3

27. Kreider BL, Benezra R, Rovera G, Kadesch T. Inhibition of myeloid differentiation by the helix-loop-helix protein id. *Science* (1992) 255(5052):1700–2. doi: 10.1126/science.1372755

28. Quesenberry PJ, Iscove NN, Cooper C, Brady G, Newburger PE, Stein GS, et al. Expression of basic helix-loop-helix transcription factors in explant hematopoietic progenitors. *J Cell Biochem* (1996) 61(3):478–88. doi: 10.1002/(SICI)1097-4644(19960601)61:3<478::AID-JCB15>3.0.CO;2-F

29. Cooper CL, Brady G, Bilia F, Iscove NN, Quesenberry PJ. Expression of the id family helix-loop-helix regulators during growth and development in the hematopoietic system. *Blood* (1997) 89(9):3155–65. doi: 10.1182/blood.V89.9.3155

30. Cooper CL, Newburger PE. Differential expression of id genes in multipotent myeloid progenitor cells: Id-1 is induced by early-and late-acting cytokines while id-2 is selectively induced by cytokines that drive terminal granulocytic differentiation. *J Cell Biochem* (1998) 71(2):277–85. doi: 10.1002/(SICI)1097-4644(19981101)71:2<277::AID-JCB12>3.0.CO;2-I

31. Jen Y, Weintraub H, Benezra R. Overexpression of id protein inhibits the muscle differentiation program: *in vivo* association of id with E2A proteins. *Genes Dev* (1992) 6(8):1466–79. doi: 10.1101/gad.6.8.1466

32. Desprez PY, Hara E, Bissell MJ, Campisi J. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein id-1. *Mol Cell Biol* (1995) 15(6):3398–404. doi: 10.1128/MCB.15.6.3398

33. Leeanansaksiri W, Wang H, Gooya JM, Renn K, Abshari M, Tsai S, et al. IL-3 induces inhibitor of DNA-binding protein-1 in hemopoietic progenitor cells and promotes myeloid cell development. *J Immunol* (2005) 174(11):7014–21. doi: 10.4049/jimmunol.174.11.7014

34. Cochrane SW, Zhao Y, Welner RS, Sun XH. Balance between id and e proteins regulates myeloid-versus-lymphoid lineage decisions. *Blood* (2009) 113 (5):1016–26. doi: 10.1182/blood-2008-06-164996

35. Jankovic V, Ciarrocchi A, Boccuni P, DeBlasio T, Benezra R, Nimer SD. Id1 restrains myeloid commitment, maintaining the self-renewal capacity of hematopoietic stem cells. *Proc Natl Acad Sci USA* (2007) 104(4):1260–5. doi: 10.1073/pnas.0607894104

36. Singh SK, Singh S, Gadomski S, Sun L, Pfannenstein A, Magidson V, et al. Id1 ablation protects hematopoietic stem cells from stress-induced exhaustion and aging. *Cell Stem Cell* (2018) 23(2):252–65.e8. doi: 10.1016/j.stem.2018.06.001

37. Perry SS, Zhao Y, Nie L, Cochrane SW, Huang Z, Sun XH. Id1, but not Id3, directs long-term repopulating hematopoietic stem-cell maintenance. *Blood* (2007) 110(7):2351–60. doi: 10.1182/blood-2007-01-069914

38. van Galen P, Kreso A, Wienholds E, Laurenti E, Eppert K, Lechman ER, et al. Reduced lymphoid lineage priming promotes human hematopoietic stem cell expansion. *Cell Stem Cell* (2014) 14(1):94–106. doi: 10.1016/j.stem.2013.11.021

39. Suh HC, Leeanansaksiri W, Ji M, Klarmann KD, Renn K, Gooya J, et al. Id1 immortalizes hematopoietic progenitors *in vitro* and promotes a myeloproliferative disease *in vivo*. *Oncogene* (2008) 27(42):5612–23. doi: 10.1038/onc.2008.175

40. Buitenhuis M, van Deutekom HW, Verhagen LP, Castor A, Jacobsen SE, Lammers JW, et al. Differential regulation of granulopoiesis by the basic helix-loop-helix transcriptional inhibitors Id1 and Id2. *Blood* (2005) 105(11):4272–81. doi: 10.1182/blood-2004-12-4883

41. Morrow MA, Mayer EW, Perez CA, Adlam M, Siu G. Overexpression of the helix-Loop-Helix protein Id2 blocks T cell development at multiple stages. *Mol Immunol* (1999) 36(8):491–503. doi: 10.1016/S0161-5890(99)00071-1

42. Kim D, Peng XC, Sun XH. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol Cell Biol* (1999) 19(12):8240–53. doi: 10.1128/MCB.19.12.8240

43. Sun XH. Constitutive expression of the Id1 gene impairs mouse b cell development. *Cell* (1994) 79(5):893–900. doi: 10.1016/0092-8674(94)90078-7

44. Belle I, Zhuang Y. E proteins in lymphocyte development and lymphoid diseases. *Curr topics Dev Biol* (2014) 110:153–87. doi: 10.1016/B978-0-12-405943-6.00004-X

45. Mercer EM, Lin YC, Benner C, Jhunjhunwala S, Dutkowski J, Flores M, et al. Multilineage priming of enhancer repertoires precedes commitment to the b and myeloid cell lineages in hematopoietic progenitors. *Immunity* (2011) 35(3):413–25. doi: 10.1016/j.immuni.2011.06.013

46. Mercer EM, Lin YC, Murre C. Factors and networks that underpin early hematopoiesis. *Semin Immunol* (2011) 23(5):317–25. doi: 10.1016/j.smim.2011.08.004

47. Ikawa T, Masuda K, Huijskens M, Satoh R, Kakugawa K, Agata Y, et al. Induced developmental arrest of early hematopoietic progenitors leads to the generation of leukocyte stem cells. *Stem Cell Rep* (2015) 5(5):716–27. doi: 10.1016/j.stemcr.2015.09.012

48. Kee BL, Rivera RR, Murre C. Id3 inhibits b lymphocyte progenitor growth and survival in response to TGF-beta. *Nat Immunol* (2001) 2(3):242–7. doi: 10.1038/85303

49. Pan L, Sato S, Frederick JP, Sun XH, Zhuang Y. Impaired immune responses and b-cell proliferation in mice lacking the Id3 gene. *Mol Cell Biol* (1999) 19 (9):5969–80. doi: 10.1128/MCB.19.9.5969

50. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. *Immunity* (2000) 12(1):17–26. doi: 10.1016/S1074-7613(00)80155-7

51. Thal MA, Carvalho TL, He T, Kim HG, Gao H, Hagman J, et al. Ebf1-mediated down-regulation of Id2 and Id3 is essential for specification of the b cell lineage. *Proc Natl Acad Sci USA* (2009) 106(2):552–7. doi: 10.1073/pnas.0802550106

52. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. *Mol Cell Biol* (1997) 17(12):7317–27. doi: 10.1128/MCB.17.12.7317

53. Suh HC, Ji M, Gooya J, Lee M, Klarmann KD, Keller JR. Cell-nonautonomous function of Id1 in the hematopoietic progenitor cell niche. *Blood* (2009) 114(6):1186–95. doi: 10.1182/blood-2008-09-179788

54. Yang J, Li X, Morrell NW. Id proteins in the vasculature: from molecular biology to cardiopulmonary medicine. *Cardiovasc Res* (2014) 104(3):388–98. doi: 10.1093/cvr/cvu215

55. Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, et al. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* (1999) 401(6754):670–7. doi: 10.1038/44334

56. Gadomski S, Singh SK, Singh S, Sarkar T, Klarmann KD, Berenschot M, et al. Id1 and Id3 maintain steady-state hematopoiesis by promoting sinusoidal endothelial cell survival and regeneration. *Cell Rep* (2020) 31(4):107572. doi: 10.1016/j.celrep.2020.107572

57. Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, et al. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. *J Biol Chem* (2004) 279(31):32941–9. doi: 10.1074/jbc.M403344200

58. Fraidenraich D, Stillwell E, Romero E, Wilkes D, Manova K, Basson CT, et al. Rescue of cardiac defects in id knockout embryos by injection of embryonic stem cells. *Science* (2004) 306(5694):247–52. doi: 10.1126/science.1102612

59. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature* (2014) 505(7483):327–34. doi: 10.1038/nature12984

60. Wei Q, Frenette PS. Niches for hematopoietic stem cells and their progeny. *Immunity* (2018) 48(4):632–48. doi: 10.1016/j.immuni.2018.03.024

61. Kusumbe AP, Ramasamy SK, Itkin T, Mae MA, Langen UH, Betsholtz C, et al. Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* (2016) 532(7599):380–4. doi: 10.1038/nature17638

62. Itkin T, Gur-Cohen S, Spencer JA, Schajnovitz A, Ramasamy SK, Kusumbe AP, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature* (2016) 532(7599):323–8. doi: 10.1038/nature17624

63. Comazzetto S, Shen B, Morrison SJ. Niches that regulate stem cells and hematopoiesis in adult bone marrow. *Dev Cell* (2021) 56(13):1848–60. doi: 10.1016/j.devcel.2021.05.018

64. Rafii S, Butler JM, Ding BS. Angiocrine functions of organ-specific endothelial cells. *Nature* (2016) 529(7586):316–25. doi: 10.1038/nature17040

65. Kobayashi H, Butler JM, O'Donnell R, Kobayashi M, Ding BS, Bonner B, et al. Angiocrine factors from akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* (2010) 12(11):1046–56. doi: 10.1038/ncb2108

66. Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* (2014) 507(7492):323–8. doi: 10.1038/nature13145

67. Ding BS, Cao Z, Lis R, Nolan DJ, Guo P, Simons M, et al. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature* (2014) 505(7481):97–102. doi: 10.1038/nature12681

68. Ding BS, Nolan DJ, Butler JM, James D, Babazadeh AO, Rosenwaks Z, et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature* (2010) 468(7321):310–5. doi: 10.1038/nature09493

69. Poulos MG, Guo P, Kofler NM, Pinho S, Gutkin MC, Tikhonova A, et al. Endothelial jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Rep* (2013) 4(5):1022–34. doi: 10.1016/j.celrep.2013.07.048

70. Kfoury Y, Scadden DT. Mesenchymal cell contributions to the stem cell niche. *Cell Stem Cell* (2015) 16(3):239–53. doi: 10.1016/j.stem.2015.02.019

71. Li H, Dai M, Zhuang Y. A T cell intrinsic role of Id3 in a mouse model for primary sjogren's syndrome. *Immunity* (2004) 21(4):551–60. doi: 10.1016/j.immuni.2004.08.013

72. Li J, Maruyama T, Zhang P, Konkel JE, Hoffman V, Zamarron B, et al. Mutation of inhibitory helix-loop-helix protein Id3 causes gammadelta T-cell lymphoma in mice. *Blood* (2010) 116(25):5615–21. doi: 10.1182/blood-2010-03-274506

73. Ji M, Li H, Suh HC, Klarmann KD, Yokota Y, Keller JR. Id2 intrinsically regulates lymphoid and erythroid development *via* interaction with different target proteins. *Blood* (2008) 112(4):1068–77. doi: 10.1182/blood-2008-01-133504

74. Hock H, Hamblen MJ, Rooke HM, Schindler JW, Saleque S, Fujiwara Y, et al. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature* (2004) 431(7011):1002–7. doi: 10.1038/nature02994

75. Zeng H, Yucel R, Kosan C, Klein-Hitpass L, Moroy T. Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. *EMBO J* (2004) 23(20):4116–25. doi: 10.1038/sj.emboj.7600419

76. Li H, Ji M, Klarmann KD, Keller JR. Repression of Id2 expression by gfi-1 is required for b-cell and myeloid development. *Blood* (2010) 116(7):1060–9. doi: 10.1182/blood-2009-11-255075

77. Kim W, Klarmann KD, Keller JR. Gfi-1 regulates the erythroid transcription factor network through Id2 repression in murine hematopoietic progenitor cells. *Blood* (2014) 124(10):1586–96. doi: 10.1182/blood-2014-02-556522

78. Jakubison BL, Sarkar T, Gudmundsson KO, Singh S, Sun L, Morris HM, et al. ID2 and HIF-1α collaborate to protect quiescent hematopoietic stem cells from activation, differentiation, and exhaustion. *J Clin Invest* (2022) 132(13). doi: 10.1172/JCI152599

79. Takubo K, Goda N, Yamada W, Iriuchishima H, Ikeda E, Kubota Y, et al. Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell Stem Cell* (2010) 7(3):391–402. doi: 10.1016/j.stem.2010.06.020

80. Barone MV, Pepperkok R, Peverali FA, Philipson L. Id proteins control growth induction in mammalian cells. *Proc Natl Acad Sci USA* (1994) 91(11):4985–8. doi: 10.1073/pnas.91.11.4985

