frontiers | Research Topics

MYC as a disease target beyond cancer

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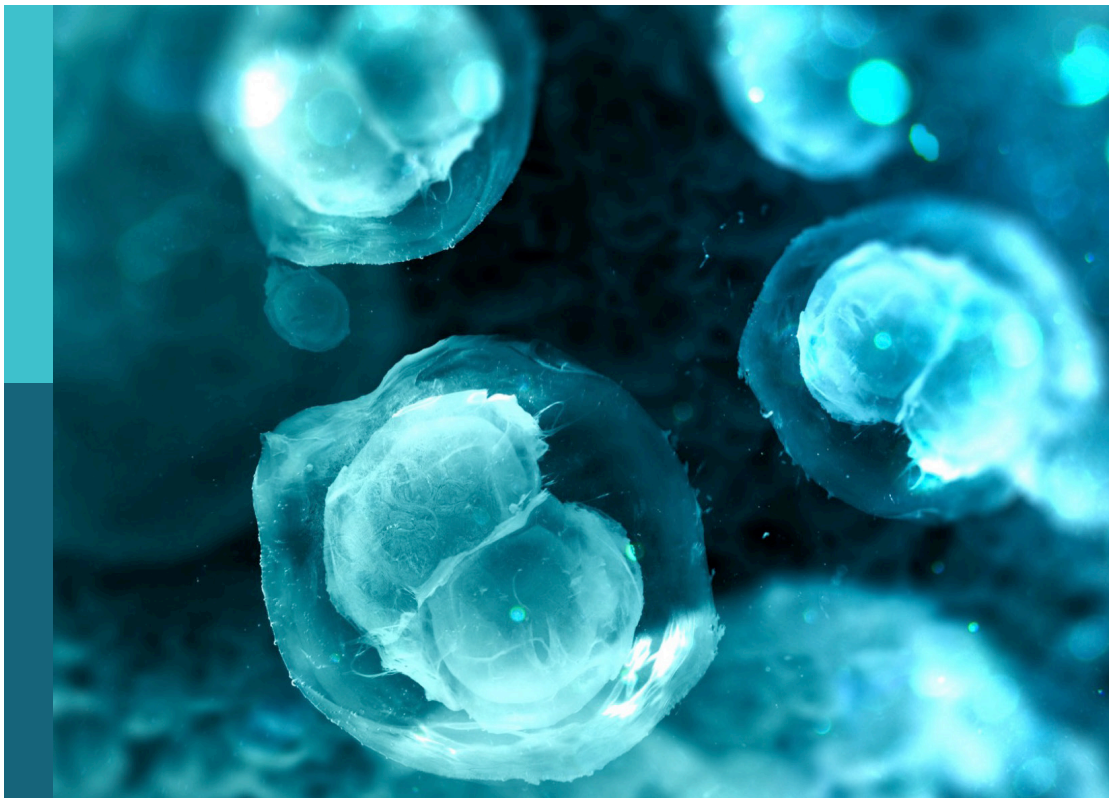

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MYC as a disease target beyond cancer

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Editorial: MYC as a disease target beyond cancer

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KEYWORDS

MYC, targeting, transcription factor, non-oncologic diseases, therapy

Editorial on the Research Topic
MYC as a disease target beyond cancer

MYC is a highly pleiotropic transcription factor involved in multiple cellular and developmental processes, but it is most renowned for its role in driving tumorigenesis. Hence, it is currently subject to intense efforts towards its targeting for the treatment of cancer, and while it was long considered undruggable, there are now 3 direct inhibitors in clinical trials, one of which recently successfully passed Phase I. There are many existing reviews on its role in cancer and strategies for its targeting, therefore here we focus on all the other diseases and conditions to which its inhibition–or activation or overexpression in some cases–could be applied.

There are a significant number of publications linking MYC to a wide variety of diseases, some with only preliminary data showing modulated MYC expression in disease models or patient samples, while others describe inhibition, knock-down or overexpression of MYC to demonstrate a key role in disease development or progression. As MYC inhibitors advance in their clinical testing, one can hope that they will soon be applied beyond cancer patients. It is curious that some of the earliest trials were indeed in a non-oncological setting, using MYC antisense for the treatment of heart restenosis.

With this intense focus on MYC inhibition in cancer, it is easy to lose track of all the other diseases in which MYC is key. The objective of this Research Topic was to bring together reviews and original research papers that link non-oncological pathologies with MYC.

This Research Topic kicks off with a review of the literature linking MYC to various diseases and conditions beyond cancer (Zacarías-Fluck et al.). This is an overview of MYC and its multiple physiological functions, which are also described in more detail in an additional review in this Research Topic by Kumar Jha et al., that extensively discusses the MYC amplifier model. Our introductory review (Zacarías-Fluck et al.) then describes how MYC often becomes a central hub used by oncogenic drivers to modulate many cellular processes, and that these same multiple and pleiotropic functions of MYC implicate it in the aetiology of a wide variety of diseases. These range from conditions of 'normal development gone wrong', as in the case of bone development disorders, to those where mutations in upstream signalling pathways drive de-regulated MYC expression or activation and the development of a pathological condition.

Then, the Research Topic dives into specific conditions, with reviews on MYC's role in regeneration, aging, mitochondrial diseases, obesity, and endometriosis. Indeed, MYC has a well-known role in regeneration across the animal kingdom, and this is discussed by Ascanelli et al. along with the potential to activate MYC in non-regenerative tissues for therapeutic purposes. This is extended to *Drosophila* in a review by Serras and Bellosta, who focus on the regenerative process in flies, and the utility of this model for understanding human tissue repair (Serras and Bellosta).

MYC in aging is a rather more controversial topic with seemingly contrasting data between MYC haploinsufficient and MYC KO models, mentioned in the review by Zacarías-Fluck et al., and with the MYC KO data discussed at length in an additional review by Prochownik and Wang.

MYC also plays a role in stem cell renewal, and the MYC-SIRT1 axis is described by Fan and Li to have a part to play both in cancer and normal embryogenesis, the latter suggesting that MYC could be a therapeutic target in developmental diseases.

In addition, a perspective piece by Nothnik et al. discusses MYC's role in endometriosis, a disease that affects many women, causing pain and reduced quality of life, and presents some preliminary data showing that MYC inhibition reduces endometriotic cell proliferation and viability *in vitro*.

Another article of the Research Topic sheds light on the relatively unexplored role of MYC in mitochondrial diseases. In fact, while the association of MYC upregulation with mitochondrial dysfunction is quite clear, Purhonen et al. review 2 decades of literature and the role of MYC in various mitochondrial diseases, identifying key questions that are still unanswered.

Two articles in the Research Topic discuss obesity. Nevzorova and Cubero refer to "moonlighting" MYC due to its many jobs within a cell, and describe mechanisms for the development of obesity and the implication of MYC in them. Given the rapidly increasing incidence of obesity–and the subsequent impact on health and healthcare systems–it is provocative to think that MYC inhibitors could have a place in its treatment. However, an original research paper included in this Research Topic suggests that inhibition of MYC is associated with weight gain. In this article, knockout of MYC in mouse endothelial cells leads to progressive increase in body weight during aging, while overexpression of MYC attenuates diet-induced obesity (Machi et al.). On the other hand, oral administration of the small molecule MYC inhibitor 10058-F4 to obese mice was previously shown to reduce obesity (Luo et al., 2021). It appears that further studies with additional obesity models and MYC inhibitors are still needed to clarify this topic.

Additional original research papers in the Research Topic also provide new data pointing to a role for MYC in polycystic kidney disease and neonatal lung disease, as well as to its involvement in the process of self-renewal, and the control of nucleolar function and the somatotropic axis. The study by Harafuji et al. for example, relates to autosomal recessive polycystic kidney disease, a severe hepato-renal disorder that causes childhood morbidity. The authors show that MYC is overexpressed in kidneys from disease patients and find an association between MYC expression levels and renal cyst development in mouse models. The next step will be to show that MYC inhibitors can modulate disease progression.

Intra-amniotic inflammation is associated with morbidity at an even earlier age, causing pre-term births and chronic lung disease of prematurity. In a research article, Tan et al. use the MYC inhibitor 10058-F4 to treat a model of intra-amniotic inflammation in pregnant rats caused by LPS. Here, MYC expression is associated with the intra-amniotic inflammation in neonatal tissues, and treatment with the MYC inhibitor ameliorates many of the effects of LPS.

Furthermore, roles of MYC in additional processes may hint at new disease applications, for example, its control of nucleolar function could be linked to cancer and ribosomopathies (Manara et al.), while a role in self-renewal of oesophogeal epithelium basal cells (Hishida et al.) could link to regeneration after injury or for repair, as also mentioned above.

Finally, a role in the regulation of the somatotropic axis through miRNA-mediated IGF1 downregulation suggests that the link between MYC, aging and several diseases (such as cancer, cardiovascular disease, diabetes, osteoporosis, and neurodegeneration) is a convoluted one and requires further investigation to more clearly define it (Petrashen et al.).

Overall, it is an exciting and optimistic time for cancer researchers in the MYC inhibitor field, and hopefully clinical success there will soon lead to the application of MYC inhibitors in multiple and diverse diseases. There has so far been far less focus on MYC overexpression or activation for disease modulation, but as described in the Research Topic, this may be therapeutic in conditions requiring tissue regeneration, and as such may be a new challenge for the MYC field. We are eagerly looking forward to seeing all the research into MYC making a difference for as many diseases as possible.

Author contributions

JW: Conceptualization, Project administration, Writing–original draft, Writing–review and editing.

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Conflict of interest

JW is a shareholder in Peptomyc S.L.

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ORIGINAL RESEARCH
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Myc Supports Self-Renewal of Basal Cells in the Esophageal Epithelium

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It is widely believed that cellular senescence plays a critical role in both aging and cancer, and that senescence is a fundamental, permanent growth arrest that somatic cells cannot avoid. Here we show that Myc plays an important role in self-renewal of esophageal epithelial cells, contributing to their resistance to cellular senescence. Myc is homogeneously expressed in basal cells of the esophageal epithelium and Myc positively regulates their self-renewal by maintaining their undifferentiated state. Indeed, Myc knockout induced a loss of the undifferentiated state of esophageal epithelial cells resulting in cellular senescence while forced MYC expression promoted oncogenic cell proliferation. A superoxide scavenger counteracted Myc knockout-induced senescence, therefore suggesting that a mitochondrial superoxide takes part in inducing senescence. Taken together, these analyses reveal extremely low levels of cellular senescence and senescence-associated phenotypes in the esophageal epithelium, as well as a critical role for Myc in self-renewal of basal cells in this organ. This provides new avenues for studying and understanding the links between stemness and resistance to cellular senescence.

Keywords: MYC, cancer, senescence, aging, mitochondria highlights

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HIGHLIGHTS

- Esophageal epithelia show resistance to senescence in mice.
- c-Myc is homogeneously expressed in basal cells of the esophageal epithelium.
- Myc is required for stemness-associated inhibition of senescent characteristics in basal cells of the esophageal epithelium.
- Basal cells of the esophageal epithelium have low levels of mitochondrial activity.

INTRODUCTION

Most cells do not proliferate indefinitely, but instead enter cellular senescence, a permanent cell cycle arrest triggered by excessive rounds of cell division, oncogenic stimuli, or genotoxic stresses (Kuilman et al., 2010). Senescence is thought to be a fundamental feature of somatic cells, and

is known to contribute to organismal aging and prevention of cancer initiation (Hanahan, 2022). However, there are cell-type specific differences in the induction and effectiveness of senescence (Lopez-Otin et al., 2013). For example, rodent glia cells (rat Schwann cells) do not exhibit replicative senescence *in vitro* (Mathon et al., 2001), nor do cultured epidermal stem cells that have been derived from the footpad (Stern and Bickenbach, 2007; Doles et al., 2012). Thus, some cell types, such as adult/tissue stem cells and progenitors, show resistance to senescence. However, it is unknown whether there is tissue *in vivo* that can similarly evade senescence.

Here we found that the esophagus did not exhibit aging features in mice. Forced-expression of MYC induced oncogenic cell proliferation while MYC knockout reduced the self-renewal capacity of esophageal epithelial cells, which resulted in cellular senescence, indicating the importance of MYC in preserving their self-renewal. It was also suggested that MYC is necessary for proliferating cells to keep an undifferentiated state and maintain a low level of mitochondrial superoxide. Taken together, these data revealed an essential role of MYC on the stemness of esophageal epithelial cells, which are highly resistant to senescence.

MATERIALS AND METHODS

Mice

Myc$^{\text{Myc-GFP/Myc-GFP}}$ (Huang et al., 2008), *Sox2*$^{\text{CreER/WT}}$ (Arnold et al., 2011), *Sox2*$^{\text{GFP/WT}}$ (Arnold et al., 2011), *ROSA*$^{\text{LSL-GFP/LSL-GFP}}$ (Mao et al., 2001), tetO-*MYC* (Felsher and Bishop, 1999) and *ROSA*$^{\text{LSL-rtTA-IRES-GFP/LSL-rtTA-IRES-GFP}}$ (Belteki et al., 2005) have been previously described. LMNA$^{\text{G609G}}$ mice were generated by Carlos López-Otín at the University of Oviedo, Spain and kindly donated by Brian Kennedy at the Buck Institute. We generated Myc cdKO mice from Myc cdKO ESCs (Varlakhanova et al., 2010). Genotyping was performed by using the primer set which are suggested to be used in The Jackson Laboratory. Genotyping for Myc was performed as described previously (Varlakhanova et al., 2010). We used both male and female mice for this study but the same gender was used for each experiment unless otherwise stated. To activate Cre in the mice carrying CreER, TAM, dissolved in corn oil, was given orally (50 mg/ml) to 3- to 12-week-old animals for 3 consecutive days, if not otherwise stated. Dox was administered in drinking water (0.5 mg/ml), starting with TAM treatment. All animal experiments were approved by the Salk Institute for Biological Studies IACUC and conform to regulatory standards.

Ki67 Immunostaining and Cell Quantification

Esophagi and small intestines were dissected from young, old and LMNA G609G mice and washed with PBS, followed by fixation with 4% paraformaldehyde for 24 h at 4°C. The tissues were then soaked in 15% sucrose in PBS for at least 12 h and 30% sucrose in PBS for 24 h before being embedded in optimal cutting temperature (OCT) (Tissue-Tek) prior to cryo-sectioning. The prepared sections (8–10 μm) were washed twice in Tris-buffered saline (TBS, *pH* = 7.0) to remove OCT followed by antigen retrieval using HistoVT One (Nacalai tesque) according to manufacturer's instructions. Sections were then blocked for 1–2 h in 6% normal horse serum in TBST (TBS + 0, 5% Triton X-100) and incubated overnight at 4°C with primary antibodies anti-Ki67 (Cell signaling, 12,202, 1:100). Alexa Fluor 488-conjugated donkey anti-Rabbit IgG (Molecular Probe, 1:200) was used as a secondary antibody and nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI). Images were acquired using a Zeiss LSM 780 laser-scanning microscope (Carl Zeiss Jena) at 10×, 20×, and 63× magnification. For the quantification, several images were taken at 20× or 63× and underwent the stitching function of ImageJ (National Institute of Health) to reconstruct the whole tissue. At least, three sections per tissue from three animals were used for the analyses. Cells positive for each marker were counted in a blinded manner using ImageJ. For the esophagus, the percentage of Ki67^{+} cells was assessed on nine sections from three mice of each group (young, old and LMNA G609G mice). For the small intestine, the number of Ki67^{+} cells was counted from at least 16 crypts from three mice of each group.

IHC and SA-βGal Staining

For IHC, tissues were harvested, fixed in 10% neutralized Formalin for 2 days and then stored in 70% ethanol until further processing. H&E staining, PAS staining and IHC on paraffin-section were performed following standard protocols. The following antibodies were used for IHC: anti-GFP (Abcam, 6673, 1:200; Clontech, JL-8, 1:100); Ki67 (Cell signaling, 12202, 1:200). SA-βgal staining was performed as previously described (Debacq-Chainiaux et al., 2009).

RNA Isolation and Quantitative-PCR

Total RNAs were isolated using TRIzol reagent (Invitrogen) and RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA samples were treated with RNase-Free DNase Set (Qiagen). RT was performed with SuperScript III (Invitrogen) followed by qPCR using Platinum SYBR Green quantitative PCR super mix (Invitrogen) in a thermocycler. The levels of expression of respective genes were normalized to corresponding GAPDH values or Nat1 values, and the normalized values were divided by those of the corresponding standard samples (Young Esophagus for **Figures 1D,F**; Untreated (-4OH) samples for **Figure 4F**; Control samples for **Supplementary Figure S2**). Primer sequences are listed in **Supplementary Table S1**.

Esophageal Cell Culture

mEPCs were derived as previously described (Kalabis et al., 2008). Briefly, the esophagi were isolated, opened longitudinally, washed in PBS followed by Dispase (1 U/ml) for 15–20 min at 37°C. The opened esophagi were minced with forceps and incubated with trypsin for 10 min at 37°C. After inactivation of trypsin with FBS, the cell suspension was filtrated through 100-μm and 40-μm cell strainers. The obtained cells were centrifuged and re-suspended in SAGM (LONZA) containing 1 μM A-83-01, 1 μM DMH-1,

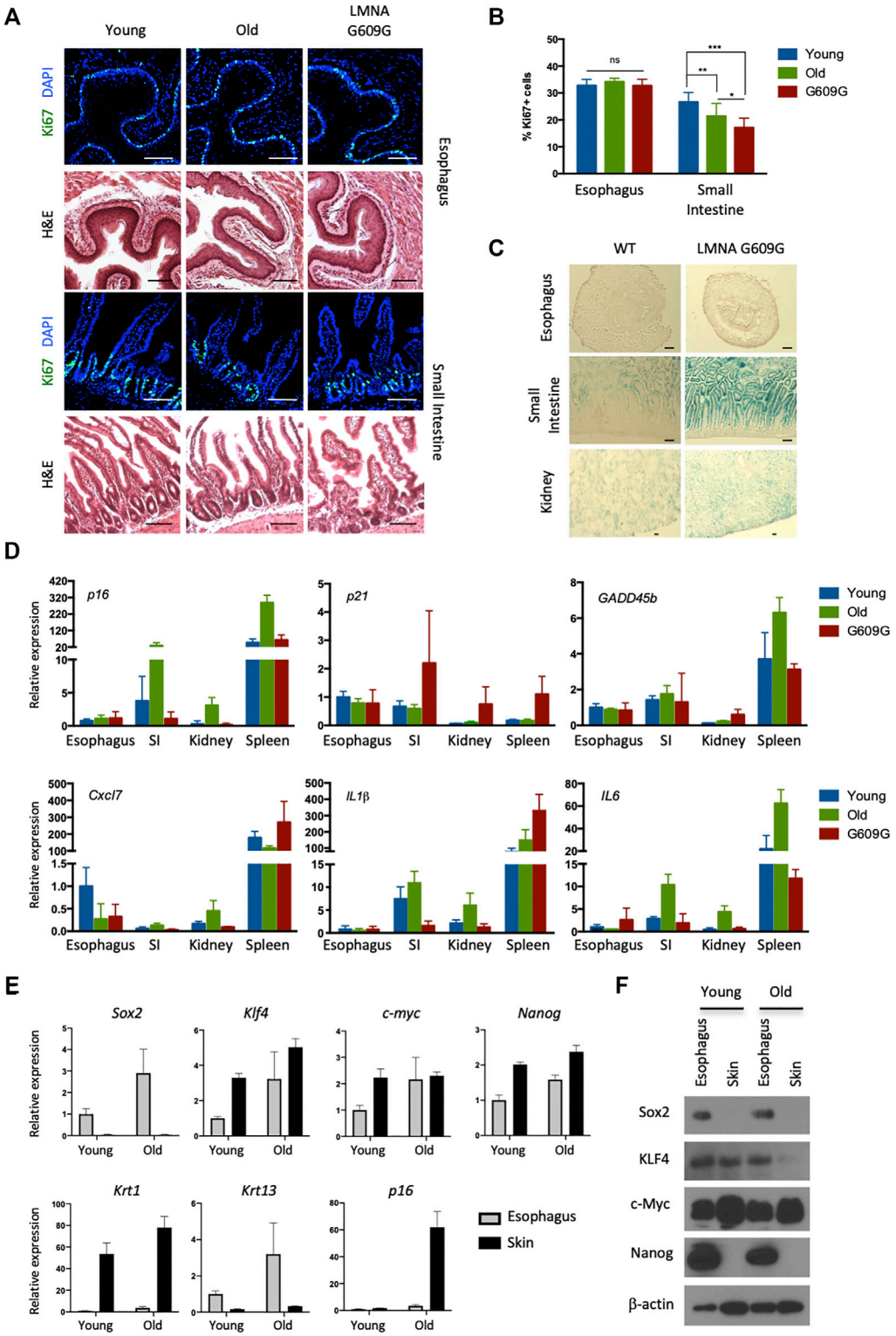

FIGURE 1 | No visible senescence of esophageal epithelial cells expressing pluripotency factors. **(A)** Representative immunofluorescent pictures of Ki67 (green) staining and H&E staining in the esophagus and small intestine for young (3 months old), old (22 months old) and LMNA G609G HGPS mouse model (3 months old). Scale bars, 100 μm. **(B)** Proliferative index of the esophagus and small intestine. We quantified Ki67$^+$ cells in the esophagus and small intestine from three mice for each group. Data represent the mean with SD. *ns* = non-significant, $^*p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$. **(C)** SA-βgal staining in the esophagus, small intestine and kidney for both young (3 months old) and LMNA G609G HGPS mouse models (3 months old). Scale bars, 100 μm. **(D)** qPCR analysis for aging markers. SI: Small Intestine. Data represent the mean with SE ($n = 3$). **(E)** qPCR analysis for the esophagus and the skin from young and old mice. Krt1 and krt13 were used as specific keratins for the skin and the esophagus, respectively. Data represent the mean with SE ($n = 3$). **(F)** Western blotting for pluripotency factors. Epithelial cells were isolated from the indicated tissues and cultured for 1 week before cell lysate preparation.

3 μM CHIR99021 and 10 μM Y-27632, followed by plating on matrigel-coated plates. To activate CreER or ER-Ras^{V12}, the cells were treated with 0.1 μM 4OH. For cell cycle analysis, EdU-647 was used together with cell-cycle 405 according to manufacture's protocol (ThermoFisher). Human primary esophageal epithelial cells were described in previous papers (Harada et al., 2003; Takaoka et al., 2004). 10058-F4 (Sigma) was used as a Myc inhibitor as needed.

Plasmid Constructions and Viral Production

The ORF of ER-Ras^{V12} was amplified from pLNCX2-ER-Ras^{V12} (Addgene, #67844) and subcloned to pMX-IB with In-Fusion (Clontech). pMX-retroviral plasmids were transfected to PLAT-E cells using Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions. For tetO-MYC-2A-puro, the amplified ORF of MYC was subcloned to ptetO-2A-puro lentiviral vector, generated from pTetO-Ngn2-puro (Addgene, #52047). Viral supernatants were collected around 48 h after transfection and passed through a 0.45 μm filter to remove cellular debris and then the supernatants freshly prepared were incubated for 1 h while spinning at 800 × g, followed by changing to fresh medium. Two days after infection, the cells were selected with 20 μM blasticidin or 2 μg/ml puromycin for 6 days.

Nanostring

Differential gene expression profiling was carried out with purified RNA using the Nanostring nCounter Pan Cancer Profiling Panel (Nanostring, Seattle, WA) according to manufacturer's instructions.

Live-Imaging and Image Analysis

Cell tracking experiments was performed using IncuCyte imaging system (Essen Bioscience). Images were automatically acquired from 6-well plates at ×10 magnification every 30 min for 80 h. Raw images were processed with two types of filter sets according to time period. For 0–40 h, the filter set 1 for non-confluent cells were applied as follows: (Step 1) Contrast enhancement by original source code in R (version 3.1.0) (R Development Core Team, https://www.r-project.org/). Pixel higher than 140 were converted into 255. (Step 2) Texture enhancement, (Step 3) Segmentation, (Step 4) Removal of small objects, and (Step 5) Fill holes (under area 20) were processed. Then recognized cellular objects were counted. During this period, individual cells were recognized sharply for cellular region. However, flat enlarged cells can only be recognized with their center nuclei area, and the cell recognition accuracy for their edge was not sharp. Therefore, during this period, only the total cell counts were used as measurement data. For the period after 40 h, the filter set 2 for confluent cells were applied as follows: From Step 1 to Step 3, the same processing was applied as filter set 1. (Step 4) Fill hole (under 20), (Step 5) Erode (2 pixels) were added to the processed images. Finally the recognized cellular objects were counted, labeled, and measured for their area size. The image processing was applied by CL-Quant (Nikon corp. Tokyo, Japan). To illustrate time-course growth, bar-whisker plots, and the size distribution in measured cells, original source code by R was applied.

For the detailed morphological analysis on four conditions (Control, +Ca, −ADCY, and −ADCY + Ca), the phase contrast images taken by phase contrast microscopy (OLYMPUS, IX51) were manually traced to measure the accurate morphology in both normal and flat enlarging cells. The detailed image processing is described in **Supplementary Note S2**.

For SA-βgal positive cell measurement, SA-βgal-stained color image and phalloidin-stained fluorescent image from the same FOV by fluorescent microscopy (OLYMPUS, IX51) were processed by CL-Quant. First, the SA-βgal stained images were converted into Blue image, and binalized (threshold > 100 intensity). From the binalized image, their stained area was constructed as a image mask. Second, the phalloidin stained fluorescent images were binalized (threshold 120), and remaining intensity = 96 pixels were converted by 255 to enhance the regional contrast per cells. Then the binalized images were constructed as the second image mask. These two image masks were merged, and recognized for cell labeling. In each cellular object, the SA-βgal stained area, covered by the first mask, were measured to calculate the SA-βgal positive area per cells. From the recognized cells, 300 cellular objects were randomly selected in the data processing, and used for their distribution analysis. All data analysis was done by original source code by R.

Mitochondrial Analysis

Isolated, trypsinized cells were incubated with 100 nM TMRM (ThermoFisher) and 500 nM MitoSpy Green FM (BioLegend) at 37°C for 30 min in PBS containing 0.5% BSA and washed with PBS once, followed by FACS analysis.

Statistic Analysis

For comparisons, unpaired *t* test or one-way ANOVA with Tukey's *post hoc* analysis were used with GraphPad Prism 8 unless otherwise stated. Values with $p < 0.05$ are considered statistically significant.

RESULTS AND DISCUSSION

A Lack of Senescence in the Esophagus

Recent deep- and micro-sequencing-based mapping of genetic mutations has revealed that normal esophageal epithelial cells exhibit age-dependent expansion of mutated clones, as well as higher levels of mutations than seen with sun-exposed skin cells. This suggests that esophageal epithelial cells robustly proliferate and survive long enough to accumulate many somatic mutations without cellular senescence (Martincorena et al., 2018; Yokoyama et al., 2019). We have also observed that basal esophageal epithelial cells continue to proliferate after exposure to oncogenic insults, namely Kras^{G12D} (Hishida et al., 2019) and PIK3CA^{H1047R} (data not shown). Based on these data, we speculated that esophageal epithelial cells may possess a unique ability to resist senescence. To address this hypothesis, we analyzed the proliferative capacity of esophageal cells in aged

wild-type (WT) mice (24 months old), and in a murine model of Hutchinson-Gilford Progeria Syndrome, a human condition that results in premature aging. These mice carry the c.1827C>T; G609G mutation in the *Lamin A* gene, which causes aberrant splicing and accumulation of a truncated form of Lamin A called progerin (Osorio et al., 2011). We analyzed the small intestines as a control. In the small intestinal crypt, the number of cells expressing Ki67, a marker of proliferation, was reduced in aged and G609G mice compared with young WT mice (2 months old). In contrast, age did not affect the number of $Ki67^+$ cells in the esophagus (**Figures 1A,B**). Similar results were obtained using another premature aging mouse model, PolG mice (Kujoth et al., 2005) (data not shown). We next analyzed senescence-associated beta-galactosidase (SA-βgal) activity, a canonical marker of senescence. Although SA-βgal activity was detected in the small intestines and kidneys of G609G mice, none was detected in the esophagus (**Figure 1C**). Moreover, age-associated induction of aging-related cyclin-dependent kinase inhibitors (p16 and p21), a p53-responsible, stress-inducible gene (GADD45b) and senescence associated secretory phenotype-related factors (Cxcl7, IL1β, and IL6) were not observed in the esophagus (**Figure 1D**). Notably, esophageal cells expressed pluripotency factors, regardless of the age of the mouse. Among these factors, SOX2 and NANOG proteins were quite specific to esophageal cells (**Figures 1E,F**), consistent with previous reports (Liu et al., 2013; Piazzolla et al., 2014). As reported, NANOG protein levels was lower in skin compared to those in the esophagus although mRNA levels seem differently regulated, which may reflect the complexity of post-transcriptional regulations of Nanog (Saunders et al., 2013; Piazzolla et al., 2014). Taken together, these results indicate that the esophageal epithelium expresses pluripotency factors and does not undergo aging-associated senescence *in vivo*.

The Role of Myc in Self-Renewal of Esophageal Epithelial Cells *In Vitro*

We next derived primary mouse esophageal progenitor/basal cells (mEPCs) to analyze their ability to resist senescence in detail (Extended Data **Supplemenary Figure S1**). We optimized culture conditions based on previous reports (DeWard et al., 2014; Mou et al., 2016) and found that mEPCs could be homogeneously cultured on matrigel for > 50 passages (100 days) in SAGM medium that included A-83-01 (an ALK4/5/7 inhibitor), DMH-1 (an ALK2 inhibitor), CHIR99021 (a GSK-3β inhibitor), and Y-27632 (a ROCK inhibitor), hereafter referred to as ADCY. When cultured in ADCY, mEPCs propagated and expressed pluripotency factors. Withdrawal of ADCY resulted in rapid morphological changes and downregulation of markers of undifferentiation (Sox2, Nanog, and p63, a marker of basal epithelial cells), and telomere-related factors (mTert and mTerc), as well as the induction of Involucrin, a differentiation marker (Extended Data **Supplementary Figure S2**). These changes were seen in the presence or absence of Ca^{2+}, which is known to induce keratinocyte differentiation, while a combination of Ca^{2+} addition and ADCY withdrawal synergistically induced Involucrin. We were able to derive mEPCs even from 24-month-old mice without any noticeable difference in derivation efficiency and cell morphology, compared to those derived from 2-month-old mice. This was not the case for skin keratinocytes and tongue epithelial cells, which were difficult to derive from aged mice (Extended Data **Supplementary Figure S3**). These results suggest that mEPCs do not exhibit replicative senescence. However, when challenged with Ras activation [*via* the retroviral overexpression of an activated form of Ras fused to the estrogen receptor (ER: Ras^{V12}) and administration of the ER ligand, 4-hydroxytamoxifen (4OH)], mEPCs exhibited reduced levels of proliferation, larger cell size, loose cell-cell contacts, and a more differentiated state (Extended Data **Supplementary Figures S4A–C**). This observation implies that an oncogenic insult can trigger a senescence program, namely oncogene-induced senescence (OIS), which is in agreement with a previous report (Takaoka et al., 2004). The negative impact of Ras activation on cell proliferation was reversed by partial inhibition of the MAPK pathway, which was achieved by treating cells with of 0.01 μM PD0325901, a potent MEK inhibitor (Bain et al., 2007). Strong inhibition of the MAPK pathway (by treating cells with 1 μM PD0325901) dramatically impeded cell proliferation, suggesting that fine-tuning of MAPK, at least in part, contributes to EPC self-renewal (Extended Data **Supplementary Figure S4D**).

To determine whether pluripotency factors play an important role in mEPC self-renewal, we next manipulated the levels of Nanog, which was abundantly expressed in the esophagus and reported to have an oncogenic function in stratified epithelia (Piazzolla et al., 2014). RNAi-mediated knockdown of Nanog resulted in the poor propagation of mEPCs (Extended Data **Supplementary Figure S5**), as has been seen with Sox2 (DeWard et al., 2014). Thus, pluripotency factors are important for EPC self-renewal. Taken together, these results indicate that mEPCs express pluripotency factors and are deficient for replicative senescence, but are still capable of protecting themselves from tumor initiation *via* OIS.

We next sought to understand the mechanism by which esophageal basal cells avoid replicative senescence. Using a Myc-GFP knock-in mouse model, we noticed that Myc is expressed in adult stem cells and progenitor cells associated with several tissues, including the esophagus and forestomach (**Figure 2A**, data not shown). Myc expression was relatively homogeneous in the basal layer of the esophageal epithelium, even though it is widely believed that Myc is transiently expressed during the G1 to S transition (Blackwood et al., 1991) and is relatively unstable. The MYC protein localizes to human esophageal basal cells at higher levels than seen in skin cells [according to the public data set from the Human Protein Atlas (Uhlen et al., 2015)] (**Figure 2B**). Myc is known to regulate self-renewal of pluripotent stem cells (PSCs) (Smith et al., 2010; Varlakhanova et al., 2010; Hishida et al., 2011). Notably, Myc depletion was reported to induce a pluripotent dormant state, indicating that Myc determines cell proliferation and growth arrest in PSCs. In addition, previous reports showed that c-Myc inactivation is associated with senescence in some cancer cells (Wu et al., 2007; Tabor et al., 2014; Alimova et al., 2019).

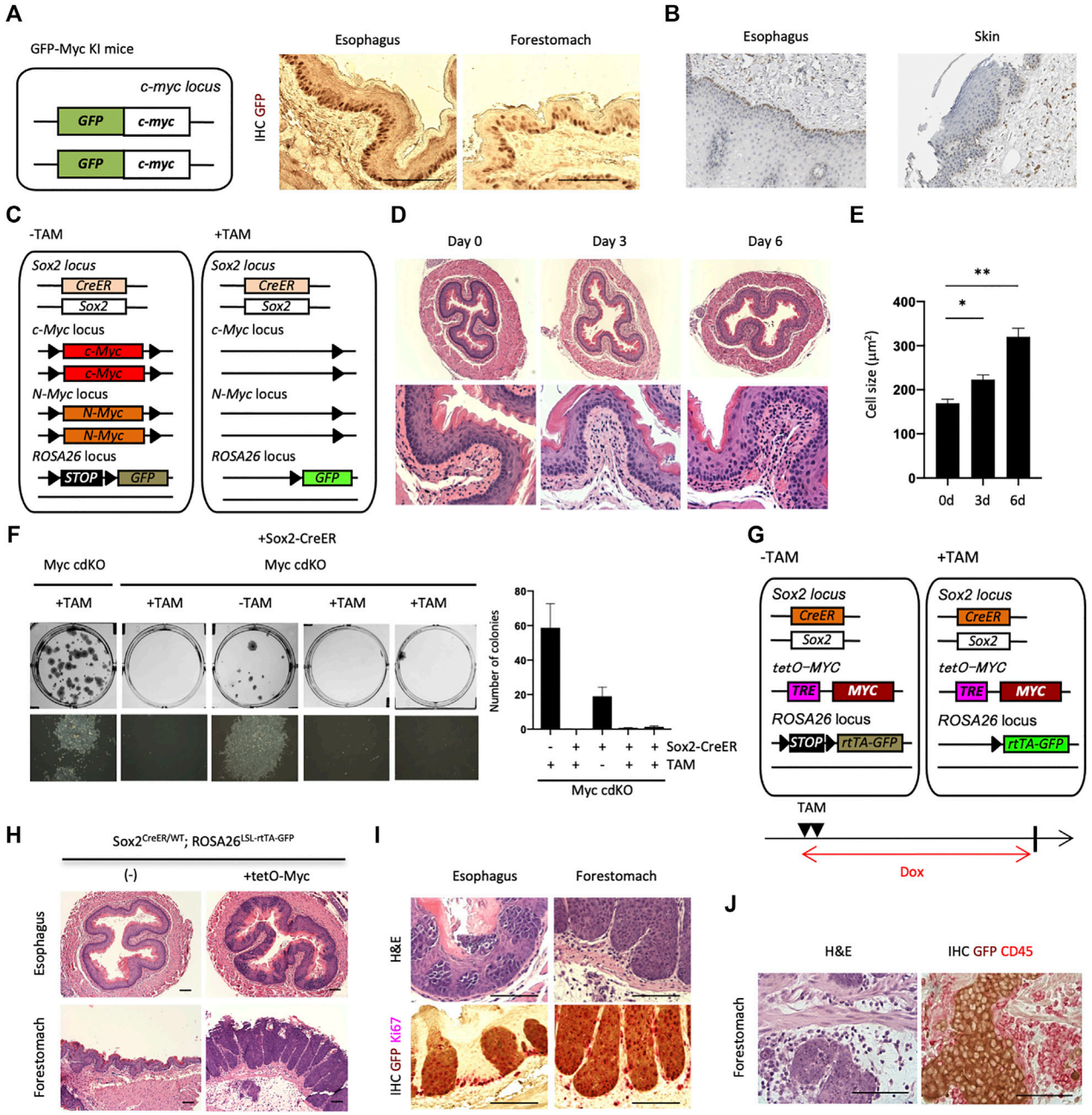

FIGURE 2 | Loss-of-function and gain-of-function of Myc in the esophagus. **(A)** IHC for GFP on paraffin-embedded sections of the esophagus and forestomach from GFP-Myc Knock-in (KI) mice. Scale bars, 100 μm. Two mice were analyzed. **(B)** IHC image of MYC staining for the human esophagus and skin. MYC is largely expressed in esophageal basal layer. The data were kindly provided by the Protein Atlas Project publicly available (www.proteinatlas.org). **(C)** Schematic representation of Sox2^{+} cell-specific Myc conditional double knockout mice, *Sox2*$^{CreER/WT}$; *cMyc*$^{Flox/Flox}$; *nMyc*$^{Flox/Flox}$; *ROSA*$^{LSL\text{-}GFP/WT}$ (Myc cdKO). GFP can be used for lineage tracing purpose. **(D)** H&E staining for esophagi from Myc cdKO mice treated with TAM. Two mice were analyzed for each condition. **(E)** Cell size of esophageal basal cells. Two mice were used for each condition. Data represent the mean with SE. $^{*}p < 0.05$, $^{**}p < 0.0001$. **(F)** Clonogenic colony-forming assays. After cell isolation, 2,500 live cells were seeded per well in 6-well plate in ADCY medium. Twelve days after seeding, the cells were stained with Leishman's stain to count the number of colonies. Each culture was derived from each indicated mice. Left, representative image of each condition. Right, quantification of colony number. Data represent the mean with SD (n = 3). **(G)** Schematic representation of *Sox2*$^{CreER/WT}$; *tetO-MYC*; *ROSA*$^{LSL\text{-}rtTA\text{-}GFP/LSL\text{-}Luc}$ mouse. TAM treatment eliminates STOP cassette in front of rtTA-IRES-GFP in *ROSA26* locus, which can activate transgenic *MYC* expression in a tetracycline- or doxycycline (Dox)-dependent manner. GFP expression allows for lineage tracing. The mice were collected 10 days after 2-days TAM treatment. Dox was administered to the mice in their drinking water (0.5 mg/ml). **(H,I)** H&E **(H)** and IHCs **(I)** on paraffin-embedded sections of the indicated tissues corrected from SMP$^{WT/WT}$. Three mice were analyzed. Scale bars, 100 μm. **(J)** Inflammation along with tumor invasion in forestomach. Scale bars, 100 μm.

Collectively, these results encouraged us to further investigate Myc's function in supporting stemness.

To understand Myc's role in inhibiting replicative senescence in mEPCs, we combined inducible Myc loss-of-function alleles [both c-Myc and N-Myc, as they are functionally redundant (Smith et al., 2010; Varlakhanova et al., 2010)] with Sox2-CreER (**Figure 2C**). We then treated $Sox2^+$ cell-specific Myc conditional double knockout mice [$Sox2^{CreER/WT}$; $cMyc^{Flox/Flox}$; $nMyc^{Flox/Flox}$ (Myc cdKO)] with tamoxifen (TAM). Treated mice were dead within 7 days when TAM was administered continuously (data not shown). We therefore administered TAM for 3 days, collected esophagi, and performed H&E staining (**Figure 2D**). Esophageal basal cells were more sparsely larger in TAM-treated mice compared with untreated controls (**Figure 2E** and Extended data **Supplementary Figure S6**), which may reflect the loss of undifferentiated state as stated below. Further detailed analysis may provide deeper insight into contribution of Myc to differentiation and loss of stemness. To test whether Myc deletion affects self-renewal, we performed clonogenic colony-forming assays (**Figure 2F**). Only a few colonies were observed following Myc deletion, unlike that seen with controls, indicating that Myc is important for EPC self-renewal. We next examined whether Myc overexpression enhances mEPC proliferation, leading to tumors. To do so, we generated $Sox2^{CreER/WT}$; *tetO-MYC*; $ROSA^{LSL\text{-}rtTA\text{-}GFP/LSL\text{-}Luc}$ mice (**Figure 2G**) and treated them with TAM for 2 days and doxycycline (Dox, a tetracycline derivative) for 10 days, resulting in overexpression of *MYC* in $Sox2^+$ cells. GFP was used to label *MYC* overexpressing cells in this mouse model. Ten days following Dox treatment, we observed the proliferation of GFP^+ cells with abnormal morphologies in the esophagus and forestomach (**Figures 2H,I**). Invasive tumors were observed in the forestomach, with these invasive regions containing inflammatory cells, as assessed by H&E staining and localization of CD45, a marker of inflammatory cells (**Figure 2J**). An oncogenic role for Myc is also supported by the Oncoprint plot generated by cBioPortal (Cerami et al., 2012; Gao et al., 2013) (https://www.cbioportal.org/) from the Cancer Genome Atlas (TCGA), which indicates that MYC is frequently amplified (27%) in esophageal cancers (Extended data **Supplementary Figure S7**). Taken together, these results indicate that Myc is required for mEPC self-renewal while overexpression of Myc results in tumor formation.

The Role of Myc in Self-Renewal of Esophageal Epithelial Cells *In Vitro*

We next sought to understand Myc's role in EPC self-renewal in more detail by deriving mEPCs from Myc cdKO mice. We confirmed that 4OH treatment induced recombination at both the *c-myc* and *N-myc* loci, effectively knocking out both *Myc* genes (**Figures 3A,E,F**). Live-imaging experiments using the IncuCyte system revealed that 4OH-treated cells showed less proliferation (**Figure 3B**). As observed *in vivo*, enlarged cells (>1,000 μm^2) also appeared and became more prevalent after several rounds of cell division (**Figures 3C,E** and Extended data **Supplementary Figure S8**). Enlarged cells were largely positive for SA-βgal staining (**Figures 3D,F**) and most of them could not undergo cell division (Extended Data **Supplementary Figure S9**). Western blotting revealed decreased levels of pluripotency factors, such as Sox2 and Nanog, whereas p63 levels were not affected (**Figure 3G**). We did not detect an accumulation of p53 protein, nor an increase in Caspase-3 cleavage, implying that apoptosis was not induced by Myc deletion. Rather, cyclin-dependent kinase inhibitors, as well as Cyclin D and E, were upregulated, whereas Cyclin A and B were downregulated (**Figure 3H**), as assessed by gene expression profiling using Nanostring technology. These analyses also showed that Myc deletion affected genes associated with MAPK and PI3K (Extended Data **Supplementary Figure S10**). In agreement with the dysregulation of cell cycle-associated genes, cell cycle analysis revealed fewer cells in S-phase and more endoreplication (**Figure 3G**), which is consistent with the emergence of multinuclear cells (**Figure 3C**). These multinuclear cells were not able to undergo cell division, as revealed by live-imaging (data not shown). Endoreplication with an enlarged morphology is typical of differentiated keratinocytes (Gandarillas et al., 2000). Multinuclear enlarged cells were also found in the absence of 4OH treatment, albeit in small numbers, and may therefore reflect spontaneous differentiation. Thus, the emergence of these cells cannot be attributed to Myc knockout, but may result from loss of an undifferentiated state after 4OH treatment. The dependence on Myc for self-renewal was also observed in human esophageal epithelial cell lines (EPC1 and EPC2) (Extended Data **Supplementary Figure S11**). Taken together, these results indicate that Myc is required for EPC self-renewal associated with the resistance to senescence.

The Role of Mitochondria in Esophageal Cells on Suppressing Cellular Senescence

We next investigated the mitochondrial status within esophageal cells, as mitochondria play important roles in the induction of senescence (Gallage and Gil, 2016). Esophageal epithelial cells had lower membrane potential than skin epidermal epithelial cells (Extended Data **Supplementary Figure S12**), as assessed using TMRM, an indicator of membrane-potential-dependent mitochondria mass. This result encouraged us to analyze mitochondria in mEPCs. FACS analysis using MitoSpy and TMRM (for membrane potential-independent and -dependent mitochondria mass, respectively), showed that mEPCs had less mitochondrial membrane potential compared to Myc-deleted or Ca^{2+}-treated cells (**Figure 4A** and Extended Data **Supplementary Figure S13**). Increases in membrane potential are known to produce reactive oxygen species and therefore we assessed mitochondrial superoxide levels following 4OH treatment. 4OH treatment increased mitochondrial superoxides, as assessed by MitoSox indicator (**Figure 4B**). Importantly, Mitotempo, a mitochondria-targeted antioxidant, reduced the number of SA-βgal positive cells, and resulted in smaller cells, while it did not affect total cell number (**Figures 4C,D**). Mitotempo did not affect loss of the undifferentiated state

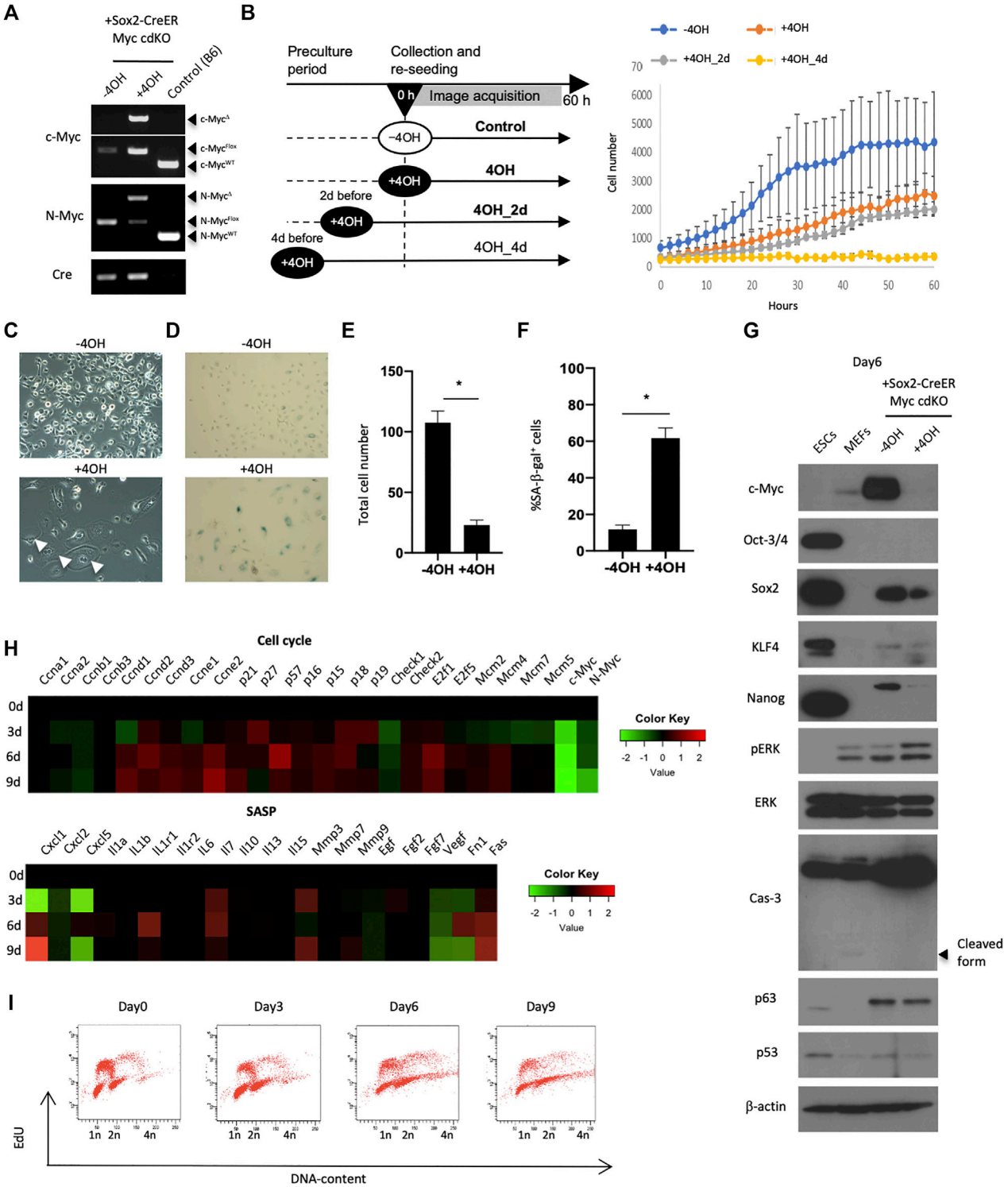

FIGURE 3 | Requirement of Myc for preserving self-renewal of esophageal epithelial cells. **(A)** Genotyping to confirm Myc knockout. The cells were treated with 0.1 μM 4OH for 3 days and lysed for genomic DNA purification. PCR reactions were performed using purified genomic DNA for WT, Flox and deleted (Δ) alleles of c-myc and N-Myc. **(B)** Live-imaging of Myc cdKO mEPCs. Left, image acquisition scheme. Right, image-based cell count. **(C)** Image of untreated and the Myc cdKO mEPCs treated with 4OH for 9 days. White arrow indicates multinuclear cells. **(D)** SA-βgal staining in Myc cdKO mEPCs treated with 4OH for 9 days. Data represent the mean with SE ($n = 6$). *$p < 0.0001$. **(E)** Quantification of total cell number in **(D)**. Data represent the mean with SE ($n = 6$). *$p < 0.0001$. **(F)** Quantification of SA-βgal-positive cells in **(D)**. Data represent the mean with SE ($n = 6$). *$p < 0.0001$. **(G)** Western blotting for Myc cdKO mEPCs treated with 4OH for 6 days. **(H)** Nanostring-based gene expression analysis. Myc cdKO mEPCs were treated with 4OH and samples were collected at the indicated time-points. RNAs were isolated and subjected to Nanostring RNA detection. **(I)** Cell cycle analysis of Myc cdKO mEPCs by FACS.

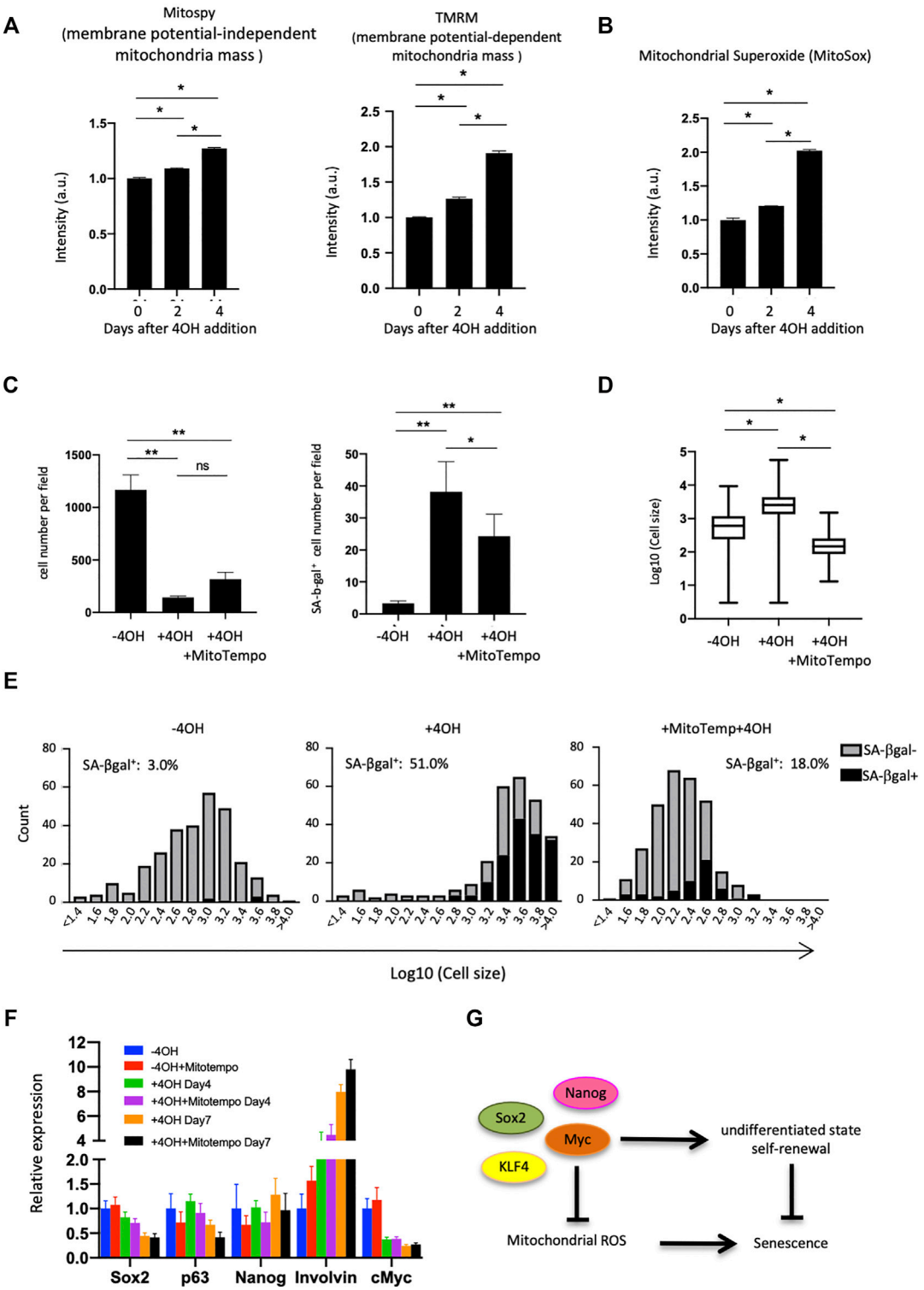

FIGURE 4 | Mitochondrial analysis in Myc cdKO mEPCs. **(A)** Mitochondrial quantity and activity in Myc cdKO mEPCs treated for 2 days. Data represent the mean with SD (n = 3). *p < 0.0001. **(B)** Mitochondrial superoxide levels in 4OH-treaed cells. Data represent the mean with SD (n = 3). *p < 0.0001. **(C)** Rescue effect of Mitotempo. Left, representative image of each condition. Right, quantification of total cell number. Data represent the mean with SE. *ns* = non-significant, *p < 0.05, **p < 0.0001. **(D)** Rescue effect of Mitotempo on cell size. *p < 0.0001. **(E)** Histogram of the cell size distribution. **(F)** qPCR analysis for Mitotempo-rescued cells. Data represent the mean with SD (n = 3). **(G)** Proposed model of Myc function supporting self-renewal of the esophageal basal cells.

(**Figure 4E**), suggesting that an increase in mitochondrial membrane potential may be a consequence of the loss of an undifferentiated state, while helping to induce cellular senescence.

Senescence was thought to be a fundamental cellular process; however it has gradually been recognized that susceptibility to senescence is cell-type specific and indeed stem cells and progenitors are highly resistant to senescence. Our findings have revealed that esophageal epithelia are deficient for replicative senescence *in vivo*. Epithelial stem cells themselves are known to be resistant to aging; however skin epithelial stem cells do exhibit senescence in aged mice. Thus, esophageal epithelial cells may possess distinct mechanisms of self-renewal, which needs to be clarified. A previous report showed that human esophageal epithelial cells do not exhibit telomere shortening during aging, partly because of telomerase activity (Takubo et al., 1999). This supports our finding of the resistance to senescence in the esophageal epithelium. It is tempting to speculate that esophageal cells may have evolved characteristics of "perpetual youth" because they are turned over rapidly and must face damage and stress caused by continuous exposure to food and drink.

Of interest, Myc is homogeneously expressed in esophageal basal cells. This is a unique feature because Myc expression largely depends on the cell-cycle phase (Blackwood et al., 1991). Similar to MYC, Sox2 and Nanog are also expressed in esophageal epithelial cells, as reported (Liu et al., 2013; Piazzolla et al., 2014). These pluripotency factors might be key to sustaining the negligible senescence feature of esophageal epithelial cells. Indeed, Sox2 deletion lost self-renewing propensity (DeWard et al., 2014). It needs to be elucidated how MYC expression is homogenously sustained in basal cells of the esophageal epithelium.

Mechanistically, Myc inhibited senescence in the esophagus, and thereby functions as a double-edged sword, both inhibiting senescence and promoting tumorigenesis when overexpressed. This study is the first to show the physiological role of Myc (and Nanog) on preserving stemness in the esophageal epithelia by loss-of-function studies, presumably by working cooperatively with other pluripotency factors in this physiological context (**Figure 4G**), thus revealing a link between stemness and cellular aging.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Salk Institute IACUC.