81. Ganuza M, McKinney-Freeman S. Hematopoietic stem cells under pressure. *Curr Opin Hematol* (2017) 24(4):314–21. doi: 10.1097/MOH.0000000000000347

82. Termini CM, Chute JP. Hematopoietic stem cell stress and regeneration. *Curr Stem Cell Rep* (2020) 6(4):134–43. doi: 10.1007/s40778-020-00181-3

83. Boettcher S, Manz MG. Regulation of inflammation- and infection-driven hematopoiesis. *Trends Immunol* (2017) 38(5):345–57. doi: 10.1016/j.it.2017.01.004

84. Caiado F, Pietras EM, Manz MG. Inflammation as a regulator of hematopoietic stem cell function in disease, aging, and clonal selection. *J Exp Med* (2021) 218(7). doi: 10.1084/jem.20201541

85. Singh S, Jakubison B, Keller JR. Protection of hematopoietic stem cells from stress-induced exhaustion and aging. *Curr Opin Hematol* (2020) 27(4):225–31. doi: 10.1097/MOH.0000000000000586

86. Kleppe M, Spitzer MH, Li S, Hill CE, Dong L, Papalexi E, et al. Jak1 integrates cytokine sensing to regulate hematopoietic stem cell function and stress hematopoiesis. *Cell Stem Cell* (2018) 22(2):277. doi: 10.1016/j.stem.2017.12.018

87. Zhao Y, Ling F, Wang HC, Sun XH. Chronic TLR signaling impairs the long-term repopulating potential of hematopoietic stem cells of wild type but not Id1 deficient mice. *PloS One* (2013) 8(2):e55552. doi: 10.1371/journal.pone.0055552

88. Barreyro L, Chlon TM, Starczynowski DT. Chronic immune response dysregulation in MDS pathogenesis. *Blood* (2018) 132(15):1553–60. doi: 10.1182/blood-2018-03-784116

89. Trowbridge JJ, Starczynowski DT. Innate immune pathways and inflammation in hematopoietic aging, clonal hematopoiesis, and MDS. *J Exp Med* (2021) 218(7). doi: 10.1084/jem.20201544

90. Craver BM, El Alaoui K, Scherber RM, Fleischman AG. The critical role of inflammation in the pathogenesis and progression of myeloid malignancies. *Cancers (Basel)* (2018) 10(4):104–20.

91. Prabhu S, Ignatova A, Park ST, Sun XH. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and id proteins. *Mol Cell Biol* (1997) 17(10):5888–96.

92. Rothschild G, Zhao X, Iavarone A, Lasorella A. E proteins and Id2 converge on p57Kip2 to regulate cell cycle in neural cells. *Mol Cell Biol* (2006) 26(11):4351–61. doi: 10.1128/MCB.01743-05

93. Mern DS, Hoppe-Seyler K, Hoppe-Seyler F, Hasskarl J, Burwinkel B. Targeting Id1 and Id3 by a specific peptide aptamer induces e-box promoter activity, cell cycle arrest, and apoptosis in breast cancer cells. *Breast Cancer Res Treat* (2010) 124(3):623–33. doi: 10.1007/s10549-010-0810-6

94. Zheng W, Wang H, Xue L, Zhang Z, Tong T. Regulation of cellular senescence and p16(INK4a) expression by Id1 and E47 proteins in human diploid fibroblast. *J Biol Chem* (2004) 279(30):31524–32. doi: 10.1074/jbc.M400365200

95. Semerad CL, Mercer EM, Inlay MA, Weissman IL, Murre C. E2A proteins maintain the hematopoietic stem cell pool and promote the maturation of myelolymphoid and myeloerythroid progenitors. *Proc Natl Acad Sci USA* (2009) 106(6):1930–5. doi: 10.1073/pnas.0808866106

96. Yang Q, Esplin B, Borghesi L. E47 regulates hematopoietic stem cell proliferation and energetics but not myeloid lineage restriction. *Blood* (2011) 117(13):3529–38. doi: 10.1182/blood-2010-07-297689

97. Dias S, Mansson R, Gurbuxani S, Sigvardsson M, Kee BL. E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity* (2008) 29(2):217–27. doi: 10.1016/j.immuni.2008.05.015

98. Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* (2000) 287(5459):1804–8. doi: 10.1126/science.287.5459.1804

99. Santos PM, Ding Y, Borghesi L. Cell-intrinsic *in vivo* requirement for the E47-p21 pathway in long-term hematopoietic stem cells. *J Immunol* (2014) 192(1):160–8. doi: 10.4049/jimmunol.1302502

100. Nair R, Teo WS, Mittal V, Swarbrick A. ID Proteins regulate diverse aspects of cancer progression and provide novel therapeutic opportunities. *Mol Ther: J Am Soc Gene Ther* (2014) 22(8):1407–15. doi: 10.1038/mt.2014.83

101. Radich JP, Dai H, Mao M, Oehler V, Schelter J, Druker B, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. *Proc Natl Acad Sci USA* (2006) 103(8):2794–9. doi: 10.1073/pnas.0510423103

102. Rucker FG, Bullinger L, Schwaenen C, Lipka DB, Wessendorf S, Frohling S, et al. Disclosure of candidate genes in acute myeloid leukemia with complex

karyotypes using microarray-based molecular characterization. *J Clin Oncol* (2006) 24(24):3887–94. doi: 10.1200/JCO.2005.04.5450

103. Zhou JD, Ma JC, Zhang TJ, Li XX, Zhang W, Wu DH, et al. High bone marrow ID2 expression predicts poor chemotherapy response and prognosis in acute myeloid leukemia. *Oncotarget* (2017) 8(54):91979–89. doi: 10.18632/oncotarget.20559

104. Zhou JD, Yang L, Zhu XW, Wen XM, Yang J, Guo H, et al. Clinical significance of up-regulated ID1 expression in Chinese *de novo* acute myeloid leukemia. *Int J Clin Exp Pathol* (2015) 8(5):5336–44.

105. Tochareontanaphol C, Sinthuwiwat T, Buathong B, Thita T, Promso S, Paca-Uccaralertkun S. New mutations of the id1 gene in acute myeloid leukemia patients. *Pathobiology* (2015) 82(1):43–7. doi: 10.1159/000370243

106. Klarmann K, Ji M, Li H, Satyanarayana A, Kim W, Bowers E, et al. Novel targets in myelogenous leukemia: the id family of proteins. myeloid leukemia – basic mechanisms of leukemogenesis. Koschmieder S, Krug U editors. Rijeka, Croatia: InTech (2011). 215–38 p.

107. Bera R, Chiu MC, Huang YJ, Lin TH, Kuo MC, Shih LY. RUNX1 mutations promote leukemogenesis of myeloid malignancies in ASXL1-mutated leukemia. *J Hematol Oncol* (2019) 12(1):104. doi: 10.1186/s13045-019-0789-3

108. Liquori A, Ibanez M, Sargas C, Sanz MA, Barragan E, Cervera J. Acute promyelocytic leukemia: A constellation of molecular events around a single pml-rara fusion gene. *Cancers (Basel)* (2020) 12(3):624–6. doi: 10.3390/cancers12030624

109. Sayar H, Liu Y, Gao R, Zaid MA, Cripe LD, Weisenbach J, et al. Consecutive epigenetically-active agent combinations act in ID1-RUNX3-TET2 and HOXA pathways for Flt3ITD+ve AML. *Oncotarget* (2018) 9(5):5703–15. doi: 10.18632/oncotarget.23655

110. Eisfeld AK, Kohlschmidt J, Mrozek K, Volinia S, Blachly JS, Nicolet D, et al. Mutational landscape and gene expression patterns in adult acute myeloid leukemias with monosomy 7 as a sole abnormality. *Cancer Res* (2017) 77 (1):207–18. doi: 10.1158/0008-5472.CAN-16-1386

111. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* (2004) 350(16):1617–28. doi: 10.1056/NEJMoa040465

112. Tam WF, Gu TL, Chen J, Lee BH, Bullinger L, Frohling S, et al. Id1 is a common downstream target of oncogenic tyrosine kinases in leukemic cells. *Blood* (2008) 112(5):1981–92. doi: 10.1182/blood-2007-07-103010

113. Tang R, Hirsch P, Fava F, Lapusan S, Marzac C, Teyssandier I, et al. High Id1 expression is associated with poor prognosis in 237 patients with acute myeloid leukemia. *Blood* (2009) 114(14):2993–3000. doi: 10.1182/blood-2009-05-223115

114. Wang L, Gural A, Sun XJ, Zhao X, Perna F, Huang G, et al. The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation. *Science* (2011) 333(6043):765–9. doi: 10.1126/science.1201662

115. Wang L, Man N, Sun XJ, Tan Y, Garcia-Cao M, Liu F, et al. Regulation of AKT signaling by Id1 controls t (8,21)Leukemia initiation and progression. *Blood* (2015) 126(5):640–50. doi: 10.1182/blood-2015-03-635532

116. Man N, Sun XJ, Tan Y, Garcia-Cao M, Liu F, Cheng G, et al. Differential role of Id1 in MLL-AF9-driven leukemia based on cell of origin. *Blood* (2016) 127 (19):2322–6. doi: 10.1182/blood-2015-11-677708

117. Wojnarowicz PM, Escolano MG, Huang YH, Desai B, Chin Y, Shah R, et al. Anti-tumor effects of an ID antagonist with no observed acquired resistance. *NPJ Breast Cancer* (2021) 7(1):58. doi: 10.1038/s41523-021-00266-0

118. Soroceanu L, Murase R, Limbad C, Singer E, Allison J, Adrados I, et al. Id-1 is a key transcriptional regulator of glioblastoma aggressiveness and a novel therapeutic target. *Cancer Res* (2013) 73(5):1559–69. doi: 10.1158/0008-5472.CAN-12-1943

119. McAllister SD, Christian RT, Horowitz MP, Garcia A, Desprez PY. Cannabidiol as a novel inhibitor of id-1 gene expression in aggressive breast cancer cells. *Mol Cancer Ther* (2007) 6(11):2921–7. doi: 10.1158/1535-7163.MCT-07-0371

120. Kovtonyuk LV, Fritsch K, Feng X, Manz MG, Takizawa H. Inflamm-aging of hematopoiesis, hematopoietic stem cells, and the bone marrow microenvironment. *Front Immunol* (2016) 7:502. doi: 10.3389/fimmu.2016.00502

121. Ko J, Patel N, Ikawa T, Kawamoto H, Frank O, Rivera RR, et al. Suppression of e-protein activity interferes with the development of BCR-ABL-mediated myeloproliferative disease. *Proc Natl Acad Sci U S A* (2008) 105 (35):12967–72. doi: 10.1073/pnas.0805073105

122. Ghisi M, Kats L, Masson F, Li J, Kratina T, Vidacs E, et al. Id2 and e proteins orchestrate the initiation and maintenance of MLL-rearranged acute myeloid leukemia. *Cancer Cell* (2016) 30(1):59–74. doi: 10.1016/j.ccell.2016.05.019

123. Nigmatullina L, Norkin M, Dzama MM, Messner B, Sayols S, Soshnikova N. Id2 controls specification of Lgr5(+) intestinal stem cell progenitors during gut development. *EMBO J* (2017) 36(7):869–85. doi: 10.15252/embj.201694959

124. Russell RG, Lasorella A, Dettin LE, Iavarone A. Id2 drives differentiation and suppresses tumor formation in the intestinal epithelium. *Cancer Res* (2004) 64 (20):7220–5. doi: 10.1158/0008-5472.CAN-04-2095

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EDITED BY
Michele Kay Anderson,
University of Toronto, Canada

REVIEWED BY
Juan Carlos Zuniga-Pflucker,
University of Toronto, Canada
Hai-Hui Xue,
Hackensack University Medical Center, United States
Jinfang Zhu,
National Institute of Allergy and Infectious Diseases (NIH), United States

*CORRESPONDENCE
Xiao-Hong Sun
xiao-hong-sun@omrf.org

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The divergence between T cell and innate lymphoid cell fates controlled by E and Id proteins

Aneta Pankow[1,2] and Xiao-Hong Sun[1,2,3]

[1]Program in Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK, United States, [2]Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, United States, [3]Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, United States

T cells develop in the thymus from lymphoid primed multipotent progenitors or common lymphoid progenitors into αβ and γδ subsets. The basic helix-loop-helix transcription factors, E proteins, play pivotal roles at multiple stages from T cell commitment to maturation. Inhibitors of E proteins, Id2 and Id3, also regulate T cell development while promoting ILC differentiation. Recent findings suggest that the thymus can also produce innate lymphoid cells (ILCs). In this review, we present current findings that suggest the balance between E and Id proteins is likely to be critical for controlling the bifurcation of T cell and ILC fates at early stages of T cell development.