AUTHOR CONTRIBUTIONS

TH designed the research, performed most experiments and analyzed/interpreted data. TH, DO, and JI wrote the manuscript. EV-F performed experiments and analyzed/interpreted data. YH-N and FH. helped perform experiments and/or analyzed data. TY, KY, and RK analyzed/interpreted live imaging data. JP, SS, YT, PR, CR, and HN provided critical advices, interpreted data and aided in manuscript preparation. PK, EN, AC, and JC provided critical advices and reagents. JI designed and supervised the experiments, provided financial and administrative support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.786031/full#supplementary-material

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The SIRT1-c-Myc axis in regulation of stem cells

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SIRT1 is the most conserved mammalian NAD^+-dependent protein deacetylase. Through deacetylation of transcriptional factors and co-factors, this protein modification enzyme is critically involved in metabolic and epigenetic regulation of stem cells, which is functionally important in maintaining their pluripotency and regulating their differentiation. C-Myc, a key member of Myc proton-oncogene family, is a pivotal factor for transcriptional regulation of genes that control acquisition and maintenance of stemness. Previous cancer research has revealed an intriguing positive feedback loop between SIRT1 and c-Myc that is crucial in tumorigenesis. Recent literature has uncovered important functions of this axis in regulation of maintenance and differentiation of stem cells, including pluripotent stem cells and cancer stem cells. This review highlights recent advances of the SIRT1-c-Myc axis in stem cells.

KEYWORDS

c-Myc, SIRT1, stem cells, deacetylation, pluripotency, differentiation, c-Myc/Max heterodimer, positive feedback loop

1 Introduction

Stem cells, including pluripotent stem cells (PSCs), adult stem cells (ASCs), and cancer stem cells (CSCs), possess the ability to self-renew and to differentiate to give rise to all cell types in organs, tissues, or tumors. Embryonic stem cells (ESCs) and induced pluripotent stem cell (iPSCs) are two types of PSCs. ESCs are derived from the inner cell mass of a blastocyst (early stage of preimplantation embryos). iPSCs can be induced *in vitro* from adult somatic cells, such as murine embryonic fibroblasts (MEFs) or human somatic cells, through simultaneous overexpression of core pluripotent factors including OCT4, SOX2, KLF4, and c-Myc (Takahashi and Yamanaka, 2006; Smith and Dalton, 2010). These cells can be unlimitedly expanded *in vitro* while maintaining their pluripotency indefinitely.

C-Myc is one of the key pluripotent factors. C-Myc was firstly discovered as an oncogene that belongs to the Myc family of proton-oncoproteins. This family of proton-oncoproteins contains three main transcription factors, c-Myc, N-Myc, and L-Myc. They are basic-helix-loop-helix/leucine zipper (bHLH) DNA binding proteins and are known to be

Abbreviation: DBC1, deleted in breast cancer 1; Dnmt3l, DNA methyltransferase 3-like; FLT3, Fms-likes tyrosine kinase; iPSCs, induced pluripotent stem cells; ITD, internal tandem duplication; LIF, leukemia inhibitory factor; LSC, leukemic stem cells; MAL, acute myeloid leukemia; MAT2, methionine adenosyltransferase; MAT, methionine adenosyltransferase; MEFs, murine embryonic fibroblasts; mESCs, murine embryonic stem cells; NAD, nicotinamide adenosine dinucleotide; NAMPT, nicotinamide-phosphoribosyltransferase; NSCs, neural stem cells; PSCs, pluripotent stem cells; SAM, S-adenosylmethionine; SM, sphingomyelin; SMPDL3B, sphingomyelin phosphodiesterase acid like 3B; STAT3, Signal transducer and activator of transcription 3; Tert, Telomerase reverse transcriptase; TSS, Transcription start site.

fundamentally important for a number of cellular activities, such as metabolism, apoptosis, proliferation and differentiation (Prendergast, 1999; Meyer and Penn, 2008; Dang, 2013; Bretones et al., 2015). In healthy cells, maintaining an appropriate abundance and activity of MYC proteins is critical for these cellular programs. Aberrations or upregulation of MYC-related pathways by alternate mechanisms are observed in the vast majority of cancers (Dhanasekaran et al., 2021). Specifically, dysregulations of MYC proteins are associated with 70% of human cancers, and a wealth of evidence suggests that aberrantly expressed MYC proteins are closely related with both tumor initiation and maintenance (Llombart and Mansour, 2022). As the first member discovered in Myc family, c-Myc contributes to the genesis of many human cancers and is associated with alteration of cellular metabolism (Dang et al., 2009). Mechanistically, c-Myc controls global gene expression, especially genes involved in the biogenesis of ribosomes and mitochondria. These actions in turn impact cell proliferation, differentiation, cell cycle, apoptosis, as wells as metabolism of glucose and glutamine in cancer cells (Dang et al., 2009). In PSCs, c-Myc also acts as a transcriptional factor to regulate several thousand genes involved in cell reprogramming as well as maintenance and establishment of the pluripotent state (Chappell and Dalton, 2013). Additionally, c-Myc is important in embryogenesis. Its expression is maintained at the highest level during embryonic stage, declines over development, and eventually stays relatively low in mature organs (Elbadawy et al., 2019).

The activation of c-Myc is modulated by post-translational modifications, such as phosphorylation, de/acetylation and ubiquitination (Gregory and Hann, 2000; Faiola et al., 2005). For instance, c-Myc is acetylated by HATs (histone acetyltransferase) and its acetylation status has a complex impact on its protein stability and subsequent transcriptional activity (Faiola et al., 2005). SIRT1, a highly conserved nicotinamide adenosine dinucleotide (NAD^+) dependent class III histone deacetylase, is able to interact with and deacetylate c-Myc in cancer cells, which in turn increase its stability and activity (Mao et al., 2011; Menssen et al., 2012).

SIRT1 is the most conserved mammalian member of the Silent Information Regulator 2 (Sir2) family known as sirtuins (Calvanese et al., 2010; Vassilopoulos et al., 2011). The deacetylation activity of sirtuins is strictly dependent on NAD^+, a cofactor for hundreds of metabolic reactions in all cell types. Sirtuins deacetylate target proteins by transferring a wide range of lipid acyl-groups, such as acetyl, succinyl, malonyl, glutaryl, or long-chain acyl-groups, from their protein substrates to the ADP-ribose moiety of NAD^+(He et al., 2012; Choudhary et al., 2014; Wagner and Hirschey, 2014; Imai and Guarente, 2016). This exclusive NAD^+ requirement makes SIRT1 an important cellular metabolic sensor and regulator. It can sense the alteration of cellular energy status to modulate the functions of a wide range of protein substrates, including transcription factors and co-factors, histones, metabolic enzymes, and cell membrane proteins (Fang et al., 2019).

SIRT1 is highly expressed in both mouse ESCs (mESCs) and human ESCs (hESCs) (Calvanese et al., 2010; Vassilopoulos et al., 2011; Tang et al., 2017). Recent studies have shown that through deacetylation of transcription factors and co-factors, particularly c-Myc, SIRT1 plays important roles in normal embryogenesis and mouse embryonic stem cell pluripotency maintenance (Tang et al., 2017; Fan et al., 2021). Intriguingly, activation of c-Myc can enhance expression, stability, and activation of SIRT1. SIRT1 and c-Myc thereby form a positive feedback loop for regulation of tumorigenesis (Menssen et al., 2012). This review article summarizes the latest knowledges on the SIRT1-c-Myc axis in regulation of acquisition and maintenance of stemness, the capability of self-renewal potential and multi-lineage differentiation, differentiation of stem cells, and embryogenesis.

2 C-Myc is critical for the self-renewal and pluripotency of ESCs and normal embryogenesis

C-Myc is a critical regulator of normal embryogenesis in mice. Early studies showed that mouse embryos derived from the homozygous *c-myc* mutant mESCs display the embryonic lethality between 9.5 and 10.5 days of gestation. The homozygous *N-myc* mutant mESCs derived mouse embryos are also embryonic lethal at around 11.5 days of gestation. Both *c-myc* and *N-myc* mutant embryos have severe multi-organ development defects (Yoshida, 2018). In mESCs, although neither *c-myc* nor *N-myc* is required for their maintenance and functions, mESCs with *c-myc* and *N-myc* genes simultaneously knocked out exhibit severe disruption in their self-renewal and pluripotency. These cells have reduced survival, along with enhanced differentiation (Varlakhanova et al., 2010). Consistently, chimeric embryos generated by injection of *c-myc* and *N-myc* doubly KO mESCs most often completely fail to develop or, in rare cases, survive but with severe defects (Varlakhanova et al., 2010). Therefore, *c-myc* and *N-myc* together are important in maintaining the pluripotency of mESCs by suppressing early stage differentiation (Yoshida, 2018).

At the molecular level, c-Myc is important for maintaining self-renewal and pluripotency of mESCs by interacting with leukemia inhibitory factor (LIF)/Signal transducer and activator of transcription 3 (STAT3) signal pathway (Cartwright et al., 2005). Specifically, LIF actives c-Myc via two mechanisms (Figure 1): elevates the transcription of *c-myc* through the Janus kinase (JAK)-STAT3 pathway and prevents GSK3β-mediated phosphorylation of c-Myc T58 and subsequent degradation (Cartwright et al., 2005). Moreover, the stability of c-Myc is sensitive to growth factors such as fibroblast growth factor 4 (FGF-4), which activates extracellular signal-regulated kinase (ERK1/2), a mitogen-activated protein kinase (MAPK). ERK phosphorylates c-Myc at Ser 62, leading to its stabilization (Sears et al., 2000; Lee et al., 2008; Ying et al., 2008). The phosphorylated c-Myc then interacts with Myc-associated protein X (Max) to form a heterodimer complex. This complex then binds to the "E-box" sequence in the target gene promoter region, thereby activating or repressing the transcription of target genes (Yoshida, 2018). Importantly, the c-Myc/Max heterodimer complex acts as a central node of the regulatory network which prevents loss of stemness of mESCs and subsequent apoptosis (Figure 1). Firstly, this complex inhibits the p-ERK, which forms a negative feedback loop to prevent the MARK signaling induced loss of stemness (Hishida et al., 2011). Secondly the c-Myc/Max complex can directly suppress expression of primitive endoderm master regulator, GATA6, to maintain stemness (Smith et al., 2010).

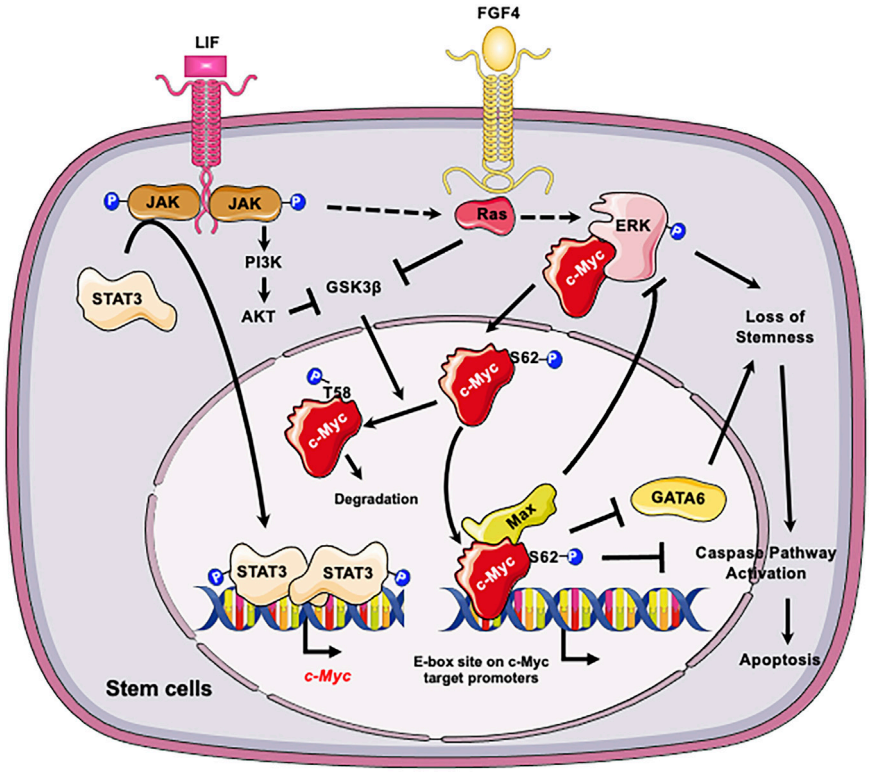

FIGURE 1
C-Myc is critical for the self-renewal and pluripotency of ESCs. LIF promotes the transcription of *c-myc* through JAK-STAT3 pathway and prevents GSK3β-mediated phosphorylation of c-Myc T58 and subsequent degradation. In parallel LIF and FGF4 activate the ERK1/2 signaling cascade, resulting in phosphorylation of c-Myc at S62. The phosphorylation enhances the stability of c-Myc, thereby promoting its interaction with Max and the formation of c-Myc/Max heterodimer complex. The c-Myc/Max complex in turn prevents loss of stemness of mESCs by feedback inhibition of p-ERK and suppression of GATA6 and suppresses subsequent apoptosis. Figures were created using images downloaded and adapted from Service Medical ART: SMART (https://smart.servier.com/image-set-download/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/)."

Consistently, depletion of *Max* gene in mESCs results in loss of the undifferentiated state, upregulation of linage markers, and induction of apoptosis/death with Caspase-3 activation (Hishida et al., 2011). All these are primarily caused by activation of MAPK signaling, because inhibiting MAPK kinase signaling significantly blocks the decline of pluripotency genes and eliminates differentiated cells (Hishida et al., 2011).

3 SIRT1 regulates stem cell maintenance and embryogenesis at multiple levels

SIRT1 is highly expressed in the pre-implantation embryos and ESCs compared with adult tissues/cells (Tang et al., 2017). It plays an important role in maintaining normal embryogenesis and animal development. Mice with germline deletion of *Sirt1* display severe development defects, such as neonatal lethality, defective germ cell differentiation, developmental defects of the retina and heart, bone developmental delay, and intrauterine growth retardation (Cheng et al., 2003; McBurney et al., 2003; Wang et al., 2008; Tang et al., 2014; Liu et al., 2017).

Accumulating evidences indicate that SIRT1 regulates embryogenesis, animal development, and ESC pluripotency maintenance through multilevel mechanisms, which strictly rely on its protein deacetylation activity (Fang et al., 2019). The deacetylation substrates of SIRT1 in stem cells include a key component of core pluripotency network OCT4, tumor repressor p53, histones, and epigenetic regulator DNA methyltransferase 3-like (DNMT3L). For instance, it has been shown that SIRT1-mediated deacetylation of OCT4 is required to maintain the naïve state of mESCs, whereas SIRT1 reduction-induced acetylation of OCT4 leads to naïve-to-primed transition (Zhang et al., 2014; Williams et al., 2016). SIRT1 also modulates DNA methylation in stem cells through antagonizing *Dnmt3l* transcription and protein stability by deacetylation of histones and DNMT3L itself (transcriptionally and post-transcriptionally) (Heo et al., 2017). These actions of SIRT1 control the expression of imprinted and germline genes and the differentiation potential of mESCs, which are important in maintaining the normal neurogenesis and spermatogenesis (Heo et al., 2017). Moreover, SIRT1 represses the transcription of differentiation genes in ESCs through direct deacetylation of histones. Consequently, the reduction of SIRT1 reactivates those development genes during

embryo developments (Calvanese et al., 2010). Furthermore, SIRT1 suppresses retinoic acid receptor (RAR)-mediated activation of differentiation genes in mESCs by deacetylation of a cellular retinoic acid binding protein II (CRABPII). Deacetylation recycles CRABPII from the nucleus out to the cytosol, thereby terminating the retinoic acid signaling (Tang et al., 2014). Additionally, SIRT1 is important to maintain healthy pluripotent ESCs. In response to endogenous reactive oxygen species (ROS), SIRT1 deacetylates p53 and promotes its mitochondrial translocation from the nucleus. This action of SIRT1 sensitizes mESCs to mitochondrial p53-induced apoptosis while inhibiting nuclear p53-mediated suppression of *Nanog* expression (Han et al., 2008). Together, by deacetylation of key regulators, SIRT1 acts as a pivotal regulator to orchestrate metabolic and epigenetic signal pathways to maintain pluripotent ESCs and normal embryogenesis.

4 The SIRT1-c-Myc feedback loop in regulation of tumorigenesis in cancer cells

The link between SIRT1 and c-Myc was first observed in cancer cells. It has been previously shown that both c-Myc and SIRT1 are highly elevated in major types of cancer cells, where c-Myc may elicit apoptosis or premature senescence through p53-dependent pathway (Vafa et al., 2002; Dominguez-Sola et al., 2007; Menssen et al., 2007; Campaner et al., 2010). Since SIRT1 is known to inhibit p53 through deacetylation (Luo et al., 2001; Vaziri et al., 2001; Langley et al., 2002), SIRT1 may regulate c-Myc activation through p53. Subsequent studies revealed that SIRT1 could directly activate the transactivation activity of c-Myc. To activate the transcription of its target genes, c-Myc needs to form a heterodimer with Max to recognize the E-box sequence in the target promoters (Yoshida, 2018; Singh et al., 2022). Mao et al. (2011) showed that SIRT1 binds to and deacetylates the C-terminal bHLH-ZIP motif containing region of c-Myc, which is directly involved in the formation of c-Myc/Max heterodimer. Deacetylation of c-Myc by SIRT1 increases its binding affinity to Max, presumably due to deacetylation induced conformation changes. The enhanced c-Myc/Max dimerization consequently facilitates the transcription of c-Myc target genes, such as human telomerase reverse transcriptase (*hTERT*), cyclinD2 (*CCND2*) and Lactate Dehydrogenase A (*LDHA*), thereby promoting cell proliferation (Mao et al., 2011). Deacetylation of c-Myc by SIRT1 also affects its stability in immortalized or cancer cells. Previous reports have shown that acetylation of c-Myc by PCAF and TIP60 inhibits its ubiquitination and subsequently increases its stability (Vervoorts et al., 2003; Patel et al., 2004). Consistently, Yuan et al. (2009) reported that SIRT1 deacetylates c-Myc at K323 and decreases its stability in immortalized cells. However, Menssen et al. (2012) reported that deacetylation of c-Myc by SIRT1 increases its stability and enhances its transcriptional activity. C-Myc can be conjugated with both lysine-48 (K48)- and lysine-63 (K63)-linked polyubiquitin chains, and K63-linked ubiquitination of c-Myc does not lead to its degradation. Instead, it is required for recruitment of the coactivator p300, transactivation of multiple target genes, and induction of cell proliferation by c-Myc (Adhikary et al., 2005). Menssen et al. (2012) showed that SIRT1-mediated deacetylation increases the conjugation of K63-linked ubiquitin chains to c-Myc, which in turn stabilizes c-Myc by competing with K48-likned degradative ubiquitination. The reasons for the discrepancies between studies of Yuan et al. (2009) and Menssen et al. (2012) are still not completely clear.

Conversely, c-Myc has also been reported to enhance the activity of SIRT1 through several different mechanisms. Firstly, c-Myc increases the NAD^+/NADH ratio by transcriptional activation of nicotinamide-phosphoribosyltransferase (NAMPT), the rate-limiting enzyme of the amidated NAD^+ salvage pathway (Menssen et al., 2012). Menssen et al. (2012) showed that the NAMPT promoter contains "E-box" binding motifs of c-Myc in the vicinity of the transcription start site (TSS). Activation of c-Myc transcriptionally increases the mRNA levels of NAMPT, which elevates cellular NAD^+ salvage and subsequently promotes the activity of SIRT1. Secondly, c-Myc can enhance the activity of SIRT1 by sequestering its inhibitor deleted in breast cancer 1 (DBC1) (Menssen et al., 2012). DBC1 binds to the active site of SIRT1 and inhibits SIRT1–substrate interaction (Kim et al., 2008; Zhao et al., 2008). c-Myc also interacts with DBC1, which protects SIRT1 from interaction with DBC1, resulting in reduced inhibition of SIRT1 (Koch et al., 2007; Menssen et al., 2012). Finally, c-Myc can directly binds to the conserved "E-box" DNA binding motif on the *Sirt1* promoter and induces its transcription (Yuan et al., 2009). Interestingly, this transcriptional activation can be inhibited by p53, as p53 shares the response element with c-Myc and blocks the c-Myc recruitment on the *Sirt1* promoter (Yuan et al., 2017).

Collectively, in cancer cells, SIRT1 and c-Myc could form a positive feedback loop, in which activation of c-Myc increases the expression and activity of SIRT1 to deacetylate c-Myc. Deacetylation of c-Myc increases its stability and transactivation activity (Figure 2). This axis of SIRT1-c-Myc positive feedback may orchestrate cellular response to endogenous or exogenous stimulations.

5 The SIRT-c-Myc axis is important in metabolic and epigenetic regulation of mESCs and mouse embryonic development

Given the importance of SIRT1 and c-Myc in regulation of stem cell self-renewal, pluripotency, and differentiation, it is not surprising that the SIRT1-c-Myc axis revealed in cancer research is also functionally important in stem cell biology and animal embryonic development.

The stemness of PSCs, including ESCs, is sustained by their specific metabolic programs and epigenetic status (Folmes et al., 2012; Zhang et al., 2012; Ito and Suda, 2014; Teslaa and Teitell, 2015). These special metabolic programs, including high glycolytic flux under aerobic condition, consumption of high levels of exogenous glutamine, as well as high dependence on one-carbon catabolism, are required to produce precursors and ATP for the high proliferation of PSCs. Moreover, the intermediate products of these metabolic processes, such as acetyl-CoA, NAD^+, α-ketoglutarate, and S-adenosylmethionine (SAM), can also act as cofactor or co-substrates of enzymes which participate epigenic regulation of chromatin and gene expression in PSCs (Takahashi and

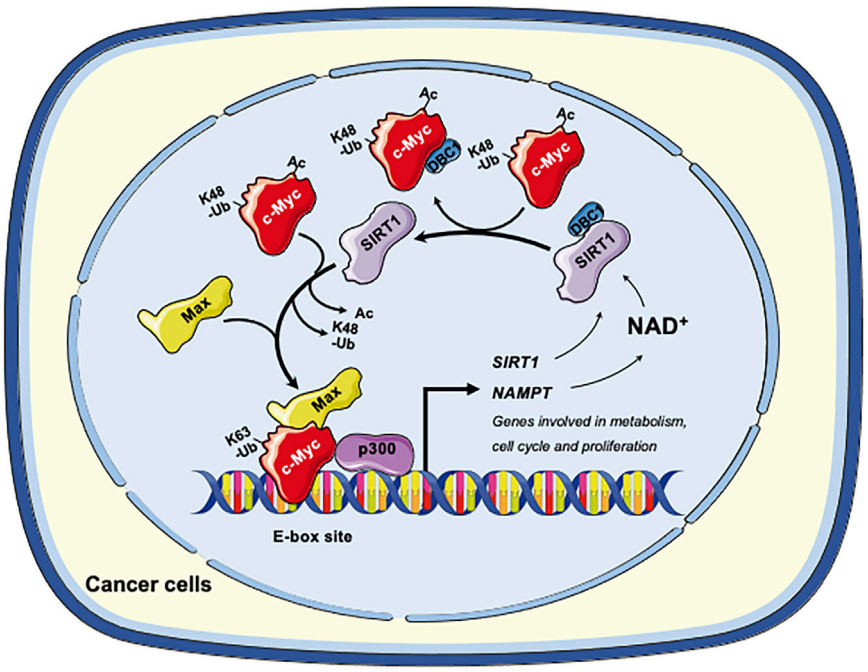

FIGURE 2
The SIRT1-c-Myc positive feedback loop in cancer cells. SIRT1-mediated deacetylation of c-Myc increases its binding affinity to Max. Deacetylation of c-Myc by SIRT1 also increases K63-linked polyubiquitination while repelling degradative K48-linked polyubiquitination, enhancing the stability of c-Myc and increasing recruitment of p300. Both mechanisms facilitate the transactivation of c-Myc target genes, including genes involved in metabolism, cell cycle and cell proliferation. Conversely, c-Myc also enhances the activity of SIRT1. Firstly, c-Myc increases the cellular NAD^+ by transcriptional activation of NAMPT, the rate-limiting enzyme in the amidated NAD^+ salvage pathway. Increased NAD^+ enhances the deacetylase activity of SIRT1. Secondly, c-Myc sequesters the SIRT1 inhibitor DBC1, thereby increasing SIRT1-substrate interaction. Finally, c-Myc directly increases the transcription of SIRT in p53 deficient cells. Figures were created using images downloaded and adapted from Service Medical ART: SMART (https://smart.servier.com/image-set-download/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/)."

Yamanaka, 2006; Wellen et al., 2009; Cai et al., 2011; Xu et al., 2011; Shyh-Chang et al., 2013; Moussaieff et al., 2015). Consequently, the distinctive metabolic programs in PSCs are directly linked to their unique epigenetics and gene expression profiles, thereby strongly influencing the self-renewal and pluripotency of PSCs (Folmes et al., 2011; Carey et al., 2015).

One metabolic pathway that is critically involved in epigenetic regulation of stem cell pluripotency is methionine metabolism. As a sulfur-containing essential amino acid, methionine is a key component of dietary proteins important for protein synthesis, sulfur metabolism, epigenetic modification, antioxidant defense, and signaling (Mato et al., 2008). Specifically, SAM, the methyl-donor for histone methyltransferases, is produced from methionine by oligomeric enzyme methionine adenosyltransferase (MAT2) in ESCs (Halim et al., 1999; Shiraki et al., 2014). It has been shown that altered methionine or threonine metabolism induce the fluctuation of intracellular SAM. Such fluctuation influences histone methylation in both mESCs and hESCs, thereby modulating their fate (Shyh-Chang et al., 2013; Shiraki et al., 2014). Through a large scale unbiased metabolomic analysis of SIRT1 KO and control WT mESCs, Tang et al. (2017) discovered that one of primary metabolic defects in SIRT1 deficient mESCs is methionine metabolism, particularly the conversion of methionine to SAM. As a result, SIRT1 deficient mESCs have a reduced cellular SAM abundance and decreased histone methylation levels. Particularly, the levels of H3K4me3, a histone activation mark that is sensitive to methionine deprivation/restriction, is significantly reduced in SIRT1 KO mESCs. This reduction is associated with a dramatic alteration of global gene expression profiles, including reduced expression of a number of pluripotent genes (e.g., *Nanog*). It is also associated with a hypersensitivity to methionine depletion/restriction-induced differentiation and apoptosis. Mechanistically, Tang et al. (2017) showed that SIRT1 promotes SAM production in part through Myc-mediated transcriptional activation of *Mat2a*, which encodes the catalytic subunit of Mat2. Deletion of SIRT1 leads to hyperacetylation of both N- and c-Myc proteins. Hyperacetylation in turn leads to instability of c-Myc and reduced recruitment of both factors to the promoter of *Mat2*, and thereby reducing expression of this enzyme (Figure 3). In support of this notion, adding back MAT2A rescues the reduction of H3K4m3 and *Nanog* mRNA, enhances differentiation, and increases apoptosis upon methionine restriction in SIRT1 KO mESCs. Therefore, the epigenetic homeostasis of mESCs, comprising the methylation status of core histone protein (H3K4me3) and profiles of gene expression, is maintained by the SIRT1-c-Myc axis through regulation of methionine metabolism. Importantly, SIRT1 KO mouse embryos have reduced *Mat2a* expression and histone methylation and are sensitive to maternal methionine restriction-induced lethality. Conversely, maternal methionine supplementation increases the

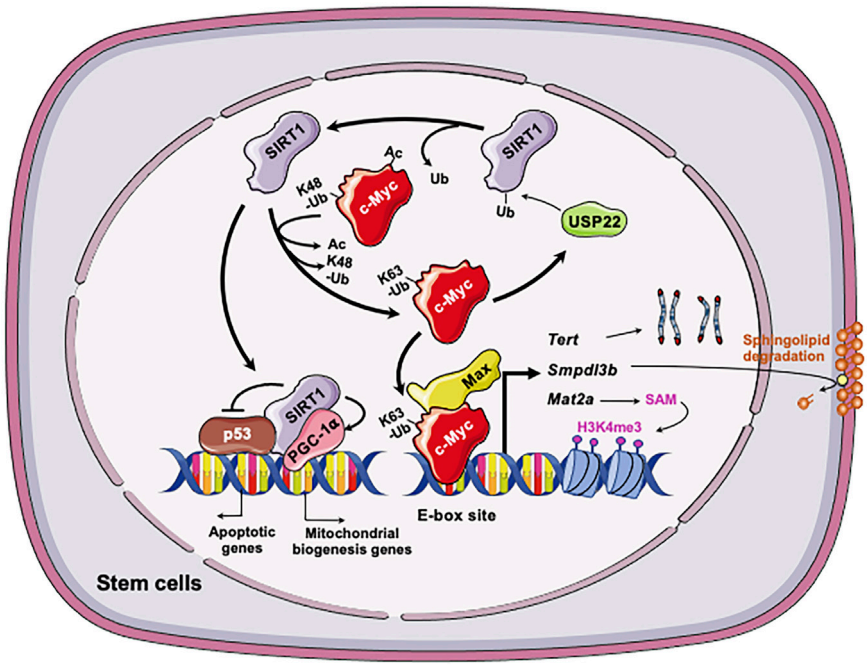

FIGURE 3
The SIRT1-c-Myc axis in regulation of stem cells. Both SIRT1 and c-Myc are highly expressed in stem cells, including mESCs, iPSCs, and LSCs. In all three types of stem cells, SIRT1 deacetylates c-Myc, which increases stability, presumably via reported exchange of K63-linked vs. K48-linked polyubiquitination chains. Increased stability of c-Myc enhances the transcription of c-Myc target genes in stem cells, including *Mat2a*, *Smpdl3b*, *Tert*, and *Usp22*. In mESCs, increased expression of MAT2A induces the production of SAM from methionine, which in turn increases H3K4me3 on pluripotent genes and induces their expression. This action is important for the maintenance of pluripotent stem cells. C-Myc in mESCs also induces the expression of SMPDL3B to remodel sphingolipids on the plasma membrane, which impacts membrane fluidity and signaling pathways involved in neuronal differentiation. In post-reprogrammed iPSCs, c-Myc activates the transcription of Tert to promote telomere elongation. In LSCs, c-Myc posttranscriptionally induces overexpression of USP22, a protein deubiquitinating enzyme that can stabilize the SIRT1. This regulation enhances SIRT1-mediated inhibition of p53 while stimulating PGC-1α-mediated mitochondrial biogenesis, promoting LSC survival and proliferation. Figures were created using images downloaded and adapted from Service Medical ART: SMART (https://smart.servier.com/image-set-download/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/)."

survival of SIRT1 KO newborn mice. All those observations suggest that the defective methionine metabolism is partially responsible for SIRT1 deficiency-induced developmental defects in mice (Tang et al., 2017).

Metabolomic analysis revealed that SIRT1 deficient mESCs also exhibit dramatic accumulation of sphingomyelin independently of the defects in methionine metabolism as previously reported by Tang et al. (2017). Sphingomyelin is a type of sphingolipids, which is a class of natural lipids enriched in central nervous system (Merrill et al., 2007; Chen et al., 2010; Rao et al., 2013). In addition to be main structural components of cell membrane, sphingolipids act as important signaling molecules controlling many cellular events such cell growth, differentiation, and apoptosis (Hannun and Obeid, 2008; van Meer et al., 2008). The significance of sphingolipids for human health is best demonstrated by the observation that many neurodegenerative diseases, such as Niemann-Pick's, Alzheimer's, and Parkinson's, are associated with defects in sphingolipids degradation enzymes and impaired sphingolipid metabolism (Brice and Cowart, 2011; Czubowicz et al., 2019). Particularly, sphingolipids are bioactive lipids critical for survival and differentiation of stem cells (Bieberich, 2008). Fan et al. confirmed that different SIRT1 deficient mESC lines have significantly increased levels of sphingomyelin, primarily due to a marked reduction of sphingomyelin phosphodiesterase acid like 3B (SMPDL3B) (Fan et al., 2021). SMPDL3B is a GPI-anchored plasma membrane bound sphingomyelin phosphodiesterase that degrades sphingomyelin into ceramide. Utilizing ChIP-qPCR assay, promoter analysis, luciferase reporter assay, and sgRNA/dCas9-mediated *in situ* gene expression perturbation, they further found that the *Smpdl3b* promoter is located within a bivalent chromatin domain targeted by c-Myc and EZH2, a H3K27me3 transferase. SIRT1 actively modulates this bivalent domain, primarily through deacetylation and stabilization of c-Myc. Loss of SIRT1 decreases c-Myc binding to the *Smpdl3* promoter, which in turn increases EZH2 recruitment and H3K27me3, resulting in silencing of *Smpdl3b* (Figure 3). Functionally, accumulation of sphingomyelin in SIRT1 KO mESCs disrupts the integrity of cell membrane and subsequently increases the membrane fluidity. The increase of cell membrane fluidity does not significantly impact pluripotency of mESCs, but instead markedly delays and impairs *in vitro* differentiation of mESCs into neural progenitors and mature neurons (Fan et al., 2021). When analyzed *in vivo*, Fan et al. (2021) showed that maternal high-fat diet feeding elevates sphingomyelin contents in all brain regions of SIRT1 KO embryos. This metabolic defect is associated with reduced expression of many markers of intermediate progenitors and mature neurons and delaying intrauterine growth of embryos. This study uncovers a

novel function of the SIRT1-c-Myc axis in maintaining sphingolipid homeostasis and normal neural differentiation of mESCs, which are important for normal mouse embryonic development.

Both studies highlight the importance of the SIRT1-c-Myc axis in metabolic and epigenetic regulation of mESC pluripotency, differentiation, and mouse embryogenesis.

6 The SIRT1-c-Myc axis promotes telomere elongation of iPSCs

In vertebrates, telomeric repeats (TTAGGG tandem repeats), which constitute a telomere, are synthesized by telomerase expressed mainly in the period of embryonic development and in adult stem cells (de Lange, 2005; Flores et al., 2006a; Liu et al., 2007). During reprogramming of MEFs into iPSCs, telomeres are elongated, and telomere elongation has been recognized as a hallmark of an iPSC (Takahashi and Yamanaka, 2006). The SIRT1-c-Myc axis has been reported to promote telomere elongation of iPSCs (De Bonis et al., 2014).

SIRT1 is extremely highly expressed in mESCs compared with adult stomatic cells and differentiated cells such as MEFs (Calvanese et al., 2010; Tang et al., 2017). De Bonis et al. (2014) showed that during reprogramming from MEFs to iPSCs, the expression of SIRT1 is continuously induced and eventually reaches to a comparable level with that in mESCs. The increased expression of SIRT1 is coupled with the formation of the hyper-long telomeres. Specifically, utilizing loss-of-function ($Sirt1^{-/-}$) and gain-of-function ($Sirt1^{Super}$) MEFs, they showed that the expression level of SIRT1 does not affect the reprogramming of MEFs. However, telomeres in $Sirt1^{-/-}$ iPSCs are significantly shorter than those in $Sirt1^{+/+}$ iPSCs, whereas the length of telomeres in $Sirt1^{Super}$ is 20% in average longer than that in $Sirt1^{-/-}$ iPSCs. Moreover, telomeres in $Sirt1^{+/+}$ iPSCs elongate more progressively in the stage of post-reprogramming than those in $Sirt1^{-/-}$ iPSCs. Therefore, SIRT1 is required for telomere elongation in the stage of post-reprogramming.

In cancer cells, c-Myc activates the transcription of mouse telomerase reverse transcriptase (*mTert*), the catalytic subunit of telomerase (Wang et al., 1998; Flores et al., 2006b). De Bonis et al. showed that in late-passage iPSCs, SIRT1 increases the stability of c-Myc, which in turn promotes the transcription of *mTert* and telomere elongation (De Bonis et al., 2014). Consequently, SIRT1 deficient iPSCs accumulate chromosomal aberrations and display a derepression of telomeric heterochromatin. Therefore, SIRT1 positively regulates the expression of TERT by enhancing the stability of c-Myc protein (Figure 3).

7 The SIRT1-c-Myc axis promotes the maintenance and drug resistance of leukemia stem cells

In acute myeloid patients (AML), self-renewing leukemic stem cells (LSCs) generate a bulk of leukemic cells and correlate with low prognosis (Eppert et al., 2011; Patel et al., 2012). In AML patients containing the internal tandem duplication (ITD) in the Fms-like tyrosine kinase (*FLT3*) gene, lack of elimination of LSCs due to their strong drug resistance is presumably responsible for failed treatment with the small molecules of FLT3 tyrosine kinase inhibitors (TKIs) (Levis, 2011; Horton and Huntly, 2012; Smith et al., 2012).

Li et al. (2014) reported that the positive feedback between SIRT1 and c-Myc contributes to the maintenance and drug resistance of FLT3-ITD AML LSCs. Li et al. (2014) found that SIRT1 is overexpressed in the primary human FLT3-ITD AML LSCs due to c-Myc induced overexpression of USP22, a protein deubiquitinating enzyme that can stabilize SIRT1 (Lin et al., 2012). Increased SIRT1 protein in LSCs in turn inhibits p53 and enhances PGC-1α-mediated mitochondrial biogenesis, promoting LSC survival and proliferation (Li et al., 2014). Conversely, SIRT1 knockdown or inhibition by its inhibitor Tenovin-6 (TV6) increases c-Myc acetylation, enhancing its degradation and subsequent reduction in transcriptional activity in FLT3-ITD cells (Figure 3). In support of the notion that the positive SIRT1-c-Myc feedback loop contributes to partial maintenance of FLT3-ITD AML LSCs after treatment with TKI, inhibition of SIRT1 expression or activities reduces their growth and significantly enhances their sensitivity to TKIs (Li et al., 2014). The findings from this study suggest that targeting the SIRT1-c-Myc axis using the small molecule inhibitors of SIRT1 could potentially improve outcomes of TKI-based treatment of FLT3-ITD AML.

8 Concluding remarks

While c-Myc is a well-known oncoprotein, the impact of SIRT1 on tumorigenesis is distinct at different stages depending on its deacetylation substrates, which include both tumor suppressors and oncogenic proteins (Garcia-Peterson and Li, 2021). The positive feedback loop between SIRT1 and c-Myc has been reported to suppress senescence and apoptosis in established cancer cells (Menssen et al., 2012). Recent studies revealed that this positive feedback loop is particularly important in maintenance, proliferation, and stress resistance of stem cells, including PSCs and CSCs. In PSCs, these actions are crucial for the maintenance of their pluripotency, self-renewal, and differentiation, which are ultimately important for normal embryogenesis. In CSCs, the impacts of SIRT1-c-Myc axis could result in drug resistance, relapse, and metastasis of tumors, thereby directly influencing therapeutic outcomes. Future studies are still needed to better understand the functional importance of the SIRT1-c-Myc axis in different type of stem cells. In particular, the maintenance and early lineage specification of primed hESCs are regulated by signaling pathways such as FGF and Activin/Nodal signaling (Brown et al., 2011; Fathi et al., 2017). Yet the potential role of the SIRT1-c-Myc axis in regulation of these signaling in hESCs remains unknown. Future research along this line could provide molecular basis for novel therapeutic strategies against developmental diseases and cancers.

Author contributions

WF and XL conceived and designed the review, wrote, edited and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Lessons in aging from Myc knockout mouse models

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Despite *MYC* being among the most intensively studied oncogenes, its role in normal development has not been determined as *Myc*−/− mice do not survival beyond mid-gestation. *Myc* ± mice live longer than their wild-type counterparts and are slower to accumulate many age-related phenotypes. However, *Myc* haplo-insufficiency likely conceals other important phenotypes as many high-affinity Myc targets genes continue to be regulated normally. By delaying *Myc* inactivation until after birth it has recently been possible to study the consequences of its near-complete total body loss and thus to infer its normal function. Against expectation, these *"Myc*KO" mice lived significantly longer than control wild-type mice but manifested a marked premature aging phenotype. This seemingly paradoxical behavior was potentially explained by a >3-fold lower lifetime incidence of cancer, normally the most common cause of death in mice and often Myc-driven. *Myc* loss accelerated the accumulation of numerous "Aging Hallmarks", including the loss of mitochondrial and ribosomal structural and functional integrity, the generation of reactive oxygen species, the acquisition of genotoxic damage, the detrimental rewiring of metabolism and the onset of senescence. In both mice and humans, normal aging in many tissues was accompaniued by the downregulation of Myc and the loss of Myc target gene regulation. Unlike most mouse models of premature aging, which are based on monogenic disorders of DNA damage recognition and repair, the *Myc*KO mouse model directly impacts most Aging Hallmarks and may therefore more faithfully replicate the normal aging process of both mice and humans. It further establishes that the strong association between aging and cancer can be genetically separated and is maintained by a single gene.

KEYWORDS

cancer, glycolysis, MLX, mitochondria, progeria, ribosomes, ROS, senescence

1 Introduction

1.1 The *MYC* oncogene and its role as a transcription factor in cancer

MYC bears the distinction of being among the first transforming retroviral oncogenes (v-myc) that was discovered before it cellular counterpart (c-Myc) (Duesberg and Vogt, 1979; Roussel et al., 1979; Sheiness et al., 1980; Hayward et al., 1981; Ramsay et al., 1990). Its long and storied history, combined with its well-documented involvement in many human cancers, provides ample reason as to why it persists after nearly 50 years as being among the most intensely studied of all mammalian oncogenes (Meyer and Penn, 2008). Myc's widespread role in human cancer pathogenesis also explains why efforts to identify and

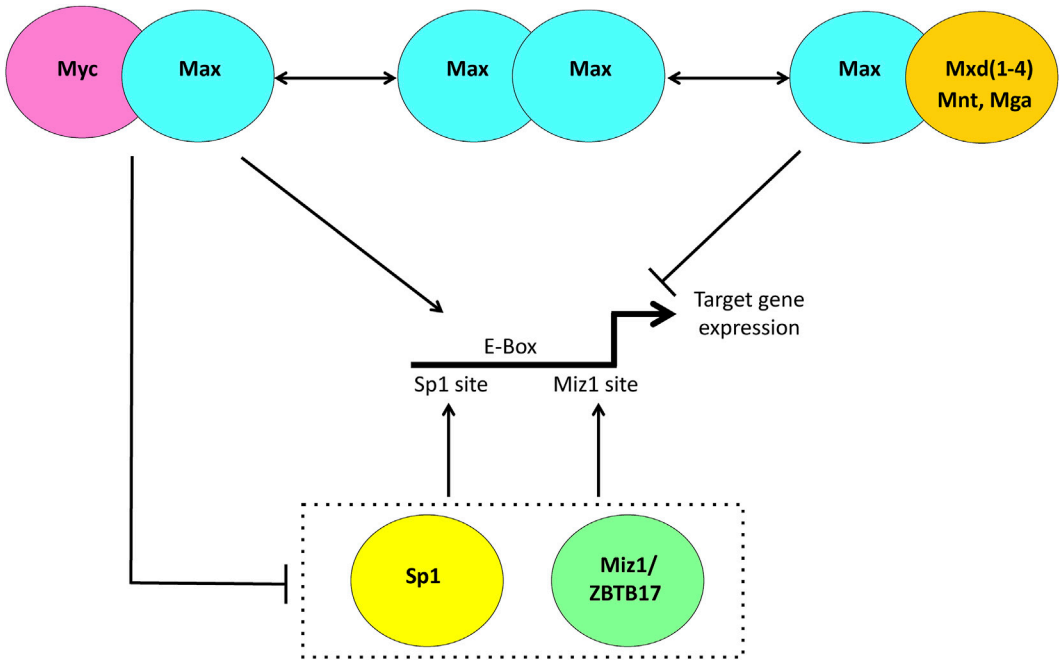

FIGURE 1
Transcriptional regulation via the Myc Network. When Myc is abundant, such as during proliferation, it heterodimerizes with the bHLH-ZIP protein Max, binds to consensus E box elements, usually located in the proximal promoters of its target genes and facilitates transcription. When Myc levels are low such as in quiescent cells, Max is more likely to heterodimerize with members of the Mxd family, comprised of the related bHLH-ZIP factors Mxd1-4 and the more distantly related Mnt and Mga. These can compete with and displace Myc-Max heterodimers from E boxes and silence gene expression. Negative regulation of Myc target genes is achieved indirectly as a result Myc-Max heterodimers binding to and suppressing the positively-acting transcription factors Sp1/3 and Miz1/ZBTB17.

develop effective inhibitors remain a major priority despite the frustratingly difficult nature of this task (Weber and Hartl, 2023).

The Myc protein is a bHLH-ZIP transcription factor that regulates thousands of downstream target genes or perhaps even the entirety of the genome by serving as a more general transcriptional amplifier of gene expression (Eilers and Eisenman, 2008; Nie et al., 2012; Carroll et al., 2018; Patange et al., 2022). Positive regulation is achieved upon Myc's association with its bHLH-ZIP partner protein, Max, and binding of the heterodimer to consensus "E box" elements that are typically located in the proximal promoters of its direct target genes (Figure 1) (Eilers and Eisenman, 2008; Carroll et al., 2018; Prochownik, 2022). The general consensus is that the extent to which a gene is upregulated by Myc is determined largely, although not exclusively, by several independent and non-mutually exclusive factors. These include the intrinsic affinity of the Myc-Max heterodimer for the target gene's associated E box (es); the E box's epigenetic modification; the degree to which neighboring chromatin is itself epigenetically altered and relaxed to allow access of Myc-Max to the E box; the presence of other unrelated factors that may bind nearby and hinder or promote Myc-Max binding and the extent to which Myc-Max must compete with other E box-binding transcription factors including those between Max and members of the Mxd family, which actively oppose Myc by transcriptionally suppressing its target genes (Figure 1) (Prendergast and Ziff, 1991; Conacci-Sorrell et al., 2014; Dolezal et al., 2017; Carroll et al., 2018; Prochownik, 2022; Prochownik and Wang, 2022). Collectively the integrated interplay and cooperation among these various factors serve to define the "functional affinity" of a binding site. This operative definition allows for changes in these affinities in ways that reflect different cell types, states of proliferation or differentiation and the dynamic nature of intracellular conditions and cues.

Among the most critical determinants of whether and to what extent Myc will upregulate a target gene is the absolute level of Myc protein itself (Dolezal et al., 2017; Wang et al., 2022b). This led to the concept of "physiologic" and "pathologic" targets (Fernandez et al., 2003; Soucek and Evan, 2010; Dolezal et al., 2017; Prochownik, 2022; Prochownik and Wang, 2022) with the former being defined as genes that respond to levels of Myc that can be achieved in normal cells during, for example, periods of log-phase growth. The binding sites in such targets might therefore be considered as being of moderate-high affinity based on the above definition. In contrast, pathologic targets bind and/or respond to the high Myc levels that are only observed in tumors or in untransformed cells with experimentally enforced Myc over-expression (Coller et al., 2000; Nesbit et al., 2000; Zeller et al., 2006; Sabo et al., 2014). These may include previous physiologic targets that are now induced to even higher levels or targets that bind Myc-Max and respond to it only when it is over-expressed. The binding sites in these targets might therefore be considered as being low-affinity. The relevance of pathologic targets is dramatically underscored *in vivo* where high-level conditional Myc induction can rapidly induce aggressive tumors and the expression of unique transcriptomes whereas subsequent Myc inactivation causes complete tumor regression and transcriptomic normalization even before the

tumor itself shows any objective gross or histologic response (Karlsson et al., 2003; Shachaf et al., 2004; Wu et al., 2007; Dolezal et al., 2017). Despite these distinctions, it is likely that both physiologic and pathologic targets contribute to transformation (Soucek and Evan, 2010; Prochownik, 2022; Prochownik and Wang, 2022).

In contrast to Myc's positive transcriptional regulation, an equal or somewhat smaller fraction of its target gene repertoire is negatively regulated via more indirect mechanisms. This is accomplished by interactions between Myc-Max heterodimers and the transcription factors Miz1, Sp1 and Sp3, thereby preventing the upregulation of target genes bearing Miz1 and/or Sp1 sites in their promoters (Figure 1) (Gartel et al., 2001; Gartel and Shchors, 2003; Eilers and Eisenman, 2008; Herkert and Eilers, 2010). While there is less direct evidence that Myc's negative targets are subject to the same types of physiologic and pathologic regulation as positive targets, this does appear to be the case as evidenced by data showing much larger numbers of Miz1 and Sp1 binding sites being co-occupied by Myc and an expansion of negative target gene responses during peaks of high physiologic or pathologic Myc expression (Encode Project Consortium, 2012; Diehl and Boyle, 2016; Wang et al., 2018; Wang et al., 2022a).

2 Myc target genes and the consequences of Myc inhibition *in vitro* and *in vivo*

The proteins encoded by Myc target genes can be broadly classified into several general functional categories (Zeller et al., 2006; Kim et al., 2008; Anczukow and Krainer, 2015; Wang et al., 2022a; Wang et al., 2022b; Prochownik, 2022). Their duties include promoting the cell cycle; overseeing the structure and function of mitochondria; regulating translation, notably, the synthesis of ribosomal subunits, tRNA, rRNAs and translation/initiation factors; coordinating non-mitochondrial metabolic pathways, particularly glycolysis, glutaminolysis, and lipid and nucleotide biosynthesis; the control of mRNA splicing and the recognition and repair of various types of DNA damage (Grandori et al., 2000; Felton-Edkins et al., 2003; Grandori et al., 2005; Li et al., 2005; Gomez-Roman et al., 2006; Mannava et al., 2008; Dang, 2010; van Riggelen et al., 2010; Dang, 2011; Carroll et al., 2018; Singh et al., 2019; Singh et al., 2021; Wang et al., 2022a; Wang et al., 2022b; Prochownik, 2022). In primary murine embryonic fibroblasts (MEFs), which undergo immediate cell cycle arrest in response to *Myc* inactivation, 2 additional sets of genes related to aging and senescence have also been recently identified (Wang et al., 2022b).

Regardless of whether *MYC* is silenced genetically or pharmacologically, its inhibition *in vitro* is almost always associated with an immediate cessation of proliferation that usually coincides with G_0/G_1 arrest, although in cancer cells this may occur in other stages of the cell cycle or even in all stages simultaneously (Trumpp et al., 2001; Huang et al., 2006; Wang et al., 2008; von Bueren et al., 2009; Wang et al., 2015; Scognamiglio et al., 2016; Wang et al., 2022b). *In vivo,* total deletion of *Myc* in the embryo is uniformly lethal at ~e10.5 (Davis et al., 1993). Embryos of *Myc* hypomorphs engineered to express progressively lower Myc levels show dose-related reductions in body size as Myc levels decline and MEFs derived from these mice also show a gradual, and eventual total loss of proliferative capacity (Trumpp et al., 2001). Examination of individual cell populations from these mice showed their overall smaller organ size to be due to reductions in the total cellular content rather than decreases in cell size (Trumpp et al., 2001). Similar defects have been demonstrated in isolated Myc-depleted T lymphocytes whose activation did not affect cell growth but did severely impair their ability to proliferate (Wang et al., 2011).

In contrast, and for unknown reasons, transient inhibition of Myc *in vivo* in older mice rarely has such dramatic effects. For example, body-wide induction in adult mice of the dominant-negative Myc inhibitor known as OmoMyc was entirely compatible with survival but did cause mild and transient bone marrow hypoplasia and flattening of the intestinal mucosa, both of which were reversible despite continued Myc suppression (Soucek et al., 2008). Neither the degree to which OmoMyc inhibited the function of endogenous Myc nor any long-term follow up of these mice was reported. On the other hand, Myc inhibition was sufficient enough to promote the regression of pre-existing Ras-driven lung tumors indicating that certain neoplasms and their responsible oncogenes can display a high Myc-dependency both *in vivo* and *in vitro* (Sklar et al., 1991; Karlsson et al., 2003; Shachaf et al., 2004; Wu et al., 2007; Soucek et al., 2008; Dolezal et al., 2017). In contrast to these findings, the hepatocyte-specific elimination of Myc did not alter the time needed for mice to regenerate a normal liver mass following 2/3rd partial hepatectomy (Baena et al., 2005; Li et al., 2006; Sanders et al., 2012). Concerned that this procedure did not provide a sufficiently strong or lengthy proliferative demand, Edmunds et al. employed the "FAH" mouse model of hereditary tyrosinemia to show that the long-term ability of transplanted wild type and *Myc*−/− donor hepatocytes to repopulate the liver, replace the diseased hepatocyte population and cure the recipient mice were in all cases equivalent (Overturf et al., 1996; Edmunds et al., 2016). Indeed, not even subtle differences were observed in the long-term repopulation by the 2 donor hepatocyte populations when they were allowed to compete in the same recipient.

The above findings raised questions as to Myc's role in initiating and/or supporting neoplastic hepatocytes proliferation mediated by other oncogenes. This was examined in a mouse model of hepatoblastoma (HB) in which tumors could be rapidly and efficiently induced via the hydrodynamic tail vein-mediated delivery of Sleeping Beauty plasmids encoding mutant forms of β-catenin and the Hippo pathway effector YAP (Tao et al., 2014; Bell et al., 2017; Zhang et al., 2019). When these vectors were delivered to the previously mentioned mice lacking *Myc* in their hepatocytes, tumor initiation remained at 100% although the ensuing growth was markedly slowed and survival was prolonged (Wang et al., 2016). It was concluded that, at least within this model neoplastic framework, endogenous Myc was not necessary to initiate tumorigenesis but was necessary to maintain maximal tumor growth rates. This was supported by the finding that, relative to *Myc*+/+ HBs, *Myc*−/− HBs expressed lower levels of transcripts encoding proteins involved in the structure and function of mitochondria and the translational machinery, including most ribosomal proteins and many translation factors. Consistent with a less pronounced Warburg effect, *Myc*−/− tumors also showed an attenuated induction of transcripts encoding glycolytic enzymes and lower levels of fatty acid β-oxidation (FAO). Perhaps as testimony to a less pronounced upregulation of Myc

target genes, global histone H3K9 acetylation was reduced in tumors from *Myc*−/− mice (Martinato et al., 2008; Wang et al., 2016). Together with the previously mentioned findings, these studies provided a more nuanced role for Myc in both normal and neoplastic development and suggested that Myc's role in the generation of these tumors was not to participate in tumor initiation (or at least in its most critical aspects) but rather to provide the necessary translational and metabolic support needed to achieve maximal rates of tumor growth.

3 Embryonal *Myc* heterozygosity extends lifespan and improves healthspan

The embryonic lethality of *Myc*−/− mice (Davis et al., 1993; Trumpp et al., 2001; Dubois et al., 2008) has until recently precluded an evaluation of Myc's role in growth and development beyond mid-gestation. Attempting to make the best of a bad situation, Hofmann et al. studied the life-long consequences of mice that had been rendered heterozygous for *Myc* (*Myc* ± mice) at the time of conception (Hofmann et al., 2015). These mice displayed several unanticipated phenotypes. First, and perhaps most importantly, they lived on average about 15% longer than their *Myc*+/+ (wild-type) counterparts with significant differences being noted between the sexes (20.9% longer for females and 10.7% longer for males). Like previously described *Myc* hypomorphs, *Myc* ± mice were also smaller at the time of birth and remained so, with the proportional mass of their individual organs being reduced accordingly so as to maintain the same relationship to total body mass as seen in wild-type mice. Both groups of mice showed a similar incidence of cancer during their lifetimes, the vast majority of which were lymphomas as has been reported in most strains of mice (Ward, 2006; Snyder et al., 2016). Thus, the increased longevity of *Myc* ± mice did not appear to be related to a lower cancer incidence, although they were reported to have smaller tumors with less extensive spread, thus perhaps reflecting the experience of Wang et al. in generating HBs in *Myc*−/− hepatocytes.

Relatively few gene expression changes were noted in the 3 tissues that were examined in both young and old *Myc* ± mice by microarray analysis, namely, liver, skeletal muscle and white adipose tissue (Hofmann et al., 2015). Indeed, the gene expression differences that were attributable to the 50% loss of Myc expression were ~10-fold fewer in number than those associated with aging. In retrospect this is perhaps not surprising given that, as discussed above, the most functionally important Myc target genes in non-neoplastic tissues might be expected to be those with the highest affinity E boxes and thus unlikely to be particularly impacted by a 2-fold loss in Myc expression. However, among the most significant and important functional classes of genes to be downregulated in *Myc* ± tissues based on gene set enrichment were those related to ribosome biogenesis and rRNAs (Hofmann et al., 2015).

Despite the relatively modest impact on Myc target gene expression, Hoffmann et al. identified a number of phenotypic changes in *Myc* ± mice that, in addition to the lifespan extension, were consistent with delayed aging and an extended health span. These included lower levels of age-related cardiac fibrosis, osteoporosis, hepatic lipid accumulation, serum cholesterol and loss of motor coordination as measured by rotarod testing. The age-related exhaustion of long-term hematopoietic stem cells was also delayed in *Myc* ± mice (Hofmann et al., 2015). Importantly, neither overall body adiposity not the proportion of senescent cells were impacted in *Myc* ± animals. Double-stranded DNA breaks (DSBs), as measured by the accumulation of 53BP1 foci in livers also increased equally with aging in the livers of both wild-type and *Myc* ± mice. This suggested that the reduced rate of aging of *Myc* ± mice could not be attributed to a lower rate of DNA damage (or at least of DSBs).

Metabolic cage studies pointed to *Myc* ± mice as having significantly higher metabolic rates, as measured by total oxygen consumption (Nie et al., 2015). Although CO_2 production rates were not reported, the implication of these studies was that the respiratory exchange ratio (RER), as determined by the VCO_2/VO_2 ratio, was lower and that *Myc* ± mice were more reliant on fatty acid oxidation as an energy source. Finally, consistent with the previously mentioned reduction in transcripts related to ribosomes and rRNAs, Hoffman et al. found evidence for a decreased rate of total liver protein synthesis as measured by the *in vivo* incorporation of radio-labeled phenylalanine (Hofmann et al., 2015).

4 Post-natal deletion of *Myc* is associated with premature aging, increased lifespan and a lower cancer incidence

Not all findings in *Myc* ± mice pointed to a slowing of the aging process and an overall healthier lifespan (Hofmann et al., 2015). For example, the reduced rates of ribosomal biogenesis, rRNA production and translation described by Hofmann et al. are actually common properties of aging whereas the presumptive increased reliance on FAO as an energy source might be indicative of another age-related phenomenon, namely glucose intolerance and the switch to fatty acids as an alternate energy source (Chang and Halter, 2003; D'Aquila et al., 2017; Gonskikh and Polacek, 2017; Woodward and Shirokikh, 2021). Although FAO normally declines with age (Toth and Tchernof, 2000), this might not be the case with *Myc* ± mice whose defects in glycolysis and mitochondrial structure and function might have forced an unnatural over-reliance on FAO, which is a more efficient means of energy extraction (Li et al., 2005; Prochownik, 2022).

As mentioned above, the most critical Myc target genes may well be those with the highest affinity binding sites that would be impacted minimally, if at all, by a 50% decline in Myc levels. Thus the phenotypes described by Hofmann et al. (Hofmann et al., 2015) may well represent only the "tip of the iceberg" and may even be quite different from those associated with a more thorough loss of Myc expression. Therefore, in response to these considerations, Wang et al. performed 2 studies in parallel, with each informing the other, that sought to characterize the long-term consequences of a more extensive and potentially more consequential body-wide knockout of Myc (Wang et al., 2022b; Wang et al., 2023). The second study in particular asked whether it was possible to achieve a more extensive loss of *Myc in vivo* while avoiding the lethality associated with *Myc*−/− embryos (Davis et al.,

1993; Trumpp et al., 2001). The question was partly motivated by the findings that OmoMyc induction in adult mice had been previously found to be compatible with at least short-term survival as well as by the observation that the viability of *Myc*−/− embryos could be extended by 2 days if Myc expression was preserved in the placenta (Dubois et al., 2008; Soucek et al., 2008). Concerns as to whether high-levels of *Myc* knockout could be achieved without compromising viability remained however given that both OmoMyc-expressing mice and *Myc*−/− embryos showed marked hematopoietic compromise (Trumpp et al., 2001; Dubois et al., 2008; Soucek et al., 2008).

In both studies undertaken by Wang et al., mice bearing 2 "floxed" *Myc* alleles were crossed with a strain bearing a CreER transgene under the control of the ubiquitously expressed Rosa26 promoter (Wang et al., 2022b; Wang et al., 2023). In the first study, e14-16 MEFs were isolated from these mice, expanded for 2-3 passages and then exposed to 4-hydroxytamoxifenn (4OHT) for 7–10 days. This resulted in a >95% excision of the *Myc* gene, a comparable loss of Myc protein expression and proliferative arrest in G_0/G_1, which, over the ensuing week became permanently consolidated in G_2/M. These "*Myc*KO" cells appeared to be larger, flatter and less spindle-shaped, all of which mimicked the previously noted characteristics of senescent primary fibroblasts and of a unique immortalized *Myc*−/− rat fibroblast line that grows at ~20% the rate of its *Myc* ± counterparts (Mateyak et al., 1997; Zhao and Darzynkiewicz, 2013; Kumari and Jat, 2021).

Growth-arrested primary *Myc*KO MEFs shared additional features with *Myc*−/− rat fibroblasts and cancer cell lines in which Myc function was inhibited genetically or by structurally diverse small molecule Myc inhibitors (Graves et al., 2012). These included increases in mitochondrial-derived reactive oxygen species (ROS) and neutral lipid content suggesting that *Myc*KO MEFs contained functionally defective mitochondria. However, unlike the above cancer cell lines and *Myc*−/− rat fibroblasts, which cannot sustain adequate ATP levels, an ~2-fold increase in mitochondrial mass observed in *Myc*KO MEFs was postulated to represent a means of compensating for energy generating defects as often occurs in association with aging, senescence and various mitochondrial stresses and diseases (Trifunovic et al., 2004; Barrientos, 2012; Miwa et al., 2022). Thus, rather than completely mimicking the properties of immortalized *Myc*−/− fibroblasts, *Myc*KO MEFs more closely recapitulated the behaviors of aging and/or senescence primary fibroblasts, whose proliferation declines and eventually ceases with continued *in vitro* passage and whose mitochondrial content increases in parallel (Hayflick, 1974; Korolchuk et al., 2017; Popay et al., 2021; Martini and Passos, 2023). Like senescent cells, growth-arrested primary *Myc*KO MEFs also showed increased lysosomal content and glucose uptake, higher levels of senescence-associated β-galactosidase and decreased translation as measured by puromycin incorporation into elongating polypeptide chains (Bittles and Harper, 1984; Sharpless and Sherr, 2015; Payea et al., 2021; Popay et al., 2021; Wang et al., 2022b).

RNAseq performed on WT primary and *Myc*KO MEFs within 10 days of *Myc* excision revealed >4300 gene expression differences, about equally divided between up- and downregulated transcripts and with nearly 2/3^{rds} of them being encoded by previously identified direct Myc target genes (Wang et al., 2022b). Gene set enrichment analysis (GSEA) categorized these into a small number of functionally-related categories that were consistent with some but not all previously described Myc target gene classifications. These functions included those dedicated to mitochondrial and ribosomal structure and function, cell cycle regulation, aging, senescence and the recognition and repair of multiple types of DNA damage. In follow-up to this latter observation, immuno-staining showed that *Myc*KO primary MEFs expressed higher levels of the DNA damage recognition and response proteins p53, 53BP1, γ-H2AX, RAD51 and Ku80 while also showing increased staining in a TUNEL assay, which, like γ-H2AX staining, identifies DSBs. Furthermore, whereas treatment of WT MEFs with the DSB-inducing chemotherapeutic drug etoposide elicited a coordinated response of the above factors, the response in *Myc*KO MEFs was suppressed and dysregulated. These findings suggested that *Myc*KO MEFs displayed more evidence of baseline genotoxic stress, not only as a result of their increased ROS production but also due to their inability to properly marshal and sustain a well-regulated DNA damage response.

While aging and senescence are thought to be at least partially driven by the accumulation of DNA damage, it is also true that old and/or senescent cells are less capable of DNA damage repair and therefore generate genotoxic lesions at faster rates than younger cells and maintain them longer (Chen et al., 2007; Collin et al., 2018; Schumacher et al., 2021; Yousefzadeh et al., 2021). To test the idea that *Myc*KO cells might be more prone to DNA damage, Wang et al. took advantage of the unexpected finding that SV40 T antigen-immortalized *Myc*KO MEFs could escape proliferative arrest in response to *Myc* inactivation and continue to replicate at about half the normal rate (Wang et al., 2022b). The RNAseq profiles of these cells also showed that they retained evidence of numerous DNA damage recognition and repair pathway defects. As a result, these cells were significantly more resistant than wild-type immortalized MEFs to genotoxic insults that, in addition to DSBs, included single-stranded breaks, oxidative base lesions and both inter- and intra-strand cross-links. Wang et al. contrasted this seemingly paradoxical behavior to that associated with monogenic disorders of DNA repair such as Fanconi's anemia and xeroderma pigmentosum, which are exquisitely sensitive to DNA damage (Black, 2016; Taylor et al., 2019). They suggested that the non-repairable lesions associated with these inherited conditions initiate a robust apoptotic response since the pathways that mediate this remain intact. In contrast, the multiple defects in *Myc*KO MEFs are so extensive that any new DNA damage is neither recognized, repaired nor able to elicit a coordinated apoptotic response. A similar loss of sensitivity to cis-platinum and etoposide has been observed in medulloblastoma cell lines following the siRNA-mediated knockdown of Myc (von Bueren et al., 2009).

The second study reported by Wang et al. utilized the above-described $Myc^{loxP/loxP}$ x Rosa26-CreER mouse strain in which individuals of both sexes were treated with 5 daily injections of tamoxifen beginning on the day of weaning (Wang et al., 2023). The timing of these injections was critical as preliminary studies had shown that treating younger mice or those weighing <15–16 grams was associated with a high incidence of fatal aplastic anemia. qPCR and qRT-PCR analysis performed with over a dozen tissues from treated mice showed that *Myc* gene excision frequencies exceeded 75%–95% in nearly all tissues with a notable exception being brain

TABLE 1 Notable phenotypic differences among mice with varying degrees of myc inactivation vs. WT controls.

	Genotype (references)		
	Myc−/− Davis et al. (1993); Trumpp et al. (2001); Dubois et al. (2008)	*Myc* ± Hofmann et al. (2015)	*Myc*KO Wang et al. (2023)
Timing of knockout	Embryonal	Embryonal	Post-natal
Extent of knockout	100%	100%	Variable (~70%–100%)
Lifespan	embryonal lethal	Extended	Extended
Lifetime cancer incidence	ND[a]	Normal	3.4-fold lower
Size of mice	Reduced	Reduced	Normal
Major organ structural defects	placenta, BM[b], vasculature	None	BM, intestine Fat:lean mass ratio
Fat:lean mass ratio	ND	ND	Prematurely Increased
Hepatic steatosis	ND	No	Yes
Alopecia	ND	No	Yes
Achromotricia	ND	No	Yes
Hyperkeratinization	ND	ND	Yes
Overall strength, endurance, balance	ND	Better	Worse
Glucose tolerance	ND	ND	T2D-like GTT
Serum cholesterol	ND	Reduced	ND
Cardiac fibrosis	ND	Less severe	ND
Osteoporosis	ND	Less severe	ND
CD4:CD8 T cell ratio	ND	Less pronounced decline	ND
Increased genotoxic stress	ND	No	Yes
Oxygen consumption	ND	Increased: day time + night time	Increased: night time only
Rate of protein translation	ND	Decreased	Decreased
Energy deficit	ND	Yes	ND

[a]ND, not determined.
[b]BM, bone marrow.

where it was 40%–60%. The observation that these "*Myc*KO" mice not only survived the major cause of embryonic mortality (Davis et al., 1993; Trumpp et al., 2001; Dubois et al., 2008) but also remained seemingly healthy allowed for other previous findings to be confirmed and further extended. For example, while >90% of mice survived and eventually normalized their peripheral counts, bone marrows remained hypoplastic and, by 4–5 months of age, resembled those of middle-aged mice (~50% cellularity). Like the OmoMyc-treated mice described by Soucek et al. *Myc*KO mice also displayed transient intestinal epithelial flattening and loss of crypt structure (Soucek et al., 2008; Wang et al., 2023). Yet, despite these obvious morphological changes, the mice showed no evidence of steatorrhea or failure to thrive indicating that the changes had minimal physiologic impact. These findings, as well as well as additional ones described below, are summarized in Table 1 where they are compared and contrasted with those from *Myc*−/− embryos and *Myc* ± mice (Davis et al., 1993; Trumpp et al., 2001; Dubois et al., 2008; Hofmann et al., 2015).

Although the body weights of *Myc*KO and wild-type mice were initially indistinguishable, the former began to acquire significantly higher fat:lean mass ratios after 5–6 months such that by 10 months of age these ratios resembled those of ~20 month old wild-type mice (Pappas and Nagy, 2019). Both groups then began to lose weight and to reduce their fat:lean mass ratios at similar rates, although both parameters remained high in *Myc*KO mice for the remainder of their lives. *Myc*KO mice also developed premature graying and loss of fur beginning at 3–5 months of age that resembled, albeit to a lesser degree, the phenotype of old mice or those with melanocyte-specific embryonal excision of *Myc* (Pshenichnaya et al., 2012). Skin samples from the alopecic regions showed epidermal thickening, hyperkeratinization and focal peri-follicular staining for senescence-associated β-galactosidase.

Testing of *Myc*KO mice for strength, fitness and coordination showed them to be generally inferior to age-matched wild-type mice, although these differences became noticeable at different times

throughout life. In older *Myc*KO females (~20 months), even normal, diurnal ambulatory activity was reduced.

A number of metabolic abnormalities consistent with premature aging also distinguished wild-type and *Myc*KO mice. Nonalcoholic fatty liver disease (NAFLD or hepatic steatosis) commonly accompanies aging, particularly in the face of co-existing conditions such as dyslipidemia, obesity and insulin resistance (Honma et al., 2011; Bertolotti et al., 2014). Although a NAFLD-like picture was previously identified in mice with *Myc*−/− hepatocytes, neither its maximal severity nor its life-long consequences were evaluated (Edmunds et al., 2016). Wang et al. confirmed that the degree of NAFLD in 5 month old *Myc*KO mice, as measured by neutral lipid and triglyceride content, was 3-4-fold higher than that of comparably aged wild-type mice and matched that of 22 month old individuals from the latter cohort (Wang et al., 2023). Thus, although the maximal levels of lipid that were accumulated by *Myc*KO mice never exceeded the highest levels attained in the oldest wild-type mice, the rate of accumulation was more rapid.

Metabolic cage studies were also performed during the lifetimes of wild-type and *Myc*KO mice while being maintained on normal diets, during fasting and after re-feeding with either normal or high-fat diets. Young wild-type mice tended to possess very high nocturnal respiratory exchange ratios (RERs) indicating that, during active feeding, they were almost totally reliant on glucose as an energy source. RERs exceeding 1.0 were observed in some cases and indicated that these mice were engaged in high levels of fatty acid synthesis, which is commonly observed in juvenile animals undergoing rapid growth (Bruss et al., 2010; Houtkooper et al., 2011). In contrast, *Myc*KO mice demonstrated a significantly greater reliance on FAO and their RERs never exceeded 1.0. These findings were interpreted as indicating one or more of at least 3 non-mutually conditions. First, the absence of Myc may have reduced the animals' ability to metabolize glucose since Myc positively regulates glycolysis (Dang, 2010; Dang, 2011; Stine et al., 2015; Prochownik, 2022). Second, it may also have impaired the efficiency of mitochondria, making them more reliant on FAO to maintain normal energy levels as had been described in *Myc*KO MEFs and other Myc-compromised cells (Wang et al., 2015; Wang et al., 2022a; Wang et al., 2022b). Increased FAO dependency may also explain the neutral lipid accumulation of *Myc*KO mice, MEFs and other cells with compromised Myc function, in which energy-rich fatty acids are taken up in excess of what is needed to maintain energy stores with the difference being stored (Edmunds et al., 2016). Other ways to explain the greater reliance of *Myc*KO cells on FAO include a reduced supply of glycolytically-derived pyruvate for the TCA cycle and/or its diversion into other, non-acetyl coenzyme A-generating pathways (Stine et al., 2015; Prochownik and Wang, 2021). Third, younger *Myc*KO animals may have either already aged beyond the point where increased fatty acid synthesis would have been observed and/or may have compromised Myc-regulated fatty acid synthetic function (Morrish et al., 2010; Singh et al., 2021). Irrespective of cause(s), the RERs of younger *Myc*KO mice tended to more closely mimic those of older wild-type mice with the differences between the 2 groups becoming more erratic and tending to converge as the 2 cohorts aged.

Consistent with their high utilization of fatty acids as an energy source, but also indicating that they may be insulin resistant, *Myc*KO mice were mildly ketotic although fasting glucose and lactate levels were normal. Glucose tolerance testing and the quantification of peripheral insulin levels in *Myc*KO mice showed that they resembled those associated with Type 2 diabetes, with exaggerated hyperglycemia and hyperinsulinemia in response to a glucose challenge. However, these defects became progressively less pronounced with aging, thus indicating that *Myc*KO mice metabolically adapted in a Myc-independent manner while also reflecting their age-dependent tendency toward RER normalization.

Mitochondrial structural and functional compromise as a result of Myc's loss could explain the above-discussed metabolic abnormalities and would be consistent with the previously documented MEF results (Wang et al., 2022b). The examination of partially purified mitochondria from age-matched wild-type and *Myc*KO livers and adipose tissues showed that, even when pyruvate was non-rate-limiting, the oxygen consumption rates of the latter were blunted, thus strongly suggesting a defective in Complex I function with Complex II responses to succinate being similar in wild-type and *Myc*KO tissues (Wang et al., 2023).

The transport of free fatty acids across the outer mitochondrial membrane requires that they first be converted to fatty acyl-CoAs and then conjugated to carnitine via the rate-limiting enzyme carnitine palmitoyl transferase I (CPTI). They are then transported across the inner mitochondrial membrane, re-transformed via CPTII into a fatty acyl coenzyme A in the matrix and enter the FAO pathway (El-Gharbawy and Vockley, 2018). Complex I defects and the ensuing inefficient oxidation of long chain fatty acids are associated with elevated serum levels of 3-hydroxy-C14-carnitine (C14-OH), which is used as a clinical marker of these disorders (El-Gharbawy and Vockley, 2018). Wang et al. measured the serum levels of 51 acyl carnitines by mass spectrometry (MS) and indeed were able to document significant elevations of C14-OH in 5 month old *Myc*KO mice. As these mice aged, C14-OH levels normalized but were replaced by 12 new changes mostly involving higher levels of longer chain serum acylcarnitines (Wang et al., 2023). This suggested a progressive loss of normal FAO that is observed in aging humans with Type 2 diabetes (Mihalik et al., 2010). It was suggested that the normalization of C14-OH in older *Myc*KO mice cohort resulted from a reduced C14 pool due to the accumulation of the longer chain fatty acyl CoA precursors and their defective oxidation to shorter chain acylcarnitines. Collectively, the findings were consistent with the previous ones indicating that *Myc*KO animals were more insulin-resistant, more dependent on FAO and prematurely developed NAFLD. Interestingly, although the above findings indicated some normalization of the mitochondrial defects that were initially observed in the youngest *Myc*KO mice, older mice from this cohort were noted to have elevated levels of C5 carnitine, which is generally considered as being diagnostic of errors in branched chain amino acid (BCAA) catabolism (Gibson et al., 1994). This suggested that, as *Myc*KO mice aged, their mitochondrial defects worsened and/or broadened so as to increase their utilization of valine, leucine and isoleucine as alternate energy sources. Indeed, a comparison of RNAseq data from the livers of 5 and 20 month old *Myc*KO mice showed enrichment for gene sets involved in FAO at both ages and BCAA catabolism in the older group, thus mirroring the results

of serum MS-based measurements and findings from aging humans with Type 2 diabetes (Mihalik et al., 2010).

Perhaps the most unexpected finding from the above studies was that, despite the *Myc*KO mice displaying so many attributes of premature aging, they actually lived significantly longer than the wild-type cohort (median survival 32.7 months vs. 28.3, $p = 1.2 \times 10^{-7}$) with the longevity difference being particularly notable among females (median survival 33.5 months vs. 27.3 months, $p = 1.5 \times 10^{-8}$). Indeed, these findings were virtually identical to those of *Myc* ± mice (Hofmann et al., 2015). Careful documentation of the various pathologies observed at the time of death revealed *Myc*KO mice to have a more than 3-fold lower incidence of cancer during their lifetimes but with no change in the tumor spectrum, which was largely comprised of high-grade lymphomas (Ward, 2006; Snyder et al., 2016; Wang et al., 2023). Analysis of lymphomas from 3 *Myc*KO mice showed that Myc re-expression could be detected in at least 2 cases and that the *Myc* gene was intact or even modestly amplified in all 3. These results were interpreted as indicating that the rare tumors arising in *Myc*KO mice likely originated from a small minority population of bone marrow cells that failed to completely excise the *Myc* gene during the initial period of tamoxifen treatment (Wang et al., 2023). It also emphasized that, unlike previously described *Myc*−/− and *Myc* ± mice that were generated at the time of fertilization, all *Myc*KO tissues were almost certainly genetically mosaic, with varying proportions of *Myc*+/+, *Myc* ± and *Myc*−/− genotypes.

Additional RNAseq studies were performed on liver, skeletal muscle and abdominal white adipose tissues from 5 to 20 month old wild-type and MycKO mice in order to obtain both a broad overview of the genes under Myc's purview in each of these tissues and an appreciation for how these responded to aging relative to those expressed in wild-type tissues. These tissues were chosen because of previously reported Myc-related changes and because they alter their gene expression profiles during normal aging (Short et al., 2005; Tchkonia et al., 2010; Honma et al., 2011; Hofmann et al., 2015; Uchitomi et al., 2019). The results, which largely agreed with those previously documented in MEFs (Wang et al., 2022b), showed enrichment for at least 7 categories of genes that pointed to their functions being coordinately downregulated and/or compromised in tissues from young *Myc*KO tissues and in aging tissues from both wild-type and *Myc*KO mice.

Transcripts encoding proteins with roles in ribosomal and mitochondrial structure and function tended to be prominently downregulated in younger *Myc*KO tissues and older wild-type tissues although the exact identities of the individual genes and their degree of dysregulation differed in tissue-specific ways (Kim et al., 2008; Edmunds et al., 2016; D'Aquila et al., 2017; Dolezal et al., 2017; Wang et al., 2022b; Wang et al., 2023). Consistent with these findings as well as previous ones pointing to mitochondrial dysfunction in Myc-compromised cells and tissues (Graves et al., 2012; Wang et al., 2016; Wang et al., 2022a; Wang et al., 2022b), a third category of gene sets with roles in the response to oxidative stress was noted to be mostly upregulated in *Myc*KO issues. This was consistent with the previously mentioned increased ROS production resulting from Complex I dysfunction, preference for the use of fatty acids as a source of energy and the increase in mitochondrial mass in at least some tissues (Wang et al., 2022a; Wang et al., 2022b).

Two additional and related gene set categories whose directions of regulation were largely consistent with the premature aging phenotypes of *Myc*KO mice were those specifically associated with aging and senescence. Specifically, members of a 79 member gene set previously shown to be nearly universally dysregulated in response to aging in both mice and humans were largely expressed in opposite directions in wild-type and *Myc*KO mice, with the overall signature pointing to an "older" profile in livers and adipose tissues from the latter group. Several large gene sets previously identified as being enriched in tissues of patients with Type 1 and 2 diabetes, were also dysregulated in all 3 tissues of younger *Myc*KO mice in ways that would have been expected for individuals with these conditions and consistent with the previously documented Type 2 diabetes-like insulin resistance of these animals. In contrast, gene sets found to be enriched in patients with cancer were regulated in opposite ways in young wild-type and *Myc*KO mice with the latter being consistent with the lower life time cancer incidence associated with this group.

The sixth functional category of gene sets that was selectively enriched in young *Myc*KO mouse tissues pertained to DNA damage recognition and its repair, with the directions of dysregulation tending to reflect those seen previously in *Myc*KO MEFs which, as mentioned above, showed much higher levels of ongoing DNA damage despite being highly resistant to a wide variety of genotoxic agents (Wang et al., 2022b). Like *Myc*KO MEF, *Myc*KO livers demonstrated much higher levels of DSBs as documented by immuno-histochemical staining for γ-H2AX (Wang et al., 2023). These studies established that the dysregulation of genes associated with premature aging syndromes due to defective DNA repair pathways were recapitulated in *Myc*KO mice only on a much larger scale.

The final category of gene sets that was significantly enriched between wild-type and *Myc*KO tissues, although only in the liver, pertained to splicing and mRNA processing that includes maturation steps such as intron-exon recognition, lariat formation and excision and exon-exon ligation (Yan et al., 2019). A search for an excess of incorrectly or incompletely spliced transcripts, which have been reported to accompany aging (Meshorer and Soreq, 2002; Deschenes and Chabot, 2017; Bhadra et al., 2020) did not reveal any differences until 20 months of age at which time liver transcripts from *Myc*KO mice contained ~3-fold more non-canonically spliced transcripts than those of wild-type livers. It was speculated that, like the above-described heterogeneous causes of DNA damage, splicing defects would not only be another sign of premature aging but might also contribute to the highly mutagenic environment of the *Myc*KO background that could be a major contributor to the aging and pro-senescence phenotypes the cells from these animals (Koh et al., 2015; Deschenes and Chabot, 2017; Bhadra et al., 2020; Wang et al., 2022b; Wang et al., 2023).

Several features of the gene expression differences between young and old wild-type and *Myc*KO mice lent further credibility to the notion that the premature aging of the latter actually represented an acceleration of otherwise normal processes. First, the gene set differences between the 2 groups were greater in the young mice than in older mice. This suggested that the same transcriptional changes were occurring between the 2 groups except that they accumulated faster in the *Myc*KO group. Second, the previously noted "universal" 79 member collection of age-related

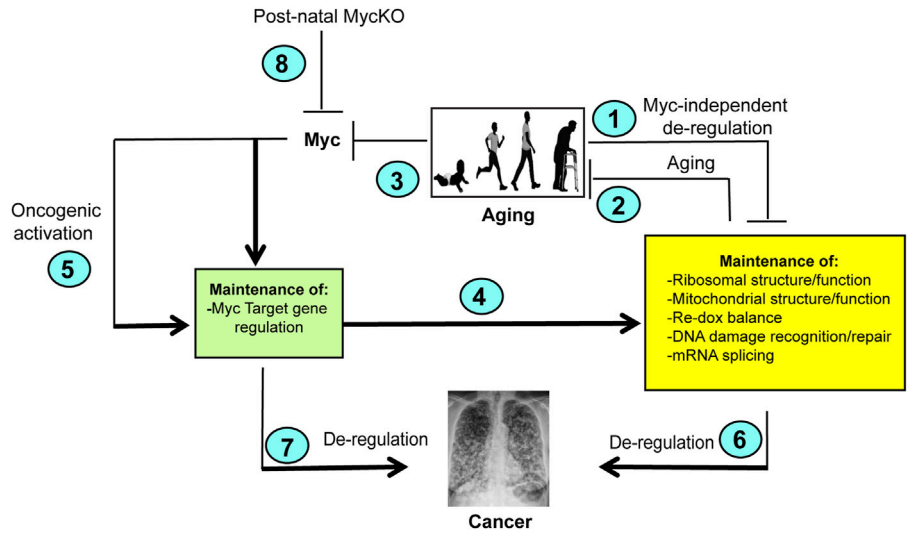

FIGURE 2
Model depicting the cooperation between normal aging and Myc. (1). Normal aging is associated with the accumulation of Myc-independent defects in a number of important cellular functions and/or an inability to maintain the regulation associated with youth. Examples include losses in translational efficiency, impaired mitochondrial function, increased ROS production, the accumulation of DNA damage and splicing (Meshorer and Soreq, 2002; Balaban et al., 2005; Park and Gerson, 2005; Short et al., 2005; Gonskikh and Polacek, 2017; Opresko and Shay, 2017). In turn, these functions may impact one another. For example, the high levels of ROS generated as a result of mitochondrial dysfunction can inhibit translation and induce oxidative DNA damage (Kirkinezos and Moraes, 2001; Vafa et al., 2002; Prochownik and Li, 2007; Rosca et al., 2012; Ghosh and Shcherbik, 2020; Molenaars et al., 2020). (2). The above-mentioned functions are also needed to maintain normal rates of aging. For example, defined defects in mitochondrial function and DNA damage recognition/repair pathways can accelerate aging (Opresko and Shay, 2017; Hahn and Zuryn, 2019; Rizza et al., 2021; Miwa et al., 2022; Shcherbakov et al., 2022). (3). Normal aging is associated with gradual declines in Myc and the ensuing dysregulation of Myc target gene expression (green box) (Wang et al., 2023). (4). As a result of declining Myc levels (3), normal aging leads to gradual declines in the expression of positively-regulated Myc target genes and increases in the expression of negatively-regulated Myc target gene (green box) (Wang et al., 2023). (5). Oncogenic activation of Myc deregulates its target genes leading to the constitutive up- or downregulation of its target genes (Figure 1), thereby driving increases in ribosome content, translation, mitochondrial mass and function, ROS production and DNA damage (Coller et al., 2000; Vafa et al., 2002; Felton-Edkins et al., 2003; Dang et al., 2006; Prochownik and Li, 2007; Prochownik, 2022). (6) The dysregulation of Myc target genes shown in (5) stabilizes or reverses the normal age-related changes in their expression and instead can drive and/or support the cellular processes necessary to maintain high levels of cancer-associated gene expression. (Shachaf et al., 2004; Dolezal et al., 2017; Prochownik, 2022). (7). Myc target genes not necessarily included in the yellow box, such as those which maintain cell cycle and impair apoptosis and senescence may independently contribute to tumor evolution when they are dysregulated as a result of Myc over-expression (Dang, 2011, 2012; Gabay et al., 2014; Prochownik, 2022). Some of these are likely to be "pathological targets" with low-affinity Myc binding sites and are activated only Myc levels exceed a certain threshold (Prochownik, 2022; Prochownik and Wang, 2022). (8). *Myc*KO mice fail to properly regulate their target genes. They therefore lose the ability to maintain the functions depicted in the yellow box. This lead to an accelerated aging phenotype, particularly in collaboration with the normal age-related declines in Myc-independent function (2). At the same time, the los of Myc eliminates major oncogenic pathways (4,5, and 7) thereby leading to an overall lifetime reduction in cancer susceptibility that contributes to longer survival even in the face of co-morbidities that are normally associated with shorter lifespans such as lipid accumulation and defective DNA damage recognition and repair. Created with BioRender.com.

genes was less dysregulated between the 20 month old groups of mice than it was between the 5 month old groups. The changes in expression were somewhat different for this gene set than for the previously described transcript sets associated with Types 1 and 2 diabetes and cancer where the differences between the wild-type and *Myc*KO groups were detected in the youngest mice and the numbers and identities of the gene set transcript members changed somewhat between the younger and older cohorts.

The remarkable degree to which a broad range of age-related phenotypes and genes was altered in both *Myc* ± and *Myc*KO mice suggested that these models were more fully integrated into the physiologic networks that oversee normal aging than were the monogenic disorders associated with DNA damage recognition and repair that are commonly used as models of aging (Kalb et al., 2006; Brosh and Bohr, 2007; Opresko and Shay, 2017; Rizza et al., 2021). Thus, using data from their own *Myc*KO mice, the ENCODE and *Tabula Muris* Consortia and elsewhere, Wang et al. next focused on Myc and its direct target genes in an assortment of tissues from normal aging mice and humans (Encode Project Consortium, 2012; Diehl and Boyle, 2016; Tabula Muris Consortium, 2020; Wang et al., 2023). Initially using RNAseq results from young and old normal mice, they first identified highly significant age-related declines in Myc expression in 12 of 90 single-cell populations isolated from 23 different tissues. Even more impressive declines in more than 60% of direct Myc target gene sets were seen in one or more of the single cell populations from most of the above tissues. In those cases where the directionality of gene expression could be ascertained, it was highly correlated with age-related reductions in Myc. These studies thus documented age-related loss of Myc expression in normal aging mouse tissues and even more extensive effects on direct Myc target genes.

Myc levels in *in vitro* propagated primary human fibroblasts decline with time, and the inevitable onset of senescence and growth inhibition can be delayed or reversed by sustaining Myc expression (Dean et al., 1986; Benanti et al., 2007; Wang et al., 2022b). In addition, a previous study with cultured primary fibroblast samples

from >650 young and old humans had shown that replicative senescence occurred more rapidly in the latter group (Trumpp et al., 2001; Smith et al., 2002; Wang et al., 2008; Wang et al., 2022b). Based on the above findings, Wang et al. therefore examined RNAseq data from a large number of normal human tissues from the Broad institute's GTEx data base and divided these into young and old cohorts (~20–40 years of age vs.~60–80 years of age). Although variable, Myc levels were on average lower in several aged tissues, most notably sigmoid colon, adipose tissue and peripheral leucocytes. As was true for murine tissues, a collection of direct Myc target gene sets from the MSigDB data base showed that the expression of positive Myc targets declined in older tissues and negative Myc targets increased.

Taken together, the studies of Wang et al. demonstrate that the abnormal findings associated with body-wide *Myc* knockout initiated shortly after birth are the consequence of a combination of the loss of this gene, dysregulation of its direct targets and the normal aging process (Wang et al., 2023). As such, these results and the previous ones obtained from *Myc* ± mice allow for the conclusion that Myc and its downstream target genes oversee the timing of many if not all of the most important aspects of normal aging (Figure 2) (Hofmann et al., 2015). It also showed that many of the findings previously associated with tissue-specific *Myc* inactivation could be recapitulated with body-wide knockout that included multiple tissue components rather than just a single one (Pshenichnaya et al., 2012; Edmunds et al., 2016; Wang et al., 2018). On the other hand, some of the most pronounced findings associated with *Myc* ± *and Myc*−/− mice such as growth retardation and smaller body size were not seen in *Myc*KO mice indicating that these phenotypes are determined prior to birth and that the substantial growth that occur post-natally is much less impacted by *Myc* loss (Davis et al., 1993; Trumpp et al., 2001; Dubois et al., 2008). On the other hand, even the low levels of Myc expression in some tissues of *Myc*KO mice may have been sufficient to rescue some of the more severe consequences that have been attributed to the total *Myc*KO and that is achievable only with embryonal targeting.

Aging and cancer are intimately linked, with advanced age being among the strongest predictors of cancer development (Pettan-Brewer and Treuting, 2011; White et al., 2014; Snyder et al., 2016). This relationship is particularly notable among individuals with monogenic disorders of DNA damage recognition and repair, known as progeroid syndromes, who despite their young chronological age, show signs of pronounced premature aging that can be reproduced in animal models (Blasco, 2005; Park and Gerson, 2005; Brosh and Bohr, 2007; Knoch et al., 2012; Opresko and Shay, 2017; Folgueras et al., 2018; Rizza et al., 2021; Rossiello et al., 2022). These disorders resemble normal aging in the sense that "aging" and the predisposition to cancer remain connected phenotypically if not chronologically. In contrast, a possible lower incidence of cancer in *Myc* ± mice described by Hofmann et al. (2015), might have been due to their overall healthier life span, such that this aspect of aging and cancer remained phenotypically linked as well. *Myc*KO mice, with a more than 3-fold lower cancer incidence despite their premature aging and increased lifespans, therefore represent a unique example in which chronological age and cancer incidence can be genetically separated and attributed to a single gene, namely *Myc*. The inextricable association between Myc and its role in driving and/or maintaining cancer, even when it is not needed to initiate tumors, likely explains the significantly lower cancer incidence of *Myc*KO mice (Karlsson et al., 2003; Wu et al., 2007; Meyer and Penn, 2008; Stine et al., 2015; Dolezal et al., 2017; Wang et al., 2023). This is underscored by the observation that the rare tumors that did arise in these animals tended to express Myc and contained at least a diploid or pseudo-diploid *Myc* DNA content, indicating that cells with incomplete *Myc* excision were selected for neoplastic transformation in aged individuals. Interestingly, an example of a human progeria syndrome that is not associated with a high incidence of cancer early in chronological life is Hutchinson-Guilford progeria (HGP), which is caused by mutations in the laminin A (*LMNA*) gene (Sarkar and Shinton, 2001; Sinha et al., 2014). While these individuals do show evidence of genomic instability and defective DNA repair, the primary *LMNA* mutations in HGP cause an abnormal nuclear architecture and loss of heterochromatin organization and its contact with the nuclear envelope (Arancio et al., 2014). We examined the catalogued RNAseq data from 2 studies that analyzed the differences between HGP and normal human fibroblast transcriptomes and found no evidence for the dysregulation of Myc or its target genes (Kohler et al., 2020; San Martin et al., 2022). Nor did the authors of these reports identify irregularities in the expression of any of the major Myc target gene categories. Thus, although aging and cancer can be dissociated in HGP as it can in *Myc*KO mice, it appears unrelated to any changes in the expression of *Myc* or its target genes.

5 The *Myc*KO mouse as a new (and improved?) model for premature aging?

Mouse models of the above-discussed progeroid syndromes have long been used as surrogates for normal human aging (Harkema et al., 2016; Koks et al., 2016; Folgueras et al., 2018; Rizza et al., 2021). However, these models are based on exceedingly rare monogenic disorders that are of questionable relevance to normal aging aside from recapitulating some of its associated phenotypes. This is because they directly impact only one or 2 of aging's so-called "Hallmarks", namely those pertaining to genomic stability and telomere maintenance (Figure 3) (Lopez-Otin et al., 2023). Thus they likely over-emphasize the roles of these 2 hallmarks while discounting the roles of others. While the *Myc*KO model is also monogenic, it differs importantly from progeroid syndrome models primarily because the loss of Myc, which is a transcription factor, is more consequential by virtue of directly impacting the vast majority of aging's hallmarks (Figure 2). As discussed above, for example, 4 of the 7 major categories of gene sets that are impacted in *Myc*KO mice and MEFs (i.e. ribosomal/mitochondrial structure and function, DNA damage response/repair and splicing directly impact 4 of the Aging Hallmarks (Figure 3) (Meshorer and Soreq, 2002; Short et al., 2005; Tchkonia et al., 2010; D'Aquila et al., 2017; Deschenes and Chabot, 2017; Gonskikh and Polacek, 2017; Bhadra et al., 2020). The categories pertaining to senescence and aging are directly related to 2 additional Aging Hallmarks and the dysregulation of the transcripts within these categories reflects the declines of Myc and its direct target genes that accompany normal aging in both mice and humans (Dean et al., 1986; Smith et al., 2002;

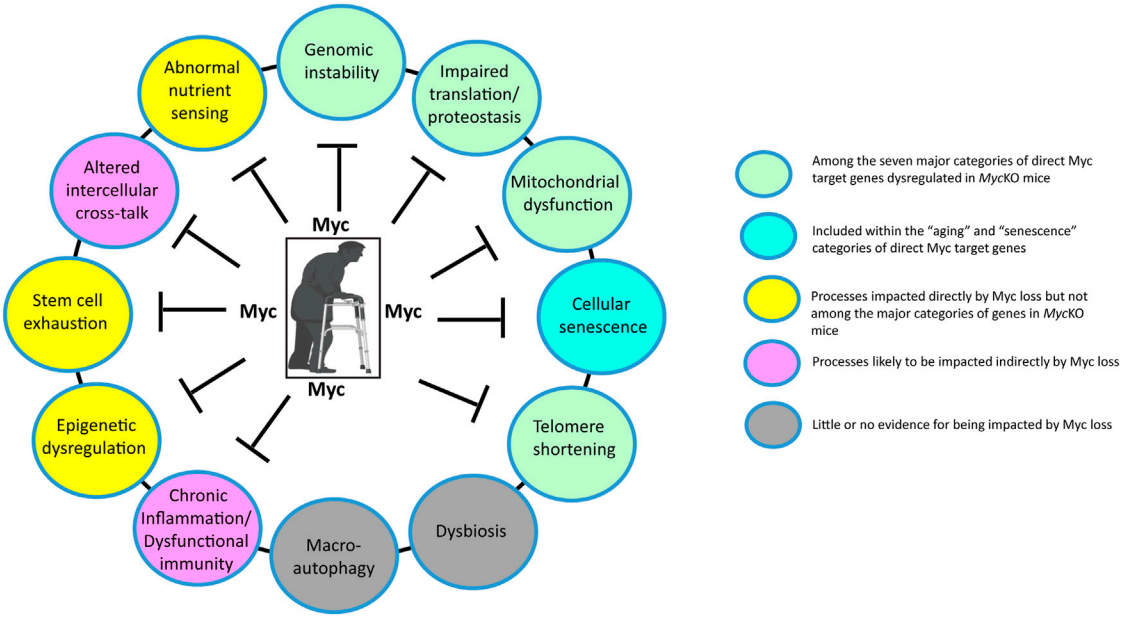

FIGURE 3
The impact of Myc on the Hallmarks of Aging. Depicted here are the major molecular, cellular and whole body changes that represent the common denominators of aging and how they are impacted by Myc (Lopez-Otin et al., 2023). Many previously described direct Myc target genes are involved in maintaining the structure and function of ribosomes, translation factors and mitochondria (Li et al., 2005; Gomez-Roman et al., 2006; Ruggero, 2009; Morrish et al., 2010; van Riggelen et al., 2010; Morrish and Hockenbery, 2014; Dolezal et al., 2017; Singh et al., 2021; Prochownik, 2022). Myc also regulates glutamine metabolism and its anaplerotic entry into the TCA cycle, while also promoting glycolysis by directly up-regulating the genes encoding most enzymes in the glycolytic pathway, particularly the rate-limiting ones (Dang, 2011; Stine et al., 2015; Prochownik, 2022; Prochownik and Wang, 2022). Both the over- and under-expression of Myc can promote genomic instability via the regulation of genes involved in DNA damage recognition and repair, telomere maintenance, the generation of genotoxic ROS and the promotion of tetraploidy (Yin et al., 1999; Vafa et al., 2002; Prochownik and Li, 2007; Wang et al., 2022b; Solvie et al., 2022; Wang et al., 2023). Myc's transcriptional control over genes involved in mRNA splicing, together with Myc-induced genomic instability, can contribute to a higher background of neo-antigen production and inflammation while altering rates of aerobic and anaerobic respiration by, for example, altering splicing choices for genes such as that encoding pyruvate kinase (Meshorer and Soreq, 2002; David et al., 2010; Koh et al., 2015; Wang et al., 2022b; Prochownik, 2022; Wang et al., 2023). Myc can also suppress senescence and maintain the stem cell niche (Dean et al., 1986; Wu et al., 2007; Dubois et al., 2008; Zhuang et al., 2008; Vilas et al., 2018) and can also impact intercellular communication by regulating the expression of cytokines, chemokines and immune checkpoints (Hayashi et al., 1998; Yi et al., 2003; Singh et al., 2006; Piddock et al., 2018). Myc's control over its target genes largely involves epigenetic re-programming, primarily at the level of histone H3/H4 acetylation and/or methylation (Knoepfler et al., 2006; Kalkat et al., 2018; Tu et al., 2018). Finally, Myc may be involved indirectly in the regulation of macroautophagy given that Miz1 appears to be involved in this process (Wolf et al., 2013). Created with BioRender.com.

Benanti et al., 2007; Wang et al., 2023). In addition, the category pertaining to oxidative stress, which is associated with high levels of ROS and ROS-mediated damage in *Myc*KO cells and tissues (Edmunds et al., 2016; Wang et al., 2022b) can accelerate the onset of other Aging Hallmarks, including genomic and mitochondrial DNA instability and impaired translation (Gonskikh and Polacek, 2017; Hahn and Zuryn, 2019; Payea et al., 2021; Renaudin, 2021; Woodward and Shirokikh, 2021; Wang et al., 2022b). While gene categories involved in nutrient sensing, stem cell maintenance and epigenetic regulation were not among the top ones identified in the tissues of *Myc*KO of mice (Wang et al., 2022b; Wang et al., 2023), Myc has long been known to play important roles in these processes; indeed Myc positively regulates transcription via its ability to recruit epigenetic modifiers to its bound sites in chromatin (Amati et al., 2001; Ba et al., 2018; Kalkat et al., 2018). Moreover, its age-related decline is likely to play some role in determining the pace at which these functions deteriorate (Figure 3) (Chappell and Dalton, 2013; Kalkat et al., 2018; Prochownik and Wang, 2022). Finally, 2 of the Aging Hallmarks pertaining to intercellular communication and chronic inflammation are likely to be indirectly regulated by Myc, which controls the expression of a number of cytokines, chemokines and immune checkpoints that contribute to these processes (Hayashi et al., 1998; Yi et al., 2003; Singh et al., 2006; Casey et al., 2018; Piddock et al., 2018). The Myc-dependent re-organization of glucose and glutamine metabolism has been shown to be essential for the expansion of activated T cells (Dang, 2010; Wang et al., 2011).

The wide-ranging consequences of *Myc* inactivation make it highly unlikely that, as is true for normal aging, its impact on any single Aging Hallmark can fully explain the premature aging profile of *Myc*KO mice (Figure 3). Just as genotoxic damage and telomere attrition drive some aspects of premature aging in progeria syndromes, so too can the interference with protein synthesis, mitochondrial DNA integrity and stem cell regulation, all of which are Myc-dependent to varying degrees (Hiona and Leeuwenburgh, 2008; Vilas et al., 2018; Shcherbakov et al., 2022). While the general categories of gene sets that are dysregulated in *Myc*KO mice are similar, the degree to which their component transcripts are enriched as well as their individual identities differ among the limited number of tissues and cell types that have been thus far surveyed, namely MEFs, liver, skeletal muscle and adipose tissues (Wang et al., 2022b; Wang et al., 2023). These seemingly

subtle distinctions may well exert significant influence over which tissues display signs of premature aging, what these signs are, when they first appear and their severity. Finally, it should be kept in mind that, unlike the generation of *Myc*−/− and *Myc* ± mice, which provide consistent levels of *Myc* knockout in each tissue (Davis et al., 1993; Trumpp et al., 2001; Hofmann et al., 2015), the degree of Myc loss in *Myc*KO mice varies both among and within tissues and individual mice, while also showing some age-related recovery (Wang et al., 2023). Indeed, even the 80%–90% levels of knockout routinely achieved may still be sufficient to allow normal or near-normal regulation of certain direct target genes with the highest affinity Myc binding sites. Considerable growth and development normally continue beyond the time of weaning that marks the point in time of *Myc* gene deletion (Wang et al., 2023). It thus remains to be determined whether even higher gene knockout efficiencies would have remained compatible with the prolonged survival noted by Wang et al. and whether other phenotypes might have emerged.

Yet to be fully explained is why mice with a 50% normal level of Myc and those with ~80->90% knockout have such different aging phenotypes (Hofmann et al., 2015; Wang et al., 2023). Here too there may be no single answer but at least 2 major and non-mutually exclusive reasons may pertain to the timing of *Myc* gene knockout and dose-related effects. The first may be related to the fact that, when *Myc* inactivation is initiated in the embryo, gene dose determines the resultant body size whereas when Myc is inactivated post-natally, no effect on overall body size is observed (Trumpp et al., 2001; Hofmann et al., 2015; Wang et al., 2023). The relationship between body size and longevity is well-known, with smaller members of the same species ranging from flies to humans tending to have longer average lifespans (Samaras et al., 2003; Khazaeli et al., 2005; Blagosklonny, 2013). Whether the increased longevity of *Myc* ± mice arises simply as a consequence of their smaller body size and is thus not a direct *Myc* gene dosage effect could be determined by engineering a precise 50% knockout of *Myc* post-natally so as to genotypically mimic *Myc* ± mice while avoiding the size disparities previously noted when haplo-insufficiency is generated in the embryo (Hofmann et al., 2015). The second reason pertaining to gene dosage involves the phenomenon of "heterozygous advantage" whereby possessing a single mutant or inactive allele can confer a selective survival advantage whereas mutational homozygosity can be deleterious or even lethal (Hedrick, 2012). Examples of such genes include those encoding the cystic fibrosis transmembrane conductance regulator, the α- and β-globins and triose phosphate isomerase (Cuthbert et al., 1995; Jones, 1997; Destro-Bisol et al., 1999; Ralser et al., 2006).

6 Questions for the future

The studies reviewed, compared and summarized here have clearly indicated that Myc plays a significant role in balancing the overall health and wellness of mice and probably of humans as well. Myc is also involved in the development and timing of tumors that appear to impact their natural life spans. However, a number of questions remain unanswered. For example, the high mortality rate associated with inactivating the *Myc* gene prior to about 1 month of age leaves open the question of its role in the considerable amount of growth and development that occurs prior to this time. The work of Wang et al. and the earlier work of Soucek et al. showed that the initial inhibition of *Myc*, even in adult mice, is accompanied by significant changes in the gastrointestinal and hematopoietic compartments (Soucek et al., 2008; Wang et al., 2023). What limits the severity of these changes and why is the latter so much more severe prior to weaning and particularly so in the embryo (Davis et al., 1993; Trumpp et al., 2001; Dubois et al., 2008)? Perhaps an even more fascinating question is what factor(s) contribute to the reversal of these initial changes and allow these highly proliferative tissues to remain so over the course of a lifetime in the face of little to no expression of Myc?

This last question remains particularly germane when considering the nearly universal requirement for Myc in maintaining the proliferation of non-transformed cells *in vitro* (Mateyak et al., 1997; Wang et al., 2022b). The critical contribution of Myc to maintaining rapid tumor cell growth and/or viability both *in vitro* and *in vivo* has also been demonstrated in many transformed cell types and a variety of neoplasms, including those arising in bone, the lymphatic system and the liver (Shachaf et al., 2004; Wu et al., 2007; Wang et al., 2008; Gabay et al., 2014; Dolezal et al., 2017). Yet, there are clear exceptions to this rule, most notably in the case of the liver where the short-term regeneration of the organ following partial hepatectomy and its longer-term repopulation by transplanted hepatocytes are entirely Myc-independent (Baena et al., 2005; Khazaeli et al., 2005; Sanders et al., 2012; Edmunds et al., 2016). In the hepatoblastoma model, where Myc is not one of the driver oncogenes, but is expressed at a high level, tumor initiation remains highly effficient in Myc's absence although survival is markedly prolonged due to slower tumor growth (Wang et al., 2016). Although no comparative studies have been done, it would appear that, both *in vitro* and *in vivo*, Myc in many cases is required to maintain cellular growth at maximum rates, particularly for tumors and even more so for those tumors in which Myc is the actual driver oncogene. Cells that proliferate relatively slowly and are not transformed may therefore be less reliant on Myc to maintain this state.

The extent to which different functions that are impacted by *Myc*'s loss contribute to premature aging (Wang et al., 2023) also remains a major question. Impaired mitochondrial function, metabolism and translation have all been described in association with aging and all of these are dependent upon Myc to balance and maintain their normal function (Li et al., 2005; van Riggelen et al., 2010; Barrientos, 2012; Graves et al., 2012; Edmunds et al., 2016; D'Aquila et al., 2017; Gonskikh and Polacek, 2017; Miwa et al., 2022; Prochownik and Wang, 2022). The degree to which these drive the premature aging phenotypes of *Myc*KO mice may well be impacted by and synergize with one another. An example of this is the relationship between what are arguably the 2 major drivers of aging, namely ROS and DNA damage, which are inextricably linked by virtue of the fact that both Myc over- and under-expression can drive ROS production which in turn can cause oxidative DNA damage (Vafa et al., 2002; Balaban et al., 2005; Brosh and Bohr, 2007; van Riggelen et al., 2010). Virtually all the major pathways that are under Myc's control are known to be associated with or to drive aging and senescence when they are deregulated (Figures 2, 3) (van Riggelen et al., 2010; Popay et al., 2021; Prochownik, 2022; Prochownik and Wang, 2022).

Two additional and related issues worth examining in future studies are whether the inactivation of Myc later in life also accelerates aging and whether normalizing Myc expression can reverse this process or at least "reset" the aging clock. These are important practical questions given the long-standing interest in inhibiting Myc as a general chemotherapeutic strategy for cancer (Llombart and Mansour, 2022). The finding that *Myc* ± mice showed both increased longevity and improved overall health initially suggested that the use of Myc inhibitors to treat various cancers might actually have additional secondary benefits and even hold promise as anti-aging therapies akin to those provided by caloric restriction or metformin (Kebbe et al., 2021). However, the more recent work of Wang et al. (2023) suggests that this might not be the case (at least in cancer) given that total Myc inhibition would be the desired therapeutic goal in treating cancer and would more likely than not accelerate rather than slow aging as one of its potential side effects. This consideration might have particular relevance for older adults who in many cases are already frail at the time they begin chemotherapy and who can ill afford to age any more rapidly (Ness and Wogksch, 2020; Shafqat et al., 2022). Perhaps even more deserving of consideration would be whether Myc inhibitors should be used in children in whom even relatively short courses of standard cancer chemotherapy can elicit features of premature aging and might collaborate with agents that deliberately lowered Myc levels (Smitherman et al., 2020; Kruseova et al., 2023). Similar concerns may be warranted over the use of such agents solely as potential lifespan and/or healthspan extenders or in the long-term treatment of non-malignant conditions associated with other Myc-dependent hyperproliferative states (Prochownik and Vogt, 2010).

Author contributions

EVP conceived the article, EVP and HW wrote the paper. All authors contributed to the article and approved the submitted version.

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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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MYC—an emerging player in mitochondrial diseases

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The mitochondrion is a major hub of cellular metabolism and involved directly or indirectly in almost all biological processes of the cell. In mitochondrial diseases, compromised respiratory electron transfer and oxidative phosphorylation (OXPHOS) lead to compensatory rewiring of metabolism with resemblance to the Warburg-like metabolic state of cancer cells. The transcription factor MYC (or c-MYC) is a major regulator of metabolic rewiring in cancer, stimulating glycolysis, nucleotide biosynthesis, and glutamine utilization, which are known or predicted to be affected also in mitochondrial diseases. Albeit not widely acknowledged thus far, several cell and mouse models of mitochondrial disease show upregulation of MYC and/or its typical transcriptional signatures. Moreover, gene expression and metabolite-level changes associated with mitochondrial integrated stress response (mt-ISR) show remarkable overlap with those of MYC overexpression. In addition to being a metabolic regulator, MYC promotes cellular proliferation and modifies the cell cycle kinetics and, especially at high expression levels, promotes replication stress and genomic instability, and sensitizes cells to apoptosis. Because cell proliferation requires energy and doubling of the cellular biomass, replicating cells should be particularly sensitive to defective OXPHOS. On the other hand, OXPHOS-defective replicating cells are predicted to be especially vulnerable to high levels of MYC as it facilitates evasion of metabolic checkpoints and accelerates cell cycle progression. Indeed, a few recent studies demonstrate cell cycle defects and nuclear DNA damage in OXPHOS deficiency. Here, we give an overview of key mitochondria-dependent metabolic pathways known to be regulated by MYC, review the current literature on MYC expression in mitochondrial diseases, and speculate how its upregulation may be triggered by OXPHOS deficiency and what implications this has for the pathogenesis of these diseases.

KEYWORDS

electron transport chain, oxidative phosphorylation, respiratory complex III, mitochondrial integrated stress response, Warburg effect, cellular senescence

1 Introduction

MYC (c-MYC, avian MYeloCytomatosis viral oncogene homolog) is a Basic-Helix-Loop-Helix-Leucine Zipper (bHLHZip)-family transcription factor that regulates a broad range of cellular functions including metabolism, growth, proliferation, differentiation, and survival (Figure 1) (Hartl, 2016). It is a major driver of cancer, being frequently overexpressed but rarely mutated. Transgenic overexpression of MYC in mice leads to increased proliferation and tumor development in multiple tissues. Conversely, inhibition or removal of MYC consistently causes growth arrest of cancer cells both in culture and *in vivo*

(Dave et al., 2017). In mammals, the MYC family also includes MYCN (N-MYC) and MYCL (L-MYC) proteins, which are highly homologous to MYC but expressed spatially differently. MYC is expressed widely but at a very low level in non-proliferating (quiescent) or postmitotic cells, whereas it is typically much more abundant in proliferating cells. Semsei et al. studied the tissue expression of *Myc* throughout mouse development and life span (Semsei et al., 1989). Its expression was highest in prenatal and newborn tissues and then decreased, reaching its lowest levels at about 6 months. The spleen and liver consistently showed the highest *Myc* expression at all ages. Interestingly, *Myc* expression did not continue to decline upon further ageing but instead progressively increased in the brain, liver, skin, and small intestine.

In contrast to the broad but low-level expression of MYC, MYCN expression mainly limits to neuronal and reproductive tissues, whereas MYCL expression restricts to the gastrointestinal tract, including pancreas and dendritic cells (Das et al., 2023). MYC and MYCN germline knockouts are embryonic lethal, whereas mice lacking MYCL do not have an overt phenotype. All three MYC family members heterodimerize with MAX (MYC-Associated factor X) to exert their transcriptional functions. MAX can homodimerize or heterodimerize with other proteins than MYC family, and these dimers compete for a common DNA sequence element called the E box, providing a complex transcriptional regulation system. After DNA binding, the MYC-MAX dimers can recruit further transcriptional cofactors and chromatin modifiers that license RNA polymerase II activation and transcription. The MYC-driven transcriptional programs are complex and almost genome wide because MYC acts as a global transcriptional amplifier that binds and increases expression at active promoters (Nie et al., 2020). Thus, MYC disproportionally upregulates highly expressed genes. Notwithstanding that its modes of conducting transcriptional activation are still somewhat obscure despite intensive research, MYC has been estimated to control the expression of at least 15% of all genes in humans, some of the most prominent categories being genes involved in cell cycle progression, metabolism, ribosomal biogenesis, and translation (Hartl, 2016).

The MYC-MAX dimerization domain function is conserved from human to zebrafish (Schreiber-Agus et al., 1993) and fruit fly (Schreiber-Agus et al., 1997). The discovery of MYC and MAX homologs in the unicellular organisms choanoflagellates revealed that MYC evolved even before the metazoa (animals) (Young et al., 2011). In *Hydra* (polyp), *Myc* mRNA is highly expressed in stem cells and other rapidly proliferating cell types, whereas in terminally differentiated cells, its expression is not detectable, suggesting an overall conserved role in cell proliferation (Hartl et al., 2010). The fruit fly (*Drosophila melanogaster*) MYC homolog (dMyc, diminutive) (Schreiber-Agus et al., 1997) has been studied quite extensively in the context of normal physiology. Loss of dMyc function impedes growth and reduces cell size, whereas dMyc overexpression boosts growth and cell size and promotes G_1/S progression but not G_2/M progression or cell division (Johnston et al., 1999). It also increases genomic rearrangements, typical of erroneous DNA double-strand break repair, and shortens lifespan. Conversely, *dMyc* haploinsufficiency decreases mutation load and extends lifespan (Greer et al., 2013), similarly to in mice (Hofmann et al., 2015), as we shall see in the next section.