KEYWORDS

E2A, HEB, innate lymphoid cells, Id2, Id3, T cells

Introduction

The E protein family of transcription factors are crucial molecules engaging in B cell development in the bone marrow and T cells differentiation in the thymus (1, 2). This family consists of proteins encoded by three genes, E2A (also called *Tcf3*), HEB (*Tcf12*) and E2-2 (*Tcf4*) (Figure 1A) (3–5). These proteins share extensive sequence homologies in the activation domains (AD1, LH) and basic helix-loop-helix (bHLH) DNA-binding domain (6–9). E proteins regulate the transcription of their target genes by forming homodimers or heterodimers and bind to E-box sequences (9). The E2A gene gives rise to two proteins, E12 and E47, due to alternative splicing of two adjacent exons, each encoding a basic helix-loop-helix (bHLH) domain (10). While E47 binds DNA avidly as homodimers, E12 does so poorly due to the presence of an inhibitory domain (11). However, both form heterodimers with other bHLH proteins such as MyoD, and bind DNA efficiently. The HEB gene encodes a full-length canonical protein (HEBCan) and a truncated alternate form (HEBAlt), which derives from a transcript initiated in the middle of the gene (12). HEBAlt lacks the AD1 transcription activation domain and has lower transcriptional activities (13). It has an Alt domain at the N-terminus with three

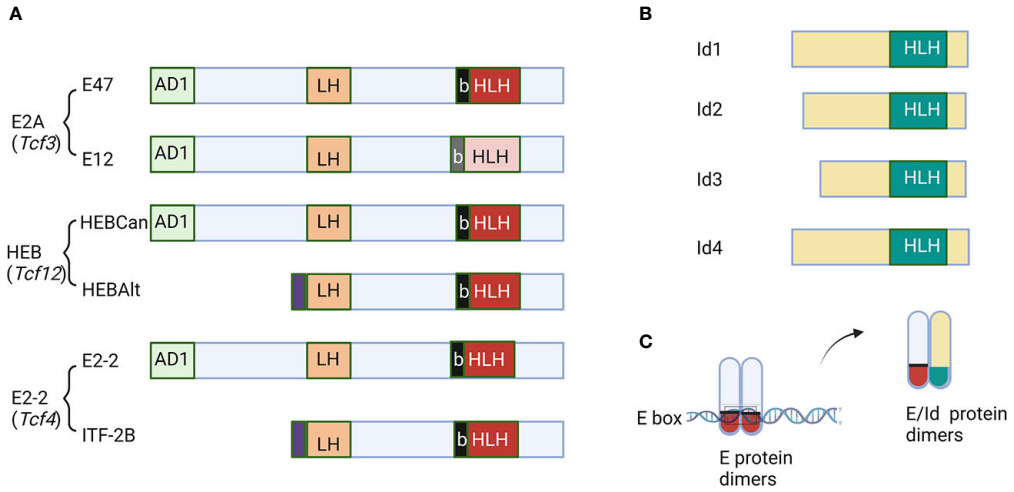

FIGURE 1
Schematic diagrams of E and Id proteins. **(A)** The function domains of E proteins are labeled. AD1 and LH are two transcription activation domains. The basic and helix-loop-helix domains are marked as b and HLH, respectively. **(B)** Id proteins with the HLH domain are shown. **(C)** The mechanism of action of Id proteins to inhibit DNA binding by E proteins. The figure was created by BioRender.com.

tyrosine residues which can be modified by phosphorylation that augments its transcriptional activity (13).

The family of inhibitor of differentiation proteins, Id1-4, antagonize E proteins by dimerizing with them *via* the helix-loop-helix domain (Figure 1B) (14–19). However, because Id proteins lack the basic amino acids necessary for DNA binding, heterodimers between E and Id proteins cannot bind to E box sequences (Figure 1C). Transcription of the E protein genes is less variable but that of the Id genes is highly dynamic. Therefore, the net E protein activity in a given cell is determined by the levels of both E and Id proteins (16, 17). In this review, we intend to highlight the roles of E and Id proteins in regulating the fate choices between T cells and innate lymphoid cells.

T cell development

Lymphoid-primed multipotent progenitors (LMPP) and common lymphoid progenitors (CLP) travel from the bone marrow to the thymus and become early T cell progenitors (ETP) (20–23). T cell developmental progression in the thymus can be generally defined by the expression of CD4 and CD8 surface markers: from CD4 and CD8 double negative (DN) to double positive (DP) and then to CD4 or CD8 single positive (SP) (24–27). During the transition from DN to DP stage, an immature CD8 single positive subset (ISP) has been described (28, 29). Within the DN compartment, four subsets (DN1 to DN4) are characterized by the expression of c-kit and CD25 in the order of maturity as c-kit$^+$CD25$^-$, c-kit$^+$CD25$^+$, c-kit$^-$CD25$^+$ and c-kit$^-$CD25$^-$ (27). ETPs are at the top of the hierarchy and included in the DN1 subset (23). They give rise to both αβ and γδ T cells, which have distinct T cell receptors (TCRs), different developmental programs and divergent functions. The E2A and HEB genes are both expressed in the thymus. Interestingly, HEBAlt is preferentially produced in the DN and ISP stages. Since E proteins are known to inhibit cell proliferation and HEBAlt acts as a hypomorph (13, 30), whether HEBAlt plays a role in tampering E protein activities during pre-TCR-triggered cell expansion is interesting to be investigated.

αβ T cells

The development of αβ T cells is largely driven by αβ TCR signaling events. However, before the formation of pre-TCRs and TCRs, the differentiation of committed T cell precursors is supported by Notch signaling and signaling from cytokine receptors such as that of IL-7 (31–35). Critical transcription factors involved in T cell commitment include TCF1, GATA3 and Bcl11b (36–40). The sequential rearrangements of TCRβ and then TCRα genes catalyzed by the RAG1 and RAG2 recombinases set the milestones of the developmental progression (24, 41–44). The TCRβ locus undergoes recombination between D to J regions and then V to DJ regions to produce functional β chains, which pair

with the pre-Tα (45, 46). The pre-TCR complex delivers signals leading to the expansion of DN3 cells and their advancement to the DP stage. The TCRα gene rearrangement occurs at the DP stage, which allows the formation of αβ TCRs, triggers the positive and negative selection and enables the generation of mature SP T cells (47–49). Mature but naive T cells leave the thymus by the upregulation of S1PR1 and CD62L (50–53).

αβ T cells possess a large repertoire of TCRs due to a collection of V regions. These TCRs recognize diverse antigens presented by the MHC molecules and elicit subsequent signaling events. $CD4^+$ and $CD8^+$ naïve T cells exit the thymus to be activated and differentiate into helper and cytotoxic effectors in peripheral lymphoid organs, respectively (54, 55). Due to the sheer quantity of thymic output of αβ T cells and their ability to proliferate in response to antigen engagement, αβ T cells are the major players of adaptive T cell immunity.

γδ T cells

The development of γδ T cells differs from αβ T cells. Firstly, unlike αβ T cells, γδ T cells do not traverse DP and SP stages during the development. Instead, they undergo γδ lineage commitment and maturation at DN2 and DN3 stages (56–58). Generation of mature γδ T cells depends on the V-J rearrangement of the TCRγ locus and V(D)J recombination in the TCRδ locus, along with Notch signaling. Since the TCRδ gene is embedded in the TCRα locus, TCRα rearrangement, triggered by pre-TCR signaling after an independent rearrangement event of the TCRβ gene, can eliminate the TCRδ gene, thus aborting the γδ T cell fate (59–61). Early precursors of effector γδ T cells in the thymus are identified as $CD24^+$ and then mature to $CD24^-$ stage (62, 63). There are three types of γδ T cells classified based on their effector functions, γδ1, γδ17 and innate-like γδ T cells, which secrete interferon γ, IL-17 and interferon γ plus IL-4, respectively (64–66). The development of γδ T cells require stronger TCR signals in the comparison to αβ T cells (67, 68). The gradients of TCR signals determines the development of specific effector subsets. The generation of innate-like γδ T cells depends on the strongest TCR signal as indicated by their higher levels of CD5 compared to other γδ subsets (69). CD5 levels are proportional to TCR signaling strength in the thymus (70, 71). Expression of PLZF transcription factor also depends on ligand ligation with TCR and PLZF is required for the effector function of innate-like γδ T cells (72). Type 1 γδ T cells also require a strong TCR signal and the T-bet transcription factor is critical for γδ1 differentiation (65, 72–75). On the other hand, type 17 γδ T cells rely on a weaker TCR signal for the differentiation (65, 72, 74, 76). In fetal organ culture, addition of activating antibodies against γδ TCR or CD3 impairs the production of γδ17 cells (76). Moreover, RORγt transcription factor is essential for γδ17 development (77). Additionally, CD73 expression marks most of γδ T cells committed to mature into effector cells in the thymus (78).

Distinct subsets of γδ T cells reside in different tissues and develop at different ages in mice (56, 57). The Vγ regions are described by two different nomenclatures. In this review, we will use the one defined by Raulet and colleagues (79). In the early fetal stage, the first wave of γδ T cells is associated with the $V\gamma3^+V\delta1^+$ subset known as the dendritic epidermal cells, which produce IFNγ (65, 80, 81). The development of the $V\gamma4^+$ subset begins at the fetal stage and lasts until birth. The generation of the $V\gamma2^+$ subset occurs in the late fetal stage and continues through adulthood. The $V\gamma2^+$ subset consists of cells producing IL-17 or IFNγ (82). The IL-17-producing cells become long-lived cells with self-renewal capabilities after birth (83). $V\gamma1.1^+$ cells develop at the prenatal stage and this persists through adult life (56). Despite the complicated developmental schemes of γδ T cell differentiation, how γδ TCRs interact with their ligands and elicit signals is less understood. To some extent, γδ T cells are thought to have properties resembling innate cells.

T cell development is a "wasteful' process". Every D-J or V-DJ combination only has one third of a chance to create an in-frame joint that result in a full-length TCR chain. It is believed that over 70% of the developing T cells do not reach the mature stage and die because they fail to form pre-TCR (β selection) at the DN3 stage or because they cannot produce a full-length TCRα chain at the DP stage (death by neglect). They can also be eliminated due to excessively strong TCR signaling (negative selection). Are there any alternative fates for these T cell "drop-outs"? Perhaps, innate lymphoid cells are some of the options.

Regulation of T cell development by E and Id proteins

E proteins play pivotal roles in governing the development of αβ T cells. Two of the E protein genes, E2A and EBCan, are expressed in T cells and they have redundant functions. The proteins encoded by these two genes include E12, E47, HEBCan and HEBAlt. Since all knock-out constructs targeted the bHLH domains, E2A or HEB deficient mice lack all of their respective proteins. Germ-line ablation of either E2A or HEB gene partially impairs T cell development by dramatically reducing thymocyte counts (84, 85). The leaky block allows the maturation of small numbers of T cells, which are predisposed to develop T cell lymphoma (84–86). HEB deficiency also reveals a novel role of HEB at the ISP stage (86). In contrast, simultaneous inhibition of all E proteins by expressing Id1 using the proximal promoter of *lck* in transgenic mice results in a complete block of T cell development, arresting thymocytes at the DN1 stage when the Id1 transgene begins to be expressed (87, 88). Likewise, inducible ablation of both E2A and HEB genes using the *plck-Cre*

transgene results in a developmental arrest at the DN3 stage when the Cre gene is expressed (89).

E protein-mediated control at these early stages of T cell development is multi-dimensional. First, E proteins are known to activate the transcription of *Notch1*, which encodes the receptor for Notch ligands such as Delta-like 4 in the thymus and ensure the differentiation and survival of T cells (90–92). Second, E proteins are found to activate the transcription of *Rag1* and *Rag2* (93, 94), which code for the enzymes essential for VDJ recombination of TCR genes. Third, E proteins facilitate TCR gene rearrangement by increasing chromatin accessibility at the TCRβ locus (95). Fourth, the binding of E2A-HEB heterodimers to *Ptcra* enhancer regulates pre-Tα expression at the DN3 stage (96–98). Finally, the interplay between E proteins and other transcription factors such as TCF1 and LEF1 also contribute to the positive regulation of early T cell development (36, 99).

Following pre-TCR signaling, the Ras-MAP kinase pathway is activated, which leads to the up-regulation of Egr transcription factors and then activation of the Id3 gene (100–103). This suggests that down-regulation of E protein activity is necessary for DN3 cells progress to the DP stage. Indeed, when *Rag1* was deleted, T cell development arrested at the DN3 stage (104). However, if E proteins are down-regulated by germline E2A deletion or *pLck-Id1* expression, *Rag1*$^{-/-}$ thymocytes can advance to the DP stage (105, 106). Another mechanism to down-regulate E proteins is to accelerate their ubiquitin-mediated degradation in the presence of Notch signals and MAP kinases activated by pre-TCR signaling (107, 108).