2 Insight into normal MYC function and regulation from genetic models

Despite the extensive knowledge about the roles of MYC in carcinogenesis and cultured cancer cells accumulated during the past 4 decades, much less is still known about its roles in normal development and tissue homeostasis (Figure 1). Greatly aiding studies on the latter, the homologous recombination-based gene knockout (KO) technology allowed the development of several

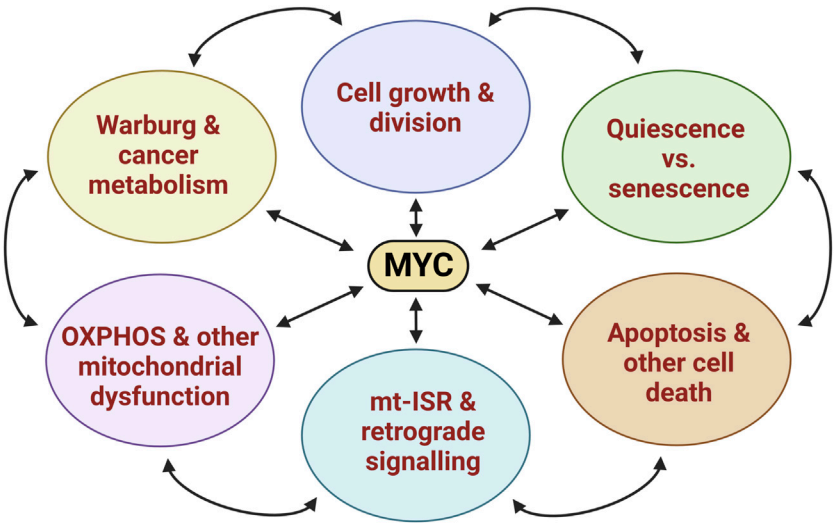

FIGURE 1
The cartoon illustrates some central physiological processes in mammalian cells and tissues to which MYC-regulated transcriptional programs contribute as driver, modulator, or adaptor. The abbreviation mt-ISR stands for mitochondrial integrated stress response. See sections 3-5 in the main text for elaboration.

TABLE 1 Models with genetic MYC manipulation and their effect on mitochondria.

Model/allele	Effect on MYC	Phenotype	Mitochondria-related changes	References
***Myc* KO in rat normal fibroblasts**	KO	Decreased growth rate, doubling time 4-5 days (WT 18-24 h)	Decreased number, size, membrane potential, OXPHOS, ATP level, CI, ATP synthase, SCs, fusion	Mateyak et al. (1997), Graves et al. (2012)
Heterozygous exon 2&3 excision (*Myc*$^{+/-}$) *in vivo*	mRNA, protein 50% of WT	Increased longevity and health span, unaltered cancer incidence	Decreased fatty acid and cholesterol synthesis	Hofmann et al. (2015)
Excised exons 2&3 with *Alb-Cre in vivo*	>90% KO in hepatocytes	No overt phenotype	Mild respiration defect in liver mitochondria. Increased FAO.	Edmunds et al. (2016)
Excised exons 2&3 with *ROSA26-CreER*	Whole body 75%-95% loss of mRNA expression	Signs of accelerated aging, fatty liver disease, decreased incidence of tumours	Increased whole-body FAO, decreased CI activity, increased ROS in MEFs	Wang et al. (2023)
(tamoxifen) *in vivo*				
Excised exons 2&3 with *ROSA26-CreER* (tamoxifen) in MEFs	>95% loss of MYC protein	Poor proliferation, G_0/G_1 arrest, flattened senescent morphology, DNA damage response	Increased mitochondrial mass, ROS production	Wang et al. (2022)

CI, complex I; SCs, supercomplexes; FAO, fatty acid oxidation; ROS, reactive oxygen species; MEFs, mouse embryonic fibroblasts.

invaluable genetic models to assess MYC function during ontogenesis (Table 1). In the early 1990s, it turned out that homozygous *Myc* knockout in mice is lethal by embryonic day 10.5 due to developmental defects in the placenta, hematopoietic system, and vasculature (Davis et al., 1993). Embryonic fibroblasts (MEFs) isolated from E9.5 KO embryos are viable but flattened and do not proliferate (Trumpp et al., 2001). A highly useful early *Myc* KO *in vitro* model was developed via homologous recombination in rat fibroblasts (Mateyak et al., 1997). These cells are highly abnormal and divide 2 to 3 times more slowly than controls, are very flattened, and show dramatically decreased or delayed cyclin D, E and A expression during the cell cycle. Their mitochondria are less abundant, much smaller in size, and show disrupted cristae patterns, indicating that MYC plays a role in the maintenance of mitochondria. Furthermore, Myc KO fibroblasts show decreased glycolysis, mitochondrial membrane potential and respiration, and low levels of oxidative phosphorylation (OXPHOS) machinery enzymes, likely explaining their 3-fold decreased ATP level.

In contrast to complete or near-complete *Myc* loss-of-function, *Myc* haploinsufficiency (*Myc*$^{+/-}$) brings no adverse effects but about 20% decrease in adult body mass and increased lifespan in mice (20% in females, 10% in males) (Hofmann et al., 2015). This increased longevity is accompanied by a lower incidence of age-associated pathologies such as osteoporosis, cardiac fibrosis, and immunosenescence. Compared to wild-type mice, the *Myc*$^{+/-}$ mice are also more active and have a higher metabolic rate. Upregulation of ribosome biogenesis is a canonical MYC-driven process, and indeed the *Myc*$^{+/-}$ mice have slightly reduced ribosomal RNA content and protein translation. The mice also have reduced serum IGF-1, increased AMPK activity, and decreased AKT and mTOR activities, altogether indicating a marked suppression of anabolic metabolism.

Despite the importance of MYC for embryonic and cancer growth, several studies have shown that MYC is largely dispensable for normal tissue growth and homeostasis in juvenile and adult stages. In adult human and mouse tissues, MYC expression is highest in rapidly proliferating compartments like the intestinal crypts and skin (Dave et al., 2017). However, conditional deletion of *Myc* in these compartments in mice does not result in a noticeable proliferation defect. For example, quite unexpectedly, MYC is dispensable for postnatal liver growth and regeneration (Edmunds et al., 2016). Nevertheless, transient induction of MYC occurs upon induced liver regeneration in various rodent liver injury models such as partial hepatectomy and hepatotoxic drug treatment (Prochownik, 2022). However, MYC's role in such experimental models of liver regeneration is less clear. In some models, the pace of liver mass restoration was unaffected by conditional loss of MYC in hepatocytes (Li et al., 2006; Sanders et al., 2012), while others claim that regeneration is compromised (Baena et al., 2005). Utilizing fumaroylacetoacetate hydrolase (FAH) mutant mice, a genetic liver disease model mimicking human type I hereditary tyrosinemia, subjected to transplantation of WT and *Myc*$^{-/-}$ hepatocytes, Edmunds et al. showed convincingly that MYC is dispensable for liver regeneration in this model (Edmunds et al., 2016).

To assess the regulation of *Myc* transcription in normal tissues and upon tumorigenesis, Dave et al. generated a series of alleles with large deletions in the *Myc* 5' regulatory sequences (Dave et al., 2017). The mice carrying the largest (>500 kb) of these (*Myc*$^{\triangle 2-540}$) show a 50%–80% decrease in basal *Myc* expression but are homozygous viable and fertile, with no overt phenotype and only a slight decrease in the number of B lymphocytes. These mice fail to induce MYC overexpression during early tumorigenesis, and cultured fibroblasts from these mice grow slowly and are unable to upregulate MYC in response to serum stimulation. In a recent paper, Wang et al. revisit the question of MYC functions in postnatal development and tissue homeostasis by generating a mouse line with tamoxifen-inducible Cre-mediated loss of *Myc* (Wang et al., 2023). Their system allowed near-complete (75%-95%) elimination of *Myc* expression with a 5-day tamoxifen regimen started at 4 weeks of age. Somewhat surprisingly, the resulting mice with extreme but not complete loss of MYC display some premature aging features, like alopecia and graying of the hair as early as 3-4 months of age, increased adiposity, and hepatic steatosis. However, the mice live significantly (median 4.6 months) longer than wild-type controls, probably due to a 4-5-fold lower cancer incidence. Transcriptional profiling of the liver, white adipose tissue and skeletal muscle showed changes related to mitochondrial and ribosomal function, cellular

senescence, DNA damage recognition and repair, and mRNA splicing upon loss of MYC (Wang et al., 2023). The results from both hypomorphic mouse models indicate that MYC is an important metabolic regulator but not critical for the normal physiology of adult mice.

3 Regulation of normal mitochondrial homeostasis and metabolism by MYC

As the main cellular site of energy metabolism and biosynthesis, mitochondria are highly dynamic and respond to the selection of available nutrients and requirements for biomass production to ensure sufficient resources for cell proliferation, tissue growth, homeostasis, and mechanical work. MYC is a major driver of cellular proliferation and growth, so it is obvious that the transcriptional programs driven by it must be tightly connected to mitochondrial homeostasis, energy metabolism and biosynthesis (O'Connell et al., 2003; Morrish and Hockenbery, 2014; Goetzman and Prochownik, 2018). To understand why and how MYC might be needed in response to mitochondrial dysfunction, such as in mitochondrial diseases, we will take a brief look at what is known about how MYC regulates some key mitochondria-related functions. It is worth bearing in mind, however, that most of these findings were made in cancer studies, or at least using cancer cell lines as a model.

3.1 Mitochondrial dynamics and biogenesis

Mitochondrial biogenesis generates new mitochondria to maintain or increase mitochondrial number and mass in a cell (Morrish and Hockenbery, 2014). It is a massive undertaking requiring the transcription, translation, mitochondrial import, and assembly of over 1,000 nuclear genome-encoded proteins and 13 proteins encoded by the mitochondrial genome (mtDNA). Numerous studies employing MYC-overexpressing or MYC-deleted cell systems have shown that nearly half of the nuclear genes encoding mitochondrial proteins can be transcriptionally upregulated by MYC (Li et al., 2005; Kim et al., 2008; Graves et al., 2012; Morrish and Hockenbery, 2014). In addition to transcript level analyses, Li. et al. demonstrated, utilizing a human lymphoblastoid cell line carrying tetracycline-repressible ectopic *MYC* and estradiol-inducible endogenous *MYC,* that MYC overexpression increases mitochondrial mass and lack of MYC has the opposite effect (Li et al., 2005).

Mitochondria cannot be built from scratch, but "new" mitochondria are always generated from existing ones by means of fission and fusion of the organelle coupled to mtDNA replication, transcription and translation. A generally accepted purpose of fusion is to mitigate stress and maximize function by mixing the contents of damaged and healthy mitochondria. In contrast, fission is a means to create new mitochondria and a quality control mechanism to remove damaged mitochondria via mitophagy (Goetzman and Prochownik, 2018). Expression of many mitochondrial proteins controlling fission and fusion, such as the mitofusins Mfn1 and Mfn2, is low in $Myc^{-/-}$ fibroblasts compared to the cells rescued by *Myc* transfection (Graves et al., 2012). MYC-deficient cells also have twofold lower rates of mitochondrial fusion compared to MYC-expressing cells, suggesting that the normally proliferating MYC-expressing cells were under pressure to ensure mitochondrial quality for sufficient energy and biosynthetic precursor production (Wang et al., 2022).

One of the first identified transcriptional targets of MYC encoding a mitochondrial protein was SURF-1, an assembly factor for respiratory complex IV (cytochrome c oxidase, CIV) (Vernon and Gaston, 2000). Direct MYC target genes also involve several other respiratory complex assembly factors, the TIM/TOM (Translocase of the Inner/Outer Membrane) proteins, and practically all mitochondrial ribosomal proteins (Morrish and Hockenbery, 2014). Most of the major transcription factors that drive mitochondrial biogenesis in response to metabolic cues are transcriptional targets of MYC, at least in some systems (Seitz et al., 2011). The best-characterized ones are TFAM (Transcription factor a, mitochondrial, a key regulator of mtDNA transcription and replication), PPARGC1A (Peroxisome proliferator-activated receptor γ coactivator 1-α, also known as PGC-1α), ESRRA/B/G (Estrogen-related receptors α/β/γ, also known as ERRα/β/γ), PPARA/D/G (Peroxisome proliferator-activated receptors α/δ/γ), NRF1 (Nuclear respiratory factor 1), GABPB1 (also called Nuclear respiratory factor 2), and PPRC1 (PPARG-related coactivator 1). Because of several reviews on the roles of these TFs in mitochondrial biogenesis (Dinkova-Kostova and Abramov, 2015; Gureev et al., 2019; Popov, 2020; Vernier and Giguère, 2021), it suffices to say here that their interplay with MYC in the context of OXPHOS dysfunction is an understudied but exciting topic.

3.2 Energy metabolism and biosynthesis

Cellular metabolism can be divided into catabolic (degradative) and anabolic (biosynthetic) branches that intertwine widely. Catabolism is the collective term for breaking complex molecules into simpler ones with concomitant release of energy from chemical bonds to drive cellular functions and to produce biosynthetic precursors. Uncovering the central role of MYC as a regulator of both catabolism and anabolism started with studies on cancer cells in the mid-1980s. To begin with, MYC plays a crucial role in the regulation of glycolysis by upregulating glucose transporters and nearly all the glycolytic enzymes and by regulating pyruvate kinase splicing (Haikala et al., 2017; Goetzman and Prochownik, 2018; Dong et al., 2020). Indeed, the basal rate of glycolysis in the aforementioned $Myc^{-/-}$ fibroblasts is about 50% of that of parental wild-type cells (Graves et al., 2012). An example of a canonical anabolic process driven by MYC is the upregulation of ribosome biogenesis and protein synthesis (van Riggelen et al., 2010).

Proliferating cells require a continuous supply of amino acids, nucleotides, and lipids as building blocks of cell mass. Apart from tumor cells this goes for embryonic and adult stem cells as well as differentiated cells, such as hepatocytes, that can enter the cell cycle for tissue regeneration. All these cell types share a reliance on glucose and glutamine to support their anabolism (Haikala et al., 2017; Goetzman and Prochownik, 2018). Glutamine is the most abundant amino acid in human blood, and proliferating cells use it

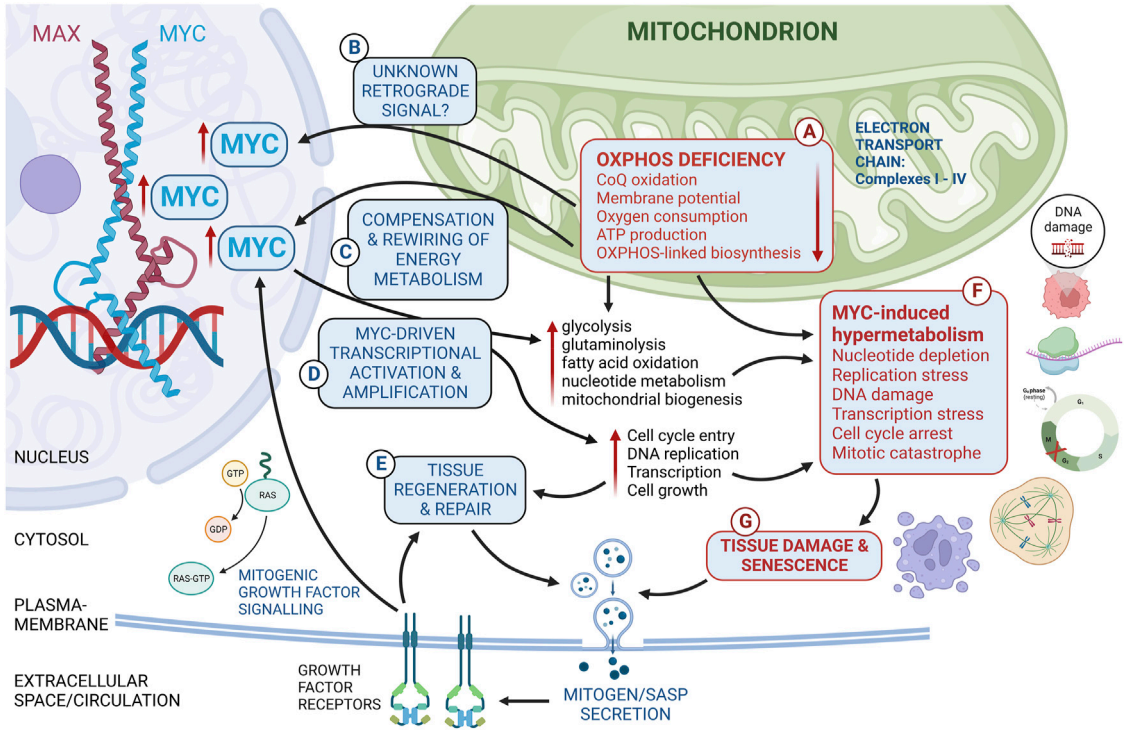

FIGURE 2
Cartoon showing connections between OXPHOS deficiency, MYC, cellular metabolism, and cell fates. Mitochondrial diseases are genetic diseases, in which a mutation in either the nuclear or mitochondrial genome compromises directly or indirectly mitochondrial ATP production by the oxidative phosphorylation (OXPHOS) machinery **(A)**. OXPHOS deficiency triggers a retrograde (mitochondrion-to-nucleus) signal **(B, C)** to adjust gene expression to restore homeostasis (anterograde signals, **(C, D)**) and to allow cell survival and tissue regeneration and repair **(E)**. The nature of the retrograde signal **(B)** triggering MYC expression in OXPHOS deficiency remains uncertain. Beneficial or neutral compensatory changes induced by OXPHOS deficiency are shown in text boxes with blue font **(C–E)**. Known or hypothetical adverse consequences of MYC induction concomitantly with OXPHOS deficiency are listed in text boxes with red font **(F, G)**. These include hypermetabolism (F, increased demand for energy and biosynthesis), potentially leading to nucleotide depletion, cell cycle arrest, genomic instability, and senescence **(G)** with senescence-associated secretory phenotype (SASP).

for energy production and as a carbon and nitrogen source for biosynthesis. MYC promotes glutamine metabolism directly and indirectly via diverse mechanisms, for example, through upregulation of glutamine synthesis, uptake, transport, and consumption. A recent review thoroughly covers the genetic and biochemical mechanisms of regulation of glutamine uptake and metabolism by MYC (Tambay et al., 2021). As the first step of glutaminolysis, the hydrolase enzymes glutaminase 1 and 2 (GLS-1, GLS-2) convert glutamine to glutamate. MYC can upregulate GLS-1 via miR-23a/b-dependent posttranscriptional mechanisms (Gao et al., 2009) and also more directly via binding to the *GLS-1* transcription start site near the 5′UTR (Haikala et al., 2018). In the cytosol, glutamate serves as a substrate for the synthesis of several amino acids (serine, alanine, aspartate, and ornithine), whereas it mainly undergoes conversion to α-ketoglutarate in the mitochondria to fuel the Krebs cycle (Haikala et al., 2017).

In contrast to proliferating cells, many differentiated permanently postmitotic tissues prefer to utilize fatty acid oxidation (FAO) over glycolysis for energy production. Loss or inhibition of MYC leads to exaggerated reliance on FAO as an energy source in several cell and tissue models (Graves et al., 2012; Zirath et al., 2013; Edmunds et al., 2014; 2016). Finally, one canonical target of MYC is nucleotide metabolism, and MYC directly binds the regulatory regions of many genes encoding enzymes involved in purine and pyrimidine nucleotide biosynthesis (Liu et al., 2008). Moreover, MYC upregulates pathways such as *de novo* serine synthesis and one-carbon metabolism that support nucleotide biosynthesis (Sun et al., 2015).

3.3 The Warburg effect

In mitochondrial diseases, cells unavoidably rewire their metabolism to compensate for the compromised OXPHOS (Figure 2). In cancer cells, glycolysis coupled to lactate production is often favored over OXPHOS even in the presence of sufficient oxygen, an effect first observed by Otto Warburg in the 1920s. Such “fermenting glycolysis” was initially suggested by Warburg to be caused by defective mitochondria (Warburg, O. et al., 1924; Warburg, 1956). His contemporary Herbert Crabtree suggested an opposite explanation: that cancer cells downregulate OXPHOS in response to increased glycolysis (Crabtree, 1929). The Warburg effect is now known to occur in both rapidly proliferating normal cells and cancer cells without any impairment in OXPHOS (Senyilmaz and Teleman, 2015; Goetzman and Prochownik, 2018; Vaupel and Multhoff, 2021). Instead, it is essentially a metabolic reprogramming resulting from characteristic normal and/or malignant proliferation-associated transcriptional and signaling alterations, such as hypoxia-inducible factor-1 (HIF-1) stabilization, oncogene activation (MYC, Ras), loss

TABLE 2 Current evidence for MYC upregulation in models of mitochondrial disease or dysfunction.

Model/modified gene	Tissue/cell line	OXPHOS activity	MYC expression/targets	References
Chemical depletion of mtDNA (ρ^0 cells)	T143B and ARPE19 cell lines, KSS fibroblasts	n.d. but presumed total loss	*MYC* mRNA up ~1.5-fold (qPCR); targets n.d	Miceli and Jazwinski (2005)
***FRDA, KSS, LHON, MERRF, NARP* mutations**	primary patient fibroblasts	n.d	*MYC* mRNA up 1.5-fold (qPCR); targets n.d	Cortopassi et al. (2006)
OXPHOS inhibitor treatment	U2OS cells	n.d. but presumed loss	MYC protein up (WB)	Gleyzer and Scarpulla (2016)
***Twnk, Tfam, Polrmt, Lrpprc* and *Mterf4* cardiospecific KO mice (*Ckmm-Cre*)**	heart	n.d	*Myc* mRNA up 4-12-fold (transcriptomics); targets n.d	Kühl et al. (2017)
***Uqcrfs1* conditional KO (*Vav-iCre*)**	fetal hematopoietic stem cells	Decreased by ~80% in fetal liver Lin^- cells	MYC targets the most highly upregulated gene signature	Ansó et al. (2017)
Tamoxifen-induced Cre-mediated loss of *Uqcrq*	primary lung endothelial cells	n.d	MYC targets the most highly upregulated gene set	Diebold et al. (2019)
***Bcs1l*$^{p.S78G}$ and *Bcs1l*$^{p.S78G}$;*mt-Cyb*$^{p.D254N}$ mice**	liver, kidney, heart, skeletal muscle (P21-150)	CIII activity 10%-50% of WT	MYC mRNA and protein up 2-40-fold (transcriptomics, qPCR, WB); cell cycle, nucleotide and one carbon metabolism up	Purhonen et al., 2017 (2023)
***Clpp* and *Fgf21* DKO mice**	heart	Mild decrease	*Myc* mRNA up 2.5-fold (transcriptomics)	Croon et al. (2022)
Mitochondrial ribosomal protein S5 *(Mrps5)* cardiospecific KO	heart	n.d	~30% increase in MYC protein	Gao et al. (2023)

of function of tumour suppressors (P53, PTEN), activated (PI3K-Akt-mTORC1, RAS-RAF-MEK-ERK, Jak-Stat3), or deactivated (LKB1-AMPK) energy signaling pathways (Senyilmaz and Teleman, 2015; Goetzman and Prochownik, 2018; Vaupel and Multhoff, 2021). At the molecular level, some of the key features of the Warburg effect are accelerated glycolytic flux, diversion of glycolytic intermediates to the biosynthesis of nucleotides, non-essential amino acids, lipids, and hexosamines, inhibition of pyruvate entry into mitochondria, increased production of lactate from pyruvate, secretion of lactate through lactate–proton symporters, and increased carbonic anhydrase activity to hydrate CO_2 from oxidative metabolism into H^+ and bicarbonate. Excessive lactate-proton export results in extracellular acidification, which may drive further malignant cancer progression (Cassim et al., 2020; Vaupel and Multhoff, 2021). In the case of mitochondrial disease, decreased tricarboxylic (Kreb's) cycle activity may decrease pyruvate oxidation, force its reduction to lactate, and result in the relatively common manifestation lactic acidemia (Hikmat et al., 2021). Even though mutations compromising OXPHOS do not underlie the Warburg effect in cancer cells, mutations causing mitochondrial diseases lead to metabolic changes that are overlapping with it. Whether this might have implications for the pathogenesis of these diseases, particularly those affecting proliferating or regeneration-competent tissues, remains to be studied. In the next section, we shall turn to what is currently known about MYC in experimental OXPHOS dysfunction and mitochondrial disease models.

4 Upregulation of MYC in OXPHOS dysfunction

Even though not widely acknowledged, mRNA expression data from several cell and mouse models of mitochondrial dysfunction show upregulation of MYC (summarized in Table 2). In the earliest of these, Miceli and Jawlinski studied the response in nuclear gene expression to loss of mtDNA (ρ0 or "rho zero" cells) in two human cell lines (T143B osteosarcoma and ARPE19 retinal pigment epithelium) and fibroblasts from an individual with Kearns-Sayre syndrome (KSS, mitochondrial myopathy due to inherited mtDNA deletions). *MYC* was among the genes commonly induced due to the loss of mtDNA in all 3 cell models. RNA interference experiments in the ARPE19 cells suggested that the induction of *MYC* was related to the upregulation of glycolysis (Miceli and Jazwinski, 2005). Cortopassi et al. performed a microarray profiling of 22 different cell lines (including lymphoblasts, fibroblasts, myoblasts, muscle, and osteosarcoma cybrids) representing five mitochondrial diseases: Leber's Hereditary Optic Neuropathy (LHON), Friedreich's ataxia (FRDA), Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), KSS, and Neurogenic ataxia and retinitis pigmentosa (NARP). The authors reported a median 1.5-fold upregulation of *MYC* in 5/22 groups of cells. They also reported upregulation of cell cycle progression- and ribosomal biogenesis-associated genes and speculated that MYC may be driving these in mitochondrial dysfunction caused by ischemia or mutations (Cortopassi et al., 2006). Ten years on and with major technical advances in high-throughput technologies ("omics"), Kühl et al. moved to *in vivo* level. They performed an impressive transcriptomics and proteomics study of the heart tissue from five conditional knockout mouse strains that develop OXPHOS deficiency and cardiomyopathy due to impaired mtDNA gene expression (*Twnk*, *Tfam*, *Polrmt*, *Lrpprc* and *Mterf4* cKO alleles). The survival of these mice varied from 6 to 21 weeks. In end-stage hearts of all five cardiospecific knockouts, *c-Myc* was induced 4-12-fold. The authors also reported the upregulation of several known target gene sets of MYC, the most highly upregulated of them being related to the one-carbon metabolism. Kühl et al. did not study the role of MYC beyond mRNA expression but devoted a chapter to discussing the implications

of this finding. In brief, they propose that MYC contributes to the remodeling of metabolism under severe mitochondrial dysfunction (Kühl et al., 2017).

Chandel's group studied the role of OXPHOS in hematopoietic stem cell maintenance in mice by deleting the respiratory complex III (CIII) subunit UQCRFS1 from mid-gestation (Ansó et al., 2017). Transcriptome analysis of isolated fetal liver hematopoietic stem cells revealed that MYC targets were the most significantly upregulated pathway upon the loss of CIII function. However, the authors did not discuss the implications of this finding. MYC came up also in another study by this group, investigating the role of endothelial cell energy metabolism in angiogenesis. To this end, they generated a mouse model with endothelial cell-specific tamoxifen-inducible loss of the CIII subunit UQCRQ. Induction of Cre expression immediately after birth resulted in lethality between 2 and 4 weeks of age due to impaired angiogenesis. Transcriptomics from CIII-deficient primary lung endothelial cells isolated from these mice revealed significant upregulation in pathways associated with anabolism and cellular proliferation, including MYC target genes (Diebold et al., 2019). Additional circumstantial evidence for the involvement of MYC in mitochondrial disease pathogenesis came from a study assessing the regulation of the mitochondrial integrated stress response by the mitokine FGF21 in mitochondrial cardiomyopathy (Croon et al., 2022). In mitochondrial matrix protease *Clpp* KO heart, showing mild OXPHOS deficiency, *Myc* mRNA was ~2.5-fold upregulated and this was blunted by FGF21 loss.

We first noted several years ago that in transcriptomics data from 6-week-old CIII-deficient *Bcs1l*$^{p.S78G}$ knock-in mice, *Myc* expression was highly upregulated (12-fold) and that it was the top predicted transcriptional regulator explaining the overall gene expression changes induced by CIII deficiency in the liver (Purhonen et al., 2017). These mice carry the GRACILE syndrome patient mutation, causing one of the most severe known OXPHOS deficiency phenotypes with fetal onset (Fellman et al., 1998; Hikmat et al., 2021). In five months-old *Bcs1l*$^{p.S78G}$ mice, the *Myc* induction wanes, but the mRNA is still significantly upregulated in the liver (5.4-fold), kidney (4.1-fold), and heart (2.3-fold), the three tissues studied. Notably, the heart is presymptomatic at this age, dilating cardiomyopathy developing by postnatal day 200 (P200) (Rajendran et al., 2019). We recently (Purhonen et al., 2023) extended the studies on the MYC upregulation into juvenile (postanal day 21–35) *Bcs1l*$^{p.S78G}$ mice and found a staggering level of MYC induction, 30-40-fold, in the P30 liver (symptomatic) at both mRNA and protein level. In the P30 kidney (symptomatic), *Myc* mRNA was upregulated about 10-fold. In the skeletal muscle, which has very low CIII activity (~25% of wild-type) but no clear myopathy (Purhonen et al., 2020), *Myc* mRNA was upregulated more modestly, about 2-fold (Purhonen et al., 2023). Interestingly, MYC was induced presymptomatically, immediately after weaning (P18-P25) in the liver and possibly also in the other tissues. MYC is known to be transiently induced in liver regeneration, but the level of induction in the CIII-deficient mice was at least an order of magnitude higher than in typical liver injury models, such as 2/3 hepatectomy and bile duct ligation (Sekine et al., 2007; Zhang et al., 2019), despite that their liver disease is relatively mild when the MYC upregulation first appears. This suggests a mechanism for the MYC induction by CIII deficiency that is not solely related to the tissue regeneration need.

5 Is MYC a component of the mitochondrial retrograde signal and/or the integrated stress response (mt-ISR)?

MYC is seldom mutated in cancer but rather upregulated via transcriptional induction due to chromosomal translocation or gene copy number amplification. Alternatively, MYC can be activated by excessive growth factor signaling due hyperactivating mutations in or amplification of growth factor receptors (such as the epidermal growth factor receptor, EGFR) or signaling proteins such as Ras (Hartl, 2016). Again, much less is known about the induction mechanism of MYC in non-cancerous diseases but, presumably, the above-mentioned mitogenic signaling mechanisms, hijacked by cancer cells, are at play. How and why would mitochondrial dysfunction lead to MYC induction? In the liver of the juvenile *Bcs1l*$^{p.S78G}$ mice, we see 50- to 600-fold upregulation of the EGFR ligand amphiregulin (AREG) (Purhonen et al., 2023), a recently identified mitokine (Hino et al., 2022). A simple explanation would be that upregulation of EGFR ligands due to tissue growth and regeneration pressure drives Ras-MAPK signaling and the MYC induction in the CIII-deficient tissues (Figure 2).

Aside from the canonical growth factor signaling paradigm, there are some other possibilities for how mitochondria could communicate more directly with the nucleus to regulate MYC expression (Figure 2). Retrograde signaling refers to the process where a signal travels backwards from an organelle to the nucleus (Bilen et al., 2022). For example, signals from the mitochondria are relayed to the nucleus via small molecules and/or proteins and/or protein modifications to regulate nuclear gene expression. The mitochondrion-nucleus retrograde signaling has been thoroughly characterized in the yeast (*Saccharomyces cerevisae*). In this organism, Retrograde regulation protein 1 (Rtg1), the key conveyor of mitochondrial stress signals to the nucleus, has been suggested to be a MYC homolog (Jazwinski and Kriete, 2012). The yeast Rtg1 and Rtg3 proteins are bHLH/zip transcription factors that heterodimerize analogously to MYC and MAX (Jia et al., 1997b). Although their overall sequence similarity is low, structural modeling revealed conservation of key bHLH/zip residues between Rtg1 and MYC and Rtg3 and MAX (Srinivasan et al., 2010). In support of involvement of MYC in mammalian mitochondrial retrograde signaling, Gleyzer and Scrapulla found concurrent upregulation of the mitochondrial biogenesis-related transcription factor PGC-1-related coactivator (PRC) and MYC upon loss of OXPHOS, mainly induced by the protonophore CCCP in a human osteocarcinoma cell line U2OS (Gleyzer and Scarpulla, 2011; Gleyzer and Scarpulla, 2013; Gleyzer and Scarpulla, 2016). They also found that MYC induction is largely required for PRC stabilization and accumulation in response to mitochondrial stress (Gleyzer and Scarpulla, 2016). Furthermore, they showed that AKT phosphorylation-dependent steps are involved. Other key upstream players in MYC upregulation and universality of the findings of Gleyzer and Scrapulla in other cell lines and *in vivo* remain, however, yet to be clarified.

Various mitochondrial insults trigger a common transcriptional program called mitochondrial integrated stress response (mt-ISR) (Costa-Mattioli and Walter, 2020; Mick et al., 2020; Bilen et al., 2022). It has a resemblance to the more general integrated stress response (ISR), which is launched, for example, by amino acid starvation or ER stress. A convergent event in all integrated stress responses is the phosphorylation of Ser51 of the alpha subunit of

eukaryotic initiation factor 2 (eIF2α), blocking 5′cap-dependent translation initiation. This results in suppressed global translation but increased translation of selected mRNAs that contain inhibitory upstream open reading frames, leading to translation of ISR mediators such as ATF4, ATF5, and CHOP (DDIT3). Noteworthily, *MYC* mRNA contains an internal ribosomal entry segment and an alternative in-frame start codon, enabling MYC translation under cellular stressors that suppress global protein synthesis (Subkhankulova et al., 2001). Four distinct serine/threonine kinases can perform the eIF2α phosphorylation: PKR (interferon-induced double-stranded RNA-dependent eIF2α kinase, induced by viral infection), PERK (PKR-like endoplasmic reticulum resident kinase, induced by endoplasmic reticulum stress), GCN2 (general control nonderepressible 2, induced by amino acid starvation), and HRI (heme-regulated inhibitor kinase, induced by heme deficiency but also by various other stressors) (Costa-Mattioli and Walter, 2020; Bilen et al., 2022). Of these, HRI undergoes activation in mitochondrial stress by OMA1-cleaved and retrotranslocated mitochondria-resident DELE1 protein (Fessler et al., 2020; Guo et al., 2020; Sekine et al., 2023). In addition, mitochondrial complex I (CI) inhibition can activate GCN2 due to asparagine depletion (Mick et al., 2020).

Mt-ISR drives an adaptive rewiring of metabolism to restore homeostasis. The most notable cellular processes that mt-ISR drives are *de novo* serine biosynthesis, one-carbon metabolism, and the transsulfuration pathway (Bao et al., 2016; Khan et al., 2017; Quirós et al., 2017). These processes are important, for example, for the synthesis of nucleotides, and phospholipids, and for the maintenance of redox balance. Not surprisingly, cancer cells exploit similar metabolic rewiring for survival and growth (Yang and Vousden, 2016). Most transcriptional programs driven by mt-ISR have been attributed to the transcription factor ATF4 (Quirós et al., 2017). However, very similar changes occur upon upregulation of MYC (Liu et al., 2008; Sun et al., 2015), which frequently accompanies mt-ISR (Table 2). Moreover, these two transcription factors share a high proportion of overlapping DNA-binding sites (Tameire et al., 2019). Observations from cancer cells that MYC-driven excessive anabolic metabolism can trigger ISR brings additional complexity into decoding ATF4- and MYC-driven transcriptional responses (Tameire et al., 2019).

Our data from the *Bcs1l*$^{p.S78G}$ mice with progressive loss of CIII function showed that, similar to cancer cell lines, MYC upregulation precedes eIF2-α phosphorylation (Purhonen et al., 2023). This observation suggests that MYC is not necessarily part of mt-ISR but potentially a component of a separate retrograde signaling and potential augmenter or even a trigger of ISR in these mice. In this study, we utilized transgenic *Ciona intestinalis* alternative oxidase (AOX) to interrogate the OXPHOS-dependent mechanisms. AOX is a mitochondrial enzyme from lower animals like yeasts, sea squirt (*C. intestinalis*) and plants and can transfer electrons directly from the coenzyme Q (CoQ) pool to oxygen when the CIII-CIV segment of the respiratory electron transfer is defective (Banerjee et al., 2021; Jacobs and Ballard, 2022). Surprisingly, we found that AOX robustly blunted the MYC-induction and mt-ISR—a highly paradoxical finding given that AOX did not improve any parameters directly linked to OXPHOS system such as ATP production and levels, mitochondrial membrane potential, or NADH/NAD$^+$ ratio (Purhonen et al., 2023). Improved growth, prevention of liver and kidney pathology, and tripling of survival accompanied the suppressed MYC induction and mt-ISR in the AOX-expressing CIII-deficient mice. AOX also suppressed the MYC induction in the skeletal muscle, indicating that the mechanism is general also for postmitotic tissues.

6 Does MYC drive excessive anabolism and aberrant cell proliferation in mitochondrial diseases?

What are the consequences of MYC upregulation in tissues affected by mitochondrial disease? Some adaptive responses driven by MYC, such as mitochondrial biogenesis and enhanced glutaminolysis and glycolysis likely help cells to cope with defective OXPHOS. Nevertheless, in some sense, MYC upregulation is a paradoxical response to compromised energy metabolism due to the many energy-consuming processes it promotes. Elucidation of MYC's beneficial adaptive and potentially pathological roles in mitochondrial diseases requires *in vivo* modulation of MYC. Very limited experiments to that end, however, exist. The most robust evidence for potential pathological role of MYC induction in mitochondrial disease comes from our studies on the CIII-deficient *Bcs1l*$^{p.S78G}$ mice, as reviewed above (Purhonen et al., 2023). A common feature of excessive MYC levels in both cancerous and normal cells is facilitated evasion of metabolic checkpoints, replication stress, DNA damage, and genomic instability (Felsher et al., 2000; Rohban and Campaner, 2015). Indeed, we showed that affected parenchymal cells in tissues that renew via cell cycle entry of differentiated cells, such as hepatocytes in the liver, induce a DNA damage response upon loss of CIII activity (Purhonen et al., 2023). As expected from the degree of MYC upregulation, severe nucleotide depletion did not suppress cell cycle entry or progression to the S-phase in the liver or kidney of *Bcs1l*$^{p.S78G}$ mice, suggesting MYC-driven illicit cell cycle progression. Similar to serum-starved fibroblasts forced to proliferate by MYC overexpression (Felsher et al., 2000), proliferating CIII-deficient hepatocytes of *Bcs1l*$^{p.S78G}$ mice showed cell cycle arrest at G2 phase and almost never reached mitosis. Those CIII-deficient hepatocytes that entered mitosis showed frequent aberrations such as multipolar mitotic spindles and anaphases, anaphase bridges, and lagging or dispersed chromatin, in other words cytological hallmarks of genomic instability. Suppression of MYC function with the dominant negative mutant fragment of MYC called Omomyc was sufficient to alleviate the DNA damage in CIII-deficient hepatocytes.

Inevitably, the replication issues in *Bcs1l*$^{p.S78G}$ mice led to widespread cellular senescence (Purhonen et al., 2023). Cellular senescence is often accompanied by senescence-associated secretory phenotype (SASP), which involves secretion of a variety cytokines, chemokines, growth factors, and proteases by the senescent cells (Xu et al., 2019). One possible driver or amplifier of the MYC upregulation in the *Bcs1l*$^{p.S78G}$ mice are the SASP-related EGFR ligands, the excessive secretion of which could lead to a circle of mitogenic stimulation (Figure 2). Mitochondrial stress as such can also induce the expression of the EGFR ligand amphiregulin (AREG) (Hino et al., 2022). Areg is one of most upregulated genes in the liver and kidney of the *Bcs1l*$^{p.S78G}$ mice (Rajendran et al., 2019; Purhonen et al., 2023).

Presumably, the blockade of mt-ISR and MYC induction by AOX prevented the CIII deficiency-induced changes in the

expression of proliferation-associated genes, plunged proliferation markers to below WT level, and abolished DNA damage and cellular senescence. These findings indicate that limiting cell cycle entry can prevent tissue pathology caused by CIII deficiency (Purhonen et al., 2023). Intriguingly, we found that also low carbohydrate-high fat ketogenic diet, which we previously showed to have an unexpected beneficial effect on the liver disease in the *Bcs1l*$^{p.S78G}$ mice (Purhonen et al., 2017), dampened the MYC induction, limited the DNA damage, and moderated excessive hepatocyte proliferation (Purhonen et al., 2023). Further studies are, however, needed to elucidate therapeutic effects of MYC inhibition in this model. In the whole organism, the above-described pathological issues of cellular proliferation result in a premature aging (progeroid) disease similar to laminopathic and DNA repair-deficient juvenile progeroid syndromes. Interestingly, several other progeroid phenotypes due to mutations in genes encoding mitochondrial proteins have been reported relatively recently (Ehmke et al., 2017; Writzl et al., 2017; Elouej et al., 2020; Garg et al., 2022).

The *Bcs1l*$^{p.S78G}$ mice are a very severe model of OXPHOS deficiency. It is an interesting question whether MYC upregulation has pathological and perhaps targetable roles in less severe mitochondrial diseases and their experimental models. Proliferating, proliferation-capable, and terminally post-mitotic cells are necessarily very differently affected by MYC upregulation. In post-mitotic cells, such as in skeletal muscle of mitochondrial myopathy patients, MYC upregulation potentially drives adaptive metabolic rewiring and cellular hypertrophy but would not lead to replication issues and DNA damage. One potential consequence of MYC upregulation is a hypermetabolic state. Intriguingly, a recent study identified hypermetabolism and increased energy expenditure as common features of patients with mitochondrial diseases (Sturm et al., 2023). The contribution of potential MYC upregulation to this hypermetabolic state of OXPHOS dysfunction remains, however, speculative at present. MYC is a notorious oncogene, yet mitochondrial disease patients do not show increased cancer risk (Lund et al., 2015). Further studies on the *Bcs1l*$^{p.S78G}$ mice, displaying in some tissues by far the most staggering "cancer-like" MYC upregulation of all mitochondrial disease models, could shed light on the role OXPHOS dysfunction in carcinogenesis if crossed with mice carrying mutant alleles of major tumor suppressor pathways such as p53 or APC (Adenomatosis Polyposis Coli tumor suppressor).

7 Concluding words

Here, we argue that MYC could be a functionally important player in mitochondrial diseases, hoping to stimulate researchers of mitochondrial medicine and physiology to study MYC in their models. Given the overarching roles of MYC as a regulator of energy metabolism in cancer and in normal tissue homeostasis, this proposition is not that surprising and has perhaps been hiding in plain sight during the almost 3 decades that mitochondrial diseases have been studied with modern molecular biology tools and methods. What is less clear at this point is the role of MYC in cell proliferation with respect to the widely varying manifestations of mitochondrial diseases in continuously proliferating (e.g., bone marrow) *versus* regeneration-capable (e.g., liver) *versus* permanently postmitotic (e.g., skeletal muscle, brain) tissues that are also metabolically quite different. Our recent findings in the *Bcs1l*$^{p.S78G}$ knock-in mouse model of severe CIII deficiency provide evidence that MYC forces illicit cell cycle entry against depletion of energy and biosynthetic precursors like nucleotides—with catastrophic consequences leading to cellular senescence and progeroid disease. However, the clinical phenotypes of CIII deficiencies caused by other *BCS1L* mutations or by mutations in other genes differ markedly from each other, and there is currently no knowledge about similar mechanisms in these phenotypes. Some key questions that remain to be studied are 1) What signals induce MYC in OXPHOS deficiency, 2) How does MYC contribute to the metabolic shift upon OXPHOS deficiency, and 3) Are the potentially harmful effects of MYC induction a general phenomenon in mitochondrial diseases or do they restrict to those diseases affecting proliferating or regenerating tissues? If MYC-driven DNA damage and cellular senescence occur also as a consequence of other mitochondrial disease mutations than those causing severe CIII deficiency, understanding the role of MYC could enable several novel therapeutic options. While MYC has a reputation of being an undruggable target in cancer, several MYC inhibitors have been developed and some of them have proceeded to clinical trials. Understanding the upstream factors leading to MYC upregulation would further diversify the options to target MYC in mitochondrial disease and potentially also in cancer.

Author contributions

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Conflict of interest

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Regulation of the somatotropic axis by MYC-mediated miRNA repression

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The transcription factor MYC is overexpressed in many human cancers and has a significant causal role in tumor incidence and progression. In contrast, *Myc*$^{+/-}$ heterozygous mice, which have decreased MYC expression, exhibit a 10–20% increase in lifespan and a decreased incidence or progression of several age-related diseases. *Myc* heterozygous mice were also reported to have decreased mTOR and IGF1 signaling, two pathways whose reduced activity is associated with longevity in diverse species. Given MYC's downstream role in these pathways, the downregulation of mTOR and IGF1 signaling in *Myc* heterozygotes suggests the presence of feedback loops within this regulatory network. In this communication we provide further evidence that the reduction of *Myc* expression in *Myc*$^{+/-}$ heterozygous mice provokes a female-specific decrease in circulating IGF1 as well as a reduction of IGF1 protein in the liver. In particular, reduced *Myc* expression led to upregulation of miRNAs that target the *Igf1* transcript, thereby inhibiting its translation and leading to decreased IGF1 protein levels. Using Argonaute (AGO)-CLIP-sequencing we found enrichment of AGO binding in the *Igf1* transcript at the target sites of let-7, miR-122, and miR-29 in female, but not male *Myc* heterozygotes. Upregulation of the liver-specific miR-122 in primary hepatocytes in culture and in vivo in mice resulted in significant downregulation of IGF1 protein, but not mRNA. Reduced levels of IGF1 increased GH production in the pituitary through a well-documented negative-feedback relationship. In line with this, we found that IGF1 levels in bone (where miR-122 is not expressed) were unchanged, consistent with the decreased incidence of osteoporosis in female *Myc* heterozygotes, despite decreased circulating IGF1.

KEYWORDS

MYC proto-oncogene, miRNA regulation, IGF1 signaling, somatotrophic axis, gender effects, osteoporosis

Introduction

MYC is a transcription factor that directly regulates 20%–30% of the genome, and indirectly influences many metabolic processes (Fernandez et al., 2003; Li et al., 2003; Patel et al., 2004; Dang et al., 2006). Deregulation of MYC is implicated in 60%–70% of all human cancers, including Burkitt's lymphoma, breast cancer, osteosarcoma, and hepatocellular carcinoma (Greer et al., 2013). MYC has been proposed to act as a master regulator of metabolism, cell growth, and cell division (Miller et al., 2012). MYC thus appears to be a central point of metabolic regulation, integrating intrinsic growth factor signals with nutrient signals from the environment in order to

determine whether the cell should grow, divide, differentiate, or undergo apoptosis (Grandori et al., 2000).

While upregulation of MYC has been extensively implicated in the context of cancer, downregulation of MYC is associated with increased health span and lifespan (Greer et al., 2013; Hofmann et al., 2015). Homozygous deletion of MYC is embryonic lethal; however, *Myc* heterozygous ($Myc^{+/-}$) mice show a 10% lifespan extension in males, and 20% in females (Hofmann et al., 2015). These findings are in agreement with other lifespan extending interventions which have shown that reduction of translation, energy production, oxidative phosphorylation, and ribosome biogenesis, all of which are under positive regulation by MYC, extend lifespan (Brown et al., 2008; Gems and Patridge, 2013; Johnson et al., 2013). Furthermore, upregulation of MYC results in increased generation of reactive oxygen species (ROS) and DNA damage, which are both associated with aging (Vafa et al., 2002; Hoeijmakers, 2009). Together, this large body of evidence indicates that MYC upregulation promotes cancer and aging, while downregulation promotes healthy aging and increased lifespan.

$Myc^{+/-}$ mice are 10%–20% smaller than their wild-type siblings, but show no changes in developmental timing or reproductive ability despite an approximately 50% reduction in MYC levels across all analyzed tissues (Hofmann et al., 2015). $Myc^{+/-}$ mice have increased health span, evidenced by significant amelioration of age-related phenotypes such as cardiac fibrosis, bone density loss, dysregulation of lipid metabolism and immunosenescence, and increased rotarod performance. They also display significantly higher metabolic rates and activity at both young and old ages (Hofmann et al., 2015). These observations indicate a strong impact of decreased MYC activity on age-regulated pathways.

Interestingly, liver gene expression patterns in $Myc^{+/-}$ mice do not overlap strongly with other life-extending interventions such as caloric restriction, resveratrol, and metformin (Hofmann et al., 2015; Ma and Gladyshev, 2017). However, several age-associated pathways are downregulated in $Myc^{+/-}$ mice, including insulin-like growth factor 1 (IGF1), protein kinase B (AKT), and mechanistic target of rapamycin (mTOR) signaling pathways. While these pathways are canonically upstream of MYC activity, we recently showed that MYC regulates mTOR activity by modulating glutamine uptake through direct transcriptional regulation of the amino acid transporters *Slc1a5* and *Slc7a5*, suggesting that negative feedback loops are present within these systems (Zhao et al., 2019).

IGF1 is a downstream effector of the somatotropic axis which regulates organismal growth and development in response to environmental clues such as nutrient availability, sleep, daylight, and exercise through modulation of growth hormone levels (Kato et al., 2002). IGF1 modulates somatic growth and cellular proliferation through both endocrine and autocrine/paracrine effects, with most of the endocrine-functioning hormone produced in the liver in response to growth hormone stimulation (Sullivan et al., 2002). IGF1 produced in the liver is secreted into the serum where it is found in circulation in a complex with one of seven IGF binding proteins (IGFBP1-7) and the acid-label subunit (ALS) (Rosenfeld et al., 2000). IGF1 is also produced by other tissues in both a growth hormone dependent and independent manner, but this tissue-specific production does not contribute significantly to overall IGF1 serum levels, suggesting an alternative purpose for extrahepatic IGF1 production (Le Roith et al., 2001).

Reduced IGF1 signaling is associated with increased longevity in many animal models, including nematodes, *Drosophila*, and mice, and has been correlated with longer lifespan in humans (Kenyon et al., 1993; Clancy et al., 2001; Tatar et al., 2001; Junnila et al., 2013). Decreased IGF1 signaling is however also associated with several age-related diseases such as osteoporosis, cardiovascular disease, skeletal muscle wasting and atrophy, as well as neurological ailments such as dementia (Liu et al., 2008; Elis et al., 2011). Many of these aging-related diseases can be alleviated through administration of either growth hormone or IGF1, suggesting a causal link between IGF1 decrease and development of these diseases (Yakar and Isaksson, 2015). This seeming contradiction between decreased IGF1 being simultaneously associated with increased lifespan and increased risk of age-related diseases has not been resolved but suggests that optimal health and lifespan rely on tight regulation of IGF1.

Myc heterozygous mice have decreased serum IGF1 levels, are long-lived, and are resistant to the development of osteoporosis, thus presenting a unique model system to address whether reducing IGF1 signaling can increase lifespan without deleterious effects on health span. We provide evidence that this effect is caused by the upregulation of specific miRNAs, which are normally repressed by Myc, and that increased levels of these miRNAs reduce the translation of the IGF-1 mRNA.

Methods

Use and treatment of animals

Mice were produced and housed in a specific pathogen-free AAALAC-certified barrier facility. All females used in studies were virgins. Animals of both genotypes and the same sex were housed together. Animals were kept on a 12 h light, 12 h dark light cycle with free access to food and water. The generation of $Myc^{+/+}$ mice was described (Hofmann et al., 2015). Animals for all experiments were produced by mating $Myc^{+/-}$ males with C57BL/6NCrl females purchased from Charles River. Females were purchased at 12 weeks of age and bred immediately. No animals were lost to fighting or accidental death. Dermatitis did occur in very few of the animals but was successfully treated. 48 animals (12 $Myc^{+/+}$ males, 12 $Myc^{+/+}$ females, 12 $Myc^{+/-}$ males and 12 $Myc^{+/-}$ females) were sacrificed at approximately 4 months of age, and another 48 animals (12 $Myc^{+/+}$ males, 12 $Myc^{+/+}$ females, 12 $Myc^{+/-}$ males and 12 $Myc^{+/-}$ females) were sacrificed at approximately 24 months of age for the collection of tissue specimens, at which time they were in apparent good health.

Harvesting of tissues

Mice were euthanized between 11 a.m. and 1 p.m. Animals were euthanized one by one prior to dissection. Animals were first anesthetized by IP injection of ketamine/xylazine. Cardiac puncture was performed, and blood was collected into tubes containing heparin. Animals were then immediately euthanized by cervical dislocation. Blood samples were centrifuged at 2,200 rpm for 10 min and plasma was collected into fresh tubes

and flash frozen in liquid nitrogen. Liver, pituitary, and hypothalamus tissues were quickly dissected, and flash frozen in liquid nitrogen. Soft tissue was removed from femurs and tibia, the bones were cut crosswise, and marrow was removed by centrifugation. Marrow and bone tissue were flash frozen separately. The entire dissection of each mouse was performed in under 10 min by several trained staff members working in concert on one mouse. All flash-frozen samples were subsequently stored at −80°C.

Cell lines and culture conditions

AML-12 cells were cultured under normoxic conditions (air supplemented with 5% CO2), in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (Hyclone, SH30243.01) and Ham's Nutrient Mixture F12 (Hyclone, SH30026.01), supplemented with 10% FBS (Hyclone, SH30071.03), ITS supplement containing 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium (Corning, 354350), and 40 ng/mL dexamethasone (MP Biomedicals, 0219456125).

Hepatocyte isolation, growth hormone stimulation, and transfection

Hepatocyte isolation was performed using a two-step perfusion method as previously described with some modification (Klaunig et al., 1981). Mice were anesthetized using IP injection of ketamine/xylazine mixture as described in section 2.4. Perfusion was done through cannulation of the inferior vena cava with drainage through the portal vein. First, approximately 40 mL at a flow rate of 6 mL/min of HBSS with 0.5 mM EGTA (without calcium or magnesium) was perfused to flush the liver. Second, 40 mL of digestion media (low-glucose DMEM with 200 mg/mL calcium, 20 mM HEPES, and 80 U/mL collagenase IV (Worthington)) was perfused until liver was digested. Liver was then excised, and the cells liberated from the capsule through gentle mincing. Hepatocytes were then filtered through a 70 µm filter and washed three times with cold isolation media (high-glucose DMEM with 10% FBS, pen/strep, 200 mM glutamine). Cell viability and number was assessed with Trypan blue staining, and cells were plated at 600,000 cells/well in 6-well Primaria plates (Corning). After a 2 h incubation to allow cells to attach, media was replaced with culture media (low-glucose DMEM, pen/step, 200 mM glutamine, insulin, transferrin, selenium, dexamethasone, epidermal growth factor) with or without transfection reagents. Transfection was carried out using Fugene HD according to manufacturer's protocol with a 4:1 ratio of DNA to reagent in RNase and DNase-free sterile water using a 10 min incubation time to allow for the formation of complexes prior to addition into cell media. miRNA miRcury mimics (Qiagen) were added at 20 nM concentration with DNA carrier for a total DNA concentration of 1 ug. After 4 h of incubation with transfection reagents, media was replaced with culture media to reduce cytotoxicity and cells were incubated for 24 h prior to harvesting. Growth hormone stimulation (where performed) was carried out in the final 2 hours or incubation by replacement of media with culture media containing 50 nM mouse recombinant growth hormone. For harvesting, cells were washed twice with ice-cold PBS, then lifted with a cell scraper and pelleted by centrifugation at max speed for 2 minutes. Cell pellets were stored at −80°C.

Preparation of RNA

20–50 mg fragments of tissue were removed from −80°C, weighed, and homogenized in 1 mL Trizol reagent (Invitrogen) using a Fisher PowerGen 125 motorized homogenizer at room temperature. 200 µL chloroform was added, the samples were vortexed, and incubated at room temperature for 2–3 min (as per manufacturer's protocol). Samples were then centrifuged at 12,000 x G for 15 min, and the resulting aqueous layer was further purified using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA quality and concentration was accessed using a NanoDrop 2000 spectrophotometer. For RNA used in RNA-Sequencing experiments, RNA quality was further accessed using an Agilent 2100 Bioanalyzer. Only samples with a RIN of greater than 9 were used in sequencing experiments.

RT-qPCR

1 µg of RNA was reverse transcribed into cDNA in 50 µL reactions using the Taqman kit (Applied Biosystems), according to the manufacturer's protocol. 1 µL of this reaction was used in subsequent qPCR reactions for the assessment of mRNA abundance, which were performed using the SYBR Green system (Applied Biosystems) on the ABI 7900 Fast Sequence Detection instrument, according to manufacturer's specifications. All primer sequences are listed in Supplementary Table S1. mRNA expression was normalized to GAPDH (primer pair 4) and verified using beta actin (primer pair 5) and Beta-2 microglobulin (primer pair 6). miRNA RT-qPCR was performed as described previously (Busk *2011* BMC Biotechnology). Briefly, 500 ng of RNA was reverse transcribed into cDNA in 50 µL reactions containing 5 µL 10x Poly(A) polymerase buffer, 0.1 mM ATP, 1 µM of RT primer (was 5′-CAGGTCCAGTTTTTTTTTTTTTTTVN, where V is A, C and G and N is A, C, G and T.), 0.1 mM of each deoxynucleotide (dATP, dCTP, dGTP, and dTTP), 500 units MuLV reverse transcriptase (New England Biolabs), and five units of poly(A) polymerase (New England Biolabs). The reaction was incubated for 1 h at 42°C, followed by enzyme inactivation at 95°C for 5 min qPCR was performed using the Sybr Green system as above. Snord 70 (primer pair 42) was used for normalization.

Assessment of miRNA abundance using the nanostring platform

Total RNA was isolated as above and diluted to a concentration of 33.3 ng/uL. 3 uL of each sample was run on the Nanostring platform using the nCounter Mouse v1.5 miRNA panel according to manufacturer's instructions.

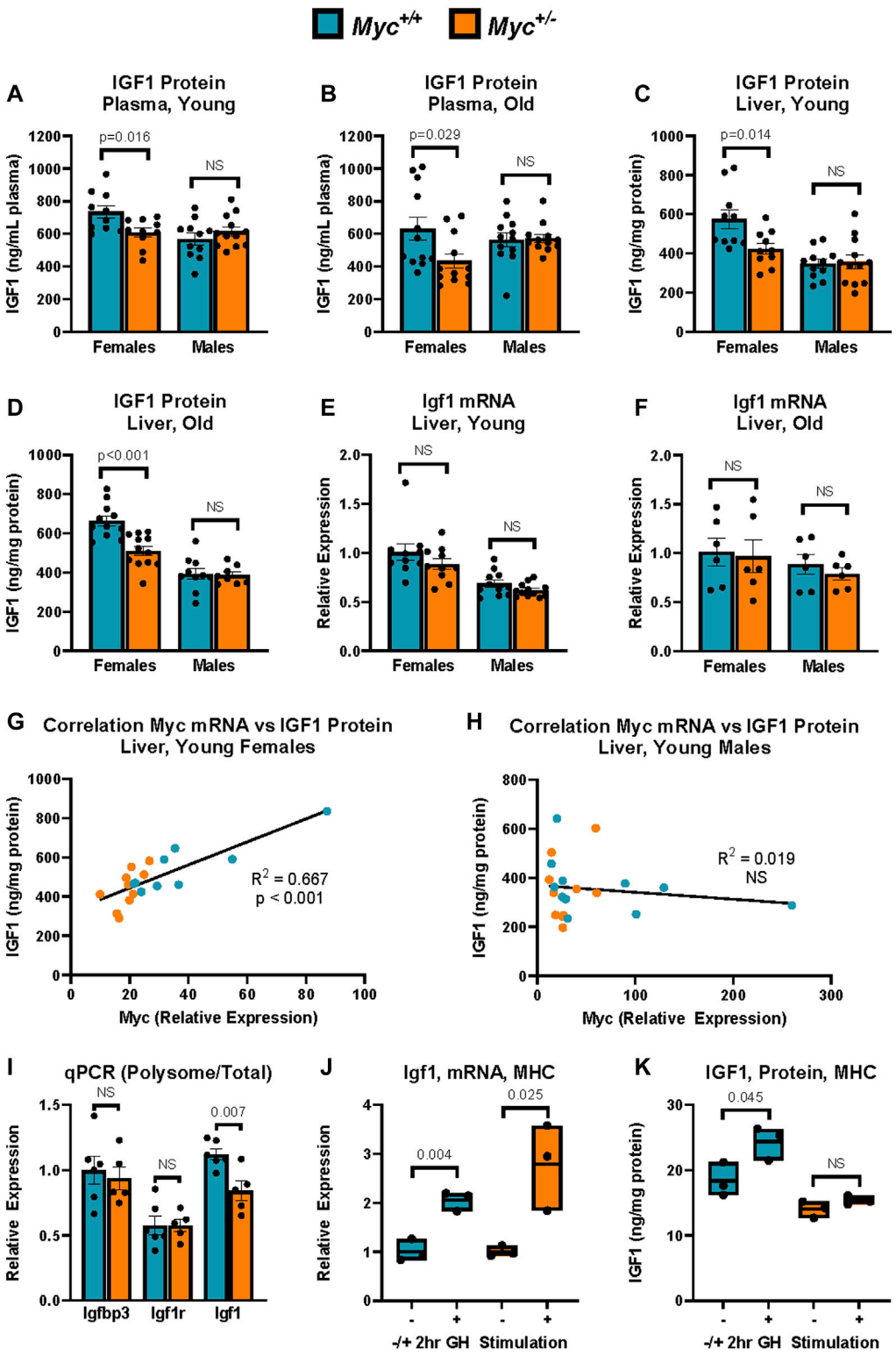

FIGURE 1
IGF-1 Expression in *Myc*$^{+/+}$ and *Myc*$^{+/-}$ mice at young and old ages. **(A)** Total plasma IGF1 protein levels as assayed by ELISA. *n* = 10–12, 3 months, males and females. **(B)** Total plasma IGF1 protein levels as assayed by ELISA. n = 10–12, 24 months, males and females. **(C)** Total liver IGF1 protein levels as assayed by ELISA. n = 10–12, 3 months, males and females. **(D)** Total liver IGF1 protein levels as assayed by ELISA. n = 10–12, 24 months, males and females. **(E)** *Igf1* mRNA levels (primer pair 7) as measured by RT-qPCR in liver. n = 10–12, 3 months, males and females. **(F)** *Igf1* mRNA levels (primer pair 7) as measured by RT-qPCR in liver. n = 10–12, 24 months, males and females. **(G, H)** *Myc* mRNA levels (primer pair 3) as measured by RT-qPCR relative to corresponding liver IGF1 protein levels assessed as in **(B)** in females **(G)** and males **(H)**. *n* = 9–11, 3 months. **(I)** Polysome-associated mRNA was isolated by sucrose-gradient centrifugation. Transcript abundance in the polysome-bound mRNA fraction was quantified by RT-qPCR for *Igfbp3* (primer pair 5), *Igf1r* (primer pair 6), and *Igf1* (primer pair 7), and expressed as fraction of transcript abundance in total mRNA, relative to GAPDH (primer pair 4). n =
(*Continued*)

FIGURE 1 (Continued)
five to six, females, 4 months. **(J)** Igf1 mRNA expression (primer pair 7) as measured by RT-qPCR in primary mouse hepatocytes (MHC) of $Myc^{+/-}$ and $Myc^{+/+}$ mice with and without treatment with 100 nM recombinant mouse growth hormone for 2 h n = 3, females, 6–8 weeks. **(K)** IGF1 protein expression as measured by ELISA in primary mouse hepatocytes (MHC) of $Myc^{+/-}$ and $Myc^{+/+}$ mice with and without treatment with 100 nM recombinant mouse growth hormone for 2 h. Normalized to total protein. n = 3, females, 6–8 weeks. Statistical significance was computed using Student's t-test **(A–F and I–K)**. Correlation for **(G, H)** was computed using Pearson's product-moment correlation sample estimate. Error bars represent SEM.

Protein extraction for enzyme-linked immunosorbent assays

Liver and pituitary protein extracts were prepared by homogenizing 5–10 mg of tissue in 60 µL/mg of tissue extraction buffer containing 100 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1X cOmplete mini protease inhibitor cocktail (Sigma-Aldrich 11836153001). Samples were homogenized using either the Fisher PowerGen 125 motorized homogenizer, or by passage through a 26 G needle. Homogenized extracts were incubated on ice for 20 min, then centrifuged at maximum speed for 10 min at 4°C in a microcentrifuge. The resulting supernatant was diluted 1:20 with MilliQ water prior to assessment of concentration using the Qubit Protein Assay kit (Qiagen Q33211).

Immunoblotting

Liver protein extracts used for immunoblotting were prepared by homogenizing 30–50 mg of tissue in 1 mL laemmli sample buffer (60 mM Tris (pH 6.8), 2% SDS, .05% bromphenol blue, 10% glycerol, 100 mM DTT, and 1X cOmplete mini protease inhibitor cocktail (Sigma-Aldrich 11836153001)). Samples were homogenized using the Fisher PowerGen 125 motorized homogenizer, then boiled for 5 min, cooled, and centrifuged at maximum speed for 10 min at 4°C. Protein concentration in the resulting supernatant was quantified using the Qubit Protein Assay kit (Qiagen Q33211), samples were diluted to 10 µg/µL protein, and stored at −80°C. For the assessment of IGF binding protein abundance, samples were boiled for 5 min, then run at 100 µg protein/well on 15% polyacrylamide gels. Gels were transferred to low-fluorescence PVDF membrane (Invitrogen, 22860). Membranes were blocked in PBS containing 5% BSA with 0.2% Tween-20, then stained with the following primary antibodies; ribosomal protein S6, S6K (Cell Signaling Technologies #2317), insulin-like growth factor binding protein 3, IGFBP3 (Santa Cruz sc-9028), enhancer of zeste homologue 2, EZH2 (Cell Signaling Technologies # 5246), and glyceraldehyde-3-phosphate dehydrogenase GAPDH (Millipore #G8795).

Quantification of IGF1 protein by enzyme-linked immunosorbent assay

Liver and plasma total IGF1 protein abundance was quantified using the Abcam IGF1 ELISA kit (Abcam ab100695) as per manufacturer's protocol. Liver and cell extracts (Figures 1B, E; Figures 3C, G) and plasma samples (Figures 1A, D; Figure 3I) were diluted 1:1 and 1:100, respectively, in assay buffer. All samples were run in duplicate. Absorbance readings were normalized to a standard curve generated from readings of standard solutions of known IGF1 concentration. IGF1 measurements for liver extracts were normalized to protein concentration as determined by the Qubit Protein Assay kit (Qiagen Q33211).

Quantification of growth hormone by enzyme-linked immunosorbent assay

Pituitary and plasma growth hormone abundance was quantified using the EMD Millipore Rat/Mouse Growth Hormone ELISA kit (EMD Millipore, EZRMGH-45 K). All samples were run in duplicate. Pituitary extracts were prepared as described above and diluted 1:5000 in sample buffer for the assay. Blood was collected every 48 h for a total of three time points between 10 and 11a.m. by saphenous vein blood collection. Samples were centrifuged at 2,200 rpm for 10 min at 4°C, and plasma was removed to a clean tube. 10 uL of undiluted plasma per mouse per timepoint was used in the assay. Absorbance readings were normalized to standard curve generated from readings of standard solutions of known growth hormone concentration. Pituitary growth hormone measurements were normalized to protein concentration as determined by the Qubit Protein Assay kit (Qiagen Q33211).

Bone density measurements

L4 vertebrae were scanned using a Scanco Medical Micro-CT 40 system to acquire approximately 250 slices per sample at 10 µm resolution. The volume containing trabecular bone (cortical bone was omitted) was selected by someone blind to the age or genotype of the mouse. The morphometric parameters of bone volume per total volume, trabecular spacing, and trabecular number, were computed for each vertebra, and the average, standard error, and p-value (Student's t-test) were determined for each cohort.

Polyribosome profiling

Polysome enriched fractions were obtained by dounce homogenizing 1 g of liver tissue per animal in 3 mL homogenization buffer (50 mM HEPES pH 7.4, 250 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 200 U/mL RNasin, and 1 µg/mL microcystin). Samples were cleared by centrifugation at 3,000 X G, 4°C, for 15 min. For each mL of supernatant, 100 µL of 10% Triton X-100 and 100 µL of 13% sodium deoxycholate (NaDOC) was

added. Samples were loaded onto 10%–50% sucrose gradients and centrifuged at 22,500 rpm for 19 h, 9 min in a Beckman SW-28 rotor at 4°C (acceleration and deceleration set to 7). Columns were fractionated using an Isco Density Gradient Fractionator at a flow rate of 2 mL/min while absorbance at 254 nm was monitored using an Isco UA-5 Absorbance/Flourescence Detector. Fractions determined from the spectral graph to contain polyribosomes were then pooled for RNA extraction. To each sample 500 mM EDTA was added to achieve a final concentration of 20 mM, prior to incubation for 5 min at room temperature. 20% SDS was added to a final concentration of 0.5%, and samples were incubated for 10 min at room temperature. An equal volume of RNase-free water was added, followed by an equal volume of acid phenol:chloroform (Ambion, 9722). Samples were then centrifuged at 12,000 X G for 30 min at 4°C, and the aqueous layer reserved. RNA was precipitated overnight at −80°C using 2.5 volumes of 100% ethanol and 0.1 volume of 5M NH_4Oac (Ambion, 9071), washed twice with 75% ethanol, and resuspended in water. RT-qPCR was conducted as described above and compared to RNA from unfractionated liver.

Argonaut crosslinking immunoprecipitation followed by sequencing sample preparation

Argonaute CLIP-Sequencing libraries were prepared as previously described (Moore et al., 2014). Frozen liver samples were ground under liquid nitrogen in a mortar and pestle to a fine powder. A small fraction of powder was reserved for total RNA extraction. Ground tissue was then irradiated on dry ice at 400 mJ per cm^2 and then again at 200 mJ per cm^2 using a Stratlinker XL-1500 (Stratagene) UV cross-linker and stored at −80°C until further use. Cross-linked tissue was resuspended in three times volume of PXL buffer (1X PBS containing 1% Igepal/NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) and incubated on ice for 10 min. Samples were then treated first with 30 µL of DNase I per mL of lysate (5 min at 37°C with agitation at 1,000 rpm) then with 10 uL per mL of lysate of 1:10,000 dilution of RNase A (5 min at 37°C with agitation at 1,000 rpm). RNase digestion was stopped with 2.5 µL per mL of lysate of RNAsin Plus. Lysates were then centrifuged at maximum speed for 20 min at 4°C. Beads for immunoprecipitation were prepared by washing 200 µL (per sample) of Dynabeads A three times in PBS with 0.02% Tween-20, incubating with 50 µg of rabbit anti-mouse IgG bridging antibody for 30 min at room temperature with end-to-end rotation, repeating the wash steps, then incubating with 4 µL anti-Ago 2A8 antibody in PBS with 0.02% Tween-20 with end-to-end rotation. Beads were then washed three times in PXL buffer prior to addition of cross-linked tissue lysates. Lysate/bead mixtures were then rotated end-to-end for 2 h at 4°C. Beads were then washed three times with cold PXL buffer, then twice with 5 PXL buffer (PXL buffer with 5X PBS), then twice with PNK buffer (50 mM Tris-HCL pH 7.5, 10 mM $MgCl_2$, and 0.05% Igepal/NP40). To prepare RNA 3′ ends for linker ligation, beads were resuspended in 80 µL of dephosphorylation buffer containing 3 U of CIAP and RNAsin Plus inhibitor and incubated for 20 min at 37°C with shaking at 1,000 rpm for 15 s every 2 min. Beads were washed once with PNK buffer, once with PNK buffer plus 20 mM EGTA, then twice with PNK buffer. Radiolabeled 3′ linkers were prepared using T4 polynucleotide kinase following manufacturer's instruction using 25 µL 32P-γ-ATP and 200 pmol of a dephosphorylated and 3′inverted ddT blocked L32 RNA linker (sequence: GUGUCAGUCACUUCCAGCGG/3InvdT/, IDT) and incubated for 30 min at 37°C. 2 µL of 1 mM ATP was added and the mixture incubated for another 5 min to drive the reaction to completion. To purify the reaction from free nucleotides, the reaction was passed through a G-25 column following manufacturer's instructions. To ligate the radiolabeled linker, beads were resuspended with T4 RNA ligase as per manufacturer's instruction with 12 pmol of radiolabeled linker and incubated at 16°C with shaking at 1,000 rpm for 15 s every 2 min. After 1 hour, an additional 60 pmol of unlabeled, phosphorylated linker was added and the reaction allowed to proceed overnight. Following 3′ linker ligation, beads were washed twice with PXL, twice with 5X PXL, and twice with PNK buffers. To restore the 5′ phosphate, beads were resuspended in 80 µL of T4 PNK phosphorylation mix according to manufacturer's instruction and incubated for 20 min at 37°C with shaking at 1,000 rpm for 15 s every 2 min. The beads were then washed three times with PNK plus 20 mM EGTA buffer. To elute protein:RNA complexes, beads were resuspended in 100 µL of LDS sample buffer with 10% reducing agent, then incubated at 70°C for 10 min with constant shaking at 1,000 rpm. Samples were then loaded onto an 8% Novex NuPAGE Bis-Tris gel in SDS-MOPS buffer run at 175 V, and transferred to Protran BA-85 nitrocellulose using a Criterion blotter at 90 V in NuPAGE transfer buffer containing 10% (vol/vol) methanol. Nitrocellulose membrane was rinsed in PBS and exposed to Biomax MR film (Kodak) at −80°C overnight. Regions corresponding to 110–150 kd were then excised and RNA liberated from the nitrocellulose by incubation with 4 mg/mL proteinase K in PK buffer (100 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM EDTA) for 20 min at 37°C with constant agitation at 1,000 rpm. 200 µL of 7M urea in PK buffer was added and the incubation proceeded for another 20 min. RNA was then extracted using acid phenol:chloroform and precipitated overnight at −20°C with two times volume of 1:1 ethanol:isopropanol. RNA was pelleted, washed twice with 75% ethanol, and resuspended in 6 µL water. A T4 RNA ligase 5′ ligation reaction was prepared according to manufacturer's protocol with 20 pmol of RL5D linker (sequence:/5InvddT/AGGGAGGACGAUGCGGNNNNG, IDT) in a total volume of 10 µL and allowed to proceed overnight at 16°C. DNase digestion was then performed using RQ1 DNase according to manufacturer's instructions with an incubation of 20 min at 37°C in a total reaction volume of 100 µL. RNA was then reprecipitated as described above. RT-PCR of was carried out using SuperScript III (Invitrogen) following the manufacturer's instructions using the DP3 primer for reverse transcription (sequence: CCGCTGGAA GTGACTGACAC). PCR was performed immediately after RT using 27 µL Accuprime Pfx (Invitrogen), and 333 pmol each of DP3 and DP5 (sequence: AGGGAGGACGATGCGG) primers for each 2.5 µL of RT reaction. PCR conditions were 95°C for 2 min, 27 cycles of 95°C for 20 s denature, 58°C for 30 s anneal, and 68°C for 30 s extension. PCR reactions were cleaned up using PureLink Quick PCR Purification Kit (Invitrogen, K310001) and eluted in 30 µL of elution buffer. Next, sequencing adapters were added by PCR using Accuprime Pfx with 333 pmol each of TSP5 and TSP7.1-TSP7.12 primers for each 2 µL of sample. DNA was then size selected by gel

purification on 10% polyacrylamide gels. Regions between 190–300 kd were excised and DNA was extracted using the Qiaquick Gel Extraction kit and eluted in 30 µL. Library quality and concentration was analyzed using an Agilent 2100 Bioanalyzer. Multiplexed sequencing was performed on a NextSeq550 High Throughput Benchtop Sequencer (Illumina) as 75 bp single-end reads with 15% PhiX spike.

Argonaute CLIP-Seq bioinformatic analysis

Bioinformatic analysis of CLIP-Seq data was performed using the galaxy suite of bioinformatic tools (http://galaxyproject.org/ (Goecks et al., 2010)). FASTQ files were filtered for reads with quality score of 20 or greater in 80% or more base pairs. Reads were then collapsed to eliminate sequencing and PCR duplicates. Cutadapt was used to trim 3′ and 5′ linker sequences, as well as discard reads shorter than 18 nucleotides. Reads were aligned to the mm10 genome with STAR using default parameters and a permissible mismatch rate of 0.3 per read. miRNA target sites in the *Igf1* transcript were downloaded from TargetScan and reads overlapping these sites were counted. Counts at each miRNA seed sequence were determined by FeatureCounts, and differential abundance and significance was assessed by DeSeq2.

In vivo miRNA delivery

For assessment of the *in vivo* effects of miRNA upregulation, 3 month-old female C57Bl/6N mice purchased from Charles River were tail vein injected with Qiagen miRcury miRNA mimics of let-7i (Catalog #YM00471739-AGA), miR-122 (Catalog #YM00470430-AGA) or scrambled control 5 (Catalog #YM00479904-AGA). Injections were prepared by combining 1 nmol of each miRNA mimic or scrambled control with invivofectamine complexation buffer prior to addition to an equal volume of invivofectamine (Catalog #IVF3005, ThermoFisher). Complexes were allowed to form by incubation for 30 min at 50°C according to manufacturer's instructions. Complexes were then diluted with PBS to achieve a final volume of 200 µL and the full volume was injected via the tail vein. Mice were euthanized at 4 days post-injection, liver was perfused with PBS via cannulation of the IVC and snipping of the portal vein, and tissues were harvested and flash frozen as described above.

Statistical analysis

Data are shown as means with SEM (unless stated otherwise). N indicates the number of animals per test group; age and sex are also noted.

Results

In circulation, ~95% of IGF1 is bound by one of seven IGF binding proteins (IGFBPs) and the acid-labile subunit (ALS) (Rosenfeld et al., 2000). This tight binding of IGF1 to IGFBPs impedes detection by antibodies used in common ELISA assays, thus additional steps are required to assess total IGF1 levels. In order to assess whether total IGF1 levels were decreased in $Myc^{+/-}$ mice, we prepared sample dilutions in a buffer containing an excess of IGF2, which is not expressed at significant levels in adulthood, but has equal affinity for IGFBPs. This allows for IGF2 to outcompete IGF1 in binding to the present IGFBPs, thus freeing IGF1 to allow detection by anti-IGF1 antibodies. Using this approach, we determined that consistent with previously published results (Hofmann et al., 2015), total plasma IGF1 levels were decreased in young and old female $Myc^{+/-}$ mice by ~20%–30% (Figures 1A, B).

Interestingly, we observed no difference in IGF1 plasma levels in male $Myc^{+/-}$ mice, suggesting a sexual dimorphism in the effect of MYC on IGF1. We next analyzed *Igf1* transcript and protein levels in liver, which is the main site of synthesis of circulating IGF1 (Sjogren et al., 1999). Consistent with the plasma data, we saw an ~20% decrease in IGF1 protein levels in young and old female $Myc^{+/-}$ mouse liver tissue compared to $Myc^{+/+}$ mice, and no significant change in male $Myc^{+/-}$ mouse liver (Figures 1C, D). However, *Igf1* transcript levels were unchanged in either sex in both young and old mice (Figures 1E, F). In line with this, IGF1 protein corresponded to *Myc* transcript abundance in female, but not male, mice in both $Myc^{+/-}$ and $Myc^{+/+}$ genotypes (Figures 1G, H). While we find that *Myc* transcript levels exhibit significant overlap between the two genotypes, particularly in males, our previously published data shows that $Myc^{+/-}$ mice exhibit an approximately 50% decrease in MYC protein levels in liver of male and female mice (Hofmann et al., 2015). Taken together, these results suggest that MYC positively regulates IGF1 protein levels post-transcriptionally in a sex-specific manner, and that decreased MYC expression in female $Myc^{+/-}$ mice results in lower IGF1 protein levels in the liver, which results in decreased levels of circulating IGF1.

To assess whether IGF1 translation is regulated in female $Myc^{+/-}$ mice, we isolated polysome-bound mRNA from liver extracts using sucrose density centrifugation. We found that while related transcripts such as *Igfbp3* and *Igf1r* showed no changes in polysome association, the *Igf1* transcript was reduced by ~20% in the polysome-associated mRNA fraction in the $Myc^{+/-}$ *mice* (Figure 1I). This result suggests that MYC regulates IGF1 by inhibiting its translation. MYC is a known regulator of genes involved in translation, and $Myc^{+/-}$ mice do show a slight decrease in overall rates of translation (Hofmann et al., 2015). However, the lack of significant translational repression on transcripts related to and regulated by the same pathways as *Igf1,* such as its binding proteins and receptor, suggests that the regulation of *Igf1* translation by MYC is a targeted effect, rather than a global one.

To determine whether hepatocytes from female $Myc^{+/-}$ mice respond to growth hormone stimulation as efficiently as $Myc^{+/+}$ hepatocytes, mouse primary hepatocytes (MHC) were isolated using a two-step perfusion protocol and allowed to adhere for 24 h in cell culture. Hepatocytes from both genotypes were then treated with 100 nM of mouse recombinant growth hormone for 2 h prior to harvest and extraction of mRNA and protein. We found that *Igf1* transcript levels were upregulated to a similar extent in $Myc^{+/-}$ and $Myc^{+/+}$ hepatocytes (Figure 1J). However, while $Myc^{+/+}$ hepatocytes show a 30% increase in IGF1 protein in response to growth hormone stimulation, treatment of $Myc^{+/-}$ hepatocytes with growth hormone did not result in significant IGF1 upregulation (Figure 1K). Together, these

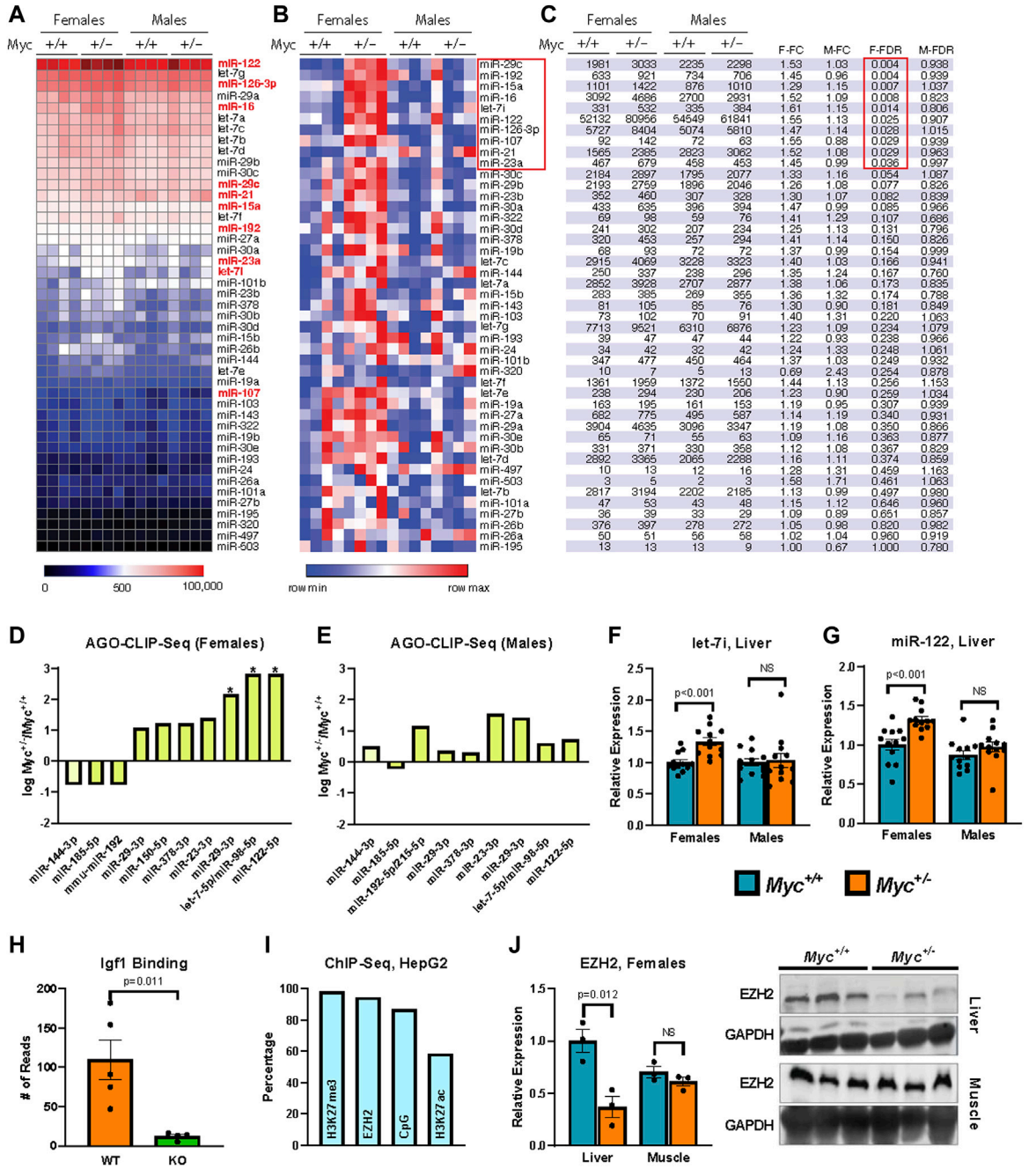

FIGURE 2
Regulation of miRNA expression by MYC **(A, B)** Heatmaps showing relative expression of candidate miRNA genes quantified by the Nanostring platform on total RNA extracted from liver of female and male mice are shown reflecting global levels of expression **(A)**, or for each miRNA across conditions (row min-max **(B)**). *n* = 4, 3 months old, males and females. **(C)** miRNA gene expression in table format showing the significant changes (FDR-corrected *p*-value <0.25). **(D, E)** Enrichment of miRNA target sequences in the *Igf1* transcript as determined by sequencing of AGO-bound RNA isolated from liver of $Myc^{+/-}$ and $Myc^{+/+}$ female **(D)** and male **(E)** mice. *n* = 3–4, 3 months old. **(F)** Expression of let-7i as determined by RT-qPCR (primer pair 11) in liver of $Myc^{+/-}$ and $Myc^{+/+}$ mice. Normalized to Snord70 (primer pair 13). *n* = 10–12, females and males, 3 months old. **(G)** Expression of miR-122 as determined by RT-qPCR (primer pair 12) in liver of $Myc^{+/-}$ and $Myc^{+/+}$ mice. Normalized to Snord70 (primer pair 13). *n* = 10–12, females and males, 3 months old. **(H)** Enrichment of miR-122 target sequence in the *Igf1* transcript in WT and miR-122 KO mouse liver, dataset originally generated by (Luna et al., 2017). *n* = four to five, females and males, 5 months old. **(I)** Percentage of candidate miRNA gene promoters (defined as ± 1,000 bp of transcription start site) with enrichment for H3K27me3, EZH2 and H3K27ac (ENCODE datasets doi:10.17989%2FENCSR000AOL, doi:10.17989%2FENCSR000ARI and doi:10.17989%2FENCSR000AMO, respectively) as well as proximity to CpG islands as determined by analysis of available ChIP-Seq datasets in HepG2 cells. **(J)** EZH2 liver and muscle protein levels were determined by immunoblot and quantified using ImageJ. Expression normalized to GAPDH. *n* = 3, 30-month old, females. The image of the immunoblot is shown to the right of the graph. Statistical significance was computed using Student's t-test and followed by FDR correction in the case of multiple comparisons **(C–E)**. Error bars represent SEM.

data suggest that low MYC levels impede the translation of the *Igf1* transcript.

Given the known role of MYC in regulating the expression of miRNA genes, as well as the ~6 kb length of the *Igf1* 3′UTR, we next assessed whether MYC-regulated miRNAs could target the *Igf1* transcript. TargetScan prediction identified 117 conserved miRNA binding sites in the *Igf1* 3′UTR, including those of 11 of the most expressed miRNAs (Lewis et al., 2005). 56 of these miRNAs have been previously shown to be repressed by MYC and are thus predicted to be downregulated in $Myc^{+/-}$ mice (Chang et al., 2008). To assess whether miRNAs known to be repressed by increased MYC levels and predicted to target the *Igf1* transcript are indeed upregulated in $Myc^{+/-}$ mice, we analyzed their expression in total RNA extracts from livers of 3-month-old $Myc^{+/-}$ and $Myc^{+/+}$ mice using the Nanostring platform.

Of the 56 candidate miRNAs identified above, 45 were found to have detectable expression in liver tissue of 3-month-old mice (greater than five counts in at least one sample). We found 10 candidate miRNAs to be significantly (FDR-adjusted p-value <0.05) upregulated in female, but not male $Myc^{+/-}$ mice (Figures 2A–C). Interestingly, no downregulated miRNAs were found for either sex. These results are consistent with the literature on miRNA regulation by MYC, which documents that the majority of miRNA genes are downregulated in the context of MYC overexpression (Chang et al., 2008).

In order to validate that upregulated candidate miRNAs in $Myc^{+/-}$ female mice lead to increased targeting of the *Igf1* transcript *in vivo*, we performed AGO CLIP-Seq on liver tissue of both genotypes and sexes (Moore et al., 2014). Briefly, flash frozen liver was pulverized and cross-linked using UV-light prior to protein extraction in the presence of RNase inhibitors. Argonaute immunoprecipitation was then carried out, and Argonaute: miRNA:mRNA complexes were isolated by gel electrophoresis using a radiolabeled 3′ linker for visualization. A 5′ linker containing a degenerate sequence was ligated to the isolated RNA tags to allow for PCR amplification and subsequent sequencing and filtering of duplicate reads. After filtering for duplicate and low-quality reads, sequences were aligned to the mm10 genome, and MACS was used to call peaks (Zhang et al., 2008). miRNA seed sequences were identified within 50 bp of peak centers using Targetscan (Lewis et al., 2005).

We found that in female $Myc^{+/-}$ mice, AGO binding of the *Igf1* transcript was significantly enriched at the target sites of miR-122, let-7, and to a more modest degree miR-29 (Figures 2D, E). These three miRNA families are among the most highly expressed miRNAs in the liver, and all three were found to be significantly upregulated in female $Myc^{+/-}$ mice in our Nanostring analysis. Given these results, we validated our Nanostring results of let-7i and miR-122 expression by RT-qPCR modified for miRNA detection (Balcells et al., 2011; Busk, 2014). We found that both let-7i and miR-122 are upregulated by ~30% in liver of female, but not male $Myc^{+/-}$ mice (Figures 2F, G), consistent with our previous analysis.

miR-122 is a liver-specific miRNA that compromises ~70% of the total miRNA species in mouse liver (Jopling, 2012). Furthermore, as decreased miR-122 expression has been linked to hepatocellular carcinoma, its effects have been investigated in the liver, and an AGO CLIP-Seq dataset is available from a miR-122 liver-specific knockout mouse model (Luna et al., 2017). We analyzed this available dataset and found that miR-122 knockout significantly reduces AGO binding at the predicted miR-122 target site in the *Igf1* transcript, thus further validating this site as a *bona fide* miR-122 target (Figure 2H). Given that our AGO-CLIP-Seq data identified the target sites for let-7 and miR-122 as the most enriched for AGO binding in female $Myc^{+/-}$ mouse liver, we chose these two miRNAs for further analysis.

MYC has previously been implicated as a positive regulator of the enhancer of zeste homologue 2 (EZH2) (Ito et al., 2018). EZH2 is a methyltransferase that, as part of the Polycomb Repressive Complex 2 (PRC2), di-/tri-methylates histone 3 lysine 27 (H3K27) to promote the heterochromatization of target regions (Bracken and Heln, 2009). We thus analyzed available Encode ChIP-Seq datasets from the human HepG2 hepatocyte cell line and found that the promoter regions of *Igf1*-targeting miRNAs (including members of the let-7 family and miR-122) are enriched for EZH2 and H3K27me3, and are frequently found in CpG-rich chromatin regions (Figure 2I). These results suggest that many of the miRNAs that target *Igf1* might be regulated by polycomb group repression. Quantification of EZH2 protein levels in aged females showed decreased EZH2 expression in liver, but not muscle in $Myc^{+/-}$ *versus* $Myc^{+/+}$ mice (Figure 2J). Although these results point to a possible involvement of EZH2 in the regulation of miRNA genes by MYC, more work remains to be done to confirm this hypothesis.

To further elucidate the effects of upregulating miR-122 and let-7 in the liver, we transfected LNA-modified miRNA mimics of these miRNAs, both alone and in combination, into primary hepatocytes isolated from wild type C57Bl/6 mice. Transfection resulted in a significant intracellular increase in both let-7i and miR-122 as measured by RT-qPCR. Specifically, transfection with 20 nM miRNA mimics resulted in a 30 to 40-fold increase in let-7i (Figure 3A). Transfection with miR-122 increased its expression to 1.7-fold over scrambled control, though due to the very high expression of miR-122 in hepatocytes, this increase translates to a significant upregulation of the miR-122 miRNA (Figure 3B). Transfection with let-7i or miR-122 decreased IGF1 protein levels by ~20% but did not achieve significance, while transfection with both let-7i and miR-122 in combination significantly decreased IGF1 protein levels by ~40% relative to scrambled control (Figure 3C). Transfection with let-7i, miR-122, or let-7i/miR-122 combined did not affect *Igf1* transcript levels as assessed by RT-qPCR in primary hepatocytes (Figure 3D). These results are consistent with our *in vivo* data in $Myc^{+/-}$ vs. $Myc^{+/+}$ female mice which showed a decrease in IGF1 protein, but not transcript, levels (Figures 1A–C). Together, these results show that upregulation of let-7i and miR-122 can decrease IGF1 protein levels while not significantly affecting *Igf1* mRNA levels.

To assess whether upregulation of let-7 or miR-122 *in vivo* can mediate translational repression of *Igf1* we injected wild-type C57Bl/6 mice with 1 nmol of each miRNA either alone or in combination by tail-vein injection using invivofectamine as a carrier. Tissues were harvested at 4 days post-injection, with retrograde perfusion of the liver prior to harvest. Injection of 1 nmol of let-7i increased its expression 12-fold relative to scrambled control, while injection of the same amount of miR-122 increased its expression 1.2-fold (Figures 3E, F). In line with our data in primary hepatocytes, upregulation of let-7i alone resulted in a slight decrease in IGF1 protein levels which did not achieve statistical significance.

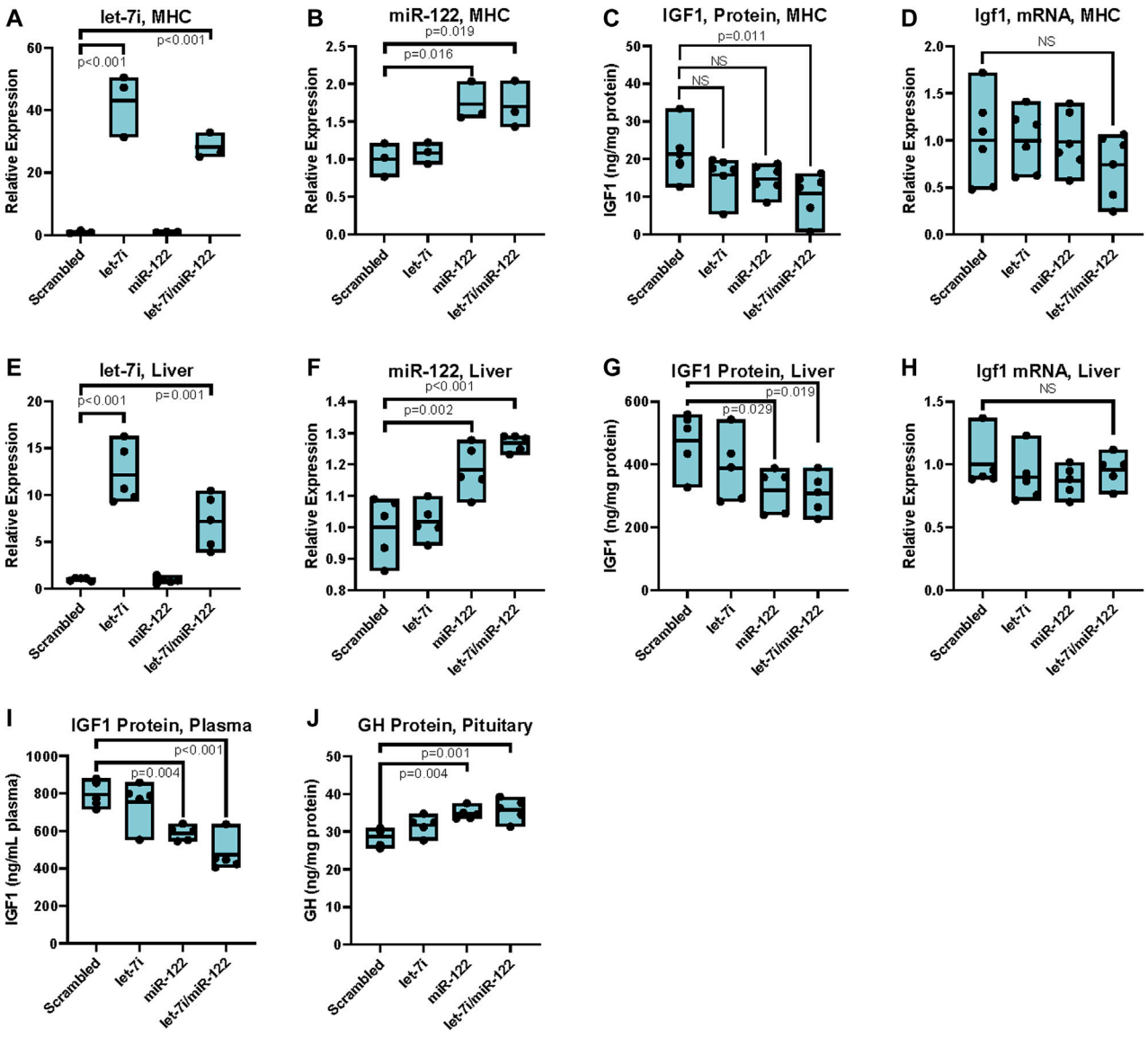

FIGURE 3
Upregulation of miR-122 inhibits IGF-1 translation *in vitro* and *in vivo* in females. **(A)** Let-7i expression in primary mouse hepatocytes (MHC) post transfection with 20 nM mimics as indicated, measured by RT-qPCR (primer pair 11), normalized to Snord 70 (primer pair 13). *n* = 3, females, 6–8 weeks. **(B)** miR-122 expression in MHC post transfection with 20 nM mimics as indicated, measured by RT-qPCR (primer pair 12), normalized to Snord 70 (primer pair 13). *n* = 3, females, 6–8 weeks. **(C)** IGF1 protein levels as assessed by ELISA in MHC) transfected with 20 nM of indicated miRNA mimics. n = 6, females, 6–8 weeks. **(D)** Igf1 mRNA levels (primer pair 7) as assessed by RT-qPCR normalized to GAPDH (primer pair 4) in primary MHC transfected with 20 nM of indicated miRNA mimics. *n* = 6, females, 6–8 weeks. **(E)** Let-7i expression in liver tissue of mice injected with 1 nmol indicated miRNA mimics via the tail vein, measured by RT-qPCR (primer pair 11), normalized to Snord 70 (primer pair 13). *n* = 5, 3 months old, females. **(F)** miR-122 expression in liver tissue of mice injected with 1 nmol indicated miRNA mimics via the tail vein, measured by RT-qPCR (primer pair 12), normalized to Snord 70 (primer pair 13). *n* = 5, 3 months old, females. **(G)** IGF1 protein levels as assessed by ELISA normalized to total protein in liver tissue of mice injected with 1 nmol indicated miRNA mimics via the tail vein. *n* = 5, 3 months old, females. **(H)** *Igf1* mRNA levels (primer pair 7) as assessed by RT-qPCR normalized to GAPDH (primer pair 4) in liver tissue of mice injected with 1 nmol indicated miRNA mimics via the tail vein. *n* = 5, 3 months old, females. **(I)** IGF1 protein levels as assessed by ELISA in plasma of mice injected with 1 nmol indicated miRNA mimics via the tail vein. *n* = 5, 3 months old, females. **(J)** GH protein levels as assessed by ELISA in pituitary extracts of mice injected with 1 nmol indicated miRNA mimics via the tail vein. n = 5, 3 months old, females. Statistical significance was computed using one-way ANOVA followed by Dunnett's *post hoc* test. Bars represent mean, minimum, and maximum values.

Compared to scrambled control, injection of miR-122 either alone or in conjunction with let-7i significantly reduced IGF1 protein levels by almost 50% in the liver, without significantly affecting *Igf1* transcript levels (Figures 3G, H). Furthermore, treatment of mice with miR-122 or combined let-7i/miR-122 significantly decreased plasma levels of IGF1 (Figure 3I). In turn, growth hormone levels were increased in the pituitary, in line with the known negative-feedback loop between these two hormones (Figure 3J).

While decreased IGF1 levels have been associated with longevity in numerous model organisms, as well as in humans, decreased IGF1 expression with age has also been correlated with increased risk of osteoporosis, muscle-wasting, and dementia (Obermayr et al., 2005;

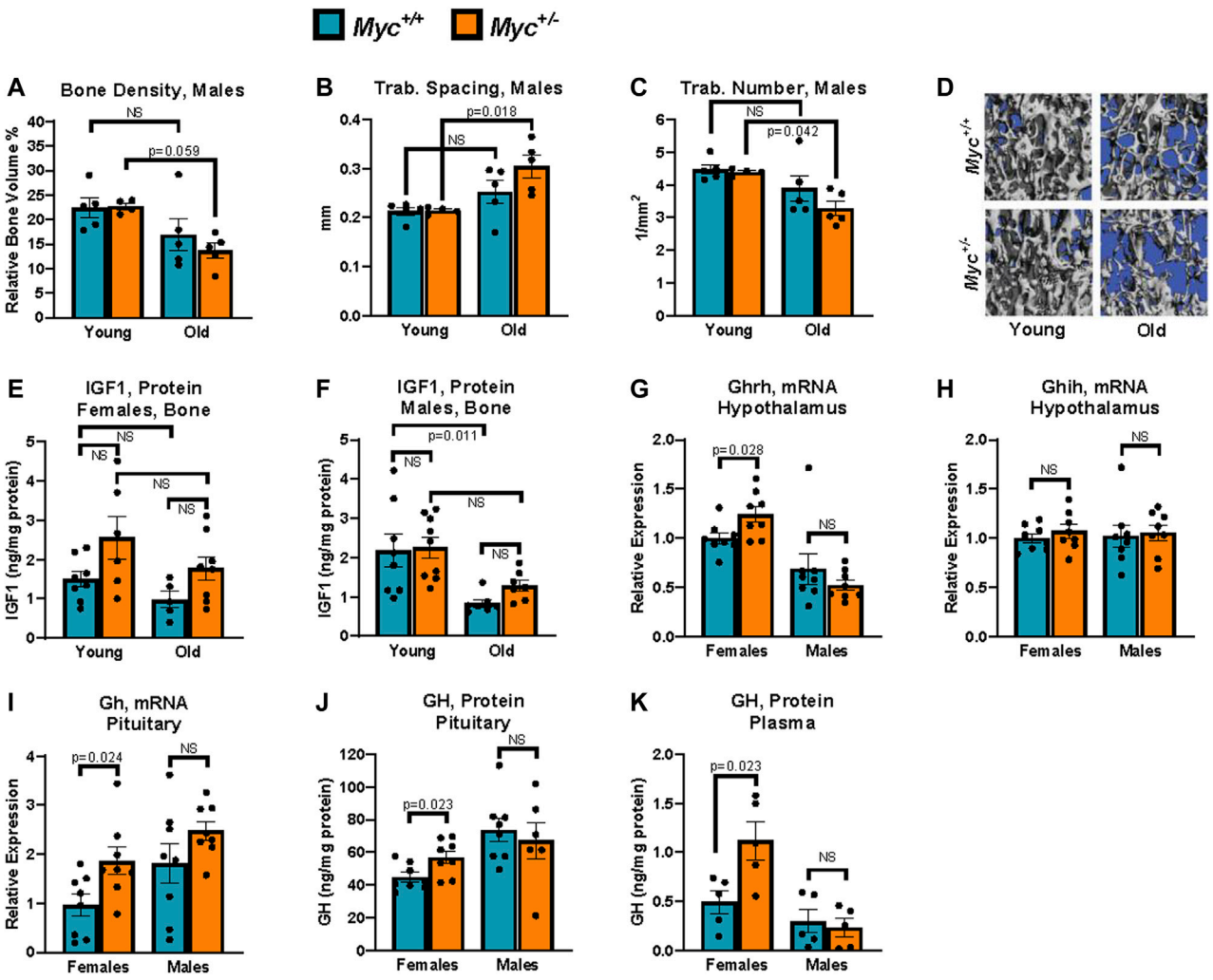

FIGURE 4
Growth hormone is upregulated through known negative-feedback loops by reduced liver IGF1 in $Myc^{+/-}$ relative to $Myc^{+/+}$ mice. **(A)** Bone density in young and old male mice measured by micro-CT. *n* = 4–5, 3 and 24 months. **(B)** Trabecular spacing (mm) in young and old male mice measured by micro-CT. *n* = 4–5, 3 and 24 months. **(C)** Trabecular number (per mm^2) in young and old male mice measured by micro-CT. *n* = 4–5, 3 and 24 months. **(D)** Representative images used for micro-CT analysis in panels **(B, C, E)** Femur IGF1 protein levels assayed by ELISA in young and old females. *n* = 5–8, 3 and 24 months. **(F)** Femur IGF1 protein levels assayed by ELISA in young and old males. *n* = 7–9, 3 and 24 months. **(G)** *Ghrh* mRNA levels (primer pair 10) assayed by RT-qPCR normalized to GAPDH (primer pair 4) in the hypothalamus. *n* = 8, 3 months, males and females. **(H)** *Ghih* mRNA levels (primer pair 9) assayed by RT-qPCR normalized to GAPDH (primer pair 4) in the hypothalamus. *n* = 8, 3 months, males and females. **(I)** *Gh* mRNA levels (primer pair 8) assayed by RT-qPCR normalized to GAPDH (primer pair 4) in the hypothalamus. *n* = 8, 3 months, males and females. **(J)** GH protein levels assayed by ELISA in the pituitary. *n* = 6–8, 3 months, males and females. **(K)** GH protein levels assayed by ELISA in plasma. Three blood samples harvested 48 h apart were averaged for each animal. Readings greater than 3 standard deviations higher than average were discarded. *n* = 5, 4 months, males and females. Statistical significance was computed by Student's *t*-test **(A–C and G–K)**, and one-way ANOVA followed by Tukey's *post hoc* test **(E, F)**. Error bars represent SEM.

Perrini et al., 2010; Westwood et al., 2014). Several mouse models with decreased growth hormone (and consequently IGF1) levels have been shown to have decreased bone mineral density, as well as increased trabecular spacing in old age (Palmer et al., 2009; Yakar Isaksson, 2015). Interestingly, mice with a liver-specific *Igf1* deletion that was essentially complete by 10 days of age did not show decreased femoral or body length (Sjogren et al., 1999; Yakar et al., 1999), suggesting that local, rather than endocrine, levels of IGF1 may be more important for the preservation of bone health into old age. In contrast to this data, *Igf1* liver-specific deletion at 1 year of age reduced circulating IGF1 levels by 70% and significantly reduced trabecular number, but not bone density (Gong et al., 2014). Together, these results suggest that the decrease of circulating IGF1 levels with age, rather than decreased lifetime levels of IGF1, may be responsible for age-related bone loss while low levels of circulating IGF1 from birth do not significantly impair skeletal health.

Female $Myc^{+/-}$ mice, with decreased IGF1 at all ages, show a remarkable resistance to age-related bone loss, and in fact show no decrease in bone density, trabecular number, or trabecular spacing at 22 months of age compared to $Myc^{+/+}$ mice (Hofmann et al., 2015). Though osteoporosis is a disease which typically affects females, we nevertheless extended our analysis to include a measurement of these parameters in male $Myc^{+/-}$ mice. While micro-CT analysis of

the L4 vertebrae in wild-type male mice showed small trends for a decrease in bone density, increase in trabecular spacing, and decrease in trabecular number, none reached significance (Figures 4A–D). In contrast, these changes were exacerbated male $Myc^{+/-}$ mice and reached significance in two out of three parameters. Thus, while reduced levels of MYC positively impact skeletal health with age in females, the opposite was true for males.

To assess whether local levels of IGF1 in bone tissue were affected by *Myc* heterozygosity, we extracted protein from femurs of male and female $Myc^{+/+}$ and $Myc^{+/-}$ mice at 3 and 24 months of age. Interestingly, in both sexes, $Myc^{+/-}$ mice IGF1 levels in the bone tended to be higher, as opposed to the decreases we found in the liver (above), although these trends were not significant (Figures 4E, F). The lack of decreased IGF1 expression in $Myc^{+/-}$ mouse bone tissue suggests that MYC regulation of IGF1 is liver-specific. However, we found a significant decrease of IGF1 protein levels in males (Figure 4F), which might explain the decrease in bone health.

Mice with liver-specific ablation of *Igf1,* resulting in greater than 70% reduction in circulating IGF1, show significantly increased levels of circulating growth hormone (Gong et al., 2014). Although the details of this negative feedback mechanism remain unclear, it has been reported that both growth hormone releasing hormone (GHRH) and growth hormone inhibiting hormone (GHIH) are regulated by IGF1 levels (Obal et al., 1991; Romero et al., 2012). We thus assessed the expression of these two factors in the hypothalamus at the mRNA level, and found a modest but significant increase in *Ghrh* in the hypothalamus of female, but not male, $Myc^{+/-}$ mice (Figure 4G), whereas *Ghih* levels were unaffected (Fig. 7H). Consistent with this, transcript and as well as protein levels of growth hormone in the pituitary were significantly increased in female but not male $Myc^{+/-}$ mice (Figures 4I, J).

Circulating growth hormone levels are difficult to assess *in vivo* due to their circadian as well as feeding-dependent fluctuations, the pulsatile, time of day-dependent fluctuations, which can range by as much as 100-fold in a matter of hours. However, through repeated measurement at consistent timepoints, it is possible to determine an average baseline level for each animal (Steyn et al., 2011). We thus collected blood via the saphenous vein at 10 a.m. every other day for a total of 3 time points from each animal and GH concentrations in each sample were measured by ELISA. We found that average growth hormone levels were significantly increased in female, but not male, $Myc^{+/-}$ mice (Figure 4K). These results indicate that decreased liver production of IGF1 in female $Myc^{+/-}$ mice, and thus reduced circulating IGF1, through a negative feedback loop increase GHRH levels in the hypothalamus, and consequently increase growth hormone production and secretion by the pituitary.

Discussion

$Myc^{+/-}$ mice show global upregulation of miRNA expression, in line with other reports showing that MYC represses most of its target miRNA genes. Many of these miRNAs have been implicated in various human diseases, including Alzheimer's, osteoporosis, muscle wasting, and hepatocellular carcinoma. As upregulation of MYC has been associated with more than 50% of all tumors, as well as other aging-related diseases, the modulation of miRNA expression by MYC is likely a significant mechanism by which MYC dysregulation leads to impaired human health (Levens, 2010). Interestingly, caloric restriction has also been shown to globally increase miRNA expression, suggesting that miRNAs may play a significant role in the regulation of lifespan (Zhang et al., 2019). Here, we have shown that downregulation of MYC in female mice induces the expression of multiple miRNAs that target the *Igf1* transcript, and that at least one of these, miR-122, significantly reduces IGF1 translation upon ectopic upregulation *in vivo*.

While the mechanisms by which MYC affects transcriptional activation are well characterized, those mediating its role in transcriptional repression are more poorly understood. Several hypotheses have been proposed and supported by experimental results, including that MYC can upregulate the expression of some transcriptional repressors (Philipp et al., 1994; Lee et al., 1996), that MYC is recruited to the promoters of its repression targets through protein-protein interactions with transcriptional regulators such as TFII-I, YY-I, Sp-I, and MIZ-1 (Schneider et al., 1997; Seoane et al., 2001), and that MYC can regulate the expression of chromatin silencing factors such as the Polycomb Repressive Complex (PRC) (Knoepfler et al., 2006).

MYC has been found to regulate both the transcription of *Ezh2* and EZH2 protein activity through phosphorylation (Bhandari et al., 2011; Neri et al., 2012), but the genes that are regulated through this PcG mechanism are not fully characterized. In line with MYC's role as a chromatin regulator, genes repressed by MYC show considerable overlap with PcG and HDAC repressed genes (Kaur and Cole, 2013; Bhadury et al., 2014). Furthermore, knockdown of *Ezh2* in glioma cells was found to upregulate 85 miRNA genes, many of which are also known to be repressed by MYC (Wang et al., 2013). In fact, many of the miRNAs upregulated in $Myc^{+/-}$ mice have already been documented to be regulated by EZH2 (So et al., 2011; Liu et al., 2013; Vella et al., 2015).

We found that, consistent with literature showing that MYC overexpression upregulates EZH2, female $Myc^{+/-}$ mice showed reduced liver EZH2 protein levels compared to $Myc^{+/+}$ mice. Analysis of available Encode ChIP-Seq datasets from the human HepG2 hepatocyte cell line showed that the promoter regions of *Igf1*-targeting miRNAs, including most members of the let-7 family and miR-122, are enriched for EZH2 and H3K27me3. These results suggest that many of the miRNAs that target *Igf1* may be regulated by PcG repression, which is regulated by MYC.

While EZH2-mediated repression is a plausible mechanism for MYC-mediated repression of miRNA genes, it was unclear why these miRNA genes were upregulated specifically in female $Myc^{+/-}$ mice. Many miRNA genes have been shown to be regulated in a sexually dimorphic manner. For example, in rat liver the IGF1-targeting miR-193a, miR-29b, and miR-122 miRNAs were found to be expressed at higher levels in females relative to males (Cheung et al., 2009). Another group found that 37% of miRNA genes were differentially expressed when MCF-7 human breast cancer cells were exposed to estrogen (Hah et al., 2011). These results were corroborated by another group which showed that estrogen induces the expression of 21 miRNAs while down-regulating 7 in MCF-7 cells (Bhat-Nakshatri et al., 2009). The miRNAs upregulated by estrogen in these cell types (the let-7, miR-30, miR-23 families), also overlap extensively with the miRNAs that target the *Igf1* transcript, as well as those upregulated in $Myc^{+/-}$ females. Interestingly, it has been shown that EZH2 can be recruited to

target gene promoters through estrogen receptor alpha, and that testosterone administration to female mice, which downregulates ER-alpha expression, results in the upregulation of several miRNAs, including members of the let-7 family, miR-122, and miR-30d (Delic et al., 2010; Ariazi et al., 2017).