At the DP stage, Id3 expression is transiently triggered by TCR signaling and is involved in the positive selection of developing thymocytes (101, 109). Deleting both Id2 and Id3 genes prevented the progression of positively selected T cells to the SP stage (110). Conversely, low levels of Id1 expression in *plck-Id1* heterozygous transgenic mice allows some T cell precursors reach the DP stage but a majority of these cells undergo apoptosis likely due to excessive responses to the normal levels of TCR stimulation (105, 111). This notion was supported by the observation of hyper-activation of NFκB upon ectopic Id1 expression (105, 112). In addition, deleting both E2A and HEB genes also impairs the generation of CD4 SP T cells (110). Collectively, E and Id proteins clearly are the central players in shaping αβ T cell development.

A strong TCR signal triggers the activation of the ERK-Egr-Id3 axis and favors γδ over αβ T cell development (73). Id3 deficiency resulted in an expansion of Vγ1.1^{+} innate-like γδ T cells, possibly due to the dampening of the strong TCR signaling which normally causes the death of these cells (113, 114). In fetal organ cultures, HEB deficiency impairs the differentiation of Vγ4 and Vγ6-containing γδ17 cells. In *et al.* postulated two pathways of γδ T cell development (115). Pathway 1, which favors γδ1 cells, depends on strong TCR signaling and up-regulation of Id3. In contrast, pathway 2 mostly occurs in the fetal stage and requires lower levels of TCR signaling and Id3 expression. HEB is necessary for Vγ6^{+}CD73^{-} γδ17 T cells in the fetal stage as well as Vγ4^{+}CD73^{+} γδ17 T cells in neonates (115). HEB and E2A are thought to activate the transcription of *Sox4, Sox13* and *Rorc* genes necessary for γδ17 differentiation (115, 116). Overall, it appears that Id3 expression plays a critical role in directing γδ T cell development through counterbalancing the function of E proteins.

Differentiation of innate lymphoid cells

Innate lymphoid cells (ILCs) are first responders in immune reactions towards environmental insults and microbial infections. ILCs are divided into three groups, ILC1 to ILC3, which play different roles during specific immune responses (117, 118). Even though ILCs share with T cells the transcriptional factors that drive their differentiation and the profiles of cytokine production, they lack T-cell receptors (TCR), thus eliciting innate immunity as opposed to adaptive immunity mediated by T cells (118–120). Each ILC subset has been increasingly recognized to be heterogenous and display different characteristics in different tissues (121). Plasticity between the three ILC subsets also exist, especially under pathophysiological conditions (118, 122). Nevertheless, the general properties and functions of these three subsets of ILCs have been established. The ILC1 group consists of helper-like ILC1s and conventional NK cells (cNK). ILC1s mediate the early immune response upon contact with intracellular pathogens like bacteria and viruses. Their effector function regarding cytokine production is similar to the that of cNK cells, namely secreting IFNγ upon pathogen exposure. However, NK cells but not helper-like ILC1s are cytotoxic and able to produce high levels of cytotoxic granules like perforin and granzymes. The T-bet transcription factor is responsible for ILC1 differentiation and function (123). ILC2s share a transcriptional network and cytokine production profiles with those of type 2 T helper cells (Th2). GATA3 is the signature transcription factor and drives the expression of cytokines including IL-5, IL-13, IL-4, IL-9, and amphiregulin (124–126). RORα is another transcription factor indispensable for ILC2 differentiation (127). ILC2s are crucial for the protection against helminth infection. They are also activated by allergens due to the release of IL-25, IL-33 and TSLP in the tissues, contributing to a number of respiratory diseases such as asthma (128). On the other hand, ILC2s have also been shown to be involved in tissue repair following influenza infection (129). The ILC3 group includes innate immune cells committed to targeting extracellular microbes. They reside mainly in the mucosal tissues and maintain their homeostasis locally. ILC3s express RORγt and produce cytokines such as IL-17A, IL-22, and GM-CSF (118, 123). Lymphoid tissue inducers (LTis) are a subset of ILC3s essential during the fetal stage for

supporting the development of lymph nodes and other lymphoid tissues (130).

Innate lymphoid cells are progenies of hematopoietic stem cells, arising from progenitors destined to become lymphoid cells such as lymphoid-primed multipotent progenitors (LMPPs) or common lymphoid progenitors (CLPs) (20, 21). These progenitors reside in fetal liver or adult bone marrow where ILCs differentiate in addition to B cells. These processes have been extensively studied as summarized below. However, LMPPs and CLPs also travel to the thymus to produce T cells. The capability of the thymus to support ILC differentiation has recently become appreciated (90, 131–133). The divergence of T cell development to ILC fates is an interesting issue to be addressed here. Finally, ILCs are also believed to be derived from tissue-resident progenitors but at what stage these progenitors seed the peripheral tissues and whether all ILC subsets utilizes this mechanism of reproduction are not fully understood.

ILC differentiation in the bone marrow and fetal liver

Innate lymphoid cells develop in the bone marrow from LMPPs or CLPs through a series of intermediate progenitors which progressively lose the potential of giving rise to B cells and then NK cells (120). The progenitors that can generate subsequent progenitors for either ILC or NK cells are called alpha LPs (αLPs), which require the NFIL3 and TOX transcription factors (134). Early innate lymphoid progenitors (EILP) characterized by TCF1 expression, also have a similar differentiation potential (135). Next, common helper ILC progenitors (CHILPs) are regulated by Id2 and responsible for the ILC but not NK subsets (136). ILC progenitors (ILCPs) controlled by PLZF are dedicated to only producing ILCs, and are found in both bone marrow and fetal liver (137). In contrast, NK progenitors (NKPs) which also express Id2 are specialized to become NK cells (120, 137). Although CHILPs or ILCPs have the potential to give rise to all three ILC subsets *in vitro* when cultured on OP9-DL1 stroma, the predominant subset detected in the bone marrow is ILC2 as well as their precursors called ILC2Ps (138). Moreover, there is also evidence that ILC1s can be generated in adult liver from fetal hematopoietic stem cells (139).

Whether the bone marrow serves as a constant source of ILC2 replenishment has not been well established. Experiments using parabionts suggested tissues such as the lung receive few ILC2s from the blood circulation (140, 141). However, recent single cell RNA sequencing (scRNAseq) data showed a population of ILC2s in the blood of wild type and athymic nude mice, which suggest that these ILC2s may come from the bone marrow or they are the recirculating ILC2s from peripheral tissues (133). IL-18R$^+$ precursors of ILC2s have also been found in the lung and shown to arrive from the blood (142, 143).

In humans, ILC progenitors with biases to different ILC subsets are readily detectable in the blood (144, 145). Likewise, committed ILC1 to ILC3 subsets are also found in the blood (122, 146). These cells are assumed to come from the bone marrow but no direct evidence is available. The frequencies of the ILC subsets are often found to be altered in different disease states, which may potentially serve as biomarkers of these diseases (147–149).

ILC differentiation in the thymus

Small numbers of ILCs, particularly ILC2s, have been found in the thymus at pre- and post-natal stages (150–154). This is consistent with the fact T cell progenitors express the transcription factors supporting ILC2 differentiation, namely GATA3, TCF1 and Bcl11b (155).

Whether the thymus is another lymphoid organ capable of exporting ILC precursors or ILCs to peripheral tissues was investigated by using scRNAseq of the lineage negative (Lin$^-$) Thy1$^+$ fraction of the blood of wild type and athymic nude mice (133). Bajana *et al.* found that about half of the ILC-containing Lin$^-$Thy1$^+$ population, was greatly diminished in the athymic nude mice, which suggest that the production of these cells is thymus-dependent, thus designated td-ILCs. These cells were fractionated into four clusters based on their distinct transcriptomic properties. All td-ILCs express genes commonly expressed in ILCs such as *Tcf7* and *Il7r* but they lack the signature transcription factors that specify ILC1 to ILC3: T-bet, GATA3 and RORγt, suggesting that td-ILCs can be ILC precursors. Indeed, when these cells were isolated as Lin$^-$Thy1$^+$CD127$^+$CD62L$^+$ from the blood and cultured on OP9-DL1 stroma, different subsets of ILCs were generated (133). Whether this population contains disparate progenitors for distinct ILC subsets or progenitors with multiple potentials is to be determined.

Interestingly, td-ILCs express *Cd3d*, *Cd3e* and *Cd3g* but no other T cell specific genes such as *Cd4, Cd8a, Rag1, Rag2* and *Dntt*. Flow cytometry analyses detected CD3ϵ by intracellular staining but not by surface staining (133). Moreover, td-ILCs do not have TCRβ or TCRδ either on the surface or in the cytoplasm, thus indicating that they are not T cells. Using intracellular CD3ϵ (icCD3ϵ) as a marker, Bajana *et al.* also detected icCD3ϵ$^+$ cells in the lung, small intestine and skin of wild type mice (133). Because these icCD3ϵ $^+$ cells are greatly diminished in nude mice, the results were interpreted to mean that icCD3ϵ marks thymus-derived cells. Like in blood td-ILCs, the icCD3ϵ$^+$ cells in the lung and small intestine do not express TCRβ or TCRδ, ruling out the possibility that they are T cells. This suggests that td-ILCs in the blood may home to peripheral tissues where they differentiate into diverse ILC subsets. In the lung, a significant fraction of icCD3ϵ$^+$ ILCs are ST2$^-$RORγt$^+$ ILC3-like cells. In contrast, the lamina

propria of small intestine harbors icCD3ϵ^{+}KLRG1^{-}T-bet^{+} ILC1-like cells. Curiously, the expression levels of GATA3 correlated inversely with those of icCD3ϵ, which suggests that ILC2 differentiation is accompanied by the down-regulation of CD3 expression (133). Although this possibility remains to be investigated, the potential down-regulation of CD3 expression makes it difficult to assess the contribution of thymus-derived ILC2s to the overall ILC2 pool. A lineage-tracing system with a *Cre* transgene that is specifically and efficiently expressed at the early stages of T cell development would greatly facilitate the estimation of the contribution of thymus-derived ILC2s and further validate the thymic origin of ILC2 subsets.

Additional evidence exist that support the notion that the thymus contributes to the ILC2 pools. Qian *et al.* showed that not only multipotent progenitors (DN1) but also committed T lineage cells (DN3) from the thymus can differentiate into functional ILC2 on OP9-DL1 stromal cells (132). Consistently, ILC2s isolated from the lung of WT but not nude mice harbor rearranged TCR genes, *Tcrb* and *Tcrg*, suggesting that at least some of the ILC2s originated from committed T lineage cells in the thymus (132, 156). While *Tcrg* rearrangement was readily detectable by electrophoresis, analyses of the D-J and V-DJ recombination in the *Tcrb* locus required Southern blotting because of the diversity of their rearrangement events. Shin *et al.* sequenced the rearranged *Tcrg* segments and found a reduced frequency of in-frame rearrangement in ILC2s compared to that in γδ T cells (156). It was thus concluded that ILC2s are derived from cells which have failed productive γδ TCR rearrangement (156, 157). However, further investigation at the single-cell level could strengthen the conclusion. Despite the rearrangement events detected, ILC2s do not express TCRβ or TCRδ either intracellularly or on the surface.

Likewise, NK cells have also been shown to arise from early T cell precursors in the thymus, suggesting a branch point between T and NK cells (158–160). It remains to be determined if this branch point is similar or different from those giving rise to ILCs.

Regulation of ILC differentiation by E and Id proteins

Id2 is expressed in ILC progenitors and plays an essential role in ILC development, which implicates the involvement of E proteins in regulating ILC differentiation (136, 161). Strikingly, down-regulation of E proteins by the ectopic expression of Id1 in transgenic thymocytes at the DN1 stage or by deletion of the E2A and HEB genes with *plck-Cre* at the DN3 stage led to dramatic increases in ILC2 production in the thymus (131, 132). As a result, large amounts of ILC2s were exported from the thymus to peripheral tissues throughout the body. The thymus was shown to be responsible for the mass production of ILC2 in Id1 transgenic mice because when the transgene was bred onto the nude background, ILC2 expansion was no longer detectable (132). ILC2s made in the thymus of Id1 transgenic and E protein deficient mice respond to IL-25 or IL-33 stimulation similarly as wild type ILC2s by secreting IL-5 and IL-13 in cultures (131, 132). *In vivo*, Id1 transgenic mice exhibited greater type 2 responses when treated with papain in the lung or during helminth infection (131). These are likely due to the presence of excessive amounts of ILC2s in Id1 transgenic mice. However, on a per cell basis, Id1 transgenic ILC2s appeared to have a less robust production of IL5 and IL-13 (131). It is not clear if this is due to a cell intrinsic difference or a limitation of stimuli available to all of the extra ILC2s in Id1 transgenic mice. Barshad *et al.* made a similar observation by treating wild type and Id1 transgenic mice with house dust mites (HDM) (162). By analyzing the chromatin accessibility, they found a reduction in AP-1 and C/EBP binding sites in open chromatins after HDM treatment in Id1 transgenic ILC2s. Whether this is due to a direct or indirect effect of E protein inhibition remained to be determined.