Estrogen also plays a significant role in the regulation of MYC targets, and miR-122 expression has been previously shown to be upregulated in females compared to males (Cheung et al., 2009). miR-122 is the predominant miRNA expressed in liver tissue, compromising more than half of the total miRNA pool in the liver, according to our analyses and those of others (Jopling, 2012). miR-122 is regulated by multiple liver-enriched transcription factors, including HNF3b (FOXA2), HNF4a, and HNF6 (Xu et al., 2010). The miR-122 regulating liver-enriched transcription factor FOXA1/2 alternatively regulates MYC depending on the presence of estrogen or androgen (Li et al., 2012). This sex-hormone specific regulation is believed to be an important contributor to the sexual dimorphism of hepatocellular carcinoma, which has a prevalence in males 2–4 times higher than in females (Hsu et al., 2012; Li et al., 2012). In fact, downregulation of several oncogenic genes by FOXA1/2 is dependent on the presence of estrogen (Li et al., 2012). While estrogen-dependent regulation of miR-122 by FOXA1/2 has not been explicitly described, it is a plausible explanation for the sex-specific upregulation of miR-122 in $Myc^{+/-}$ female mice.

Of the many miRNAs that are predicted to target the *Igf1* transcript, our results showed that miR-122 had the most profound effects on IGF1 translation in $Myc^{+/-}$ female mice. Given that miR-122 is a liver-specific miRNA, this suggests that MYC regulates IGF1 in a liver-specific manner. In line with this, we found that female $Myc^{+/-}$ mice did not have decreased IGF1 levels in bone tissue, in contrast to the significant reduction seen in the liver. In fact, IGF1 levels in femur tissue of $Myc^{+/-}$ mice trended towards increased expression both at young and old age.

IGF1 and its main regulator, growth hormone, function in a negative feedback loop such that reduced IGF1 levels in the circulation trigger an increase in growth hormone levels in the pituitary, and its secretion into circulation (Romero et al., 2012). We found increased GH expression and circulating levels in $Myc^{+/-}$ female mice, which is likely explained by the known negative-feedback loop between circulating IGF1 and GH. Increased growth hormone in circulation of $Myc^{+/-}$ female mice may then signal to peripheral tissues such as the bone to stimulate local production of IGF1, thereby mitigating bone loss with age, and possibly explaining the remarkable resistance of $Myc^{+/-}$ female mice to age-related bone loss.

Together, these results suggest that liver-specific reduction in IGF1 does not affect local production of IGF1 in peripheral tissues, and that in fact it may enhance it. Several mouse models with liver-specific disruption of IGF1 have been generated. Constitutive liver-specific *Igf1* knockout resulted in a >60% reduction in circulating IGF1 levels and had minimal effects on body weight, organ weight, and femoral length (Yakar et al., 1999). Liver-specific ablation of IGF1 at 3 weeks of age resulted in a ~75% decrease in circulating IGF1 levels, extended lifespan by 16% in females, but resulted in only minor reduction in femoral length (Sjogren et al., 1999; Svensson et al., 2011). Unfortunately, local IGF1 levels were not measured in bone tissue of either mouse model, nor was trabecular morphology assessed with age. However, the results of these studies are consistent with our data, and suggest that liver-specific disruption of IGF1 at young age does not significantly affect bone health.

In aggregate, we propose a model in which MYC-mediated activation of EZH2 causes the upregulation of multiple miRNAs (namely, miR-29, let-7, and miR-122), in an estrogen-dependent manner, which then target the *Igf1* transcript and reduce its translation. The regulation of *Igf1* translation by miR-122, which is a liver-specific miRNA that showed the most pronounced effect on *Igf1* translation, results in the reduction of IGF1 protein levels in the liver of female $Myc^{+/-}$ mice. Since circulating IGF1 is produced predominantly in the liver, the systemic effect of decreased MYC expression is reduced plasma levels of IGF1. Given that IGF1 and GH function in a negative feedback loop, such that reduced circulating IGF1 triggers an increased production and secretion of GH by the pituitary, decreased MYC expression concomitantly increases plasma levels of GH. Increased GH expression, in turn, increases the local production of IGF1 in tissues such as the bone, thereby evading some of the negative consequences of global IGF1 reduction.

Data availability statement

The AGO-CLIP-seq data presented in the study are deposited in the NCBI Sequence Read Archive (SRA) BioProject repository, accession number PRJNA1004998.

Ethics statement

All research was approved by the Brown University IACUC sommittee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AP: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing–original draft, Writing–review and editing. AV: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–review and editing. JK: Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–review and editing. JS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Validation, Visualization, Writing–review and editing.

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Conflict of interest

JS is a cofounder and SAB chair of Transposon Therapeutics and was consultant for Atropos Therapeutics, Gilead Sciences and PrimeFour.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1269860/full#supplementary-material

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MYC function and regulation in physiological perspective

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MYC, a key member of the Myc-proto-oncogene family, is a universal transcription amplifier that regulates almost every physiological process in a cell including cell cycle, proliferation, metabolism, differentiation, and apoptosis. MYC interacts with several cofactors, chromatin modifiers, and regulators to direct gene expression. MYC levels are tightly regulated, and deregulation of MYC has been associated with numerous diseases including cancer. Understanding the comprehensive biology of MYC under physiological conditions is an utmost necessity to demark biological functions of MYC from its pathological functions. Here we review the recent advances in biological mechanisms, functions, and regulation of MYC. We also emphasize the role of MYC as a global transcription amplifier.

KEYWORDS

MYC, transcription, transcription-amplifier, MYC function, MYC regulation, MYC-inhibitors, DNA-topology

Introduction

The Myc gene was first identified in the early 1980s as a cellular homolog of the retroviral v-Myc oncogene (Duesberg et al., 1977; Sheiness et al., 1978; Conacci-Sorrell et al., 2014). Its discovery led to intense research efforts to understand its function and deregulation in cancer. MYC deregulation was soon associated with genomic rearrangements including translocations in Burkitt lymphoma, gene amplification and chromosomal circles in leukemia and carcinoma, and deregulation by HPV insertion in cervical carcinoma (Dalla-Favera et al., 1982; Taub et al., 1982; Spencer and Groudine, 1991; Wasylishen and Penn, 2010; Adey et al., 2013). Subsequently, mutations that stabilize MYC protein and mRNA were recognized in malignancy (Dang, 2012). Because all these situations occur in an oncogenic setting, thousands of studies explored the cellular consequences of MYC overexpression. Upon discovering that the basic-helix-loop-helix (bHLH) protein MYC dimerizes with its bHLH partner Myc-associated factor-X referred to as MAX and binds with E-boxes (5′-CACGTG-3′) and presumed to activate transcription, the principal focus of studies to define the pathologic role of MYC revolved upon the identification of its transcriptional targets (Blackwood and Eisenman, 1991; Grandori and Eisenman, 1997; Eilers and Eisenman, 2008; Dang, 2012). The notion was that MYC programmed the expression of a discrete set of mRNAs that bypassed normal growth control leading to unrestrained proliferation. Most of these studies exploited a variety of transformed and tumor cell lines to explore pathologic MYC function. Fewer studies focused on physiological role of MYC. In the untransformed, non-oncogenic situation, MYC was found to be an immediate early gene, turned off during the G0-stationary phase of the cell cycle, but upregulated transiently during the transition to G1/S (Kelly et al., 1983; Armelin et al., 1984; Wang et al., 2008). Upon entering steady-state cell-cycle growth, MYC was stably expressed at lower levels until growth once again arrested. Survey of mRNA expression indicated that

while MYC upregulated the expression of many genes involved in cell cycle progression, it also repressed a small number of cell-cycle antagonists (Bretones et al., 2015). Sustained high level expression of MYC elicited apoptosis in non-transformed cells and so could not be maintained (Evan et al., 1992; Murphy et al., 2008). In the bulk of this review, we will consider the biological mechanisms and functions of MYC in non-transformed cells, tissues, and organisms. A description of this physiology is essential to distinguish whether the oncogenic actions of MYC arise due to an exaggeration of its normal functions or whether high level expression conjures new modes and mechanisms of MYC activity otherwise unseen.

MYC domain organization and function

The MYC family of proteins consists of three paralogs, MYC (c-MYC), MYCN (N-MYC) and MYCL (L-MYC) (Brodeur et al., 1984; Kohl et al., 1984; Nau et al., 1985). Although MYC family genes encode proteins with similar structural architecture and function, each MYC paralog is located on a different chromosome (MYCL, MYCN and MYC are in chromosomes 1, 2, and 8 respectively) and expressed at distinct times and locations during cellular differentiation (Dalla-Favera et al., 1982; Schwab et al., 1984; Zelinski et al., 1988; Ruiz-Pérez et al., 2017; Llombart and Mansour, 2022). MYCN and MYCL have tissue-specific function. MYCL is expressed and functions in dendritic cells, gastrointestinal cells, and lung cells. MYCN is expressed in neural and neuroendocrine tissue and is critical for the development of nervous system (Llombart and Mansour, 2022). MYC is composed of 439 amino acids and contains an N-terminal transactivation domain (TAD), and a C-terminal DNA-binding domain. The TAD (residue 1-143) forms an intrinsically disordered domain and is necessary for biological activity of MYC and MYC-mediated transcriptional activation (Kato et al., 1990). The C-terminal domain comprises ~80 residues and consists of a bHLH -leucine zipper (bHLH-ZIP) segment from residues 357-439. The bHLH-ZIP domain forms specific heterodimers with MAX (Blackwood and Eisenman, 1991; Amati et al., 1992; Amati et al., 1993; Kato et al., 1992). This interaction facilitates the ability of MYC' to bind DNA with preference, but not absolute specificity, for binding to the canonical E-box (5′-CACGTG-3′) (Blackwood and Eisenman, 1991; Guo et al., 2014; Carroll et al., 2018). Besides sequence recognition, a major component of MYC recruitment to the DNA are its interactions with the transcription machinery at accessible promoters (Guo et al., 2014). Initially MYC seemed to bind a wide range (2,500–25,000) of sites throughout the genome that varied according to cell type (Cawley et al., 2004). Classification and functional assessment of the programs regulated by MYC between different tissues and cells seemed complex and somewhat incoherent. The number of MYC peaks was significantly affected by the arbitrary threshold chosen to distinguish real peaks from the background and experimental conditions that most often lacked an internal control, such as "spike" chromatin from a heterologous genome, to improve quantitation (Bonhoure et al., 2014). Moreover, the normalization of gene mRNA output obscured the observation of global transcription amplification by MYC, with sensitivity to the artificial threshold used to differentiate "real" from non-specific binding (Lovén et al., 2012).

Upon binding at promoters, the transregulatory domains of MYC and its isoforms, are believed to project its influence onto target genes through patches of amino acids that share high sequence homology among the three MYC isoforms. These patches are referred to as MYC boxes (MBs). From the amino- to carboxyl terminus, there are six conserved MBs: MB0, I, II, IIIa, IIIb, and IV. They are generally unstructured and can adopt specific conformations induced upon partner-protein binding. The degree of plasticity for each MB upon complexing with different partners has not been explored. The inventory and functional roles for MB-interacting partners that have been most intensively investigated are involved in transcription and chromatin process, or control MYC turnover, has recently been reviewed (Das et al., 2023).

A sampling of the MYC-interactome shows MB0 interactions with general transcription factor TFIIF (Kalkat et al., 2018). MBI and MBII reside within the TAD and are critical for transcriptional and cell-transforming functions of MYC. MYC box I controls its proteasome mediated degradation of MYC proteins (Farrell and Sears, 2014). Aurora A, independent from its kinase activity, interacts with MBI to stabilize MYC (Dauch et al., 2016). MBII plays a crucial role in recruiting MYC transactivation coactivators such as TRRAP, GCN5, TIP48, TIP49, TIP60, CBP/p300, and SKP2 (Adhikary and Eilers, 2005; Conacci-Sorrell et al., 2014; Tu et al., 2015; García-Gutiérrez et al., 2019). Because TRRAP is a protein that participates in multiple large protein complexes engaged in chromatin remodelling and histone acetylation (Zhang et al., 2014), it may impart multiple functions when joined with a promoter-bound MYC. The central region of MYC containing MBIII and MBIV starts with a proline-rich PEST segment, followed by a calpain cleavage site (CAPN); the N-terminal fragment of this cleavage, known as "MYC-nick," lacking the nuclear localization signal (NLS) situated to the C-terminal side of the cleavage site, resides in the cytoplasm and participates in interactions and functions of the cytoskeleton (Conacci-Sorrell et al., 2010; Anderson et al., 2016). MBIII is important for transcriptional repression (Kurland and Tansey, 2008; Garcia-Sanz et al., 2014), but also interacts with WDR5 (a scaffolding protein that nucleates the assembly of histone modifier complex) and facilitates histone H3 Lys4 (H3K4) methylation which is thought to increase the interaction of MYC with active promoters (Thomas et al., 2015). MBIV is necessary for transcriptional activity of MYC and induction of apoptosis (Cowling et al., 2006) and has been shown to interact with the transcriptional coregulator HCF-1 (Thomas et al., 2016). Although each of the MBs interact with multiple partners and have been shown to modulate MYC activity, the precise role of individual MBs has not been fully ascribed.

MYC, an amplifier of transcription

Transcription activation involves the binding of transcription factors to specific DNA sequences, which recruit the transcriptional machinery, coactivators, and chromatin modifiers to form a transcriptional complex that initiates gene transcription.

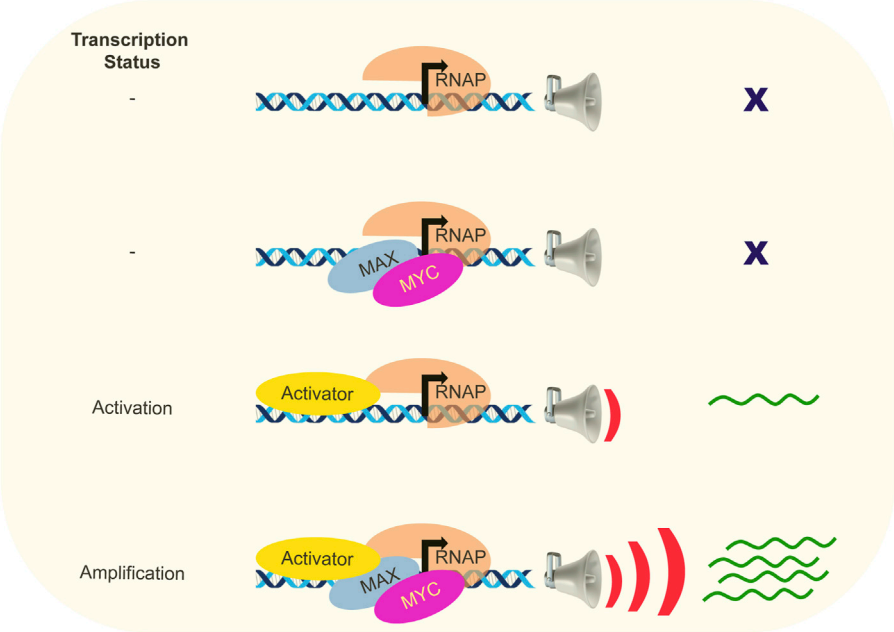

FIGURE 1
MYC is an amplifier of transcription. Schematic representation of the role of MYC as transcription amplifier is depicted. MYC exerts its influence on actively transcribed genes in the presence of activators, rather than being involved in transcriptional processes at silent genes. When MYC is not involved, activator can start transcription albeit with low outputs. Participation of MYC leads to an augmentation of gene expression beyond what would be typically anticipated solely based on the binding of transcription factors.

Transcription factors can recruit coactivators such as CBP/p300, which possess histone acetyltransferase activity and can acetylate histones to promote an open chromatin structure that allows for gene transcription. In addition, transcription factors can recruit chromatin modifiers such as SWI/SNF, which can remodel chromatin to allow access to the transcriptional machinery (Bannister and Kouzarides, 2011). Unlike transcription activation, transcription amplification refers to the process by which transcription factors globally enhance the expression of all active genes in the cell (Lin et al., 2012; Nie et al., 2012; Li et al., 2013). Transcription amplification is different from gene amplification where the number of copies of a specific gene increases without an increase in the transcription output of each copy. Gene amplification can result from DNA replication errors, chromosome translocations or gene rearrangements (Albertson, 2006; Beroukhim et al., 2010; Matsui et al., 2013; Schaub et al., 2018). In contrast, transcription amplification occurs through the recruitment of coactivator complexes or other factors that enhance the efficiency of transcriptional reinitiation and elongation, and so increase the number of RNA polymerases (RNAP) that are engaged in transcription (Wolf et al., 2015). Transcription amplification enhances the expression of a gene beyond what would be expected based on the level of transcription factor binding alone. While it was initially believed that MYC acted as a sequence-specific transcription factor, turning on genes via E-boxes (Blackwell et al., 1990; Halazonetis and Kandil, 1991; Kerkhoff et al., 1991; Prendergast and Ziff, 1991), an alternate model has been posed in which MYC acts as a global amplifier of all active genes (Lin et al., 2012; Lovén et al., 2012; Nie et al., 2012; Nie et al., 2020; Wolf et al., 2015).

When viewed a transcriptional activator, the expectation and goal were to identify specific, direct MYC target genes to provide insights into the crucial downstream targets and biological processes responsible for mediating the physiological functions and oncogenic pathology of MYC. Numerous studies were undertaken to identify MYC-regulated genes by employing techniques such as microarray or next-generation sequencing to compare RNA expression profiles and genome-wide mapping of MYC-bound chromatin. The notion that MYC and MYC-MAX complexes regulate a limited and well-defined set of target genes for their various roles has been largely challenged (Orian et al., 2003; Ji et al., 2011; Lee et al., 2012; Hurlin, 2013; Sullivan et al., 2022). Studies aimed to establish a universal signature of MYC target genes across cell types have been unsuccessful (Lee et al., 2012; Sullivan et al., 2022). Investigations across various cell types consistently revealed the presence of MYC proteins at nearly all promoters located in open chromatin regions (Chen et al., 2008). Moreover, a strong correlation between MYC binding and the presence of histone marks associated with open chromatin, particularly H3K4Me3 and H3K27Ac was observed (Nie et al., 2012). Conversely, MYC was excluded in the regions exhibiting repressive histone modifications. These results argued against the role of MYC as selective target (E box-dependent) transcription activator and led to further consideration of the transcription amplifier model, where MYC acts to globally enhance the expression of transcriptionally active genes in a nonlinear manner (Figure 1) (Lin et al., 2012; Nie et al., 2012). The transcriptional response of an active gene rises until output at the affected promoter saturates. This amplification is more efficient on highly transcribed genes, effectively raising their expression ceilings. MYC exhibited widespread binding to all promoters

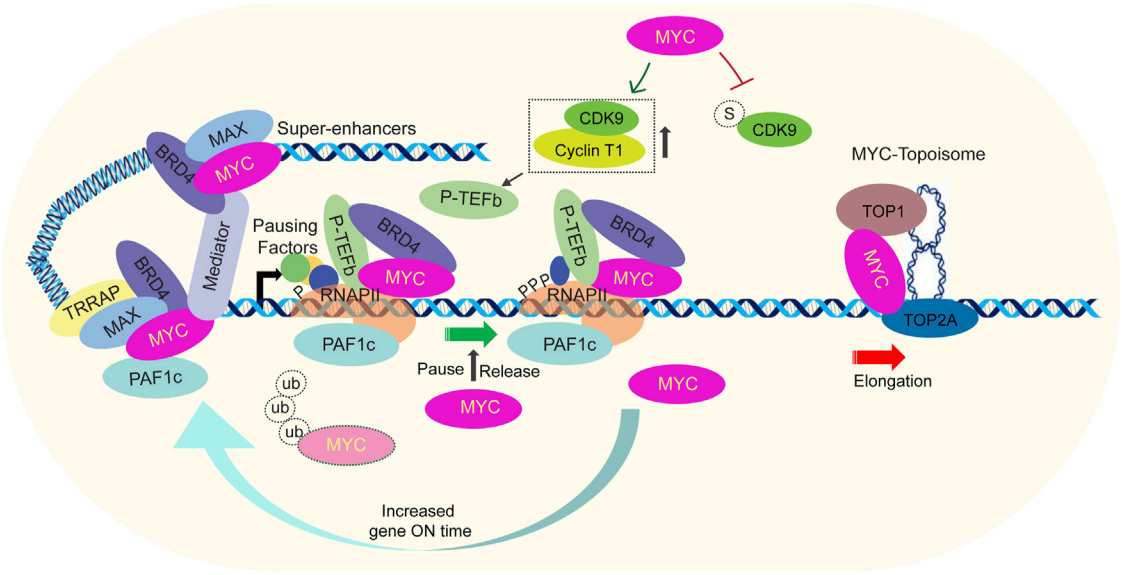

FIGURE 2
Current model for factors involved in transcription amplification by MYC. MYC interacts with essential transcription regulators involved in critical events at promoters, either coincidently or through regulated processes. MYC recruits key activators such as general transcription factors, Mediator, PAF1c, P-TEFb, DSIF, and exosome (other components omitted for simplicity). Once transcription starts, pausing factors interact with RNAP II near the start site, causing it to pause around 50 bp downstream from the initiation site. Together with cofactors like BRD4, MYC recruits P-TEFb which phosphorylates the pausing factors and RNAP II. MYC suppresses CDK9 sumolyation, facilitating active P-TEFb formation. MYC also recruits PAF1c and in association with HUWE1-mediated ubiquitylation of MYC, PAF1c is transferred to RNAP II. These events collectively trigger the release of the paused transcription complex and initiate transcription elongation. Moreover, torsional stress generated due to transcription elongation is resolved by the MYC-Topoisome complex. It activates the catalytic activity of both TOP1 and TOP2A, helping to maintain DNA supercoiling homeostasis. MYC also extends the duration of residence times of transcription machinery like TBP, SPT5 and RNAP II and this leads to the extension of transcriptional bursts (gene ON time). These events help to explain the role of MYC as an amplifier of transcription.

associated with RNAP II activity, resulting in a significant enhancement of transcription for a diverse repertoire of genes. MYC action does not entail the activation of novel genes; instead, it amplifies the expression levels of transcribed genes and so accelerates and amplifies ongoing cellular programs. Highly expressed MYC target genes tend to harbor canonical E boxes, but this is not obligatory and there is no strict correlation between MYC binding and the presence of E boxes for MYC- amplified genes in non-transformed cells (Nie et al., 2012).

The complexity of transcription amplification can be influenced biologically by input signals, cis-elements, other transcription factors, and analytically by the algorithms and pipelines used for analysis. These factors can highlight or obscure the relationship between MYC binding and promoter output in omics studies. To exclude such interfering or biased factors, minimal promoter and the reporter-based assay was designed to interrogate MYC function (Nie et al., 2020). Basal reporter expression was insensitive to MYC, and an initial activator signal was required to sensitize the promoter to MYC amplification to achieve increased transcriptional output. MYC boosted reporter gene expression to much higher levels than was attainable solely with saturating levels of transactivators. Further, MYC-mediated transcription amplification was severely attenuated by mutations in MBI and MBII but augmented by mutations in MBIII. This suggests that the MB regions coordinate with various proteins to control the chromatin opening and progression through the transcription cycle to achieve transcription amplification. The amplifier model for MYC functions is supported by the observation that MYC promotes transcription elongation by recruiting P-TEFb, PAF1c and super-elongation complexes (Jaenicke et al., 2016; Chen et al., 2017; Endres et al., 2021; Aoi et al., 2022). Increased MYC occupancy consequently led to increased P-TEFb with elevated levels of Serine 2 phosphorylation at RNAP II (a modification linked to elongation), escalated levels of elongating RNAP II, and augmented mRNA levels for active genes. Therefore, the main consequence of increased MYC is the amplification of transcription (Figure 2) (Rahl et al., 2010).

Although, it has been suggested that the binding of transcription factors to enhancer elements, super-enhancers, or other regions that drive the recruitment and activity of the transcription machinery plays a critical role in the non-linear mode of transcriptional amplification (Hnisz et al., 2016), the direct mechanism/s how MYC increases the output of expressed genes demands further investigation. A new report argues that a DNA-binding independent function of MYC helps it to function as a global amplifier (Guan et al., 2023). These authors report that MYC regulates P-TEFb availability through the inhibition of CDK9 sumoylation. CDK9 interacts with UBC9 and the PIAS family E3 ligase, specifically PIAS1, to promote CDK9 sumoylation. This modification impedes the binding between CDK9 and Cyclin T1, leading to the disruption of active P-TEFb assembly. MYC, through its independent interaction with CDK9 and UBC9/PIAS1, inhibits the association between CDK9 and UBC9/PIAS1, thereby preventing CDK9 sumoylation

(Figure 2). By facilitating the formation of the P-TEFb complex, MYC enhances the phosphorylation of Ser2 on the RNAP II CTD, promoting global transcription amplification through transcriptional elongation (Figure 2). The full extent of transcriptional functions of MYC depends on both its local and global effects, as well as its interactions with various transcriptional cofactors. In addition, the differences in transcriptional profiling and transformation potency observed between full-length MYC and truncated MYCs (Yu et al., 2018; Guan et al., 2023). MYC also indirectly amplifies transcription by inducing the expression of GCN5 that acetylates histones (chromatin opening) and PRPS2 that promotes nucleotide biosynthesis (McMahon et al., 1998; McMahon et al., 2000; Knoepfler et al., 2006; Cunningham et al., 2014).

A recent report (Patange et al., 2022) investigated the transcriptional kinetics and mechanisms through which MYC enhances gene expression in living cells. A light-controlled MYC protein was translocated from the cytoplasm to the nucleus upon blue light illumination, thereby controlling MYC activity in human cells. Photo-activation and RNA imaging enabled precise measurements of gene regulation and the MYC action on transcription factor dynamics and transcription amplification. Single-molecule fluorescence *in situ* hybridization (smFISH) in fixed cells and MS2-tagging of RNA in live cells were used to assess the immediate impact of MYC on transcription bursting. The findings demonstrate that MYC influences the length of time that other core transcriptional factors reside at promoters. Elevated MYC levels uniquely influence the dwell time of various transcriptional machinery complexes. The glucocorticoid receptor (GR) remained unchanged, while SPT5, TBP, and RNAP II exhibited increased dwell time, and MED1, a mediator component, showed decreased dwell time. Elevated MYC enhanced RNA output from its target genes and alterations in burst duration were attributed to changes in the residency of transcriptional machinery and hence altered transcription output. Overall, MYC universally extended the duration of transcriptional bursts (increased gene ON time, i.e., transcriptionally active state), without altering their frequency (Figure 2) (Patange et al., 2022). Although bursting duration was preferentially enhanced for genes with lower expression, it should be noted that the highly expressed genes, most likely, were pre-saturated with endogenous MYC.

MYC is primarily associated with transcription amplification, however, many reports have revealed that it also represses several genes. Most repression may represent an algorithmic artefact of RNA normalization by programs such as DE-seq2 when comparing samples. Yet a small number of MYC repressed targets survive the normalization correction and are truly repressed. The precise mechanism underlying transcriptional repression of MYC is not fully understood. However, it seems that MYC uses surrogates to affect repression. For example, MYC exploits transcription factors like MIZ-1 (Myc-interacting zinc-finger protein 1) or SP1 that recruit corepressor, or changes in chromatin accessibility driven by epigenetic modifications which lead to the displacement of DNA-bound coactivators to ultimately achieve gene repression (Seoane et al., 2001; Kurland and Tansey, 2008; Wiese et al., 2013; Walz et al., 2014; Lourenco et al., 2021). Further, interaction of MYC with PAF1c forms a repressive complex, inhibiting function of PAF1c as an elongation factor (Jaenicke et al., 2016). It is important to rule out the potential involvement of indirect mechanisms of repression that involve ability of MYC to amplify the expression of negative regulators of transcription, such as repressor genes and other repressive components such as microRNAs (Wolf et al., 2015; Poole and van Riggelen, 2017). Consequently, the activation of these repressive components could ultimately result in the repression of target genes. For instance, MYC has been shown to repress p53 by targeting p53-MDM2-ARF (Kung and Weber, 2022). MYC activates the expression of SENEBLOC, a lncRNA that acts as a scaffold to facilitate the binding of MDM2 with p53, leading to the downregulation of p53 (Xu et al., 2020). Furthermore, MYC also drives the expression of MILIP, another lncRNA that represses p53 by promoting p53 turnover by reducing p53 sumoylation (Feng et al., 2020). Therefore, it is essential to consider the indirect effects mediated by MYC-induced transcription amplification when studying the repression of MYC target genes (Lin et al., 2012; Nie et al., 2012).

Role of MYC in embryogenesis, cell cycle, proliferation, and apoptosis

As discussed above, MYC is an integral part of transcription progression, acting as a global amplifier, it is indispensable for both embryonic development and the maintenance of self-renewing tissues in adults (Yoshida, 2018). MYC proteins exert crucial functions mostly during embryogenesis and in tissue regenerative programs in adults (Dang, 2013; Asami et al., 2022; Asami et al., 2023). MYC was absolutely required for the immediate embryonic gene activation (iEGA). Inhibiting MYC during iEGA resulted in acute developmental arrest and caused a failure in activating approximately 95% of the upregulated genes. Further, it also changes the morphology of the embryo, and hindered the process of cytokinesis (Asami et al., 2023). In the absence of MYC, the failure of activation of 95% upregulated genes supports the notion that MYC acts as a global amplifier in developmental contexts (Lin et al., 2012; Nie et al., 2012; Nie et al., 2020). Studies have shown that knockouts of either MYC or MYCN do not survive embryonic development, whereas mice lacking MYCL are fertile and appear to develop normally (Charron et al., 1992; Stanton et al., 1992; Davis et al., 1993; Hatton et al., 1996). Mouse embryos lacking MYC experience prenatal mortality at E10.5 due to placental defects (Davis et al., 1993). However, when MYC was deleted in epiblast, the embryos demonstrate normal growth and survive until E11.5, and later develop hematopoiesis and die (Dubois et al., 2008). MYC is typically expressed at low levels, and elevated expression is almost always transient in normal cells (Levens, 2013). Deletion of certain enhancer regions that regulate MYC expression (discussed in regulation section) have examined a role for MYC in embryogenesis (Dave et al., 2017). Upon deletion of an enhancer region, MYC levels reduce by approximately 50%, but are still sufficient for normal development and tissue growth suggesting that the deleted regions were dispensable for MYC function in the placenta development and during early hematopoiesis. These mice were resistant to tumor formation suggesting that tumors demand elevated MYC levels (Dave et al., 2017). Moreover, the enhancer region known as BENC that regulates MYC abundance, plays a crucial role in precisely regulating hematopoiesis (Bahr et al., 2018).

These results support that physiological levels of MYC are a crucial factor in regulating embryogenesis.

MYC helps to regulate the cell-cycle and determine the rate of proliferation. Low MYC promotes growth of quiescence cells and controls cell cycle entrance and exit. The G1 and G2 phases of the cell cycle are lengthened in MYC-deficient rat fibroblasts compared to wild-type cells (Mateyak et al., 1997). MYC depletion using antisense oligodeoxynucleotides in human lymphoid and myeloid cells hinders entry into S-phase (Heikkila et al., 1987; Wickstrom et al., 1988). Depletion of MYC using short-hairpin RNA (sh-RNA) led to cell-cycle arrest in the G0/G1 phase in all non-transformed cells, whereas barring few, most transformed cells showed arrest in either the S phase or the G2/M phase (Wang et al., 2008). MYC regulates the expression of genes involved in cell-cycle control by activating the expression of positive regulators of cell-cycle such as Cyclin D, CDK (CDK1, 2, 4, 6), Cyclin E, Cyclin B. MYC also activates E2F target genes (Bretones et al., 2015; García-Gutiérrez et al., 2019). In addition, MYC also exerts its effect by inhibiting the negative regulators of the cell cycle, such as p15, p21, and p27 (Bretones et al., 2015; García-Gutiérrez et al., 2019). MYC represses p15 by forming a repressor complex with SP1 and SMAD in the presence of TGF-β (Seoane et al., 2001; Feng et al., 2016). Another prominent target of MYC is p21. The Interaction between MYC and MIZ-1 leads to the displacement of the transcriptional coactivators, resulting in the inhibition of MIZ-1 target genes like p21 (Wiese et al., 2013; Walz et al., 2014). Further, MYC induces the bHLH-LZ transcription factor AP4 which binds to p21 promoter and facilitates the transcriptional repression of p21 (Jung et al., 2008). It also represses p21 by activating the expression of microRNA miR-17-92 (Wong et al., 2010). MYC represses p27 at both the transcriptional and post-transcriptional levels reviewed in Ahmadi et al., 2021. MYC induces the expression of D-type cyclin, CDK4, CDK6, and components of the SCF^{SKP2} ubiquitin ligase complex, which direct the phosphorylation, degradation, and proteasome-mediated turnover of p27 (Montagnoli et al., 1999; Keller et al., 2007; Bretones et al., 2011). It should be noted that in no case has MYC been shown to directly block the expression of a cell-cycle repressor other than in specific combination with other transcription factors. Mostly simply, MYC regulates the cell-cycle, growth, and proliferation as a general amplifier of preexisting transcriptional programs inducing the expression of required genes in a timely manner.

Beyond its role in cell cycle growth and proliferation, MYC also plays a part in apoptosis. The involvement of MYC in apoptosis first became apparent in a study where elevated MYC led to apoptosis of growth factor-deprived fibroblasts (Evan et al., 1992). MYC controls apoptosis by modulating the balance between pro-survival and pro-apoptotic signals in the BCL pathway (McMahon, 2014). While modest increases in MYC levels led to increased cellular proliferation, higher MYC levels provoked apoptosis (Murphy et al., 2008). Even in normal physiological contexts, endogenous MYC was found to be an essential factor for apoptosis of self-reactive lymphocytes (Shi et al., 1992). Further, it has been shown that endogenous MYC is required for p53-mediated apoptosis in intestinal epithelial cells of mice (Phesse et al., 2014). These studies highlighted that endogenous levels of MYC maybe sufficient to induce apoptosis and based on cellular demands, nutrient levels, growth factors, *etc.* MYC can activate both p53-dependent and -independent apoptosis (Topham et al., 2015). In situations where pro-apoptotic genes are silent, the transcription of those pro-apoptotic genes must be primed before MYC further amplify their expression leading to apoptosis (Lin et al., 2012; Nie et al., 2012; 2020).

MYC in transcription and replication

MYC binds the genes transcribed by all three RNAPs- I, II, and III although with relatively lower binding to rRNA promoters (Gomez-Roman et al., 2003; Grandori et al., 2005; Oskarsson and Trumpp, 2005). MYC regulates the expression of non-coding transcripts by RNAP I and III, and most prominently mRNA expression by RNAP II (Baluapuri et al., 2019). The chromatin landscape of MYC binding sites indicates that it tends to bind primarily to active promoters or promoters linked to a preoccupied basal transcription apparatus. MYC exhibits a strong association with factors regulating RNAP II activity, including both promoter recruitment and activation of the polymerase. It directly binds to the TATA-binding protein (TBP), an essential component of the TFIID complex responsible for promoter recognition and pre-initiation complex assembly at the transcriptional start site (Wei et al., 2019). This interaction suggests a potential mechanism for TBP recruitment to MYC targets lacking a TATA box.

The rate-limiting step of transcriptional initiation, which involves the phosphorylation of Ser5 in the RNAP II C-terminal domain, is regulated by the recruitment of SPT5/SPT6, the two components of DSIF, through the influence of MYC. MYC interacts with SPT5, facilitating its recruitment to promoters and subsequent CDK7-dependent transfer to the RNAP II prior to transcription elongation. This process enables SPT5-loaded RNAP II to efficiently generate full-length transcripts through fast, continuous, and directed transcription (Baluapuri et al., 2019). When MYC is low (quiescent cells), the recruitment of SPT5 at RNAP II is insufficient, leading to a loss of directionality and processivity in RNAP II, which results in elevated production of antisense and abortive transcripts. However, it remains to elucidate the biological consequence of these antisense and abortive transcripts.

Further, MYC facilitates the formation of the P-TEFb complex and phosphorylation of Ser2 on the RNAP II CTD, to promote transcription elongation (Yu et al., 2018; Guan et al., 2023). MYC-dependent transcription activation also requires ubiquitination of MYC. It was shown that ubiquitylation of MYC is required to transfer of the PAF1c from the MYC to transcription elongation complex (otherwise repressive complex) onto RNAP II (Jaenicke et al., 2016). However, it remained unclear whether MYC ubiquitination alone was sufficient for the transfer or if it also required the involvement of P-TEFb. Excitingly, recently it has been shown that MYC recruits the PAF1c complex, and in conjunction with HUWE1-mediated ubiquitylation of MYC at the promoter, facilitates the transfer of PAF1c from MYC to RNAP II (Figure 2). This event triggers promoter escape and enables continuous elongation, which occurs downstream of the P-TEFb-dependent release of RNAP II from NELF inhibition (Endres et al., 2021). The elimination of MYC from genes is facilitated by E3-directed poly-ubiquitin pathways, which could be closely linked to its role in regulating transcription activation

and amplification. A recent study proposes that increased MYC leads to its invasion of super-enhancers (See et al., 2022). MYC utilizes various members of the KLF/SP transcription factor family, such as MAZ, ZBTB17, and EGR2 at super-enhancers. MYC interaction with super-enhancers increased the chromatin contact frequency across TADs boundaries. Further, increased MYC levels strengthen chromatin interactions between MYC binding sites at promoters and enhancers.

With MYC-driven transcription amplification, torsional stress builds up. If torsional stress is not resolved, it would quickly hinder the movement of RNAP II and stop bursts of transcription, as in bacteria (Chong et al., 2014). To maintain a high level of transcription, it is crucial to promptly reduce torsional stress (Jha et al., 2022). If MYC-driven transcription were accompanied by an increase in torsional resistance, the speed of transcription would slow down or even stop, counteracting any efforts made by MYC to boost transcription output. MYC topoisome, a recently discovered complex is a crucial regulator for the maintenance of transcription-induced torsional stress in such situations. MYC interacts with TOP1 and TOP2A and forms the MYC topoisome complex (Figure 2) in which the catalytic activities of both TOP1 and TOP2A are increased to facilitate transcription (Das et al., 2022). Notably MYCN forms a distinct topoisome incorporating TOP1 and TOP2B.

Apart from torsional stress, MYC-driven transcription amplification can also increase the chance of transcription-replication conflict. A recent finding shows that MYC forms multimers, which suppress transcription-replication conflicts (T-R conflicts) and DNA damage (Solvie et al., 2022). Through super-resolution microscopic analysis of the MYC distribution in cells revealed foci of MYC multimers. These multimers consisted of a dense MYC shell surrounding a weakly stained core. Regulators of proteasome inhibition, ubiquitylation, splicing, and transcription elongation were found to influence the formation of MYC multimers. MYC multimers drive away SPT5 from RNAP II, attenuating MYC-dependent transcription. FANCD2 and BRCA1, associated with stalled replication forks in multimers were localized near replication forks to prevent T-R conflicts. Further, HUWE1-mediated MYC polyubiquitylation drove multimerization, suppressing antisense transcription, replication-fork degradation, and double-strand DNA break formation (Solvie et al., 2022).

MYC has been shown to regulate rDNA transcription. MYC interacts with components of the SL1 complex, enhancing the association of TBP and TAF complex with the promoter and recruiting HATs to facilitate RNAP I recruitment and transcription at rDNA promoters. Consequently, the upregulation of UBF expression mediated by MYC positively influences the transcriptional activity of RNAP I, ultimately resulting in enhanced rRNA synthesis (Grandori et al., 2005; Grewal et al., 2005; Oskarsson and Trumpp, 2005). Sumoylation of MYC has been shown to regulate the MYC-mediated transcription by RNAP I as well. Sumoylation marks MYC for degradation through the proteasome pathway (Peng et al., 2019), this degradation mechanism counteracts the potential transcriptional MYC-mediated activation of RNAP I. It has been speculated MYC functions as a coordinator during differentiation, aligning the pool of active rRNA genes with the levels of RNAP I factors to tightly regulate rDNA transcription. This orchestration of gene expression ensures the proper synthesis of ribosomes to meet the changing needs of the cell throughout its differentiation process (Poortinga et al., 2011).

MYC proteins are intrinsically disordered proteins (IDPs). They tend to interact with different proteins simultaneously and has been speculated that MYC forms liquid-liquid phase separation when present at high concentration (Ann Boija et al., 2018). It has been reported that MYCN can form condensates that may be transcriptionally active, and the IDR and DNA binding domain of MYCN seem to be critical for such condensates in neuroblastoma cells (Yang et al., 2022). However, the impact of MYCN condensates on the transcriptome appears to be limited, as fewer than 6% of genes were altered among the numerous transcripts dependent on MYCN. Overall, further investigation is warranted to determine mechanisms involved for MYC condensate formation and explore its effect on gene regulation, and involvement in disease conditions if any.

Regulation of MYC

Due to its relatively unstable mRNA and protein, MYC acts as a highly efficient regulator of rapid cellular responses. MYC has one of the shortest mRNA half-lives, approximately 10–20 min (Dani et al., 1984) and protein half-lives, approximately 20 min (Hann and Eisenman, 1984), there are various mechanisms that have been shown to regulate MYC level. The regulation of MYC expression involves signalling pathways that operate at the transcriptional, post-transcriptional, and protein levels by a range of upstream and downstream mechanisms (Figure 3) (Levens, 2013). The MYC gene is transcribed from multiple promoters (P0, P1, P2, and P3), and uses different initiation sites, alternative polyadenylation sites, and the production of antisense transcripts (Nepveu et al., 1987; Chung and Levens, 2005). The mRNA transcribed by the P1 promoter represents 10%–25% of all *myc* mRNA transcripts, while the P2 promoter accounts for 75%–90% of the transcripts (Figure 3). Promoter P2 requires the presence of specific elements for initiating *c-myc* gene transcription (Hay et al., 1987; Moberg et al., 1991; Liu and Levens, 2006). The regulation of the *c-myc* locus involves DNA-level modulation through alternate non-B DNA structures (Levens, 2010). In the typical cellular environment, DNA primarily adopts the B-form, which is a classical right-handed double helix. However, a variety of non-B DNA structures have been reported both *in vitro* and *in vivo* with evident regulatory potential (Zaytseva and Quinn, 2018). One such example includes the Far Up Stream Element (FUSE) of the human MYC gene, the FUSE in the MYC promoter responds to negative supercoiling forces during transcription (Figure 3). Dynamic changes in DNA conformation are coupled with promoter output and are recognized by transcriptional factors, FIR (FUBP interacting repressor) and FUSE-binding protein (FUBP1). Transcription-generated DNA supercoiling induced melting of the FUSE region, recruits FUBP1 and the FIR to regulate the advancement of the transcription machinery through TFIIH activation. As transcription levels increase, FUBP1 facilitates progression through pausing, while further melting of FUSE recruits FIR, ultimately restoring MYC expression to basal levels (Figure 3) (Liu et al., 2006; Kouzine et al., 2008). Apart from FUBP1-FIR

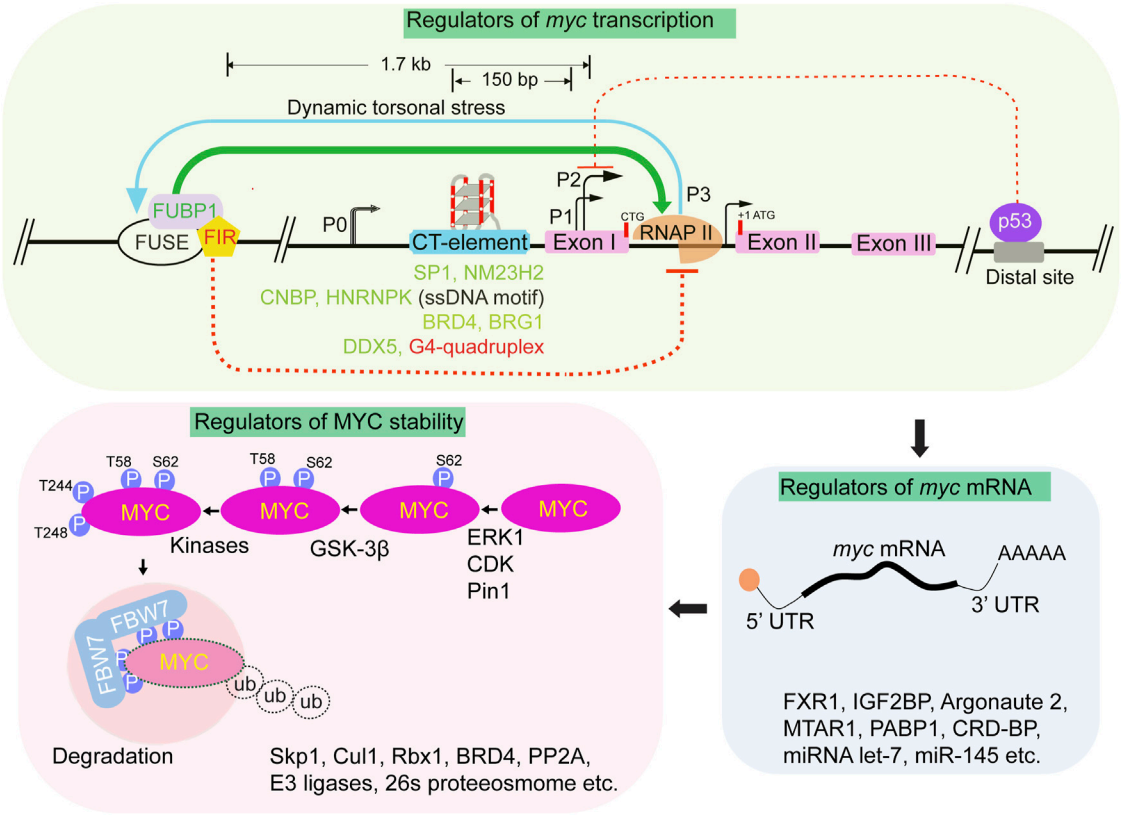

FIGURE 3
Regulation of MYC. Schematic depicting the various layers in regulation of MYC cellular levels. At the transcription level, multiple promoters (P0, P1, P2 and P3, not drawn on scale) participate in *myc* transcription. Primary *myc* transcription predominantly initiates from two major promoters, P1 and P2, contributing to roughly 10%–25% and 75%–90% of *myc* mRNA, respectively. The MYC promoter is regulated by two noncanonical cis-regulatory elements: FUSE and the CT element, induced by negative supercoiling generated during transcription activation. The FUSE element is located 1.7 kb upstream of P2, while the CT element is located between –100- and –150 bp upstream of P1. The FUSE element, which is AT-rich, melts in response to torsional stress caused by transcription activation. FUSE melting facilitates sequence-specific FUBP1 binding. Dynamic changes in DNA supercoiling regulates FUBP1 and FIR binding to the FUSE element with FUBP1 positively (Green arrow) and FIR negatively (Red dotted line) influencing *myc* transcription. The CT element which is GC-rich, facilitates the formation of alternate DNA structures. Numerous transcription factors like SP1, NM23H2, CNBP, HNRNPK, and DDX5 bind to CT element and regulate MYC transcription. Non-B DNA structures, such as G-quadruplex can form at CT elements and negatively regulate *myc* transcription. The binding of BRD4 throughout the promoter regions positively regulates *myc* transcription. Factors like Brg1 and BRD4 regulate myc transcription by influencing the interaction between enhancer and promoter regions. The binding of p53 to a distal region of MYC repress *myc* transcription. Multiple factors including RNA, RNA binding proteins and long noncoding RNAs (as indicated), regulate post-transcriptional regulation of *myc* mRNA. MYC levels are further regulated by various factors (as listed) and post-transcriptional modifications. MYC phosphorylation by known or unknown kinases at specific site sets the stage for MYC degradation. Phosphorylation of indicated sites recruit FBW7 dimer and forms the SCF complex consisting of Skp1, Cul1 and Rbx1 proteins followed by polyubiquitination of MYC and degradation by the 26s proteosome.

mediated regulation, the negative supercoiling generated during transcription can induce dynamic changes and facilitate the formation of G-quadruplexes (G4s) in the CT element region of the MYC promoter. G4 structure forms in the MYC promoter region and may impede MYC transcription by obstructing the binding of transcriptional factors, including double-stranded factor SP1, single-stranded factors CNBP, and hnRNPK (Figure 3) (Michelotti et al., 1996a). A study shows that DDX5, a potent resolvase of DNA and RNA G4s structures, unfold G4 at the MYC promoter and hence increases the MYC transcription in the cell (Wu et al., 2019, PNAS). However, the role of G4 is uncertain as it has also been claimed to activate MYC transcription (Hänsel-Hertsch et al., 2016).

These multiple transcription factors and chromatin regulators have been shown to regulate MYC expression in response to various signals. Fine-tuned control of MYC expression is dependent on sets of enhancers positioned both upstream and downstream of the gene. The *c-myc* gene is positioned within approximately a 3 Mb region that lacks other protein-coding genes and corresponds to a single topologically associating domain (MYC-TAD). The MYC-TAD harbors a multitude of super-enhancer regions that intricately regulate the expression of the MYC (Sur et al., 2012; Kieffer-Kwon et al., 2013; Uslu et al., 2014; Yashiro-Ohtani et al., 2014). These enhancers include tissue-specific enhancers that respond to diverse stimuli, along with super-enhancers (Lancho and Herranz, 2018). Removal of an enhancer present over half a megabase of DNA upstream of the *c-myc* gene (one of several different regions that have been called super-enhancers) led to a ~50% reduced MYC level (Dave et al., 2017). A MYC super-enhancer located approximately 1.7 Mb downstream of the transcription start site plays a critical role in tightly controlling MYC expression and promoting increased

chromatin accessibility (Shi et al., 2013; Mifsud et al., 2015; Bahr et al., 2018; Jia et al., 2019). The enhancer region (termed BENC) is required for MYC expression, it consists of enhancer modules that are specific to cell lineages. When these modules are deleted, it results in the downregulation of MYC expression in a cell-type-specific manner precisely correlating with gene expression (Bahr et al., 2018).

It has been shown that p53 (tumor-suppressor) regulates the expression of MYC by binding to ~50 kb downstream of the *c-myc* locus. It has been suggested that p53 binding at this site represses MYC through the involvement of a MYC enhancer (Figure 3) (Porter et al., 2017). A recent study shows that ATM represses MYC expression by promoting transcriptional-induced DNA repair at the MYC enhancer region (Najnin et al., 2023). Further, MYC regulation through enhancers appears to be a complex process and involves multiple regulatory elements, chromatin remodeling factors, RNA, and RNA binding proteins (Figure 3). For instance, FXR1 (RNA binding protein) binds to the AU rich elements (ARE) within the 3′ UTR of *myc* mRNA and improves its stability (George et al., 2021). The RGG domain of FXR1 interacts with eIF4A1 and eIF4E and facilitates recruitment of the eIF4F complex to translation initiation sites for cMYC translation ultimately increasing the total level of MYC in the cells (George et al., 2021). Another RNA binding protein, IGF2BP, can recognize and bind m^6A modified-*myc* mRNA to regulate its translation (Huang et al., 2018). MTAR1, a long noncoding RNA has been shown to facilitate IGF2BP-meditated MYC translation (Gao et al., 2022). Further, a point mutation within the intron of long noncoding RNA CCDC26 plays a role in regulating MYC expression (Yanchus et al., 2022). A risk SNP allele in a brain specific enhancer almost 2 megabase 3' of MYC, rs55705857(G), disrupts OCT2/4 binding that otherwise decreases interactions with the MYC promoter. Consequently, this SNP positively influences the regulation of MYC expression (Yanchus et al., 2022). The RNA-binding protein Argonaute 2, known for its involvement in the RNA-induced silencing complex, has been found to directly bind and stabilize *myc* mRNA (Zhang et al., 2020). RNA-binding protein hnRNPK, also controls MYC expression by directly binding to the CT-element and interacting with the transcription machinery (Figure 3) (Michelotti et al., 1996a; Michelotti et al., 1996b). Further, a recent study shows that RNA molecules originating from both MYC enhancers and promoter interact with the hnRNPK. Through its oligomerization, hnRNPK brings the MYC enhancer and promoter in proximity, thereby facilitating the elevated level of MYC (Cai et al., 2020).

The MYC amplifier role is dependent on cellular MYC levels. Slight increases in MYC levels have been shown to release cells from cell-cycle arrest, promote proliferation or trigger apoptosis. MYC levels have been observed to show an inverse correlation with cell cycle length and a direct correlation with organism size within a species (Murphy et al., 2008; Shachaf et al., 2008). Studies utilizing genetic approaches in *Drosophila* have demonstrated using developmental compartments containing a mixture of normal cells with cells expressing either double or half the normal levels of MYC, elimination of the lower MYC cells. High-MYC cells then expand, refill the compartment, and undergo normal development (De La Cova et al., 2004; Moreno and Basler, 2004; Johnston, 2014; Topham et al., 2015). The elimination of low-MYC cells in favor of high-MYC cells is termed supercompetition and underscores the critical importance of MYC levels in determining cellular fate. A recent study using exogenously expressed MYC tagged with the fluorescent protein mNeonGreen (mNG) showed that MYC expression is pulsatile, heterogeneous, and dependent on MAPK and Wnt signaling pathways (Liu et al., 2023). The heterogeneous expression of MYC leads to variable gene transcription and variable cell-cycle progression rates. Cells with high MYC, progress to S-phase rapidly and cells with low MYC have increased G0/G1 length, and so transcriptome diversity arises in the previously homogenous population. MYC, which regulates G0/G1 length and other processes, influences sensitivity to chemotherapy drugs. Reduction in MYC protein levels during doxorubicin (a chemotherapeutic agent that target topoisomerase II) treatment increased the number of surviving cells. Cells with transiently low MYC levels at the time of drug treatment were more likely to survive and proliferate. Even among cells that remained in G0/G1 throughout drug treatment, those with lower MYC immediately after treatment had higher chances of survival and proliferation. This indicates that low MYC levels limit DNA damage during gene expression. It is suggested that increasing heterogeneity of MYC may be advantageous for cancers (Liu et al., 2023). However, whether normal cells also possess heterogeneity in MYC expression and the consequences of that heterogeneity in normal physiological conditions is a matter of investigation.

The level of MYC in cells is also controlled by post-translational mechanisms such as MYC phosphorylation which plays a crucial role in controlling its turnover and degradation as recently reviewed (Sun et al., 2021). The highly conserved serine and threonine residues in MBI T58, S62, S64 and S67 undergo phosphorylation (Welcker et al., 2004). ERK kinase phosphorylates S62 within the MYC transactivation domain and enhances the stability of MYC. In contrast, GSK3β or BRD4 kinases phosphorylates MYC at threonine 58 (T58) promotes degradation of MYC (Sears et al., 2000). The dually phosphorylated form of MYC, with both S62 and T58 phosphorylation, is recognized by the phosphatase PP2A which removes S62 phosphorylation, and this event primes the recruitment of an E3 ubiquitin ligase called FBW7 (F-box/WD repeat-containing protein 7). FBW7 recognizes phosphorylated MYC and facilitates its ubiquitination, marking it for proteasomal degradation (Sears et al., 2000; Yeh et al., 2004; Arnold and Sears, 2006). However, this long-standing model for MYC degradation has been countered by a recent finding, where authors show phosphorylation of S62 does not stabilize MYC by preventing FBW7 from binding to it (Welcker et al., 2022). Instead, it enhances the interaction between MYC and FBW7, leading to degradation of MYC. Furthermore, a previously unknown dephosphorylated degron at residues T244/T248 was identified that also promotes MYC binding to FBW7. This additional degron acts alongside the T58/S62 phosphorylation to regulate MYC protein levels (Figure 3) (Welcker et al., 2022). This finding supports that stabilizing effects of pS62 may be independent of FBW7 binding (Vaseva et al., 2018) and highlight the complexity of MYC regulation and suggest that S62 phosphorylation has multiple roles beyond FBW7 binding, influencing MYC stability and function. BRD4 also regulates MYC levels by both degradation and transcriptional activation of MYC (Figure 3) (Devaiah et al., 2020). Given the significant impact of MYC levels on cellular behavior, it is crucial to understand the underlying mechanistic processes and how MYC levels are regulated. These questions remain a subject of intense investigation.

Approaches to tackle MYC

MYC is elevated in most cancers and several other pathological conditions, and so has been proposed as a drug target for decades. However, due to MYC being a general transcription amplifier in both normal and cancerous cells, directly targeting it has proven challenging. Further, MYC has "undruggable characteristics" such as the absence of an enzymatic pocket for small molecules to bind, and its predominantly nuclear localization hinders antibody access. Recent promising studies highlighted that partial depletion or inhibition of MYC may be sufficient for treatment of MYC-dependent cancers and other diseases (Hofmann et al., 2015; Wang et al., 2021). The current approaches to tackle MYC-dependent pathogenesis fall into various categories such as downregulating MYC at the transcriptional or post-transcriptional levels, and hindrance of MYC-MAX interaction.

There are many approaches to inhibit MYC at the transcription level. Inhibitors like QN-1, a difluoro-substituted quinoxaline, APTO-253, a selective p21 inducer, and CX-3543, quarfloxin, are G-quadruplex stabilizers. These inhibitors specifically stabilize the G-quadruplex at the MYC promoter and in turn repress MYC expression (Cercek et al., 2015; Local et al., 2018; Hu et al., 2019; Paul et al., 2020). Although, APTO-253 was in clinical trial for acute myeloid leukemia and high-risk myelodysplastic syndrome, it was terminated due to it lack efficacy in a phase 1. MYC expression can be targeted by inhibiting factors that activate MYC transcription, such as DDX5, BRD4, and SWI/SNF. DDX5 has been shown to activate MYC transcription by resolving G-quadruplex formation at the MYC promoter (Wu et al., 2019), thus inhibiting DDX5 might have a favorable effect on MYC-dependent diseases. Inhibitors like AZD5153, GSK525762, JQ1, and dBET1 repress MYC expression by targeting BRD4 (Wang et al., 2021). Brg1, an ATPase subunit of SWI/SNF positively regulates MYC expression by binding to an enhancer region of MYC (Shi et al., 2013). Knockdown of Brg1 or its inhibition with an ATPase inhibitor BRM014 disrupts the BENC enhancer cluster and represses MYC expression (Shi et al., 2013; Bahr et al., 2018; Rago et al., 2022; Chambers et al., 2023). These results promise continued development of SWI/SNF inhibitors in the treatment of MYC-dependent cancers and other diseases.

Another approach is to target MYC protein by direct binding-ligands. Despite, MYC lacking a precise ligand binding pocket, a recent study has emphasized the effectiveness of covalent ligand compounds that target IDR regions of MYC. For instance, EN4 is a compound that primarily interacts with cysteine (C171) within the IDR region of MYC, thereby reducing the thermal stability of MYC-MAX dimerization and subsequently its function (Boike et al., 2021). MYC functions have been indirectly challenged by targeting MYC- MAX heterodimerization. KI-MS2-008 is a drug that stabilizes the MAX homodimer to prevent MYC-MAX interaction (Struntz et al., 2019). Similarly, Omomyc (bHLH-zip domain of MYC with 4 mutations) binds to MYC bHLH-zip domain and prevents its interactions. MYCi975 is a small molecule inhibitor, which binds MYC directly to disrupt MYC-MAX interaction and increases the proteasomal degradation of MYC, and thus leads to decreased tumor growth (Han et al., 2019; Truica et al., 2021). Further, MYCi975 alters the binding of MYC as well as MYC network proteins like MAX to chromatin (Holmes et al., 2022). While the prospect of disrupting the MYC-MAX heterodimer, either by dismantling it or occupying the binding interface between the two proteins, holds promise as an alternative strategy for targeting MYC, it is important to note that the complete inhibition of MYC function by dimerization inhibition could have adverse effects on normal cells. Therefore, another approach could be the targeting of the MYC-partners that mediate its function. Recently, a specific TFIIS N-terminal domains (TNDs) and unstructured TND-interacting motifs (TIMs) binary interaction module has been established, and this module is conserved for many transcription factors including PP1-PNUTS5 (Cermakova et al., 2021; Cermakova et al., 2023). MYC protein is stabilized by the PP1 phosphatase and its regulatory subunit PNUTS. It has been shown that PNUTS interacts with MB0, and controls MYC phosphorylation, chromatin eviction, and MYC degradation. Disrupting the PNUTS-MYC interaction would enhance MYC degradation (Wei et al., 2022). This could be a new avenue to explore to limit MYC function and MYC-dependent pathological activity.

Future perspective

MYC protein is a crucial transcription regulator that plays a central role in regulating gene expression in different cellular situations. Its capacity to amplify transcriptional responses contributes to the precise control of cellular processes and the maintenance of a balanced state within cells. It is not known whether MYC exerts its pathological action from an augmentation of its normal transcription amplifier role or whether MYC neopathologic functions are elicited at supraphysiological levels. It is important to understand mechanistically how MYC regulates different kinetic steps of transcription by all three RNAPs. A deeper understanding of the mechanisms through which MYC amplifies transcription, and the factors that influence this process in physiological and pathological conditions will enhance our knowledge of gene regulation and offer valuable insights for developing targeted therapeutic approaches for MYC-related disorders.

Author contributions

RJ: Conceptualization, Visualization, Writing–original draft. FK: Conceptualization, Visualization, Methodology, Validation, Writing–review and editing. DL: Conceptualization, Visualization, Writing–review and editing, Funding acquisition, Project administration, Resources, Supervision, Writing–original draft.

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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.

The reviewer SP declared a past co-authorship with the author DL to the handling editor.

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Differential regulation of MYC expression by *PKHD1/Pkhd1* in human and mouse kidneys: phenotypic implications for recessive polycystic kidney disease

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Autosomal recessive polycystic kidney disease (ARPKD; MIM#263200) is a severe, hereditary, hepato-renal fibrocystic disorder that leads to early childhood morbidity and mortality. Typical forms of ARPKD are caused by pathogenic variants in the *PKHD1* gene, which encodes the fibrocystin/polyductin (FPC) protein. MYC overexpression has been proposed as a driver of renal cystogenesis, but little is known about MYC expression in recessive PKD. In the current study, we provide the first evidence that MYC is overexpressed in kidneys from ARPKD patients and confirm that MYC is upregulated in cystic kidneys from *cpk* mutant mice. In contrast, renal MYC expression levels were not altered in several *Pkhd1* mutant mice that lack a significant cystic kidney phenotype. We leveraged previous observations that the carboxy-terminus of mouse FPC (FPC-CTD) is proteolytically cleaved through Notch-like processing, translocates to the nucleus, and binds to double stranded DNA, to examine whether the FPC-CTD plays a role in regulating *MYC/Myc* transcription. Using immunofluorescence, reporter gene assays, and ChIP, we demonstrate that both human and mouse FPC-CTD can localize to the nucleus, bind to the *MYC/Myc* P1 promoter, and activate *MYC/Myc* expression. Interestingly, we observed species-specific differences in FPC-CTD intracellular trafficking. Furthermore, our informatic analyses revealed limited sequence identity of FPC-CTD across vertebrate phyla and database queries identified temporal differences in *PKHD1*/*Pkhd1* and *CYS1*/*Cys1* expression patterns in mouse and human kidneys. Given that cystin, the *Cys1* gene product, is a negative regulator of *Myc* transcription, these temporal differences in gene expression could contribute to the relative

renoprotection from cystogenesis in *Pkhd1*-deficient mice. Taken together, our findings provide new mechanistic insights into differential mFPC-CTD and hFPC-CTD regulation of MYC expression in renal epithelial cells, which may illuminate the basis for the phenotypic disparities between human patients with *PKHD1* pathogenic variants and *Pkhd1*-mutant mice.

KEYWORDS

ARPKD, MYC, FPC, cystin, PKHD1, Cys1

1 Introduction

The *MYC* proto-oncogene, encoding the MYC transcription factor, was first identified in patients with Burkitt's lymphoma (Taub et al., 1982). MYC contributes to the regulation of multiple cellular signaling pathways involved in cell proliferation (Gearhart et al., 2007). Aberrant MYC expression induces malignant transformation of several tumor types (Dang, 2012; Gabay et al., 2014). In addition, MYC increases the expression of inflammatory and fibrotic factors, which may significantly contribute to the pathogenesis of cystic kidney diseases (Nevzorova et al., 2013; Karihaloo, 2015; Shen et al., 2017). MYC overexpression in renal epithelia has been reported in several mouse ADPKD models as well as in *cpk* mice (Cowley et al., 1991; Burtey et al., 2008; Kurbegovic and Trudel, 2013; Wu et al., 2013). Elevated MYC appears to be a signature of renal cystic disease and may define a causative pathway (Trudel, 2015; Parrot et al., 2019). However, the role of MYC activation in the initiation and progression of autosomal recessive polycystic kidney disease (ARPKD; MIM#263200) remains incompletely understood.

ARPKD is a hereditary hepato-renal fibrocystic disorder with an estimated incidence of 1 in 26,500 live births (Guay-Woodford et al., 2014; Alzarka et al., 2017). Pathogenic variants in the polycystic kidney and hepatic disease 1 (*PKHD1*) gene, located on chromosome 6p21.1, cause all typical forms of human ARPKD. The longest *PKHD1* (MIM#606702) open reading frame (ORF) contains 67 exons, which encode a 4,074 amino acid protein called fibrocystin/polyductin (FPC) (Onuchic et al., 2002; Ward et al., 2002). Full length FPC is a single transmembrane (TM) domain protein predicted to have several immunoglobulin-like IPT/TIG conserved domains, two G8 domains, and multiple parallel beta-helix 1 (PbH1) repeats in a long extracellular segment (3,858 amino acids), and a short (192 amino acids) cytoplasmic C-terminal domain (CTD) (Supplementary Figure S5A) (Sharp et al., 2005; Wang et al., 2007). The *PKHD1* mRNA is primarily expressed in the kidney, liver, lung, and pancreas (Onuchic et al., 2002; Ward et al., 2002; Xiong et al., 2002). In adult and fetal human tissues, FPC is expressed in renal collecting ducts, thick ascending limbs of loops of Henle, bile ducts, pancreatic ducts, epididymis, and testis (Ward et al., 2003; Menezes et al., 2004). Pathogenic sequence variants in the *PKHD1* gene account for more than 80% of human ARPKD cases (Bergmann, 2017). Less than 1% of ARPKD patients have pathogenic sequence variants either in *DZIP1L* or *CYS1* genes (Lu et al., 2017; Yang et al., 2021), and the molecular cause of ARPKD in remaining patients remains to be determined (Bergmann, 2017).

Pkhd1 is the mouse ortholog of *PKHD1*. The longest ORF of mouse *Pkhd1* also contains 67 exons and encodes a 4,059 amino acid protein. The mouse and human FPC sequences are 73% identical overall but the CTD share only 55% identity (Nagasawa et al., 2002). Mouse FPC is also a single TM domain protein with 3,872 amino acid N-terminal segment and a short (187 amino acids) cytoplasmic CTD. Mouse FPC has the same numbers of conserved IPT/TIG and G8 domains as human FPC (Nagasawa et al., 2002; Bergmann, 2017). The intracellular domain of mouse FPC (mFPC-CTD), contains an 18-residue long ciliary targeting signal (CTS) that facilities delivery to the primary cilium (Follit et al., 2010). The mFPC-CTD, encoded by exons 65-67, undergoes Notch-like processing followed by regulated membrane-release and translocation to the nucleus (Kaimori et al., 2007), which is facilitated by the 25-residue long nuclear localization signal (NLS) (Hiesberger et al., 2006). Single particle electron microscopy analysis revealed that FPC-CTD forms a ring-like protein complex that binds to double stranded DNA, suggesting a role in gene expression regulation (Cameron Varano et al., 2017). Yet, the function of FPC-CTD in the nucleus remains poorly understood. Numerous rodent models of ARPKD with mutations and multiple exon deletions in *Pkhd1* have been generated. However, these models express minimal or no renal disease (Katsuyama et al., 2000; Ward et al., 2002; Masyuk et al., 2004; Moser et al., 2005; Garcia-Gonzalez et al., 2007; Woollard et al., 2007; Gallagher et al., 2008; Kim et al., 2008; Williams et al., 2008; Hu et al., 2011; Outeda et al., 2017; Ishimoto et al., 2023).

The most widely studied mouse model of ARPKD, the *cpk* mouse carries a spontaneous insertion/deletion (indel) mutation in the *Cys1* gene, encoding the cystin protein (Hou et al., 2002; Guay-Woodford, 2003; Nagao et al., 2012). The renal phenotype of *cpk* mice closely resembles human ARPKD. Mouse cystin, the product of the *Cys1* gene, is a 145-amino acid, cilia-associated protein that is mainly expressed in mouse kidney and liver ductal epithelium as early as embryonic day 14.5 (Tao et al., 2009). Mouse cystin contains a predicted N-myristylation motif (MGSGSSR) and a NLS located in the first 27 amino acids at the N-terminus. Amino acids 28-35 of mouse cystin contain a cilium-targeting motif (AxEGG) that is required for cystin trafficking to the primary cilium (Tao et al., 2009). Our previous demonstration that cystin suppresses *Myc* transcription by binding to necdin, an activator of the *Myc* P1 promoter, links renal cystogenesis in *cpk* mice to *Myc* activation and enhanced MYC levels (Wu et al., 2013; Yang et al., 2021).

Human *CYS1* encodes Cystin-1, a 158-amino acid protein (Fliegauf et al., 2003). Sequence comparison of human and mouse orthologs Cystin-1 and cystin shows 57% identity and 64% similarity (Fliegauf et al., 2003). Initial analysis of *CYS1* expression in adult human tissues revealed high *CYS1* mRNA abundance in the kidney and pancreas (Fliegauf et al., 2003).

Subsequent RNA-seq analysis of human tissues consistently revealed high *CYS1* mRNA levels in the kidney and lower expression in several other tissues including ovary, gall bladder, endometrium, pancreas, and lung (Fagerberg et al., 2014). The function of Cystin-1 is not understood, although we have reported the first genetic defect in human *CYS1* that causes the renal ARPKD phenotype (Yang et al., 2021).

In the current study, we employed immunofluorescence imaging as well as bioinformatic, molecular, and biochemical analyses to comparatively evaluate the roles of human and mouse FPC-CTDs in the regulation of MYC expression in human and mouse renal epithelia.

2 Materials and methods

2.1 Human samples

All human studies were approved by the Institutional Review Board at the Children's National Hospital or the University Hospital of Cologne. Human kidney samples were obtained from the NIDDK-funded UAB Childhood Cystic Kidney Disease Center Translational Resource at the University of Alabama at Birmingham and the University Hospital of Cologne. Kidney samples were obtained from patients ranging from 26 weeks of gestation age to 3 years of age (Supplementary Table S1).

2.2 Animal study approval

All mouse experiments were approved by the Institutional Animal Care and Use Committees at Children's National Research Institute, and experiments were carried out in accordance with relevant guidelines and regulations. Mouse colonies were maintained in the animal facility at Children's National Research Institute. All mouse kidneys were harvested from 14-day-old, 10- and 12-month-old mutants and age-matched wild-type (WT) littermates. Genetic background information for all mouse lines used in this study is shown in Supplementary Table S2.

2.3 Antibodies

All antibodies used for this study are listed in the Supplementary Table S3, unless specified in the text.

2.4 Immunohistochemistry (IHC)

Immunohistochemical staining for MYC was performed on formalin-fixed, paraffin embedded tissues using heat induced epitope retrieval solution (BOND Epitope Retrieval Solution 2, Leica Biosystems, Cat. No. AR9640) and an automated stainer (Bond-Max, Leica Biosystems). Tissues were incubated with anti-MYC antibody (Recombinant Anti-c-Myc antibody [Y69] - ChIP Grade, Abcam) at 1:25 dilution for 120 min.

2.5 Immunoblotting

Cultured cells and kidney tissues were collected, homogenized, and processed for immunoblotting as previously described (Wu et al., 2013; Dafinger et al., 2020). Immuno-reactive protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Cat. No. 34095) and images were obtained with ChemiDoc Imaging System (Bio-Rad laboratory). Densitometry was performed using Image Lab software (Bio-Rad laboratory, Version 6.0).

2.6 RNA extraction and qRT-PCR

Kidney tissue samples from 14-day-old male mice were snap frozen, transferred to a gentleMACS M tubes (Miltenyi Biotec, Cat. No. 130-093-236, RRID:SCR_020269) in Buffer RLT plus 2-Mercaptoethanol (as per RNeasy Mini Kit instructions, Qiagen, Cat. No. 74104), and homogenized using a gentleMACS Dissociator (Miltenyi Biotec) per the manufacturer's program RNA-02. Homogenized samples were transferred to microcentrifuge tubes for total RNA extraction using the RNeasy Mini Kit according to the manufacturer's instructions. Total RNA from both 5-day-postconfluent mIMCD-3 cells stably expressing FPC-CTD and hTERT-immortalized human renal epithelial cells (hTERT-HRE) transiently transfected with FPC-CTD were isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated total RNA was treated with RQ1 RNase-Free DNase (Promega, Cat. No. M6101), and then repurified using the RNeasy Mini kit. RNA samples were reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Cat. No. 18080400) and oligo dT primers as described in the manufacturer's instructions.

Quantitative RT-PCR was performed on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) using the default program. The PCR was performed with cDNA templates using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Cat. No. 4368706) and mouse primers specific for sequences of *Myc* (forward: 5′- GCC CCC AAG GTA GTG ATC CT -3'; reverse: 5′-GTG CTC GTC TGC TTG AAT GG -3′). Peptidylprolyl isomerase A (*PPIA*) was used for normalization (forward: 5′- AGC ACT GGA GAG AAA GGA TT -3'; reverse: 5′- ATT ATG GCG TGT AAA GTC ACC A-3′) (Arensdorf and Rutkowski, 2013). Overexpression of FPC-CTD in cell lines was confirmed with *Pkhd1* exon 66–67 specific primers (forward: 5′-CCA GAA GAC ATA TCT GAA TCC CAG GC-3'; reverse: 5′-AGC AAG AGA TCC TGG AAC ACA GGT-3′). Results were analyzed using QuantStudio Real-Time PCR Software and the ΔΔCt method (Livak and Schmittgen, 2001).

2.7 Conservation analysis of vertebrate *Pkhd1* gene products (fibrocystin/polyductin (FPC)) using bioinformatics tools

Protein sequences of FPC were collected from NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/) using an

advanced search with gene name, *Pkhd1* and taxonomic groups Mammalia, Aves, Reptilia, Amphibia, Caecilians, and Fish. This resulted in 102 FPC sequences from mammals, birds, reptiles, amphibians, and fish (Supplementary Table S4). FPC sequence from each species was verified by protein alignment with human FPC; the protein sequences that were significantly shorter than human FPC sequence were removed. WebLogo 3 (Crooks et al., 2004) was used to visualize FPC sequence alignment (Supplementary Figure S1) that was generated with Clustal Omega (Sievers et al., 2011). The conservation scores of FPC amino acids were extracted from the WebLogo 3 raw data (Supplementary Table S5). Conserved domains in FPC were mapped by Conserved Domain Database (CDD) (Lu et al., 2020).

Prediction of nuclear localization signals (NLSs) were performed with the human and mouse FPC-CTD construct sequences using SeqNLS (Lin et al., 2012) with 0.86 as the cut-off score.

2.8 Plasmid construction

pcDNA5/FRT/TO (pcDNA5) was obtained from Thermo Fisher Scientific (Cat. No. V652020).

pcDNA5/FRT/TO-mPkhd1-CTD-V5 (pcDNA5-mFPC-CTD): the cytoplasmic tail of mouse FPC (Follit et al., 2010) expression construct was generated from pcDNA5/FRT/TO-mPkhd1 (full length) -V5 (gift from Dr. Feng Qian) with site-directed mutagenesis (SDM) using 5′- GCT AAC TGG ACA TGA TGC TTT GCT GCT GGT TTA AGA AAA GC -3′ and 5′- GCT TTT CTT AAA CCA GCA GCA AAG CAT CAT GTC CAG TTA GC -3′ primer set.

pcDNA5/FRT/TO-mPkhd1-CTD^{delCTS}-V5 (*pcDNA5-mFPC-CTD^{delCTS}*): the ciliary targeting sequence (Follit et al., 2010) deleted mFPC-CTD expression construct was made from mFPC-CTD by SDM with 5′- GCT AAC TGG ACA TGA TGC TTG ACA TAT CTG AAT CCC AGG CT -3′ and 5′- AGC CTG GGA TTC AGA TAT GTC AAG CAT CAT GTC CAG TTA GC -3′ primer set.

pcDNA5/FRT/TO-hPKHD1-CTD-V5 (pcDNA-hFPC-CTD): the expression construct containing the hFPC-CTD, comparable to mFPC-CTD (Follit et al., 2010), and fused to V5-tag was made by LifeSct LLC. The hFPC-CTD coding sequence was cloned between *KpnI* and *NotI* sites in pcDNA5/FRT/TO.

pcDNA5/FRT/TO-hPKHD1-CTD^{delCTS}-V5 (*pcDNA-hFPC-CTD^{delCTS}*): the ciliary targeting sequence deleted hFPC-CTD expression construct was made from hFPC-CTD by SDM using 5′- CCG TGG ACA GAA TGA CTG CCG AGA TTC CTG AAT CCC AGA C -3′ and 5′- GTC TGG GAT TCA GGA ATC TCG GCA GTC ATT CTG TCC ACG G -3′ primer set.

pGL4.22 [luc2CP/Puro] vector was purchased from Promega (Cat. No. E6771).

pRL-TK vector was purchased from promega (Cat. No. E2241).

pGL4.22-mouse Myc P1 (pGL4.22-mMyc P1): the mouse *Myc* P1 promoter (chr8:127735983-127736125, GRCm38/mm10 mouse genome assembly) construct was described previously (Wu et al., 2013).

pGL4.22-human MYC P1 (pGL4.22-hMYC P1): the human *MYC* P1 promoter (chr15:61985298-61985433, GRCh38/hg38 human genome assembly), which is comparable to the mouse *Myc* P1 promoter, was amplified by PCR from HEK293 genomic DNA and cloned into pGL4.22 vector at *XhoI* and *HindIII* sites using 5′- CCG CTC GAG GAG GGC GTG GGG GAA AAG A-3′ and 5′- CCC AAG CTT AGC CAG GGA CGG CCG G -3′ primer set. Sequence alignment of human and mouse *MYC/Myc* P1 promoter shown in Supplementary Figure S2 was created by Clustal Omega (Sievers et al., 2011).

2.9 Cell culture and generation of stable cell lines expressing FPC-CTDs

Mouse TERT immortalized cortical collecting duct (mTERT-CCD) cells (Steele et al., 2010) were cultured in TERT culture medium [DMEM/F-12 medium (Thermo Fisher Scientific, Cat. No. 11330057) containing 5% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Cat. No. S11050H), 1% penicillin/streptomycin (Thermo Fisher Scientific, Cat. No. 15140163), 1x Insulin-Transferrin-Selenium solution (Thermo Fisher scientific, Cat. No. 41400045), 0.2 μg/mL dexamethasone (Sigma-Aldrich, Cat. No. D8893) and 10 nM 3,3′,5-Triiodo-L-thyronine sodium salt (Sigma-Aldrich, Cat. No. T6397)] at 37°C in 5% CO_2.

Mouse inner medullary collecting duct (mIMCD)-3 cells were purchased from American Type Culture Collection (ATCC, Cat. No. CRL-2123) and cultured in complete growth medium (CGM) [DMEM/F-12 medium (Thermo Fisher Scientific, Cat. No. 11330057) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Cat. No. S11050H) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Cat. No. 15140163)] at 37°C in 5% CO_2.

mIMCD-3 mFPC-CTD stable cell lines were generated by transfection of either pcDNA5-mFPC-CTD or pcDNA5 into mIMCD-3 cells with Lipofectamine2000 transfection reagent (Thermo Fisher Scientific, Cat. No. 11668019). At 48 hrs post-transfection, cells with spontaneously integrated plasmids were selected with hygromycin (1 mg/mL) (Thermo Fisher Scientific, Cat. No. 10687010) for 1 week. The mFPC-CTD stably overexpressing cells and the control empty vector cells were then maintained in CGM with hygromycin (200 μg/mL).

2.10 Generating human TERT-immortalized renal epithelial cell line (hTERT-HRE)

Human kidney sections were minced and immediately placed in 1% collagenase type I (Sigma, Cat. No. C0130-1G) in DMEM/F12 (Thermo Fisher, Cat No. 11330032) and incubated on a rotator for 30 min at room temperature (RT). Renal epithelial cells and tubule fragments were then transferred to a conical tube containing DMEM/F12 and centrifuged at 750 *g* for 10 min. The supernatant was removed, and the tissue was resuspended in a complete medium, containing 0.2 mg/mL dexamethasone (Sigma, Cat. No. D8893-1 MG), 5% heat inactivated FBS (Hyclone, Cat. No. SH30396-03), 2 mM glutamate (Thermo Fisher, Cat. No. 25030081), 1x insulin-transferrin-sodium selenite (ITS) (Thermo Fisher, Cat. No. 1400045), 100 U/mL penicillin/streptomycin (Thermo Fisher, Cat. No. 15140122), and 10 nM triiodothyronine (Sigma, Cat. no. T6397-100 MG) in DMEM/F12. No antibiotics were added to the complete medium in preparation for the transduction with the

hTERT lentiviral expression construct. Cells were maintained in a 37°C humidified incubator with 5% CO_2.

Lenti-hTERT-Neo Virus (Cat. No. LV622), Lenti-p53 siRNA Virus (Cat. No. G219), and Polybrene (Cat. No. G062) were purchased from Applied Biological Materials (Richmond, BC, Canada). One day prior to transfection, primary human renal epithelial cells were plated at 20%–30% confluency in a 6 well plate. The next day, the complete medium was replaced with 1 mL of transfection medium, which contained 6 μg/mL of polybrene in the complete medium. Then, 1.54×10^8 transducing units (TU)/ml of Lenti-hTERT-Neo Virus and 1×10^6 TU/mL of Lenti-p53 siRNA Virus were added to the transfection medium at a multiplicity of infection (MOI) of 7. The plate was centrifuged at 200 *g* for 30 min and then placed back in the incubator at 37°C. After 24 h culture at 37°C, 1 mL of the complete medium was added to each 6 well and cultured for an additional 24 h. Cells immortalized with the hTERT gene then were selected at 48 h post transduction, using 800 μg/mL G418 in the complete medium.

Characterization of the hTERT-HRE cells was performed using immunoblotting of E-cadherin as an epithelial marker, ZO-1 as a tight junction marker, α-ENaC as a renal epithelial cell marker, AQP1 as a renal tubule cell marker, and Keratin 17/19 as an epithelial cell marker (Supplementary Figure S3A). In addition, RT-PCR was performed for *PKHD1* as a renal epithelial marker (Supplementary Figure S3B).

2.11 Immunocytochemistry

For MYC immunofluorescence staining, cells stably overexpressing mFPC-CTD were seeded onto coverslips in 6-well plates and cultured until confluent. For FPC-CTD localization assay, mTERT-CCD or hTERT-HRE cells were transiently transfected with pcDNA-mFPC-CTD and pcDNA-mFPC-CTDd^{delCTS}, or pcDNA5-hFPC-CTD and pcDNA5-hFPC-CTD^{delCTS}, respectively. Forty-8 hrs. after transfection, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at RT and then permeabilized with 0.5% Triton X-100 in PBS for 5 min, followed by three washes with PBS before blocking with 1% BSA for 30 min. The cells were incubated with primary antibodies (anti-V5 or anti-Myc) overnight at 4°C followed by incubation with secondary antibody (Alexa Fluor 488 at 1:400 dilution) for 1 h at RT. The cells were then washed three times with PBS and mounted with ProLong Gold + DAPI (Life Technologies, Cat. No. P36935). Fluorescently labeled cells were analyzed on an Olympus FV1000 scanning laser confocal microscope configured with both an Argon Laser (5 mW, 488 nm), and a Green HeNe (10 mW, 543 nm) laser. Images were analyzed using Olympus FV10-ASW 3.0 Viewer software.

2.12 Reporter gene assay

Cells were seeded in 24-well plate, grown to ~90% confluence, and then transfected with pGL4.22-*mMyc* P1 or pGL4.22-*hMYC* P1): (0.3 μg/well) and pcDNA5-mFPC-CTD (0.6 μg or 1.2 μg/well) or pcDNA5-hFPC-CTD (0.6 μg/well) using Lipofectamine 2000. The differences in transfection efficiency were normalized by co-transfecting with 15 ng/well pRL plasmid that expressed *Renilla* luciferase (Promega) and adjusting the total amount of plasmid DNA to 1.5 ug/well by adding pcDNA5. The transfected cells were incubated for 48 h, lysed in 100 μL/well passive lysis buffer (Promega) and shaken for 20 min at RT. Firefly and *Renilla* luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega, Cat. No. E1910). The luminometer (FLUOstar OPTIMA, BMG LABTECH) was programmed using OPTIMA software to perform a 0 s delay, followed by a 5-s measurement period for each reporter assay. The 20 μL cell lysate was transferred into 96-well plate (Costar, Cat. No. 3912; white flat bottom), followed by the addition of 100 μL Luciferase Assay Reagent II and luminescence reading. After measurement of firefly luciferase activity, 100 μL of Stop&Glo reagent was added and quickly put back for reading of the *Renilla* luciferase activity. Data were collected from three independent transfections and processed using GraphPad Prism version 9.1.2 for Windows, GraphPad Software, San Diego, California United States of America, www.graphpad.com.

2.13 Chromatin immunoprecipitation (ChIP) assay

To determine the binding of FPC-CTD to the *Myc* P1 promoter, experiments were performed using Magna ChIP A/G Chromatin Immunoprecipitation Kit (MilliporeSigma, Cat. No. 17-10085), according to the manufacturer instructions and our previously published protocol (Wu et al., 2013). Because ChIP-grade anti-FPC-CTD antibodies were not available, we generated mIMCD-3 cells stably overexpressing mFPC-CTD-V5 and control cells stably transfected with empty vector and used ChIP-grade anti-V5 antibody (Abcam, Cat. No. ab15828) for immunoprecipitation. Cells were grown to 80%–90% confluence prior to experiments and processed according to the Magna ChIP A/G protocol. Following immunoprecipitation with anti-V5 antibody that recognized mFPC-CTD-V5 bound to the chromatin and subsequent protease digestion, we amplified *Myc* P1 using PCR primers specific to the full-length *Myc* P1 (forward primer: 5′- CGC TCG AGG AGA GAG GTG GGG AAG GGA GAA AG -3'; reverse primer: 5′- CCC AAG CTT AGT GAG GCG AGT CGG ACC CGG CA -3′) using the following PCR program: 94°C 3 min; 94°C 20 s, 62°C 20 s, 72°C 15 s, repeat for 40 cycles, 72°C 5 min, 10°C holding.

2.14 Visualization of gene expression profiling across developmental stages and species

Gene expression profiles of *PKHD1*/*Pkhd1*, *CYS1*/*Cys1*, *NDN*/*Ndn*, and *MYC*/*Myc* across kidney developmental stages in humans and mice, were downloaded from Evo-devo mammalian organs portal (https://apps.kaessmannlab.org/evodevoapp/) (Cardoso-Moreira et al., 2019). RPKM (reads per kilo base of transcript per million mapped reads) values were normalized using the highest value as 1 and the lowest value as 0 for each gene.

2.15 Statistical analysis

Non-parametric Wilcoxon sign rank test was used for analysis of qRT-PCR data normalized to control samples (Figure 1C, and 2D).

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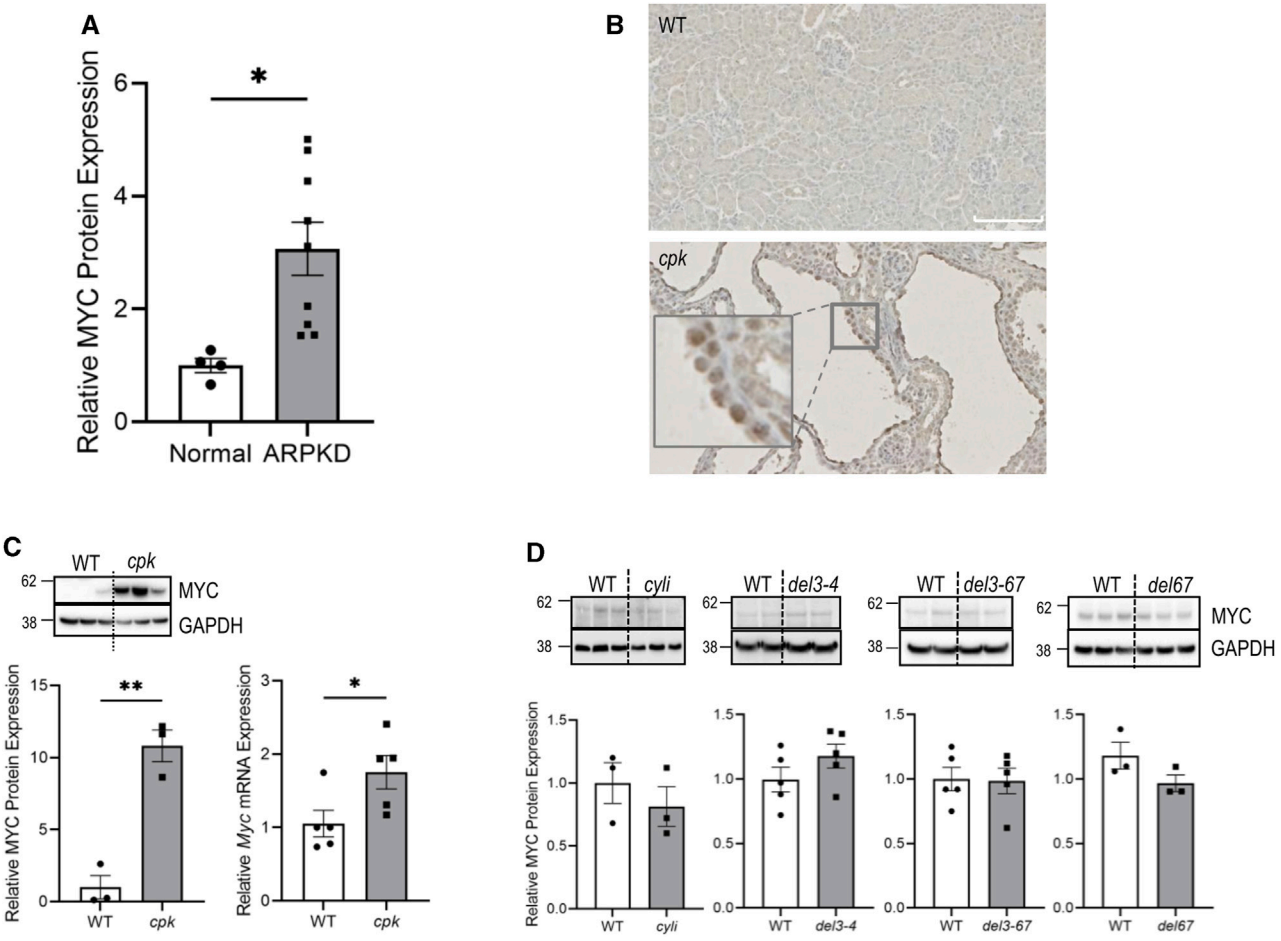

FIGURE 1
MYC/Myc overexpression is associated with renal cystic disease in human and mouse ARPKD. **(A)** Kidney lysates from normal human and ARPKD patients (Supplementary Table S1) were probed with anti-MYC and control anti-GAPDH antibodies. Relative MYC expression was normalized to GAPDH. Experiments were repeated twice independently. *t*-test $*p < 0.05$. Error bar indicates S.E.M. **(B)** Immunohistochemistry showing MYC expression (brown) in 14-day-old renal epithelial cells from wild-type (WT) and *cpk* mice. **(C)** Kidney lysates from 14-day-old WT and *cpk* mice were probed with anti-MYC and control anti-GAPDH antibodies. GAPDH was used for normalization (*cpk* mice, n = 6, *t*-test $**p < 0.01$). In parallel, *Myc* mRNA expression was quantified by qRT-PCR. The error bars indicate S.E.M. *indicates $p < 0.05$ **(D)** Kidney lysates from 14-day-old *Pkhd1* mutant mice (except 1 month old for *cyli*) and WT littermates were probed with anti-MYC and control anti-GAPDH antibodies, respectively. No significant differences were observed between groups. *cyli* (n = 3), *del3-4* (n = 5), *del3-67* (n = 5) and *del67* (n = 3). The error bars indicate S.E.M.

All other data were analyzed using either two-way Student's t-test or nonparametric test with GraphPad Prism version 9.1.2 for Windows, GraphPad Software, San Diego, California United States, www.graphpad.com.

4 Results

4.1 Elevated MYC expression in the kidneys of patients with ARPKD and in *cpk* mice with ARPKD-like kidney phenotype

MYC overexpression is a signature feature of cystic renal epithelia in human ADPKD and various mouse PKD models (Trudel, 2015). However, MYC expression in ARPKD has not been reported. In the current study, we analyzed MYC expression in ARPKD kidneys by immunoblotting. While MYC expression was marginally detectable in adult kidneys and a kidney from an infant without kidney disease (Supplementary Figure S4), we observed higher MYC abundance in all kidneys from patients with defined pathogenic variants in *PKHD1* (Figure 1A), with the highest MYC levels detected in kidneys from patients with *PKHD1* truncating pathogenic variants (Supplementary Table S1), resulting in the loss of FPC-CTD (AR1, AR2, AR3, and AR8) (Figure 1A and Supplementary Figure S4).

We then evaluated MYC expression in mouse models of ARPKD. Using IHC, we confirmed increased nuclear expression of mouse MYC protein in dilated collecting ducts from *cpk* kidneys (Figure 1B). Quantitative analysis confirmed 11-fold higher levels of MYC protein and 1.8-fold higher levels of *Myc* mRNA in the kidneys from *cpk* mice compared to WT mice (Figure 1C). In contrast, MYC protein levels were not elevated in kidneys from four different *Pkhd1* mutant mouse model lines

(*Pkhd1*^{cyli}, *Pkhd1*$^{del3\text{-}4}$, *Pkhd1*$^{del3\text{-}67}$, or *Pkhd1*^{del67}) that did not exhibit a cystic kidney phenotype (Figure 1D). These data indicate an association between high MYC expression and the renal cystic phenotype in both human ARPKD and mice with an ARPKD-like kidney phenotype. Furthermore, the lack of enhanced MYC expression in *Pkhd1* mutant mice without cystic kidney phenotype suggests differences in the function of mouse and human FPC-CTDs.

4.2 Testing the phylogenetic conservation of extracellular and intracellular FPC domains in vertebrates

We note that in each of our mouse mutant lines, the predicted *Pkhd1* translated products would be missing the FPC-CTD. Furthermore, while the mouse and human FPC sequences are 73% identical overall, the CTDs share only 55% identity (Nagasawa et al., 2002). Therefore, we analyzed phylogenetic conservation of FPC in vertebrates to better understand potential differences in the regulation of MYC expression in renal epithelia derived from patients with ARPKD and *cpk* and *Pkhd1* mutant mice. We hypothesized that the low sequence conservation of the FPC-CTDs may contribute to functional differences among FPC orthologs; an important consideration given that mouse FPC undergoes Notch-like processing that releases the FPC-CTD, which can translocate to the nucleus.

To understand phylogenetic changes in *Pkhd1,* we queried the NCBI protein database for *PKHD1* orthologs and collected FPC protein sequences of 66 mammalian, 27 bird, 5 reptile, 3 amphibian, and 1 fish (Supplementary Table S4). The large variance in the number of FPC proteins in each class may in part reflect the number of sequenced vertebrate genomes in the NCBI data base. However, while genomic data are available for multiple fish, a *PKHD1* ortholog was identified only in the genome of *Latimeria chalumnae* (a coelacanth).

After alignment of 102 vertebrate FPC protein sequences, we used the WebLogo 3 entropy scores to evaluate FPC conservation across species (Supplementary Figure S1 and Supplementary Table S5). First, we compared the WebLogo 3 entropy scores for each of the conserved domains in the extracellular portion of FPC: five IPT domains, three TIG domains, two G8 domains, two PbH1 domains, and the TM domain with entropy scores from three regions of FPC that do not correspond to any conserved domains (Supplementary Figure S5B). Since the average WebLogo 3 entropy scores were similar (Supplementary Figure S5B), we then compared the WebLogo 3 entropy scores of FPC extracellular and cytoplasmic domains. Consistent with different protein sequence identities between human and mouse extracellular and cytoplasmic portions of FPC (Nagasawa et al., 2002) the WebLogo 3 entropy scores of vertebrate FPC extracellular domain were higher than entropy scores of FPC-cytoplasmic domains (Supplementary Figure S5C). This analysis supports the hypothesis that lower sequence conservation of mouse and human FPC cytoplasmic domain compared to extracellular domain may be functionally significant and potentially contribute to the phenotypic variability observed between *PKHD1* vs *Pkhd1* mutants.

4.3 Subcellular localization of mouse and human FPC-CTD and their effects on MYC expression

Both human and mouse, FPC-CTD are encoded by *PKHD1*/*Pkhd1* exons 65, 66 and 67 (Supplementary Figure S5D). Overall, human and mouse FPC-CTD share 55% sequence identity, (Nagasawa et al., 2002). However, the CTS, localized in the human and mouse FPC-CTDs, are highly conserved (Figure 2A, red and blue highlights). Prior studies have experimentally validated one NLS in the mouse FPC-CTD (Figure 2A, purple highlight) (Hiesberger et al., 2006). However, using the web-based NLS prediction tool, SeqNLS (Lin and Hu, 2013), we identified two NLSs in mouse FPC-CTD (score >0.86), one of which overlapped with the experimentally identified NLS (Figure 2A, red bold text for predicted and purple highlight for experimentally validated NLS) (Hiesberger et al., 2006). On the other hand, human FPC-CTD had only one predicted NLS (score >0.86) (Figure 2A, red bold text). Interestingly, the two mouse, and the one predicted human NLS showed low sequence similarity.

While it has been determined that the mouse FPC-CTD translocates to the nucleus (Hiesberger et al., 2006), the intracellular trafficking of human FPC-CTD has not been determined and the nuclear function of FPC-CTD is not fully understood. Therefore, we compared intracellular localization of human and mouse FPC-CTD and tested their functions in the nucleus, concentrating on *MYC*/*Myc* regulation. Sequences the human and mouse FPC-CTD included the V5 tag and both the mouse and human FPC-CTDs contained the intracellular portion of the CTS (Figure 2A, blue highlight). The NLS sequences are shown in Figure 2A (red bold text).

By immunofluorescence, the mFPC-CTD localized primarily to the nucleus and was essentially absent from the cytoplasm (Figure 2B, left column). In contrast, the hFPC-CTD was largely excluded from the nucleus, localized to the cytoplasm and decorated the cell membrane (Figure 2B, second column from left). We suspected that nuclear trafficking of the hFPC-CTD construct with only one NLS was confounded by the CTS. Therefore, we deleted the CTS from both human and mouse constructs to generate plasmids expressing hFPC-CTD^{delCTS} and mFPC-CTD^{delCTS} respectively. Overexpression of these proteins showed strong nuclear localization for both human and mouse FPC-CTDs, although a fraction of hFPC-CTD^{delCTS} was retained in the cytoplasm (Figure 2B, right two columns).

We then investigated whether mFPC-CTD can regulate *Myc* expression in mIMCD-3 cells stably expressing the intact mFPC-CTD mouse construct. mIMCD-3 cells stably expressing the empty vector (pcDNA5) served as a control. This experimental approach allowed evaluation of the effect of mFPC-CTD on *Myc* expression in non-proliferating cells, 5 days post-confluence. The intensity of MYC immunostaining was higher in mIMCD-3 cells expressing mFPC-CTD than in the control cells (Figure 2C). Both *Myc* mRNA and MYC protein levels were higher in mIMCD-3 cells stably expressing mFPC-CTD, compared to control cells (Figure 2D). Taken together, our data provide the first evidence that overexpression of mFPC-CTD enhances MYC expression in cultured renal epithelial cells.

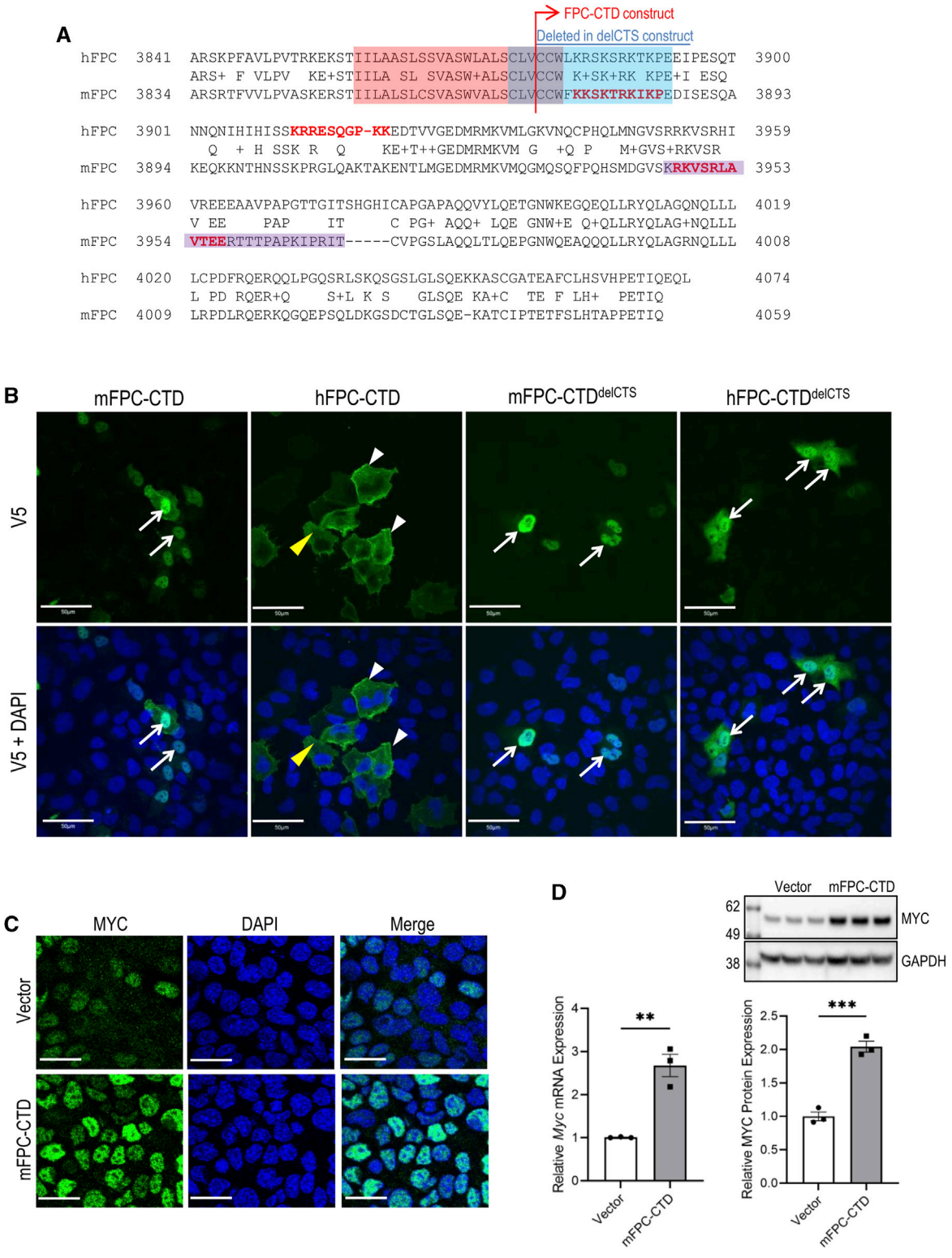

FIGURE 2
Subcellular localization of mFPC-CTD and hFPC-CTD and regulation of MYC expression by mFPC-CTD. **(A)** The alignments of human and mouse FPC-CTD. The red highlight indicates TM and blue highlight indicates the CTS. Predicted nuclear localization signals (NLSs) are shown in the red bold typeface. The purple highlight indicates experimentally tested NLS (Hiesberger et al., 2006). Blue line above the alignment indicates amino acids deleted from FPC-CTD to generate the FPC-CTD^{delCTS} constructs. **(B)** Transient transfection of V5-tagged mFPC-CTD, mFPC-CTD^{delCTS}, hFPC-CTD, and hFPC-CTD^{delCTS}, in mTERT-CCD or hTERT-HRE cells. Immunofluorescent staining was performed using anti-V5 antibody. White arrows—nucleus; yellow arrowheads—cytosol; white arrowheads—cell membrane; scale bars = 50 μM. **(C)** Immunofluorescent staining showing increased MYC expression in mIMCD-3 cells stably expressing mFPC-CTD. Green—MYC; blue—DAPI; scale bars = 20 μM. **(D)** Mouse *Myc* mRNA and MYC protein levels were increased in mIMCD-3 cells stably expressing mFPC-CTD. *Myc* mRNA expression was quantified by qRT-PCR. MYC protein expression was analysed with anti-MYC and normalized to GAPDH expression (N = 2, n = 3, *t*-test $^{**}p < 0.01$, $^{***}p < 0.001$). Error bar indicates S.E.M.

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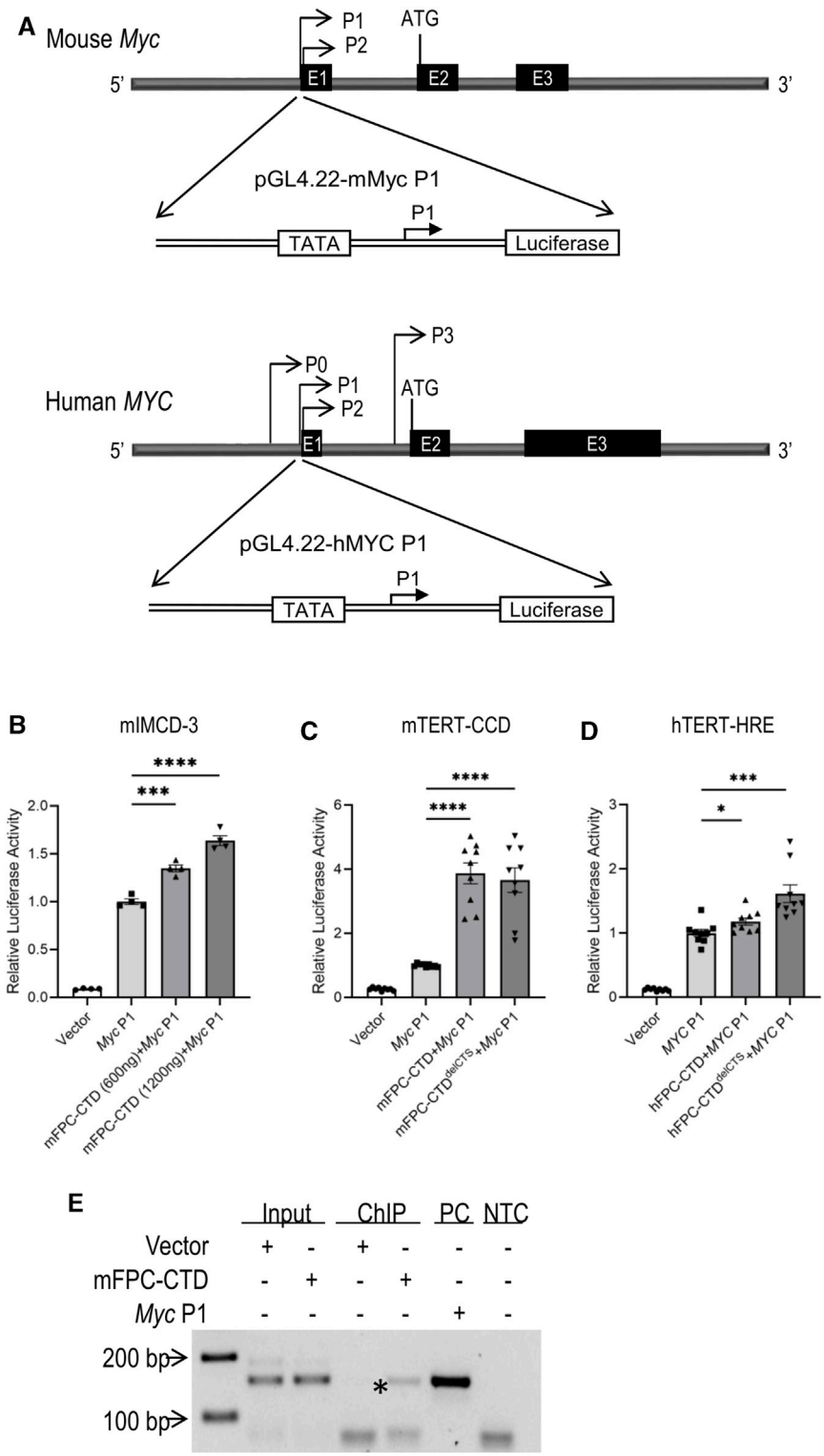

FIGURE 3
Activation of the *Myc*/*MYC* P1 promoter by m/hFPC-CTDs and binding of mFPC-CTD to the *Myc* P1 promoter **(A)** Schema of mouse (upper) and human (lower) *Myc*/*MYC* P1 luciferase reporter assay constructs. **(B)** The overexpression of mFPC-CTD increased *Myc* P1 promoter activity in mIMCD-3 cells in a dose-dependent manner. **(C)** Overexpression of mFPC-CTD and mFPC-CTD^{delCTS} increased *Myc* P1 promoter activity in mTERT-CCD cells. **(D)** hFPC-CTD^{delCTS} increased the *MYC* P1 promoter activity ~1.6 fold in hTERT-HRE cells while hFPC-CTD only increased activity ~1.1 fold. Experiments were repeated 3 times independently (N = 3) with 3 technical replicates (n = 3). The data were statistically analysed by combining all technical replicates (total n = 9) and *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. Error bar indicates S.E.M. **(E)** ChIP assay showing FPC-CTD binding to the endogenous *Myc* P1 promoter in mIMCD-3 cells. Asterisk indicates mFPC-CTD immunoprecipitation with endogenous *Myc* P1 promoter. PC indicates PCR amplification product using *Myc* P1 plasmid DNA as a template. NTC indicates negative, no template control. Experiments were repeated independently twice.

Efforts to perform these experiments with hFPC were confounded by the inability to generate an hTERT-HRE cell line that stably overexpressed either hFPC-CTD or hFPC-CTD^{delCTS}. We observed that expression of hFPC-CTD was silenced after several passages of hTERT-HRE cells under selection, suggesting that stably expressed hFPC-CTD may be cytotoxic.

4.4 Mouse and human FPC-CTD bind to the *MYC/Myc* promoter and increase MYC/Myc expression

The mechanisms that govern *Myc* transcription are complex and involve multiple promoters (P0, P1, P2, and P3) and transcription start sites (Battey et al., 1983; Bentley and Groudine, 1986; Ray et al., 1987). The P1 and P2 promoters are the predominant *Myc* regulatory elements (Albert et al., 2001). In disease states, *Myc* overexpression primarily is driven from the P1 promoter (Wierstra and Alves, 2008). Therefore, we cloned the mouse *Myc* P1 and human *MYC* P1 promoter into a pGL4.22 reporter gene construct (Figure 3A). Co-transfection of mIMCD-3 cells with constructs expressing mFPC-CTD and *Myc* P1 promoter driven reporter gene showed dose-dependent activation of the *Myc* P1 promoter by mFPC-CTD (Figure 3B). *Myc* P1 promoter activation in mTERT-CCD cells was not affected by deletion of the CTS (Figure 3C). In comparison, co-transfection of hTERT-HRE cells with constructs expressing hFPC-CTD and *MYC* P1 reporter plasmid showed only minimal, though statistically significant activation of the *MYC* P1 promoter (Figure 3D). However, deletion of the CTS from the hFPC-CTD, which enhanced its nuclear localization, resulted in a 1.6-fold activation of the *MYC* P1 promoter (Figure 3D). These data demonstrate that both human and mouse FPC-CTDs can activate the *Myc*/*MYC* P1 promoter in cultured cells. Furthermore, we tested hFPC-CTD activation of *MYC* P1 promoter in mIMCD-3 cell line and found comparable activation in this mouse line as in the human cell line, hTERT-HRE (Supplementary Figure S6). However, we note that hFPC-CTD nuclear trafficking is regulated by the CTS.

To confirm the binding of mFPC-CTD to the *Myc* P1 promoter, we performed ChIP assays. We found that mFPC-CTD-V5 bound to the endogenous *Myc* P1 promoter in non-proliferating cells (Figure 3E, asterisk). As noted above, corresponding ChIP experiments with hFPC-CTD were confounded by our inability to generate an appropriate hTERT-HRE cell line.

4.5 Temporal expression of *PKHD1*/*Pkhd1*, *CYS1*/*Cys1*, *NDN*/*Ndn* and *MYC*/*Myc* mRNAs during pre- and post-natal kidney development in human and mouse

Considering the observed differences in the nuclear trafficking and function of human and mouse FPC-CTDs, we sought to better understand how species-specific regulation of *MYC/Myc* expression may contribute to the divergent renal phenotypes in human ARPKD and the *Pkhd1* mouse models. Therefore, we analyzed cystogene expression patterns in human and mouse kidneys during intrauterine and postnatal development using the Evo-devo mammalian organs portal (http://evodevoapp.kaessmannlab.org) (Cardoso-Moreira et al., 2019). We specifically focused on genes that are known to be mutated in human ARPKD (*PKHD1/Pkhd1*), in *cpk* mice (*CYS1/Cys1*), as well as necdin (*NDN/Ndn*), which we have previously shown regulates *Myc* expression (Wu et al., 2013). We normalized the kidney developmental stages of mouse and human during pre- and postnatal development. RPKM of *PKHD1*/*Pkhd1*, *CYS1*/*Cys1*, *NDN*/*Ndn*, and *MYC*/*Myc* genes were graphed at the corresponding developmental stages of human and mouse kidneys (Figure 4). These analyses demonstrated different timing of *PKHD1*/*Pkhd1* and *CYS1*/*Cys1* mRNA expression peaks during human and mouse kidney development (Figure 4, top panels). In the fetal human kidney, *PKHD1* mRNA levels progressively increase and reach maximal expression prior to birth, whereas the progressive increase in *CYS1* mRNA lags, reaching maximum expression in the post-natal period. Conversely, in the mouse kidney, maximum expression of *Cys1* mRNA precedes the peak of *Pkhd1* mRNA expression. Expression patterns of *NDN*/*Ndn* mRNA during human and mouse kidney development were similar (Figure 4, middle panels), with expression peaking in early developmental stages (6–8 weeks post conception and e12.5–14.5 in human and mouse kidney, respectively) and decreasing thereafter. Expression of *MYC*/*Myc* mRNA also peaked during early nephrogenesis and gradually decreased thereafter in both human and mouse kidneys (Figure 4, bottom panels).