In the blood of *Tcf3*$^{fl/fl}$*Tcf12*$^{fl/fl}$*plck-Cre* mice, an extremely large population of cells (cluster 0) that belong to thymus-dependent ILC precursors was detected using scRNAseq (133). In addition, a subset (cluster 2) with characteristics of NK cells was also markedly enriched (133). These cells can give rise to different ILC/NK subsets when cultured on OP9-DL1 stroma (133). Together, these results suggest that E proteins play multiple roles in suppressing the production of ILC and NK precursors, which may arise at different developmental stages or from different T cell precursors. Whether E proteins suppress the same or different transcriptional programs governing ILC and NK differentiation remains to be investigated.

Ablating E2A and HEB genes starting at the CLP stage using *IL7r-Cre* increased the production of both ILC2s and LTi-like cells, a subset of ILC3s (90). Conversely, inducible expression of a gain-of-function mutant of E47 by *Rag1-Cre* impaired the differentiation of ILC2s from ILCP in the bone marrow (163). Furthermore, Id2$^{-/-}$ mice have been shown to be devoid of NK cells and lymph nodes which are initiated by LTi cells (130). Yet, overexpression of Id3 in human hematopoietic stem cells promoted NK differentiation (164). These findings suggest that down-regulating E protein function is instrumental for NK cell differentiation (165). It was further shown that Id2 plays a key role in regulating the production of IL-15 important for NK homeostasis (166, 167).

Transcriptional programs of E protein-mediated suppression of ILC differentiation

Inducible deletion of the E2A and HEB genes promoted ILC2 differentiation from CLP, DN1 and DN3 cells on OP9-DL1

stroma by 20-40 folds, which demonstrates a powerful cell-intrinsic suppression by E proteins (132). It is therefore interesting to elucidate the transcriptional programs that underlie the suppression of ILC2 differentiation. Miyazaki *et al.* performed RNA sequencing and Assay for Transposase-accessible Chromatin Sequencing (ATAC-seq) using DN1 (ETP) cells of fetal thymi of control and $Tcf3^{fl/fl}Tcf12^{fl/fl}$*Il7r-Cre* mice. As expected, they found the down-regulation of an array of genes important for T cell development, which include *Notch1, Ptcra, Rag1, Rag2* and *Cd3d*. On the other hand, genes known to be expressed in ILC progenitors or ILC2s were up-regulated. Examples of such genes are *Pdcd1, Il18r, Id2, Gata3, Lmo4, Rora, Tox, Est1, Il4, Il1rl1* and *Klrg1*. The chromatin accessibility assays also showed a shift from the open chromatin patterns of T cells to those of ILCs. While these findings agree with the phenotypes of E protein deficient mice, it is difficult to pinpoint the critical switches that alter the cell fates.

Likewise, Qian *et al.* conducted RNA sequencing using DN1 or DN3 cells from control and $Tcf3^{fl/fl}Tcf12^{fl/fl}Rosa26^{CreERT2}$ mice cultured on OP9-DL1 stromal cells (132). On day 4 of the culture, tamoxifen was added to the medium and the cells were collected 24 or 72 hours later. Expression of genes important for T cell development decreased whereas those crucial for ILC2 differentiation increased. Even after one day of E-protein ablation, a collection of genes coding for diverse transcription factors became activated. These include *Zbtb16, Gata3, Rora, Rxra, Klf6, Ikzf2* and *Irf4*. While it is possible that E proteins individually repress the transcription of all of these genes, a coordinated program that controls the transcription of critical factors essential for ILC2 differentiation may be at play.

A close-up look at the action of E proteins was carried out by making use of the E47-ER fusion proteins (112), which allowed instant induction of E protein activity upon addition of tamoxifen (168). ILC2s from the thymus of Id1 transgenic mice were transduced with retroviruses expressing E47-ER or empty control viruses. Transduced cells were isolated by sorting for EGFP expressed off the same retroviral vector. After expansion, these cells were then incubated with tamoxifen for 4 or 16 hours and harvested for RNA sequencing or ATAC-seq. Consistent with the function of E proteins as transcription activators, Peng *et al.* found more genes activated than repressed by E47-ER at both time points (168). Among them are three genes encoding transcriptional repressors, *Cbfa2t3, Jdp2 and Bach2* (169–171).

Interestingly, ATAC-seq data showed that a modest increase in chromatin accessibility 4 hours post induction of E47 was followed by a widespread reduction in open chromatin regions 16 hours later. Moreover, the transcription factor motifs enriched in the differential peaks shifted from those bound by bHLH and Ets1 proteins at 4 hours to those recognized by bZip and GATA factors. It is therefore possible that one of the mechanisms whereby E proteins suppress ILC2 differentiation is to control the expression of transcription repressors, which in turn negatively regulate the transcription of genes important for ILC2 differentiation or function. Although this hypothesis has not been validated through genetic complementation studies, the correlation between the alteration of gene expression in $Cbfa2t3^{-/-}$ and E protein deficient mice support this idea (168, 172). Proteins bound to bZip and GATA motifs such as Batf and GATA3 are also known to be crucial for ILC2 function (126, 173, 174).

The RORα transcription factor also plays an important role in ILC2 differentiation (127). $Rora^{-/-}$ mice lack ILC2s but have intact T cell compartments. Recently, Ferreira *et al.* showed that RORα promotes ILC2 over T cell development by activating the transcription of *Id2* and *Nfil3*, which in turn inhibit the function of E proteins (153). However, in E protein deficient thymocytes, *Rora* expression is consistently up-regulated (90, 132, 168). Thus, a positive feedback loop may perpetually cause the up-regulation of *Rora* expression during ILC2 differentiation. There are likely additional transcription factors which act in parallel or in sequence to coordinate the differentiation of ILC2s and possibly ILC progenitors. However, it is clear that E proteins and their inhibitors, Id proteins, play a central role in maintaining the balance between T cell and ILC development.

The crossroads of T cells and innate lymphoid cells

The major difference between T cells and innate lymphoid cells is the presence and absence of TCRs on their cell surface, respectively. However, there are a number of common features in the differentiation of these two types of cells (175). The thymic environment is conducive to the maturation of both T cells and ILCs (at least ILC2s and ILC3s) by supporting Notch and IL-7 signaling. The thymic progenitors equipped with transcription factors such as TCF1 and GATA3, are able to differentiate into both T cells and ILCs. Obviously, T cell production is the dominating responsibility of the thymus. This is due to the overwhelming effects of TCR-driven T cell expansion and powerful transcriptional programs in place to ensure an adequate T cell output. One of such transcriptional programs is controlled by the balance between E and Id proteins (Figure 2). When E protein activities are high, T cell development proceeds. When Id proteins overcome E proteins, ILCs can develop. Although Id2 has been shown to be expressed in ILC progenitors and play critical roles in ILC differentiation in the bone marrow, expression of Id3 is stimulated by TCR signaling in both αβ and γδ T cells (73, 106). This would create opportunities for developing T cells to divert to the ILC path. However, this possibility needs to be vigorously investigated. It is also interesting to explore whether the large numbers of developing T cells eliminated during the differentiation processes could be recycled into ILCs and used to replenish ILC pools in peripheral tissues. The E/Id axis has clearly been

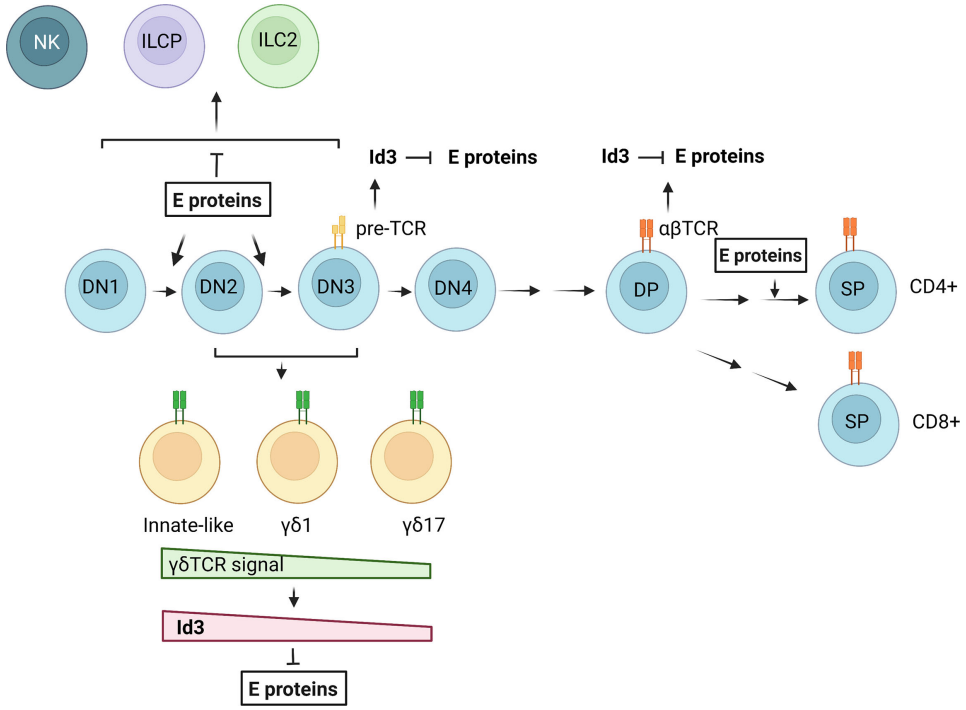

FIGURE 2
Regulation of T cell and ILC differentiation by E and Id proteins. E proteins promote T cell commitment and differentiation from DN1 to DN3 stages. Pre-TCR and TCR signaling in αβ T cells lead to transient Id3 up-regulation and E protein inhibition. In γδ T cell development, a gradient of γδ TCR signal determines the outcomes of different γδ subsets through regulation of Id3 expression and E protein activities. NKs, ILC2s and ILC precursors (ILCPs) may arise in the DN stages when E protein functions are suppressed. The figure was created by BioRender.com.

shown to be gate-keepers in the crossroads to T cell and ILC fates but the downstream transcriptional events remain to be further elucidated as the technologies and critical reagents become available.

Author contributions

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Conflict of interest

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References

1. Murre C. Helix-loop-helix proteins and lymphocyte development. *Nat Immunol* (2005) 6(11):1079–86. doi: 10.1038/ni1260

2. Lazorchak A, Jones ME, Zhuang Y. New insights into e-protein function in lymphocyte development. *Trends Immunol* (2005) 26(6):334–8. doi: 10.1016/j.it.2005.03.011

3. Murre C, McCaw PS, Baltimore D. A new DNA binding and dimerization motif in immunoglobin enhancer binding, *daughterless*, MyoD and *myc* proteins. *Cell* (1989) 56:777–83. doi: 10.1016/0092-8674(89)90682-X

4. Hu JS, Olson EN, Kingston RE. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic

regulatory factors. *Mol Cell Biol* (1992) 12(3):1031–42. doi: 10.1128/mcb.12.3.1031-1042.1992

5. Henthorn P, Kiledjian M, Kadesch T. Two distinct transcription factors that bind the immunoglobin enhancer mE5/kE2 motif. *Sci* (1990) 247:467–70. doi: 10.1126/science.2105528

6. Aronheim A, Shiran R, Rosen A, Walker MD. The E2A gene product contains two separable and functionally distinct transcription activation domains. *Proc Natl Acad Sci USA* (1993) 90:8063–7. doi: 10.1073/pnas.90.17.8063

7. Massari ME, Grant PA, Pray-Grant MG, Berger SL, Workman JL, Murre C. A conserved motif present in a class of helix-loop-helix proteins activates transcription by direct recruitment of the SAGA complex. *Mol Cell* (1999) 4 (1):63–73. doi: 10.1016/S1097-2765(00)80188-4

8. Quong MW, Massari ME, Zwart R, Murre C. A new transcriptional-activation motif restricted to a class of helix-loop-helix proteins is functionally conserved in both yeast and mammalian cells. *Mol Cell Biol* (1993) 13:792–800. doi: 10.1128/mcb.13.2.792-800.1993

9. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, et al. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* (1989) 58:537–44. doi: 10.1016/0092-8674(89)90434-0

10. Kamps MP, Murre C, Sun X-H, Baltimore D. A new homeobox gene contributes the DNA-binding domain of the t(1:19) translocation protein in pre-b ALL. *Cell* (1990) 60:547–55. doi: 10.1016/0092-8674(90)90658-2

11. Sun X-H, Baltimore D. An inhibitory domain of E12 prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell* (1991) 64:459–70. doi: 10.1016/0092-8674(91)90653-G

12. Wang D, Claus CL, Vaccarelli G, Braunstein M, Schmitt TM, Zuniga-Pflucker JC, et al. The basic helix-loop-helix transcription factor HEBAlt is expressed in pro-T cells and enhances the generation of T cell precursors. *J Immunol* (2006) 177(1):109–19. doi: 10.4049/jimmunol.177.1.109

13. Yoganathan K, Yan A, Rocha J, Trotman-Grant A, Mohtashami M, Wells L, et al. Regulation of the signal-dependent e protein HEBAlt through a YYY motif is required for progression through T cell development. *Front Immunol* (2022) 13:848577). doi: 10.3389/fimmu.2022.848577

14. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* (1990) 61:49–59. doi: 10.1016/0092-8674(90)90214-Y

15. Sun X-H, Copeland NG, Jenkins NA, Baltimore D. Id proteins, Id1 and Id2, selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol Cell Biol* (1991) 11:5603–11. doi: 10.1128/mcb.11.11.5603-5611.1991

16. Sun XH. Multitasking of helix-loop-helix proteins in lymphopoiesis. *Adv Immunol* (2004) 84:43–77. doi: 10.1016/S0065-2776(04)84002-1

17. Kee BL. E and ID proteins branch out. *Nat Rev Immunol* (2009) 9(3):175–84. doi: 10.1038/nri2507

18. Christy BA, Sanders LK, Lau LF, Copeland NG, Jenkins NA, Nathans D. An id-related helix-loop-helix protein encoded by a growth factor-inducible gene. *Proc Natl Acad Sci USA* (1991) 88:1815–9. doi: 10.1073/pnas.88.5.1815

19. Riechmann V, van Cruchten I, Sablitzky F. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucl Acids Res* (1994) 22:749–55. doi: 10.1093/nar/22.5.749

20. Adolfsson J, Borge OJ, Bryder D, Theilgaard-Monch K, Astrand-Grundstrom I, Sitnicka E, et al. Upregulation of Flt3 expression within the bone marrow lin(-)Sca1(+) c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immun* (2001) 15(4):659–69. doi: 10.1016/S1074-7613(01)00220-5

21. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* (1997) 91(5):661–72. doi: 10.1016/S0092-8674(00)80453-5

22. Perry SS, Welner RS, Kouro T, Kincade PW, Sun XH. Primitive lymphoid progenitors in bone marrow with T lineage reconstituting potential. *J Immunol* (2006) 177(5):2880–7. doi: 10.4049/jimmunol.177.5.2880

23. Allman D, Sambandam A, Kim S, Miller JP, Pagan A, Well D, et al. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* (2003) 4(2):168–74. doi: 10.1038/ni878

24. Fehling HJ, von Boehmer H. Early alpha beta T cell development in the thymus of normal and genetically altered mice. *Curr Opin Immunol* (1997) 9 (2):263–75. doi: 10.1016/S0952-7915(97)80146-X

25. Godfrey DI, Kennedy J, Suda T, Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8-triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol* (1993) 150(10):4244–52.

26. Kisielow P, von Boehmer H. Development and selection of T cells: facts and puzzles. *Adv Immunol* (1995) 58:87–209. doi: 10.1016/S0065-2776(08)60620-3

27. Porritt HE, Rumfelt LL, Tabrizifard S, Schmitt TM, Zuniga-Pflucker JC, Petrie HT. Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immun* (2004) 20(6):735–45. doi: 10.1016/j.immuni.2004.05.004

28. Shortman K, Wilson A, Egerton M, Pearse M, Scollay R. Immature CD4-CD8+ murine thymocytes. *Cell Immunol* (1988) 113(2):462–79. doi: 10.1016/0008-8749(88)90042-1

29. MacDonald HR, Budd RC, Howe RC. A CD3- subset of CD4-8+ thymocytes: A rapidly cycling intermediate in the generation of CD4+8+ cells. *Eur J Immunol* (1988) 18(4):519–23. doi: 10.1002/eji.1830180405

30. Prabhu S, Ignatova A, Park ST, Sun X-H. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and ID proteins. *Mol Cell Biol* (1997) 17(10):5888–96. doi: 10.1128/MCB.17.10.5888

31. Sambandam A, Maillard I, Zediak VP, Xu L, Gerstein RM, Aster JC, et al. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol* (2005) 6(7):663–70. doi: 10.1038/ni1216

32. Ciofani M, Knowles GC, Wiest DL, von Boehmer H, Zuniga-Pflucker JC. Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. *Immun* (2006) 25(1):105–16. doi: 10.1016/j.immuni.2006.05.010

33. He YW, Nakajima H, Leonard WJ, Adkins B, Malek TR. The common gamma-chain of cytokine receptors regulates intrathymic T cell development at multiple stages. *J Immunol* (1997) 158(6):2592–9.

34. Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immun* (1999) 10(5):547–58. doi: 10.1016/S1074-7613(00)80054-0

35. Haks MC, Oosterwegel MA, Blom B, Spits HM, Kruisbeek AM. Cell-fate decisions in early T cell development: Regulation by cytokine receptors and the pre-TCR. *Semin Immunol* (1999) 11(1):23–37. doi: 10.1006/smim.1998.0153

36. Okamura RM, Sigvardsson M, Galceran J, Verbeek S, Clevers H, Grosschedl R. Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immun* (1998) 8(1):11–20. doi: 10.1016/s1074-7613(00)80454-9

37. Anderson MK, Hernandez-Hoyos G, Dionne CJ, Arias AM, Chen D, Rothenberg EV. Definition of regulatory network elements for T cell development by perturbation analysis with PU.1 and GATA-3. *Dev Biol* (2002) 246(1):103–21. doi: 10.1006/dbio.2002.0674

38. Weber BN, Chi AW, Chavez A, Yashiro-Ohtani Y, Yang Q, Shestova O, et al. A critical role for TCF-1 in T-lineage specification and differentiation. *Nat* (2011) 476(7358):63–8. doi: 10.1038/nature10279

39. Tydell CC, David-Fung ES, Moore JE, Rowen L, Taghon T, Rothenberg EV. Molecular dissection of prethymic progenitor entry into the T lymphocyte developmental pathway. *J Immunol* (2007) 179(1):421–38. doi: 10.4049/jimmunol.179.1.421

40. Hosokawa H, Romero-Wolf M, Yang Q, Motomura Y, Levanon D, Groner Y, et al. Cell type-specific actions of Bcl11b in early T-lineage and group 2 innate lymphoid cells. *J Exp Med* (2020) 217(1):e20190972. doi: 10.1084/jem.20190972

41. Mallick CA, Dudley EC, Viney JL, Owen MJ, Hayday AC. Rearrangement and diversity of T cell receptor beta chain genes in thymocytes: A critical role for the beta chain in development. *Cell* (1993) 73(3):513–9. doi: 10.1016/0092-8674(93)90138-G

42. Hoffman ES, Passoni L, Crompton T, Leu TM, Schatz DG, Koff A, et al. Productive T-cell receptor beta-chain gene rearrangement: Coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev* (1996) 10(8):948–62. doi: 10.1101/gad.10.8.948

43. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. *Cell* (1989) 59(6):1035–48. doi: 10.1016/0092-8674(89)90760-5

44. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Sci* (1990) 248 (4962):1517–23. doi: 10.1126/science.2360047

45. von Boehmer H, Aifantis I, Feinberg J, Lechner O, Saint-Ruf C, Walter U, et al. Pleiotropic changes controlled by the pre-t-cell receptor. *Curr Opin Immunol* (1999) 11(2):135–42. doi: 10.1016/S0952-7915(99)80024-7

46. Born W, Yague J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor beta-chain genes during T-cell development. *Proc Natl Acad Sci U S A* (1985) 82(9):2925–9. doi: 10.1073/pnas.82.9.2925

47. Hayday AC, Diamond DJ, Tanigawa G, Heilig JS, Folsom V, Saito H, et al. Unusual organization and diversity of T-cell receptor alpha-chain genes. *Nat* (1985) 316(6031):828–32. doi: 10.1038/316828a0

48. Yague J, Blackman M, Born W, Marrack P, Kappler J, Palmer E. The structure of V alpha and J alpha segments in the mouse. *Nucleic Acids Res* (1988) 16 (23):11355–64. doi: 10.1093/nar/16.23.11355

49. von Boehmer H. Positive selection of lymphocytes. *Cell* (1994) 76(2):219–28. doi: 10.1016/0092-8674(94)90330-1

50. McCaughtry TM, Wilken MS, Hogquist KA. Thymic emigration revisited. *J Exp Med* (2007) 204(11):2513–20. doi: 10.1084/jem.20070601

51. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nat* (2004) 427(6972):355–60. doi: 10.1038/nature02284

52. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J Biol Chem* (2004) 279(15):15396–401. doi: 10.1074/jbc.M314291200

53. Jin R, Wang W, Yao JY, Zhou YB, Qian XP, Zhang J, et al. Characterization of the *in vivo* dynamics of medullary CD4+CD8- thymocyte development. *J Immunol* (2008) 180(4):2256–63. doi: 10.4049/jimmunol.180.4.2256

54. Singer A, Bosselut R. CD4/CD8 coreceptors in thymocyte development, selection, and lineage commitment: Analysis of the CD4/CD8 lineage decision. *Adv Immunol* (2004) 83:91–131. doi: 10.1016/S0065-2776(04)83003-7

55. Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev Immunol* (2002) 2(12):933–44. doi: 10.1038/nri954

56. Xiong N, Raulet DH. Development and selection of gammadelta T cells. *Immunol Rev* (2007) 215:15–31. doi: 10.1111/j.1600-065X.2006.00478.x

57. Munoz-Ruiz M, Sumaria N, Pennington DJ, Silva-Santos B. Thymic determinants of gammadelta T cell differentiation. *Trends Immunol* (2017) 38 (5):336–44. doi: 10.1016/j.it.2017.01.007

58. Parker ME, Ciofani M. Regulation of gammadelta T cell effector diversification in the thymus. *Front Immunol* (2020) 11:42. doi: 10.3389/fimmu.2020.00042

59. Kang J, Raulet DH. Events that regulate differentiation of alpha beta TCR+ and gamma delta TCR+ T cells from a common precursor. *Semin Immunol* (1997) 9(3):171–9. doi: 10.1006/smim.1997.0069

60. Krangel MS, McMurry MT, Hernandez-Munain C, Zhong XP, Carabana J. Accessibility control of T cell receptor gene rearrangement in developing thymocytes. the TCR alpha/delta locus. *Immunol Res* (2000) 22(2-3):127–35. doi: 10.1385/IR:22:2-3:127

61. Krangel MS. Mechanics of T cell receptor gene rearrangement. *Curr Opin Immunol* (2009) 21(2):133–9. doi: 10.1016/j.coi.2009.03.009

62. Pereira P, Zijlstra M, McMaster J, Loring JM, Jaenisch R, Tonegawa S. Blockade of transgenic gamma delta T cell development in beta 2-microglobulin deficient mice. *EMBO J* (1992) 11(1):25–31. doi: 10.1002/j.1460-2075.1992.tb05023.x

63. Kreslavsky T, Garbe AI, Krueger A, von Boehmer H. T Cell receptor-instructed alphabeta versus gammadelta lineage commitment revealed by single-cell analysis. *J Exp Med* (2008) 205(5):1173–86. doi: 10.1084/jem.20072425

64. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* (1995) 373 (6511):255–7. doi: 10.1038/373255a0

65. Jensen KD, Su X, Shin S, Li L, Youssef S, Yamasaki S, et al. Thymic selection determines gammadelta T cell effector fate: Antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immun* (2008) 29(1):90–100. doi: 10.1016/j.immuni.2008.04.022

66. Wencker M, Turchinovich G, Di Marco Barros R, Deban L, Jandke A, Cope A, et al. Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat Immunol* (2014) 15(1):80–7. doi: 10.1038/ni.2773

67. Hayes SM, Li L, Love PE. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immun* (2005) 22(5):583–93. doi: 10.1016/j.immuni.2005.03.014

68. Haks MC, Lefebvre JM, Lauritsen JP, Carleton M, Rhodes M, Miyazaki T, et al. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immun* (2005) 22(5):595–606. doi: 10.1016/j.immuni.2005.04.003

69. Kreslavsky T, Savage AK, Hobbs R, Gounari F, Bronson R, Pereira P, et al. TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gammadelta T cells with restricted TCR diversity. *Proc Natl Acad Sci U S A* (2009) 106(30):12453–8. doi: 10.1073/pnas.0903895106

70. Tarakhovsky A, Kanner SB, Hombach J, Ledbetter JA, Muller W, Killeen N, et al. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Sci* (1995) 269:535–7. doi: 10.1126/science.7542801

71. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J Exp Med* (1998) 188(12):2301–11. doi: 10.1084/jem.188.12.2301

72. Fahl SP, Coffey F, Kain L, Zarin P, Dunbrack RLJr., Teyton L, et al. Role of a selecting ligand in shaping the murine gammadelta-TCR repertoire. *Proc Natl Acad Sci U S A* (2018) 115(8):1889–94. doi: 10.1073/pnas.1718328115

73. Lauritsen JP, Wong GW, Lee SY, Lefebvre JM, Ciofani M, Rhodes M, et al. Marked induction of the helix-loop-helix protein Id3 promotes the gammadelta T cell fate and renders their functional maturation notch independent. *Immun* (2009) 31(4):565–75. doi: 10.1016/j.immuni.2009.07.010

74. Turchinovich G, Hayday AC. Skint-1 identifies a common molecular mechanism for the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T cells. *Immun* (2011) 35(1):59–68. doi: 10.1016/j.immuni.2011.04.018

75. Yin Z, Chen C, Szabo SJ, Glimcher LH, Ray A, Craft J. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-gamma by gammadelta T cells. *J Immunol* (2002) 168(4):1566–71. doi: 10.4049/jimmunol.168.4.1566

76. Sumaria N, Grandjean CL, Silva-Santos B, Pennington DJ. Strong TCRgammadelta signaling prohibits thymic development of IL-17A-Secreting gammadelta T cells. *Cell Rep* (2017) 19(12):2469–76. doi: 10.1016/j.celrep.2017.05.071

77. Barros-Martins J, Schmolka N, Fontinha D, Pires de Miranda M, Simas JP, Brok I, et al. Effector gammadelta T cell differentiation relies on master but not auxiliary Th cell transcription factors. *J Immunol* (2016) 196(9):3642–52. doi: 10.4049/jimmunol.1501921

78. Coffey F, Lee SY, Buus TB, Lauritsen JP, Wong GW, Joachims ML, et al. The TCR ligand-inducible expression of CD73 marks gammadelta lineage commitment and a metastable intermediate in effector specification. *J Exp Med* (2014) 211 (2):329–43. doi: 10.1084/jem.20131540

79. Garman RD, Doherty PJ, Raulet DH. Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell* (1986) 45(5):733–42. doi: 10.1016/0092-8674(86)90787-7

80. Xiong N, Kang C, Raulet DH. Positive selection of dendritic epidermal gammadelta T cell precursors in the fetal thymus determines expression of skin-homing receptors. *Immun* (2004) 21(1):121–31. doi: 10.1016/j.immuni.2004.06.008

81. Havran WL, Chien YH, Allison JP. Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Sci* (1991) 252 (5011):1430–2. doi: 10.1126/science.1828619

82. Narayan K, Sylvia KE, Malhotra N, Yin CC, Martens G, Vallerskog T, et al. Intrathymic programming of effector fates in three molecularly distinct gammadelta T cell subtypes. *Nat Immunol* (2012) 13(5):511–8. doi: 10.1038/ni.2247

83. Haas JD, Ravens S, Duber S, Sandrock I, Oberdorfer L, Kashani E, et al. Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. *Immun* (2012) 37(1):48–59. doi: 10.1016/j.immuni.2012.06.003

84. Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL, et al. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol* (1997) 17(8):4782–91. doi: 10.1128/MCB.17.8.4782

85. Yan W, Young AZ, Soares VC, Kelley R, Benezra R, Zhuang Y. High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. *Mol Cell Biol* (1997) 17:7317–27. doi: 10.1128/MCB.17.12.7317

86. Barndt R, Dai MF, Zhuang Y. A novel role for HEB downstream or parallel to the pre-TCR signaling pathway during alpha beta thymopoiesis. *J Immunol* (1999) 163(6):3331–43.

87. Kim D, Peng XC, Sun XH. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol Cell Biol* (1999) 19(12):8240–53. doi: 10.1128/MCB.19.12.8240

88. Wang HC, Perry SS, Sun XH. Id1 attenuates notch signaling and impairs T-cell commitment by elevating Deltex1 expression. *Mol Cell Biol* (2009) 29 (17):4640–52. doi: 10.1128/MCB.00119-09

89. Wojciechowski J, Lai A, Kondo M, Zhuang Y. E2A and HEB are required to block thymocyte proliferation prior to pre-TCR expression. *J Immunol* (2007) 178 (9):5717–26. doi: 10.4049/jimmunol.178.9.5717

90. Miyazaki M, Miyazaki K, Chen K, Jin Y, Turner J, Moore AJ, et al. The e-id protein axis specifies adaptive lymphoid cell identity and suppresses thymic innate lymphoid cell development. *Immun* (2017) 46(5):818–34. doi: 10.1016/j.immuni.2017.04.022

91. Yashiro-Ohtani Y, He Y, Ohtani T, Jones ME, Shestova O, Xu L, et al. Pre-TCR signaling inactivates Notch1 transcription by antagonizing E2A. *Genes Dev* (2009) 23(14):1665–76. doi: 10.1101/gad.1793709

92. Yuan JS, Kousis PC, Suliman S, Visan I, Guidos CJ. Functions of notch signaling in the immune system: consensus and controversies. *Annu Rev Immunol* (2010) 28:343–65. doi: 10.1146/annurev.immunol.021908.132719

93. Hsu LY, Lauring J, Liang HE, Greenbaum S, Cado D, Zhuang Y, et al. A conserved transcriptional enhancer regulates RAG gene expression in developing b cells. *Immun* (2003) 19(1):105–17. doi: 10.1016/S1074-7613(03)00181-X

94. Miyazaki K, Watanabe H, Yoshikawa G, Chen K, Hidaka R, Aitani Y, et al. The transcription factor E2A activates multiple enhancers that drive rag expression in developing T and b cells. *Sci Immunol* (2020) 5(51):eabb1455. doi: 10.1126/sciimmunol.abb1455

95. Bain G, Romanow WJ, Albers K, Havran WL, Murre C. Positive and negative regulation of V(D)J recombination by the E2A proteins. *J Exp Med* (1999) 189(2):289–300. doi: 10.1084/jem.189.2.289

96. Barndt RJ, Dai M, Zhuang Y. Functions of E2A-HEB heterodimers in T-cell development revealed by a dominant negative mutation of HEB. *Mol Cell Biol* (2000) 20(18):6677–85. doi: 10.1128/MCB.20.18.6677-6685.2000

97. Takeuchi A, Yamasaki S, Takase K, Nakatsu F, Arase H, Onodera M, et al. E2A and HEB activate the pre-TCR alpha promoter during immature T cell development. *J Immunol* (2001) 167(4):2157–63. doi: 10.4049/jimmunol.167.4.2157

98. Tremblay M, Herblot S, Lecuyer E, Hoang T. Regulation of pT alpha gene expression by a dosage of E2A, HEB, and SCL. *J Biol Chem* (2003) 278(15):12680–7. doi: 10.1074/jbc.M209870200

99. Emmanuel AO, Arnovitz S, Haghi L, Mathur PS, Mondal S, Quandt J, et al. TCF-1 and HEB cooperate to establish the epigenetic and transcription profiles of CD4(+)CD8(+) thymocytes. *Nat Immunol* (2018) 19(12):1366–78. doi: 10.1038/s41590-018-0254-4

100. Alberola-Ila J, Hogquist KA, Swan KA, Bevan MJ, Perlmutter RM. Positive and negative selection invoke distinct signaling pathways. *J Exp Med* (1996) 184 (1):9–18. doi: 10.1084/jem.184.1.9

101. Bain G, Cravatt CB, Loomans C, Alberola-Ila J, Hedrick SM, Murre C. Regulation of the helix-loop-helix proteins, E2A and Id3, by the ras- ERK MAPK cascade. *Nat Immunol* (2001) 2(2):165–71. doi: 10.1038/84273

102. Bettini M, Xi H, Milbrandt J, Kersh GJ. Thymocyte development in early growth response gene 1-deficient mice. *J Immunol* (2002) 169(4):1713–20. doi: 10.4049/jimmunol.169.4.1713

103. Haks MC, Krimpenfort P, Borst J, Kruisbeek AM. The CD3g chain is essential for development of both the TCRab and TCRgd lineages. *EMBO J* (1998) 17:1871–82. doi: 10.1093/emboj/17.7.1871

104. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1 deficient mice have no mature b and T lymphocytes. *Cell* (1992) 68:869–77. doi: 10.1016/0092-8674(92)90030-G

105. Kim D, Xu M, Nie L, Peng XC, Jimi E, Voll RE, et al. Helix-loop-helix proteins regulate pre-TCR and TCR signaling through modulation of Rel/NF-kappaB activities. *Immun* (2002) 16(1):9–21. doi: 10.1016/S1074-7613(02)00264-9

106. Engel I, Johns C, Bain G, Rivera RR, Murre C. Early thymocyte development is regulated by modulation of E2A protein activity. *J Exp Med* (2001) 194(6):733–45. doi: 10.1084/jem.194.6.733

107. Nie L, Xu M, Vladimirova A, Sun XH. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J* (2003) 22 (21):5780–92. doi: 10.1093/emboj/cdg567

108. Nie L, Perry SS, Zhao Y, Huang J, Kincade PW, Farrar MA, et al. Regulation of lymphocyte development by cell-type-specific interpretation of notch signals. *Mol Cell Biol* (2008) 28(6):2078–90. doi: 10.1128/MCB.00844-07

109. Rivera RR, Johns CP, Quan J, Johnson RS, Murre C. Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. *Immun* (2000) 12:17–26. doi: 10.1016/S1074-7613(00)80155-7

110. Jones-Mason ME, Zhao X, Kappes D, Lasorella A, Iavarone A, Zhuang Y. E protein transcription factors are required for the development of CD4(+) lineage T cells. *Immun* (2012) 36(3):348–61. doi: 10.1016/j.immuni.2012.02.010

111. Qi Z, Sun XH. Hyperresponse to T-cell receptor signaling and apoptosis of Id1 transgenic thymocytes. *Mol Cell Biol* (2004) 24(17):7313–23. doi: 10.1128/MCB.24.17.7313-7323.2004

112. Yang Y, Liou HC, Sun XH. Id1 potentiates NF-kappaB activation upon T cell receptor signaling. *J Biol Chem* (2006) 281(46):34989–96. doi: 10.1074/jbc.M608078200

113. Ueda-Hayakawa I, Mahlios J, Zhuang Y. Id3 restricts the developmental potential of gamma delta lineage during thymopoiesis. *J Immunol* (2009) 182 (9):5306–16. doi: 10.4049/jimmunol.0804249

114. Fahl SP, Kappes DJ, Wiest DL. TCR signaling circuits in alphabeta/gammadelta T lineage choice. In: J Soboloff and DJ Kappes, editors. *Signaling mechanisms regulating T cell diversity and function*. Boca Raton (FL):CRC Press. (2018). p. 85–104.

115. In TSH, Trotman-Grant A, Fahl S, Chen ELY, Zarin P, Moore AJ, et al. HEB is required for the specification of fetal IL-17-producing gammadelta T cells. *Nat Commun* (2017) 8(1):2004. doi: 10.1038/s41467-017-02225-5

116. Yang Y, Wang HC, Sun XH. Id1 induces apoptosis through inhibition of RORgammat expression. *BMC Immunol* (2008) 9:20. doi: 10.1186/1471-2172-9-20

117. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells–a proposal for uniform nomenclature. *Nat Rev Immunol* (2013) 13(2):145–9. doi: 10.1038/nri3365

118. Eberl G, Colonna M, Di Santo JP, McKenzie AN. Innate lymphoid cells. innate lymphoid cells: a new paradigm in immunology. *Science* (2015) 348(6237): aaa6566. doi: 10.1126/science.aaa6566

119. Zook EC, Kee BL. Development of innate lymphoid cells. *Nat Immunol* (2016) 17(7):775–82. doi: 10.1038/ni.3481

120. Yang Q, Bhandoola A. The development of adult innate lymphoid cells. *Curr Opin Immunol* (2016) 39:114–20. doi: 10.1016/j.coi.2016.01.006

121. Ricardo-Gonzalez RR, Van Dyken SJ, Schneider C, Lee J, Nussbaum JC, Liang HE, et al. Tissue signals imprint ILC2 identity with anticipatory function. *Nat Immunol* (2018) 19(10):1093–9. doi: 10.1038/s41590-018-0201-4

122. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell* (2018) 174(5):1054–66. doi: 10.1016/j.cell.2018.07.017

123. Artis D, Spits H. The biology of innate lymphoid cells. *Nat* (2015) 517 (7534):293–301. doi: 10.1038/nature14189

124. Hoyler T, Klose CS, Souabni A, Turqueti-Neves A, Pfeifer D, Rawlins EL, et al. The transcription factor GATA-3 controls cell fate and maintenance of type 2 innate lymphoid cells. *Immun* (2012) 37(4):634–48. doi: 10.1016/j.immuni.2012.06.020

125. Yagi R, Zhong C, Northrup DL, Yu F, Bouladoux N, Spencer S, et al. The transcription factor GATA3 is critical for the development of all IL-7Ralpha-expressing innate lymphoid cells. *Immun* (2014) 40(3):378–88. doi: 10.1016/j.immuni.2014.01.012

126. Zhu J. GATA3 regulates the development and functions of innate lymphoid cell subsets at multiple stages. *Front Immunol* (2017) 8:1571. doi: 10.3389/fimmu.2017.01571

127. Wong SH, Walker JA, Jolin HE, Drynan LF, Hams E, Camelo A, et al. Transcription factor RORalpha is critical for nuocyte development. *Nat Immunol* (2012) 13(3):229–36. doi: 10.1038/ni.2208

128. Serafini N, Vosshenrich CA, Di Santo JP. Transcriptional regulation of innate lymphoid cell fate. *Nat Rev Immunol* (2015) 15(7):415–28. doi: 10.1038/nri3855

129. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* (2011) 12(11):1045–54. doi: 10.1038/ni.2131

130. Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, et al. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nat* (1999) 397:702–6. doi: 10.1038/17812

131. Wang HC, Qian L, Zhao Y, Mengarelli J, Adrianto I, Montgomery CG, et al. Downregulation of e protein activity augments an ILC2 differentiation program in the thymus. *J Immunol* (2017) 198(8):3149–56. doi: 10.4049/jimmunol.1602009

132. Qian L, Bajana S, Georgescu C, Peng V, Wang HC, Adrianto I, et al. Suppression of ILC2 differentiation from committed T cell precursors by e protein transcription factors. *J Exp Med* (2019) 216(4):884–99. doi: 10.1084/jem.20182100

133. Bajana S, Pankow A, Liu K, Michniowska M, Urban JFJr., Chen WR, et al. Correlation between circulating innate lymphoid cell precursors and thymic function. *iSci* (2022) 25(2):103732. doi: 10.1016/j.isci.2022.103732

134. Yu X, Wang Y, Deng M, Li Y, Ruhn KA, Zhang CC, et al. The basic leucine zipper transcription factor NFIL3 directs the development of a common innate lymphoid cell precursor. *Elife* (2014) 3:e04406. doi: 10.7554/eLife.04406

135. Yang Q, Li F, Harly C, Xing S, Ye L, Xia X, et al. TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow. *Nat Immunol* (2015) 16(10):1044–50. doi: 10.1038/ni.3248

136. Klose CS, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) 157(2):340–56. doi: 10.1016/j.cell.2014.03.030

137. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nat* (2014) 508(7496):397–401. doi: 10.1038/nature13047

138. Seillet C, Mielke LA, Amann-Zalcenstein DB, Su S, Gao J, Almeida FF, et al. Deciphering the innate lymphoid cell transcriptional program. *Cell Rep* (2016) 17 (2):436–47. doi: 10.1016/j.celrep.2016.09.025

139. Bai L, Vienne M, Tang L, Kerdiles Y, Etiennot M, Escaliere B, et al. Liver type 1 innate lymphoid cells develop locally *via* an interferon-gamma-dependent loop. *Science* (2021) 371(6536):aba4177. doi: 10.1126/science.aba4177

140. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Sci* (2015) 350 (6263):981–5. doi: 10.1126/science.aac9593

141. Huang Y, Mao K, Chen X, Sun MA, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Sci* (2018) 359(6371):114–9. doi: 10.1126/science.aam5809

142. Zeis P, Lian M, Fan X, Herman JS, Hernandez DC, Gentek R, et al. *In situ* maturation and tissue adaptation of type 2 innate lymphoid cell progenitors. *Immun* (2020) 53(4):775–92. doi: 10.1016/j.immuni.2020.09.002

143. Ghaedi M, Shen ZY, Orangi M, Martinez-Gonzalez I, Wei L, Lu X, et al. Single-cell analysis of RORalpha tracer mouse lung reveals ILC progenitors and

effector ILC2 subsets. *J Exp Med* (2020) 217(3):20182293. doi: 10.1084/jem.20182293

144. Lim AI, Li Y, Lopez-Lastra S, Stadhouders R, Paul F, Casrouge A, et al. Systemic human ILC precursors provide a substrate for tissue ILC differentiation. *Cell* (2017) 168(6):1086–100. doi: 10.1016/j.cell.2017.02.021

145. Nagasawa M, Heesters BA, Kradolfer CMA, Krabbendam L, Martinez-Gonzalez I, de Bruijn MJW, et al. KLRG1 and NKp46 discriminate subpopulations of human CD117(+)CRTH2(-) ILCs biased toward ILC2 or ILC3. *J Exp Med* (2019) 216(8):1762–76. doi: 10.1084/jem.20190490

146. Mjosberg J, Spits H. Human innate lymphoid cells. *J Allergy Clin Immunol* (2016) 138(5):1265–76. doi: 10.1016/j.jaci.2016.09.009

147. Cosmi L, Liotta F, Maggi L, Annunziato F. Role of type 2 innate lymphoid cells in allergic diseases. *Curr Allergy Asthma Rep* (2017) 17(10):66. doi: 10.1007/s11882-017-0735-9

148. Ebbo M, Crinier A, Vely F, Vivier E. Innate lymphoid cells: major players in inflammatory diseases. *Nat Rev Immunol* (2017) 17(11):665–78. doi: 10.1038/nri.2017.86

149. Xiong T, Turner JE. Innate lymphoid cells in autoimmunity and chronic inflammatory diseases. *Semin Immunopathol* (2018) 40(4):393–406. doi: 10.1007/s00281-018-0670-4

150. Jones R, Cosway EJ, Willis C, White AJ, Jenkinson WE, Fehling HJ, et al. Dynamic changes in intrathymic ILC populations during murine neonatal development. *Eur J Immunol* (2018) 48(9):1481–91. doi: 10.1002/eji.201847511

151. Kernfeld EM, Genga RMJ, Neherin K, Magaletta ME, Xu P, Maehr R. A single-cell transcriptomic atlas of thymus organogenesis resolves cell types and developmental maturation. *Immun* (2018) 48(6):1258–70. doi: 10.1016/j.immuni.2018.04.015

152. Elsaid R, Meunier S, Burlen-Defranoux O, Soares-da-Silva F, Perchet T, Iturri L, et al. A wave of bipotent T/ILC-restricted progenitors shapes the embryonic thymus microenvironment in a time-dependent manner. *Blood* (2021) 137(8):1024–36. doi: 10.1182/blood.2020006779

153. Ferreira ACF, Szeto ACH, Heycock MWD, Clark PA, Walker JA, Crisp A, et al. RORalpha is a critical checkpoint for T cell and ILC2 commitment in the embryonic thymus. *Nat Immunol* (2021) 22(2):166–78. doi: 10.1038/s41590-020-00833-w

154. Liu C, Gong Y, Zhang H, Yang H, Zeng Y, Bian Z, et al. Delineating spatiotemporal and hierarchical development of human fetal innate lymphoid cells. *Cell Res* (2021) 31:1106–22. doi: 10.1038/s41422-021-00529-2

155. De Obaldia ME, Bhandoola A. Transcriptional regulation of innate and adaptive lymphocyte lineages. *Annu Rev Immunol* (2015) 33:607–42. doi: 10.1146/annurev-immunol-032414-112032

156. Shin SB, Lo BC, Ghaedi M, Scott RW, Li Y, Messing M, et al. Abortive gammadeltaTCR rearrangements suggest ILC2s are derived from T-cell precursors. *Blood Adv* (2020) 4(21):5362–72. doi: 10.1182/bloodadvances.2020002758

157. Shin SB, McNagny KM. ILC-you in the thymus: A fresh look at innate lymphoid cell development. *Front Immunol* (2021) 12:681110. doi: 10.3389/fimmu.2021.681110

158. Vargas CL, Poursine-Laurent J, Yang L, Yokoyama WM. Development of thymic NK cells from double negative 1 thymocyte precursors. *Blood* (2011) 118 (13):3570–8. doi: 10.1182/blood-2011-06-359679

159. Veinotte LL, Greenwood CP, Mohammadi N, Parachoniak CA, Takei F. Expression of rearranged TCRgamma genes in natural killer cells suggests a minor thymus-dependent pathway of lineage commitment. *Blood* (2006) 107(7):2673–9. doi: 10.1182/blood-2005-07-2797

160. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* (2006) 7(11):1217–24. doi: 10.1038/ni1395

161. Rankin L, Belz GT. Diverse roles of inhibitor of differentiation 2 in adaptive immunity. *Clin Dev Immunol* (2011) 2011:281569. doi: 10.1155/2011/281569

162. Barshad G, Webb LM, Ting HA, Oyesola OO, Onyekwere OG, Lewis JJ, et al. E-protein inhibition in ILC2 development shapes the function of mature ILC2s during allergic airway inflammation. *J Immunol* (2022) 208(5):1007–20. doi: 10.4049/jimmunol.2100414

163. Berrett H, Qian L, Roman O, Cordova A, Simmons A, Sun XH, et al. Development of type 2 innate lymphoid cells is selectively inhibited by sustained e protein activity. *Immunohorizons* (2019) 3(12):593–605. doi: 10.4049/immunohorizons.1900045

164. Heemskerk MH, Blom B, Oda K, Stegmann AP, Bakker AQ, Weijer K, et al. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* (1997) 186:1597–602. doi: 10.1084/jem.186.9.1597

165. Boos MD, Yokota Y, Eberl G, Kee BL. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of e protein activity. *J Exp Med* (2007) 204(5):1119–30. doi: 10.1084/jem.20061959

166. Schotte R, Dontje W, Nagasawa M, Yasuda Y, Bakker AQ, Spits H, et al. Synergy between IL-15 and Id2 promotes the expansion of human NK progenitor cells, which can be counteracted by the e protein HEB required to drive T cell development. *J Immunol* (2010) 184(12):6670–9. doi: 10.4049/jimmunol.0901508

167. Delconte RB, Shi W, Sathe P, Ushiki T, Seillet C, Minnich M, et al. The helix-Loop-Helix protein ID2 governs NK cell fate by tuning their sensitivity to interleukin-15. *Immun* (2016) 44(1):103–15. doi: 10.1016/j.immuni.2015.12.007

168. Peng V, Georgescu C, Bakowska A, Pankow A, Qian L, Wren JD, et al. E proteins orchestrate dynamic transcriptional cascades implicated in the suppression of the differentiation of group 2 innate lymphoid cells. *J Biol Chem* (2020) 295(44):14866–77. doi: 10.1074/jbc.RA120.013806

169. Amann JM, Nip J, Strom DK, Lutterbach B, Harada H, Lenny N, et al. ETO, a target of t (8,21) in acute leukemia, makes distinct contacts with multiple histone deacetylases and binds mSin3A through its oligomerization domain. *Mol Cell Biol* (2001) 21(19):6470–83. doi: 10.1128/MCB.21.19.6470-6483.2001

170. Jin C, Li H, Murata T, Sun K, Horikoshi M, Chiu R, et al. JDP2, a repressor of AP-1, recruits a histone deacetylase 3 complex to inhibit the retinoic acid-induced differentiation of F9 cells. *Mol Cell Biol* (2002) 22(13):4815–26. doi: 10.1128/MCB.22.13.4815-4826.2002

171. Muto A, Hoshino H, Madisen L, Yanai N, Obinata M, Karasuyama H, et al. Identification of Bach2 as a b-cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer. *EMBO J* (1998) 17(19):5734–43. doi: 10.1093/emboj/17.19.5734

172. Chyla BJ, Moreno-Miralles I, Steapleton MA, Thompson MA, Bhaskara S, Engel M, et al. Deletion of Mtg16, a target of t(16;21), alters hematopoietic progenitor cell proliferation and lineage allocation. *Mol Cell Biol* (2008) 28 (20):6234–47. doi: 10.1128/MCB.00404-08

173. Miller MM, Patel PS, Bao K, Danhorn T, O'Connor BP, Reinhardt RL. BATF acts as an essential regulator of IL-25-responsive migratory ILC2 cell fate and function. *Sci Immunol* (2020) 5(43):aay3994. doi: 10.1126/sciimmunol.aay3994

174. Xu W, Carr T, Ramirez K, McGregor S, Sigvardsson M, Kee BL. E2A transcription factors limit expression of Gata3 to facilitate T lymphocyte lineage commitment. *Blood* (2013) 121(9):1534–42. doi: 10.1182/blood-2012-08-449447

175. Cherrier DE, Serafini N, Di Santo JP. Innate lymphoid cell development: A T cell perspective. *Immun* (2018) 48(6):1091–103. doi: 10.1016/j.immuni.2018.05.010

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