With the assumption that mRNA expression serves as an appropriate proxy for protein levels, the species-specific differences in the *PKHD1*/*Pkhd1* and *CYS1*/*Cys1* developmental expression patterns and the observation that cystin may be protective of *MYC/Myc* activation suggest a mouse-specific renoprotective mechanism in mice lacking functional FPC. Therefore, we hypothesized that limiting cystin protein in kidneys from *Pkhd1* mutant mice may evoke dilatation of renal tubules and/or collecting ducts. To address this hypothesis, we crossed the *cpk* allele into mice that are homozygous for the *Pkhd1*^{cyli} mutation (*cyli*), an indel in *Pkhd1* exon 48 that causes premature termination of protein translation (Yang et al., 2023). Kidneys from 10- and 12- month-old *cyli/cyli*;*cpk*/+ mice on a mixed genetic background (D.B/11Ei; C57BL/6J, Supplementary Table S2) had mild tubular dilations that were absent in kidneys from age-matched *cyli/cyli*;+/+ mice (Supplementary Figure S7).

5 Discussion

Previous studies have demonstrated an association between MYC expression and renal cyst development in both human ADPKD and mouse PKD models (Trudel, 2015). MYC is overexpressed in cystic renal epithelial cells derived from ADPKD kidneys (Lanoix et al., 1996). Gene expression profiling studies demonstrated that the genes and pathways regulated by MYC are upregulated in kidneys from ADPKD patients (Husson et al., 2004; Song et al., 2009). Similar observations have been reported in mouse models of PKD (Trudel et al., 1998; Burtey et al., 2008; Kurbegovic and Trudel, 2013). Causality between MYC overexpression and renal cystogenesis is further suggested by observations in the SBM mouse model, in which a *Myc* transgene is driven by a β-globin promoter and SV40 enhancer (Trudel et al.,

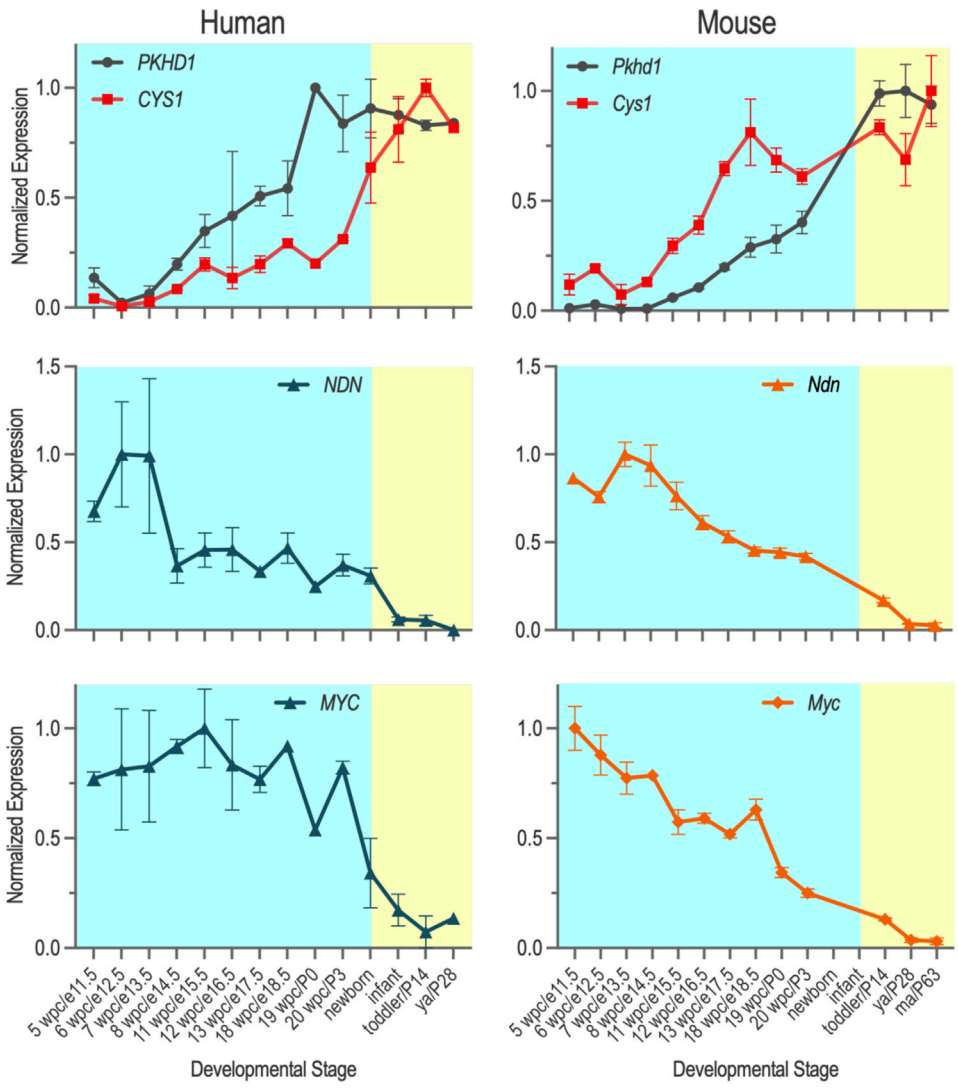

FIGURE 4
Temporal expression of *PKHD1/Pkhd1*, *CYS1/Cys1*, *CYS1/Cys1* and *MYC/Myc* mRNAs during pre- and post-natal kidney development in human and mouse were obtained from Evo-devo mammalian organs (https://apps.kaessmannlab.org/evodevoapp/), normalized, and graphed using GraphPad Prism. Y-axis shows normalized gene expression levels. X-axis shows kidney developmental stages that correspond to each other in humans and mice (human/mouse).

1991). Cystic kidney disease developed in transgenic mice overexpressing MYC, whereas mice that spontaneously lost the transgene did not develop renal cysts (Trudel et al., 1994). Additionally, *cpk* mice treated with *Myc*-antisense oligo exhibited reduced MYC protein expression, fewer renal cysts and improved renal function (Ricker et al., 2002).

In this study, we provide the first evidence that MYC is overexpressed in kidneys from patients with *PKHD1*-related ARPKD and confirm previous observations that MYC is upregulated in cystic renal epithelial cells from *cpk* kidneys (Ricker et al., 2002). In contrast, renal MYC expression levels were not altered in any of the *Pkhd1* mutant mice that lack a significant renal cystic phenotype. Our findings extend the proposition that MYC upregulation is a driver of the renal cystogenesis (Kurbegovic and Trudel, 2020).

Loss of functional FPC has different phenotypic consequences in human and mouse kidneys. In patients with pathogenic *PKHD1* sequence variants, even partial loss of FPC function can result in dramatic renal cystic disease (Cordido et al., 2021), suggesting that human FPC functions to maintain tubular integrity. In the absence of FPC, expression of MYC is aberrantly high leading to renal epithelial cell proliferation and cystogenesis. On the other hand, in mice lacking functional FPC, we show that *Myc* expression is not elevated and renal cysts are absent. A simple explanation could be that human *PKHD1* gene is important for kidney development and *MYC* homeostasis, while mouse *Pkhd1* plays a minimal role in nephrogenesis and *Myc* transcriptional regulation. This thesis is supported by a novel mouse line that was engineered to delete exon 67, which encodes most of the C-terminus, including the nuclear localization signal. Homozygous $Pkhd1^{del67}$ mice do not have a cystic

phenotype (Outeda et al., 2017). In addition, a recent report describes a new model derived from the *Pkhd1*^{del67} line such that exons 3-67 are deleted. Similar to homozygous *Pkhd1*^{del67} mice, homozygous *Pkhd1*$^{del3\text{-}67}$ mutants do not express a renal cystic phenotype (Ishimoto et al., 2023). But is the explanation for the mouse-human phenotypic disparity so simple?

We and others have shown that mouse FPC-CTD is proteolytically cleaved through Notch-like processing (Kaimori et al., 2007; Follit et al., 2010; Cameron Varano et al., 2017). The FPC-CTD traffics into the nucleus and binds to double stranded DNA as a member of a ring-structure protein complex (Kaimori et al., 2007; Follit et al., 2010; Cameron Varano et al., 2017). However, it is not clear how FPC-CTD regulates gene expression, or which genes are regulated. To address this question and to explain the phenotypic disparity across species, we hypothesized that nuclear functions of hFPC-CTD and mFPC-CTD differ, particularly with respect to transcriptional regulation of *MYC/Myc* expression. As our experimental model, we employed *in vitro* overexpression to compare the nuclear trafficking and function of the human and mouse FPC-CTD.

Using immunofluorescence, reporter gene assays, and ChIP, we demonstrate that the mFPC-CTD traffics into the nucleus, binds to the *Myc* P1 promoter, and when overexpressed can activate *Myc* expression. In reporter gene assays, hFPC-CTD and mFPC-CTD have comparable functions; both activate *MYC/Myc* P1 promoter. However, we observed differences in cellular trafficking of these intracellular FPC fragments. While mFPC-CTD largely localized to the nucleus, hFPC-CTD remained associated with plasma membrane and localized in the cytoplasm, with translocation into nucleus only upon removal of CTS. This indicates that hFPC-CTD has the functional ability to activate *MYC* P1 promoter similar to mFPC-CTD, but the nuclear transport of these two proteins differs, suggesting that intracellular transport may in part explain the species-specific differences in function.

Our experimental findings regarding the FPC-CTD nuclear function raises a new conundrum about recessive PKD pathogenesis. We show that in ARPKD loss of function of human FPC-CTD (patients AR1, AR2, AR3, and AR8: Supplementary Table S1) is associated with aberrant overexpression of MYC in the cystic kidneys, suggesting that FPC functions as a negative regulator of *MYC* to prevent cystogenesis, as was previously shown for the pro-proliferative STAT3 (Dafinger et al., 2020). But our reporter assays indicate that hFPC-CTD upregulates *MYC*. It is important to note that this apparent paradox may reflect the difference between *in vivo* mechanisms where FPC-CTD-associated proteins may dictate specific regulatory function and reductionist reporter assays demonstrating the activation of a specific promoter by an overexpressed protein. Further studies will be necessary to decipher *MYC/Myc* transcriptional activation and inhibition during kidney development and how FPC-CTD, or the lack of it may contribute to *MYC/Myc* expression regulation.

While our studies provide novel information about the nuclear trafficking and function of human and mouse FPC-CTDs, a recent study identified mitochondrial targeting sequences in both mFPC-CTD and hFPC-CTD and demonstrated the trafficking of these proteins into the mitochondria (Walker et al., 2022). These developments imply that understanding both the nuclear and mitochondrial functions of human and mouse FPC-CTDs will be necessary to better define their roles in kidney cystogenesis.

Another key to human-mouse phenotype paradox may involve the *CYS1/Cys1* gene. In the mouse, cystin negatively regulates MYC expression through binding to mouse NDN and preventing its activation of *Myc* expression (Wu et al., 2013). While the mechanism of negative regulation is not completely understood, cystin could either compete with mouse NDN for binding to the *Myc* P1 promoter, or alternatively, cystin and mouse NDN could form a complex that binds to the *Myc* P1 promoter and inhibits its activity (Wu et al., 2013). Our data mining revealed that *Cys1* expression is upregulated before *Pkhd1* during mouse kidney development. In *cpk* mice, cystogenesis is initiated in the distal portion of developing proximal tubules at e16.5–17.5 and continues after birth (Preminger et al., 1982; Nidess et al., 1984; Avner et al., 1987). The activation of *Cys1* expression before *Pkhd1* in the developing mouse kidney and suppression of *Myc* expression by cystin could explain the absence of renal cystic phenotype in mice with mutant *Pkhd1*.

To better understand FPC function during development, we extended our study to analyze gene orthologs encoding FPC across phyla. These analyses suggest that the CTD may be evolutionary innovation associated with vertebrate transition from aquatic to terrestrial life. We found a *Pkhd1* orthologue in only one fish genome, the coelacanth *L. chalumnae*. This observation is consistent with a previous study that suggested the *Pkhd1* paralogue, *Pkhd1l1,* is the ancestral gene because *Pkhd1l1* gene is present in the Fugu (puffer fish) genome, but *Pkhd1* is not (Hogan et al., 2003). *PKHD1L1* and *PKHD1* are similar; both encode proteins that have a large extracellular segment with similar arrangement of conserved structural domains (Supplementary Figure S5A) and 41.5% protein sequence identity (Hogan et al., 2003). However, their cytoplasmic segments are quite different: human and mouse *PKHD1L1*-encoded proteins have very short cytoplasmic tails, eight and six amino acids, respectively, while hFPC-CTD and mFPC-CTD have 192 and 184 amino acids (Hogan et al., 2003).

In addition, our informatic analysis revealed higher sequence conservation of the FPC extracellular than intracellular domain across phyla, which may in part explain the difference in nuclear trafficking between mFPC-CTD and hFPC-CTD. The mFPC-CTD has two predicted canonical NLSs, one of which has been experimentally validated (Hiesberger et al., 2006). In contrast, hFPC-CTD has only one predicted NLS. The difference in the number of NLSs in human and mouse FPC-CTD could explain the differences in the distribution of hFPC-CTD and mFPC-CTD in the nucleus *versus* other subcellular compartments, e.g., mitochondria. This finding raises an intriguing possibility that will require further study.

Finally, we used data mining to compare the temporal expression of *PKHD1/Pkhd1* and *CYS1/Cys1* in developing human and mouse kidneys respectively. In human kidneys, maximal expression of *PKHD1* preceded *CYS1*. However, during mouse kidney development, *Cys1* expression is upregulated before *Pkhd1* (Figure 4). Assuming similar regulation of *MYC/Myc* gene expression by human and mouse cystin in association with NDN, our data suggest that 1) *MYC/Myc* expression can be activated by human and mouse NDN during early kidney development and factors that regulate *MYC/Myc* expression in later stages of kidney development differ in humans and mice; and 2) during middle to late stages of mouse nephrogenesis, *Myc* gene expression is

downregulated by cystin in the mouse kidney but much less than in the human kidney. Therefore, the differences in the temporal expression pattens of *CYS1*/*Cys1* could contribute to the relative renoprotection from cystogenesis in *Pkhd1*-deficient mice.

In summary, we provide the first report of elevated MYC levels in *PKHD1*-deficient human kidneys. In contrast, we show that MYC abundance is unaltered in non-cystic kidneys from *Pkhd1*-deficient mice. We demonstrate several key differences between human and mouse that may explain the relative renoprotection in *Pkhd1*-deficient kidneys: 1) differences in the number of NLS in the FPC-CTD; 2) differential impact of the human and mouse CTS on intracellular trafficking and subcellular distribution of the FPC-CTD; and 3) differences in the temporal expression of *PKHD1/Pkhd1* and *CYS1/Cys1* during nephrogenesis. In addition, we observed that reduced cystin levels in *Pkhd1*-deficient mice lead to renal tubular dilatation, suggesting that in mice both cystin and FPC-CTD are necessary to maintain renal tubular architecture. Given the limited sequence identity of human vs mouse FPC-CTD, we speculate that the cytosolic cleavage peptides may have different protein interacting partner(s) and these protein complexes may differentially regulate *MYC/Myc* expression *in vivo*. Taken together, our data extend previous observations and indicate that MYC dysregulation is a central driver of renal cystogenesis in both ARPKD and ADPKD.

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 in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by Institutional Review Board at the Children's National Hospital or the University Hospital of Cologne. Human kidney samples were obtained from the NIDDK-funded UAB Childhood Cystic Kidney Disease Center Translational Resource at the University of Alabama at Birmingham and from the University Hospital of Cologne. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants legal guardians/next of kin. The animal study was approved by Institutional Animal Care and Use Committees at Children's National Research Institute. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NH: Writing–original draft, Investigation, Visualization, Project administration, Validation, Conceptualization, Data curation, Formal Analysis. CY: Conceptualization, Data curation, Investigation, Project administration, Resources, Visualization, Writing–original draft, Validation. MW: Conceptualization, Data curation, Investigation, Writing–original draft. GT: Investigation, Validation, Writing–original draft, Conceptualization, Resources. HG-D: Formal Analysis, Writing–original draft. RT: Investigation, Resources, Writing–original draft. PB: Methodology, Project administration, Resources, Supervision, Writing–original draft, Conceptualization. AR: Investigation, Methodology, Project administration, Supervision, Writing–original draft, Conceptualization, Visualization. CD: Investigation, Writing–original draft. ML: Project administration, Resources, Supervision, Writing–review and editing, Conceptualization, Data curation, Visualization. ZB: Project administration, Writing–review and editing, Data curation. LC: Conceptualization, Data curation, Methodology, Project administration, Supervision, Visualization, Writing–review and editing, Formal Analysis, Investigation, Validation. LG-W: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing–review and editing, Validation, Visualization.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2023.1270980/full#supplementary-material

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Targeting c-MYC: a potential non-hormonal therapeutic approach for endometriosis treatment

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Endometriosis is a benign gynecological disease in which eutopic endometrial tissue composed of glands and stroma grow within the pelvic cavity. The disease affects females of reproductive age and is characterized by pelvic pain, infertility and reduced quality of life. The majority of pharmacologic treatment modalities for endometriosis focus on suppression of estradiol production and/or action; an approach associated with adverse side effects. c-MYC is elevated in eutopic endometrium and endometriotic lesion tissue in patients with endometriosis and the disease shares many similar pathological characteristics with that of endometrial carcinoma. While targeting of c-MYC with Omomyc has recently gained substantial interest in the field of cancer research, there has been no recent attempt to evaluate the potential utility in targeting c-MYC for endometriosis treatment. The following perspective article compares the similarities between endometriosis and endometrial cancer and presents preliminary data suggesting that targeting c-MYC with Omomyc reduces endometriotic cell proliferation and viability *in vitro*. Future application of targeting c-MYC in endometriosis treatment and potential pros and cons are then discussed.

KEYWORDS

c-Myc, endometrial cancer, endometriosis, Omomyc, treatment

1 Introduction

Endometriosis is a chronic inflammatory disease in which endometrial tissue grows outside the uterine cavity, predominantly within the pelvic cavity (Giudice and Kao, 2004). The disease affects approximately 10%–15% of women of reproductive age and is associated with pelvic pain, dysmenorrhea, infertility, and reduced quality of life (Giudice and Kao, 2004). The etiology of endometriosis is not clear although retrograde menstruation is widely accepted (Sampson, 1927). It is postulated that menstrual overflow leaves the uterine cavity through the fallopian tubes and implants into the peritoneal cavity and the ovaries. However, most women experience retrograde menstruation, yet not all women develop the disease (Jenkins et al., 1986). Therefore, multiple factors contribute to the development and progression of endometriosis such as aberrant immune response and altered hormonal

balance (Sourial et al., 2014; MacLean and Hayashi, 2022). Given that the growth of endometrial lesions is estrogen-dependent, common pharmacologic treatments target estrogen production and subsequent estrogen action and include oral contraceptive pills (OCPs), gonadotropin-releasing hormone (GnRH) agonists, GnRH antagonists, levonorgestrel (progestin)-releasing intrauterine devices and aromatase inhibitors (Nothnick et al., 2018). We discuss below the current pharmacologic approaches for endometriosis treatment and their limitations. We then discuss the similarities between endometriosis and endometrial cancer and review targeting of Myc as a therapeutic approach in cancer treatment. Lastly, we provide preliminary evidence supporting the potential of targeting c-MYC as a non-hormonal treatment option for endometriosis.

2 Current approaches to endometriosis treatment

Oral contraceptive pills (OCPs), which contain low dose estrogens and high dose progesterone, are often the first line approach in treating endometriosis/endometriosis-associated pain (Menakaya et al., 2013; Dunselman et al., 2014). Low levels of estrogen are postulated to induced progesterone receptor expression and the progesterone/progestins contained within the OCP preparations inhibit estrogen production by the ovaries via suppression of gonadotropin release as well as via reduction of the inflammatory milieu associated with the disease. Progestins such as medroxyprogesterone acetate (MPA) are also effective in controlling pain, but a drawback associated with their use is side effects such as menstrual irregularities and weight gain (Brown et al., 2012). Levonorgestrel-releasing intrauterine system (LNG-IUS) is a popular treatment option effective in reducing dysmenorrhea, pelvic pain, deep dyspareunia as well as reducing lesion burden (Fedele et al., 2001; Bayoglu et al., 2011; Kim et al., 2022) and can be used as a long-term treatment option (Kim et al., 2022). While LNG-IUS offers many benefits, limitations include irregular uterine bleeding, vaginal bleeding and vaginal discharge. In summary, LNG-IUS treatment is an effective and feasible method to control pain as a long-term postoperative maintenance therapy for endometriosis patients. While progestin-based therapies are effective in many women, a substantial percentage of endometriosis patients exhibit progesterone resistance and therefore have insufficient therapeutic responses to these treatments.

The fact that endometriosis is an estrogen-dependent disease led to targeting production of this hormone as a means of treating the disease. Targeting gonadotropin release at the level of the hypothalamus to reduce circulating estrogen levels has proven effective and overcomes the issue of progesterone resistance. Gonadotropin hormone releasing analogs includes the use of both gonadotropin-releasing hormone (GnRH) agonists and, more recently antagonists. Both classes of drugs suppress ovarian estrogen production leading to a hypo-estrogenic state which is detrimental to endometriotic lesion survival. GnRH analogs are often prescribed when oral contraceptives and/or progestin analogues fail to produce successful outcomes. These compounds are effective in some, but not all women with endometriosis (Shaw, 1990). Further, GnRH agonist use is associated with significant side effects including altered lipid profile, hot flushes, loss of libido and reduction in bone mass/bone health (Prentice, 2001). The loss of bone mass can be overcome by estrogen add-back/hormone replacement therapy (Surrey, 2010) but this must be balanced to avoid estrogen action upon lesion survival and potential recurrence of disease and its symptomology.

One of the most common and successful therapies for endometriosis pain management is the GnRH agonist, leuprolide acetate (Geisler et al., 2004). However, while this is an advantage of GnRH agonist therapy, a disadvantage is the induction of a hypo-estrogenic state and negative impact on overall patient health. GnRH antagonists have also been used for treatment of endometriosis. Unlike GnRH agonists, they do not exhibit agonistic effects inducing estrogen "flare-up" prior to suppressing estrogen levels. Elagolix, relugolix, and linzagolix are three recent GnRH antagonist being used for endometriosis treatment (Rzewuska et al., 2023). Unlike earlier formulations, these three antagonists are taken orally, with both elagolix and relugolix already approved by the FDA and linzagolix currently under review. These GnRH antagonists may offer several benefits over older drug formulations which include lower levels of analgesics taken, significant reduction in pain scores and little to no irregular uterine bleeding. However, these GnRH antagonists are still associated with side effects including reduction in bone mineral density and risk of developing osteopenia and osteoporosis due to their induction of a hypoestrogenic state which limits their treatment regime duration (Rzewuska et al., 2023). It should be noted that combination therapy of relugolix with estradiol and norethisterone acetate for 24 weeks minimizes bone density loss and vasomotor symptoms while significantly reducing endometriosis associated pelvic pain (Giudice et al., 2022).

The use of aromatase inhibitors in the treatment of endometriosis have gained attention based upon the observation that endometriotic lesions express aromatase and are able to synthesize their own estrogen (Bulun et al., 2000). Current aromatase inhibitors prescribed include anastrozole and letrozole. Letrozole in combination with the synthetic progesterone analog, norethindrone acetate, was first reported to reduce disease burden at second look laparoscopy as well as significantly reduce pelvic pain scores in 2004 by Ailawadi and colleagues. Subsequent studies continue support the efficacy of letrozole in treatment of endometriosis-associated pelvic pain. Like letrozole, anastrozole, also inhibits estrogen production but the former is more potent in reducing levels of this steroid (Geisler et al., 2002). Anastrozole therapy combined with oral contraceptives was reported to significantly reduce pain in as little as 1 month after treatment initiation and this treatment regime was associated with minimal side effects (Amsterdam et al., 2005). However, the safety of aromatase inhibitors might be an issue especially since a recent systematic review and meta-analysis suggested an increased risk of cardiovascular events during endocrine therapy for early breast cancer (Yoo et al., 2023). None the less, aromatase inhibitors offer an additional treatment modality which is effective in treating endometriosis-associated pelvic pain.

In summary, the majority of currently prescribed endometriosis treatments rely upon reduction of estrogen production and/or estrogen action. As emphasized earlier in this article, while these treatments are effective in many women, they are not effective or well-tolerated in a large proportion of women suffering from

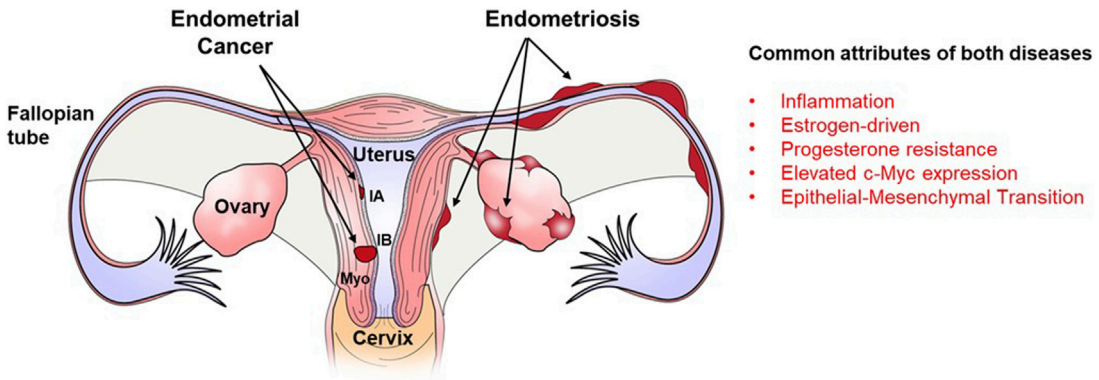

FIGURE 1
Localization of endometriotic implants and endometrial carcinoma within the female reproductive tract and major similarities between both diseases. Both endometrical cancer and endometriosis originate from the endometrial lining of the uterus. Endometrical cancer which remains confined to the endometrial lining is classified as stage IA while that which spreads into the myometrial smooth muscle (Myo) of the uterus is classified as stage IB. Endometriotic lesions develop outside of the uterus on the surface of the Fallopian tube, ovary and perimetrium of the uterus. Both diseases are characterized by inflammation, estrogen dominance, progesterone resistance, epithelial-mesenchymal transition and elevated expression of the oncogene. c-Myc.

endometriosis. These observations coupled with limitations due to side-effects and potential health complications emphasize the need for the development of novel, estrogen-sparing endometriosis treatments. In searching for such novel treatment targets, we next evaluate and compare the common mediators and mechanisms between endometriosis and cancer with the goal of identifying potential druggable targets which may be capable of reducing disease burden and symptomology associated with endometriosis.

3 Endometriosis and cancer

Both endometriosis and endometrial cancer share numerous risk factors and common pathophysiological characteristics (Figure 1). Endometrial cancer is the most commonly diagnosed form of gynecological cancer in developed nations, accounting for approximately 5% of all cancers diagnosed in women (Contreras et al., 2022). Similar to the origins of endometriotic lesions, endometrial cancer originates in the endometrium and development and progression of both diseases is associated with estrogen exposure (Yu et al., 2015). With respect to endometrial cancer, one mechanism by which estrogen may promote progression of endometrial cancer is through the activation of the NLPR3 inflammasome (Liu et al., 2019), which has also recently been proposed to play a role in the pathophysiology of endometriosis (Irandoost, et al., 2023). Like endometriosis (Ailawadi et al., 2004; Patel et al., 2017), endometrial cancer also exhibits progesterone resistance (Gunderson et al., 2012) and displays altered expression of progesterone receptors (Saito et al., 2006; Jongen, et al., 2009).

It is further postulated that the hyper-estrogen and hypo-progesterone milieus associated with both disease contribute to the enhanced proliferation and invasiveness of the endometriotic lesions/endometrial cancer. Epithelial to mesenchymal transition (EMT) is a process by which epithelial cells lose polarity and cell-to-cell contacts, undergo remodeling of the cytoskeleton, and acquire migratory abilities and a mesenchymal-like gene expression program. The EMT process is proposed to play a role in the pathophysiology of both endometrial cancer (Colas et al., 2012; Mirantes et al., 2013) and endometriosis (Yang and Yang, 2017; Konrad et al., 2020) as both diseases are associated with the migration of endometrial cells into surrounding tissues as the diseases progress. One transcription factor whose overexpression induces EMT (Qiu et al., 2016) as well as immune evasion, angiogenesis, ECM remodeling, cell migration and invasion (Masso-Valles and Soucek, 2020) is c-MYC which is overexpressed in both endometriosis and endometrial cancer.

4 c-MYC overexpression in endometriosis and endometrial cancer

The Myc family of transcription factors is composed of c-MYC, N-MYC and L-MYC (Adhikary and Eilers, 2005) whose expression is dysregulated in over 70% of human cancers and associated with poor prognosis (Wang et al., 2021). Like other cancer types, c-MYC is highly expressed in endometrial tumors (Kim et al., 2013) and immunohistochemical localization studies revealed a 78.3% positive rate of c-MYC in endometrial cancer tissues with amplified *c-MYC* in 25% of the cases (Zhang et al., 2018; Buchynska et al., 2019). From a functional standpoint, upregulation of c-MYC in endometrial cancer cells *in vitro* was shown to induce EMT, drug resistance and invasion (Lv et al., 2012; Liu et al., 2015). Qiu and colleagues (2016) used a small molecule bromodomain 4 (BRD4) inhibitor, JQ1, to target c-MYC in endometrial cancer cells using both cell culture and tumor tissue xenograft models. In that study, JQ1 inhibited endometrial cancer growth in both models and this was associated with a reduction in c-MYC protein expression as well as reduced expression of c-MYC downstream targets. Additional studies demonstrated that inactivation of c-MYC resulted in tumor regression and was associated with cellular differentiation and

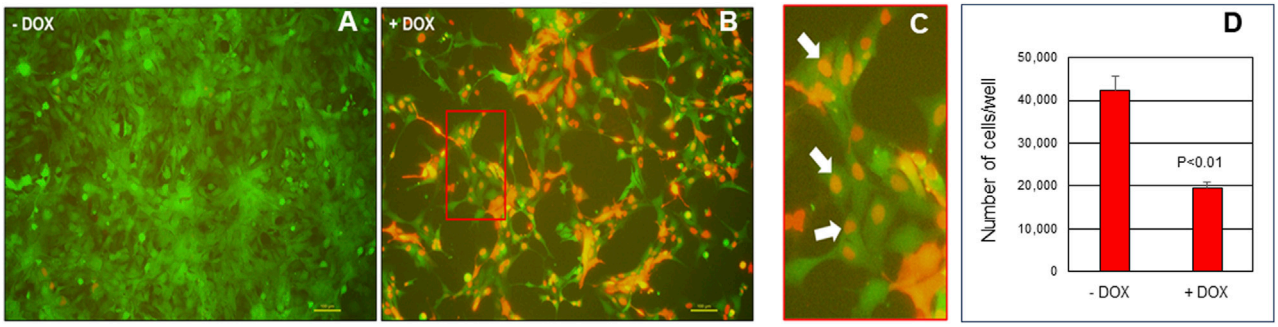

FIGURE 2
Induction of Omomyc in endometriotic epithelial 12Z cells reduces cell viability. 12Z cells were plated at 25,000 cells/mL of media for 24 h then cultured in the absence of doxycycline - DOX; **(A)** or presence of + DOX; **(B)** doxycycline to induce Omomyc expression (detected by the RFP tag). Red boxed area in panel B is enlarged and displayed in **(C)** with white arrows highlighting nuclear expression of Omomyc. Cell counts were then determined by manual counting and results are depicted in **(D)**. Cell count data were analyzed by unpaired t-tests on three separate replicates independent experiments (N = 3) and are displayed as the mean +/- SEM with $P < 0.01$.

apoptosis in transgenic mouse models (Jain et al., 2002; Tansey, 2014). Lastly, a more recent study using JQ1 demonstrated that JQ1-mediated reduction of c-MYC was associated with suppressed tumor growth in a xenograft mouse model as well as reduced proliferation and enhanced apoptosis of endometrial carcinoma cell lines *in vitro* (Pang et al., 2022). Thus, in addition to being a well-established target in multiple types of cancer, c-MYC also appears a plausible target for endometrial carcinoma.

Similar to expression levels in endometrial carcinoma, c-MYC expression is also elevated in endometriotic lesion tissue as well as matched eutopic endometrium from women with endometriosis (Schenken et al., 1991; Schneider et al., 1998; Johnson et al., 2005; Pellegrini et al., 2012; Proestling et al., 2015). Unfortunately, outside of these descriptive studies, little advancement has been made on our understanding as to why c-MYC is elevated in endometriotic lesion tissue and if this overexpression contributes to the pathophysiology of the disease. Based upon the similarities between endometrial cancer and endometriosis and the fact that c-MYC appears to a common transcription factor with augmented expression and signaling in both endometrial cancer and endometriosis, it may also be a viable, non-hormonal treatment for endometriosis. To date, the potential utility of targeting c-MYC as a therapeutic approach for endometriosis treatment has not been reported.

5 c-MYC as a therapeutic target for endometriosis treatment

Given the aforementioned similarities between endometrial cancer and endometriosis and the necessity to identify novel, non-hormonal targets for endometriosis treatment, we conducted the following preliminary studies. To begin to evaluate potential targeting of c-MYC in endometriosis therapy, we utilized the well-characterized human endometriotic epithelial cell line, 12Z which expressed eGFP (Bulun et al., 2000) to evaluate the impact of blocking c-MYC signaling on cell proliferation and survival. To do so, 12Z-eGFP cells were transduced with lentiviral particles expressing a doxycycline (DOX)-inducible pTRIPZ-Omomyc-RFP (Omomyc) plasmid (Annibali et al., 2014) and were then subjected to puromycin-selected. Puromycin resistant Omomyc plasmid-infected 12Z cells (25,000 cells/mL) were cultured in Dulbecco's Minimum Essential Medium (DMEM)/Ham's F12 (Fisher Scientific, Pittsburgh, PA) containing 10% TET-FBS and Pen-Strep for 24 h in 10 cm tissue culture dishes after which the media was replaced with fresh media containing 2% TET-FBS with or without doxycycline (DOX, 2.0 μg/mL; Takara Bio catalog# 631311) to induce Omomyc expression. Cells were incubated for 48 h after which they were trypsinized and cell counts determined using a hemocytometer and all counts were conducted on duplicate dishes for 3 separate trials (N = 3). Compared to controls (-DOX), DOX induction (+DOX) of Omomyc resulted in a significant reduction in number of cells after 48 h of treatment. Figure 2 depicts representative immunofluorescence for Omomyc localization with white arrows highlighting nuclear expression of Dox-induced Omomyc expression. Based upon these preliminary studies, Omomyc reduces endometriotic epithelial cell survival *in vitro*. These preliminary observations are encouraging and may warrant further investigation into use of Omomyc to treat endometriosis and its associated symptomology using three-dimensional *in vitro* cell culture models and *in vivo* mouse models of experimental endometriosis routinely employed in our laboratory (Alali et al., 2020).

6 Potential c-MYC signaling pathways common to endometrial cancer and endometriosis

To interrogate potential down-stream pathways relevant to c-MYC signaling in the pathogenesis of endometriosis, we generated a list of c-MYC targets which have been reported in cancer (Zeller et al., 2003) and endometrial adenocarcinoma; Peterson et al., 2023) to those reported to be dysregulated in a similar manner in endometriosis. Down-stream targets of c-MYC relevant to endometriosis pathophysiology may include upregulated targets such as cyclin E1 (CCNE1; Park et al., 2019; Park et al., 2020), enolase 1 (ENO1; Nabeta et al., 2009), fatty acid synthase (FASN; Turathum et al., 2022), lactate dehydrogenase A (LDHA; Zheng et al., 2021), microsomal glutathione S-transferase 1 (MGST1; Ferrero et al., 2019), 60S acidic riboprotein 1 (RPLP1; Alali et al., 2020), and tumor protein p53 (TP53; Toki and

Nakayama, 2000). c-MYC targets which are downregulated in cancer (Zeller et al., 2003) and endometriosis include cyclin dependent kinase 1A (CDKN1A; Kim et al., 2009) and fibronectin 1 (FN1; Holzer et al., 2020).

7 Discussion

c-MYC has long been proposed to play a functional role in the pathophysiology of cancer. However, due to c-MYC's unique properties with respect to a lack of a defined three-dimensional structure, nuclear localization and absence of a targetable enzymatic pocket, targeting c-MYC for cancer treatment has presented a challenge. Omomyc is a mutant basic helix–loop–helix leucine zipper (bHLHZip) domain which acts as a dominant negative and sequesters c-MYC in complexes preventing active transcription of c-MYC target genes while also allowing transcriptional repression (Masso-Valles and Soucek, 2020). Omomyc has been shown to be a potent inhibitor of tumor growth in multiple cancer types in both *in vitro* and *in vivo* studies (Soucek et al., 2004; Soucek et al., 2008; Sodir, et al., 2011; Soucek et al., 2013; Whitfield, et al., 2014; Fiorentino et al., 2016; Alimova et al., 2019; Sodir et al., 2020). Considering the similarities between cancer and endometriosis (Figure 1) and the limitations with current, anti-estrogen based treatment options for endometriosis, we evaluated the potential utility of Omomyc for endometriosis treatment. To do so, we transduced 12Z cells (a well-characterized endometriotic epithelial cell line) with lentiviral particles containing pTRIPZ-Omomyc-RFP (Omomyc) plasmid and treated them with DOX to induce Omomyc expression. Induction of Omomyc was associated with a reduction in cell viability (as reflected in total cell counts) compared to cells not treated with DOX. Although preliminary, these studies are encouraging and warrant further, more detailed studies. One limitation of current endometriosis treatments is the induction of a hypo-estrogenic state and unwanted side effects associated with it. For Omomyc to be an advancement over current therapies, it will be essential to assess if Omomyc could reduce disease burden and symptomology *in vivo* while concurrently avoiding induction of a hypo-estrogenic state. This would be one critical necessary first step in evaluating this c-MYC inhibitor for endometriosis treatment.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the University of Kansas Medical Center Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from gifted from another research group. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

Conceptualization: WN; Methodology: WN, PM, SA, ES, and AG; Investigation: WN, PM, SA, ES, and AG; Resources: SA and ES; Writing Original Draft: WN; Writing–Reviewing and Editing: WN, PM, SA, ES, and AG; Supervision: WBN and EBS; All authors contributed to the article and approved the submitted version.

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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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Obesity under the moonlight of c-MYC

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The moonlighting protein c-Myc is a master regulator of multiple biological processes including cell proliferation, differentiation, angiogenesis, apoptosis and metabolism. It is constitutively and aberrantly expressed in more than 70% of human cancers. Overwhelming evidence suggests that c-Myc dysregulation is involved in several inflammatory, autoimmune, metabolic and other non-cancerous diseases. In this review, we addressed the role of c-Myc in obesity. Obesity is a systemic disease, accompanied by multi-organ dysfunction apart from white adipose tissue (WAT), such as the liver, the pancreas, and the intestine. c-Myc plays a big diversity of functions regulating cellular proliferation, the maturation of progenitor cells, fatty acids (FAs) metabolism, and extracellular matrix (ECM) remodeling. Moreover, c-Myc drives the expression of a wide range of metabolic genes, modulates the inflammatory response, induces insulin resistance (IR), and contributes to the regulation of intestinal dysbiosis. Altogether, c-Myc is an interesting diagnostic tool and/or therapeutic target in order to mitigate obesity and its consequences.

KEYWORDS

c-Myc, obesity, MASLD, gut-liver axis, T2DM

Introduction

Obesity is defined by the World health organization (WHO) as an excessive fat accumulation that impairs health with a diagnosis of a body mass index (BMI) ≥30 kg/m^2.

Since 1975, the global prevalence of obesity has almost tripled and has continued to increase at an epidemic rate. In the past decades, obesity has been revisited and now it is considered a multisystemic disease affecting many multiple organs. Since it is a chronic, systemic and relapsing disorder, obesity triggers a significant number of metabolic disorders and co-morbidities. Obesity considerably elevates the risk of suffering type 2 diabetes mellitus (T2DM), metabolic-associated steatotic liver disease (MASLD), hypertension, myocardial infarction, stroke, obstructive sleep apnoea, dementia, osteoarthritis, and several cancers, thereby decreasing both quality and life expectancy (Bluher, 2019; Gjermeni et al., 2021; Lin and Li, 2021).

Recent studies revealed a clear link between obesity and urbanisation, demonstrating the crucial role that environment plays in the development of this disease. However, the considerable variation in body weight between individuals, further suggests that obesity is influenced by complex interactions between environmental developmental, behavioural, epigenetics and genetic stimuli (Thaker, 2017; Zaiou, 2022).

In recent years, it has become evident that the highly pleiotropic, multifunctional super-transcription factor (TF) c-Myc controls a variety of cellular functions by targeting up to 15% of all genes, with broad effects on cell proliferation, differentiation, apoptosis, angiogenesis, adhesion and metabolism (Dang, 2012). Different cytokines and hormones can promote

stabilization of c-Myc protein levels and subsequently activate nuclear transactivation of c-Myc-dependent target genes. Among these, genes involved in cell cycle regulation such as cyclins D1, D2, B1, cyclin-dependent kinase 4 (CDK4) and p21, p27 inhibitors of CDK (Dang, 2012).

Additionally, c-Myc also attenuates the differentiation of a great number of cell types during development, thus preserving the "stemness" of these cells (Leon et al., 2009). In spite of its association with cell proliferation and differentiation, c-Myc also promotes apoptosis and provides an additional level of regulation against uncontrolled cell growth or when the growth factors are limited (McMahon, 2014; Madden et al., 2021).

Metabolism is regulated by c-Myc through enolase A, lactate dehydrogenase A, phosphofructokinase, hexokinase II, and glucose transporter I. c-Myc expression stimulates glutaminolysis and glycolysis (Goetzman and Prochownik, 2018). Both pathways promote cellular proliferation by increasing the synthesis of nucleotides, ATP and fatty acids (FA) that serve as building blocks for cells (Dang, 2013).

Through the activation of peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1), protein kinases, mitochondrial TF, and mitochondrial receptors, c-Myc encourages mitochondrial biogenesis and enhances mitochondrial function (Dang, 2013).

In order to increase cell mass before division, c-Myc stimulates global protein expression, via the activation of RNA polymerase I, II, and III and of genes that participate in ribosomal, tRNA and rRNA biosynthesis (Dang, 2013; Rosselot et al., 2021).

Therefore, c-Myc carries out a great number of biological functions that are essential for survival, expansion, and normal cell function. Generally, c-Myc expression is tightly regulated; however, its deregulation is often observed in human cancer and is considered a poor prognostic factor. Therefore, it was termed "the oncogene from hell", given its ability to induce genomic instability, accelerate tumour progression and coordinate the crosstalk with microenvironment, thus inducing tumor growth (Whitfield and Soucek, 2012).

Additionally to its role in carcinogenesis, c-Myc appears to be involved in the control of multiple metabolic pathways from glycolysis and glutaminolysis, to nucleotide and lipid synthesis across many different cell types, especially as almost all cells basally express metabolic genes (Stine et al., 2015). Emerging evidence also suggests that c-Myc is pivotal in driving the expression of a broad range of immune cell metabolism, regulating their development, differentiation, activation and coordination of metabolic programs to support immune functions (Gnanaprakasam and Wang, 2017). As a master regulator of immunity and metabolism, c-Myc is implicated in autoimmune, inflammatory, metabolic and other non-cancerous disorders (Zheng et al., 2017), even though this is still a poorly understood topic with a huge unmet need for preclinical and clinical research (Madden et al., 2021).

In this review, we aimed to highlight and summarize the potential roles of multifunctional moonlighting c-Myc in obesity and its related metabolic diseases, including T2DM and MASLD. Indeed, the complexity of the etiopathogenesis of obesity is responsible for the dysfunction of multiple tissues and organs, including the white adipose tissue (WAT), pancreas, liver and intestine. All of the above makes the understanding of the complex role of c-Myc in the development of obesity increasingly challenging. Here, we provide a comprehensive view of c-Myc-related disturbances present in obesity and their direct and indirect effects on the different organs of the body.

WAT –holding the key of obesity

White adipose tissue (WAT) is crucial for the regulation of lipid homeostasis and energy balance. In a healthy state, WAT serves a variety of purposes, including storing energy as fat, protecting vital organs, and assisting with the endocrine system and immune response. The adipose tissue consists of adipocytes, endothelial cells, fibroblasts, immune cells, and adipose stem cells (ASCs) (Richard et al., 2000).

Obesity is the result of storing excess energy intake, thus bringing about an enlargement of the adipose tissue. Diet, genetics, and their interaction contribute to obesity (Jo et al., 2009). The expansion of the WAT associated with obesity is linked to an elevation of the adipogenesis activity. The coordinated activation of TFs and epigenetic modifications control the lipogenic and adipogenic programmes. The complicated regulatory mechanisms, however, are not yet fully understood (Longo et al., 2019).

The increase in size of existing adipocytes (hypertrophy) or in number (hyperplasia) (Jo et al., 2009) is characteristic of WAT expansion. An imbalance in caloric intake *versus* expenditure leads to the accumulation of hypertrophic and dysfunctional adipocytes. While hypertrophic growth is more closely linked to obesity-associated metabolic complications, expansion through hyperplasia is associated with a benign metabolic profile. Numerous adipogenic processes, such as the proliferation, recruitment, and/or differentiation of new fat cells, are responsible for mediating hyperplasic WAT, whereas hypertrophy is mainly governed by size increase of already present adipocytes (Choe et al., 2016). Adipogenesis and transition of adipose tissue mesenchymal stem cells to mature adipocytes is regulated by an extensive cooperative network of transcription factors (TFs), that control the expression of dozens of downstream protein-coding genes and long noncoding RNAs (Ambele and Pepper, 2017; Bjork et al., 2021).

The stromal vascular fraction of subcutaneous WAT is the source of human ASCs. ASCs are multipotent, fibroblast-like mesoderm lineage cells with the ability to differentiate into multiple lineages, much like bone marrow-derived mesenchymal stem cells. In adult WAT, the turnover of adipocytes at approximately a rate of ~10% of cells per year maintains the balance between cell renewal and death. In accordance to several studies, ASCs play an essential role in the development of obesity and obesity-related metabolic disorders (Hajer et al., 2008). Furthermore, ASC quantity and function can also change in an obese state due to adipocyte dysfunction, which can result in abnormal adipose tissue remodeling and affect the microenvironment of expanded WAT (Choe et al., 2016).

The positive energy balance provokes the proliferation of ASCs, and when adipocytes reach a volume limit, the newly formed ASCs are utilized for *de novo* adipogenesis to further increase energy

storage capacity of adipose tissue (Wang et al., 2013; Jeffery et al., 2015). The capacity of mature white adipocytes to dedifferentiate into multipotent ASCs is another feature. The functions of ASCs change in an obese condition, which causes a rise in the production of white fat and a whitening of thermogenic brown and beige fat (Shin et al., 2020). Additional research is still required to uncover the mechanism how ASCs generate new adipocytes in obesity, and the impact of environmental and genetic factors on this response.

It has been shown that c-Myc is positive regulator of ASCs fate and plays a crucial role in regulating adipocyte differentiation. Deregulated c-Myc expression prevents adipocytes and other cell types from achieving terminal differentiation (Spalding et al., 2008).

The expression of the c-Myc protein and transcript rises during the early stages of ASC differentiation and is thought to be involved in adipogenesis and the maintenance of a terminal phenotype. siRNA mediated knockdown of c-Myc in ASCs lead to inhibition of adipogenesis and dysregulation of pathways related to cytoskeletal remodelling and cell adhesion. These findings show that c-Myc is essential for driving multipotent ASCs into the adipogenic lineage (Deisenroth et al., 2014).

Overexpression of c-Myc in 3T3-L1 preadipocytes facilitates normal expression of early response regulators CCAAT/enhancer binding proteins C/EBPβ and C/EBPδ during the course of differentiation. However, the expression of downstream regulators, C/EBPα, peroxisome proliferator-activated receptor γ2 (PPARγ2), and later markers of differentiation is suppressed (Heath et al., 2000b). This suggests that c-Myc may act by blocking C/EBPβ- and C/EBPδ-directed activation of C/EBPα and PPARγ2 expression and demonstrates that c-Myc specifically inhibits the terminal stages of the adipogenic program. However, the particular molecular mechanism is not fully understood, yet (Heath et al., 2000b; Deisenroth et al., 2014). Interestingly, comparable outcomes were shown in hematopoietic stem cells, where c-Myc maintains the balance between stem cell differentiation and self-renewal via the regulation of cell-ECM interactions (Wilson et al., 2004). Importantly, c-Myc's role in cell cycle progression and transformation is functionally different from the way it induces the suppression of adipocyte differentiation (Heath et al., 2000a).

There is still a lack of clarity in the associated signaling pathways that could be used as potential therapeutic targets for c-Myc-driven adiposity. The mammalian Sirtuins (SIRT1–7) are a family of conserved NAD^+-dependent protein deacetylases. A growing body of evidences has shown that Sirtuins and their prominent substrates participate in a variety of physiological and pathological processes, including cell cycle regulation, glucose and lipid metabolism, mitochondrial biogenesis and function, energy homeostasis insulin action and inflammatory responses (Guarente, 2006; Chen et al., 2022). The nuclear sirtuins (SIRT1, SIRT6, and SIRT7), the mitochondrial sirtuins (SIRT3, SIRT4, and SIRT5), and the cytosolic sirtuin (SIRT2) regulate diverse metabolic functions. For example, SIRT1 controls several physiological processes in adipose tissue, such as inflammatory responses, mitochondrial biogenesis, cellular senescence, and apoptosis/autophagy (Hwang et al., 2013). SIRT2 regulates adipocyte development, gluconeogenesis, insulin action, and inflammatory responses (Gomes et al., 2015). By regulating mitochondrial biogenesis and function, SIRT3 plays regulating roles in a variety of metabolic processes, including acetate metabolism and thermogenesis (Shi et al., 2010).

It has been reported that Sirtuins are affected by HFD and environmental stress (Jokinen et al., 2017). In WAT of mice, pigs, and humans, restriction of nutrients causes SIRT1 upregulation, leads to changes in NAD^+ levels and act by deacetylating forkhead box protein (FOXO), peroxisome proliferator activated receptor gamma coactivator1 (PGC-1α), PPARγ and Nuclear factor kappa b (NF-κB). In contrast obesity is linked to lower levels of SIRT1(Lakhan and Kirchgessner, 2011). For instance, in comparison to obese women, thin women exhibited over two times the SIRT1 expression (Pedersen et al., 2008). In WAT of obese HFD-fed mice and db/db mice SIRT1 expression is low (Chalkiadaki and Guarente, 2012). Mechanistically, adipogenesis is boosted when SIRT1 is downregulated in WAT. In contrast, adipogenesis is suppressed and lipolysis is promoted when SIRT1 expression in WAT is high (Picard et al., 2004).

Whole-body SIRT1 overexpression protects against genetically-induced obesity and from age-induced glucose intolerance (Herranz et al., 2010). Genetic deletion of SIRT1 from adipocytes leads to increases adiposity, exaggerated insulin resistance, glucose intolerance, inflammation and predisposes to metabolic disfunction in mice on short-term HFD (Mayoral et al., 2015). Less inflammation, improved glucose tolerance, and virtually total protection against hepatic steatosis are the advantages of SIRT1 over-expression, indicating that SIRT1 is crucial in preventing the adverse metabolic effects of obesity (Banks et al., 2008). Furthermore, SIRT1 activation causes weight loss without a reduction in calorie intake (Feige et al., 2008; Pfluger et al., 2008).

3T3-L1 preadipocytes from SIRT1-deficient mice differentiate into tiny, dysfunctional, inflamed, hyperplastic adipocytes with increased proliferative potential. Remarkably, in SIRT1-silenced preadipocytes c-Myc is hyperacetylated and activated leading to, uncontrolled cell proliferation and the development of hyperplastic, defective adipocytes. Additionally, SIRT1-silenced human SW872 preadipocytes and proliferating SIRT1 knockdown MEFs have shown the increased proliferation. Preadipocytes' inability to undergo hyperplasia when both SIRT1 and c-Myc expression were simultaneously reduced suggests that SIRT1 controls adipocyte hyperplasia through c-Myc regulation. Therefore, the SIRT1/c-Myc axis controls the quantity of adipocytes and their functional integrity (Abdesselem et al., 2016). It seems that c-Myc and SIRT1 form a negative-feedback loop that inhibits c-Myc-induced cellular transformation. On one hand, c-Myc binds to the SIRT1 promoter and induces SIRT1 expression. However, SIRT1 in turn deacetylates and downregulates c-Myc, resulting in decreased c-Myc stability, reduced target gene expression and cellular transformation (Yuan et al., 2009). The functional relationships between SIRT1 and c-Myc in the control of adipocyte proliferation and differentiation will be intriguing to further explore.

Another surprising and important functional link has been described between c-Myc and mammalian target of rapamycin (mTOR) (Pourdehnad et al., 2013). mTOR regulates eukaryotic cell growth and metabolism in response to environmental variables including nutrition and growth factors. It is an important regulator of lipid metabolism and obesity (Ricoult and Manning, 2013). mTOR complex 1 (mTORC1) has been implicated in the regulation of adiposity since the discovery that genetically- or diet-induced obese mice display elevated activity of this complex

in adipose tissue. Consequently, either WAT-specific knock-out of mTORC1 (Polak et al., 2008) or pharmacological mTORC1 inhibition (Houde et al., 2010) with rapamycin reduces adiposity and protect mice from diet-induced obesity. Additionally, mTORC1 is necessary for the maturation of 3T3-L1 preadipocytes and the activation of pro-lipogenic Sterol Regulated Element-Binding Protein (SREBP1).

Importantly, moderate, in contrast to full mTORC1 inhibition, aggravates HFD-induced obesity and adipogenesis raising the hypothesis that chronic mTORC1 overactivation in adipocytes is inhibitory to fat accretion and adiposity (Laplante et al., 2012). Accordingly, mice with constitutive mTORC1 activation in adipocytes induced by tuberous sclerosis complex (TSC1) deletion in differentiated, mature adipocytes significantly reduces visceral adiposity. Mechanistically, this phenomenon can be connected, at least in part, to a reduced adipocyte size and number, increased lipolysis, mitochondrial oxidative activity and browning (Magdalon et al., 2016).

Given the significance of c-Myc and mTOR in the regulation of growth, the presence of a direct regulatory link between them is probably crucial. Indeed, c-Myc is a direct repressor of TSC expression. In turn, TSC loss de-represses c-Myc protein, creating feed-forward regulatory loop (Schmidt et al., 2009). Downstream effectors of c-Myc-Cyclin D-CDK4/6- also phosphorylates and inactivates TSC2, resulting in mTORC1-activation. Conversely, inhibition of CDK4/6 led to decreased mTORC1 activity and reduced protein synthesis. Consistent with this, the anti-proliferative effect of CDK4/6-inhibition was reduced in cells lacking TSC2 (Romero-Pozuelo et al., 2020). It should be noted that the relevance of these various mechanisms in the context of human obesity and obesity-related comorbidities is unclear and requires further studies to elucidate the full-range of the dynamic molecular interaction. The possible clinical application of small-molecule Cyclin D-CDK4/6 inhibitors in metabolic disorders is another largely unexplored area (Fassl et al., 2022).

A mechanistic link between glucocorticoid signalling and c-Myc expression has been demonstrated. Dexamethasone, a synthetic glucocorticoid hormone, is a crucial adipogenic *in vitro* component that induces c-Myc transcription (Deisenroth et al., 2014). It appears that glucocorticoid stimulation is crucial for c-Myc induction in a concentration-dependent manner. Interestingly, dexamethasone treatment of 3T3-L1 preadipocytes was previously connected to the regulation of wingless-type MMTV integration site family (WNT) and transforming growth factor beta (TGF- β) genes and the induction of C/EBPα and PPARγ (Pantoja et al., 2008). This shows a molecular link between glucocorticoid signaling and c-Myc expression. Glucocorticoids might stimulate the differentiation of ASC into adipocytes, alter the lipid metabolism through reduced lipogenesis and increased lipolysis in mature adipocytes. These effects ultimately increase the adipose cell number, thereby leading to obesity, and inducing imbalance in the lipid metabolism of adipose tissue, which contributes to the development of IR (Ayala-Sumuano et al., 2013).

In fact, Cushing's syndrome-related elevation of endogenous glucocorticoid cortisol is linked to obesity (Chaudhry and Singh, 2023). In addition, a characteristic side effect of long-term glucocorticoid therapy is an increase in central adiposity, which is partly attributed to an increase in hyperplasia inside adipose depots (Ayala-Sumuano et al., 2013).

These results are particularly intriguing because it has previously been demonstrated that glucocorticoids can cause lymphoid cell G1 arrest acting in part via inhibition of c-Myc expression. Similar effects have been reported in some fibroblastic cells (Ma et al., 2000). In fact, different cell types preferentially employ different modes of c-Myc control depending on their physiological status. Additionally, the cellular and tissue environment controls the functional activities of glucocorticoids. For instance, glucocorticoids are powerful anti-inflammatory agents in the immune system, whereas in the developing lung they are essential for normal maturation. If we understand the mechanisms of how this tissue specific activity is achieved, we should be able to develop more targeted therapeutic interventions with fewer side effects for a wide range of diseases that are either resistant to current therapy or for which glucocorticoid therapy produces unacceptable side effects (Feldman, 2009). Undoubtedly, the interaction between glucocorticoids and c-Myc is an area that need more study.

Key points

- c-Myc is essential in ASCs adipogenesis.
- c-Myc inhibits the terminal stages of adipocyte differentiation.
- The SIRT1/c-Myc axis regulates both the quantity and functional integrity of adipocytes.
- Dexamethasone induces the transcriptional activity of c-Myc in adipocytes.

MASLD- the nexus with obesity

MASLD, formerly known as non-alcoholic fatty liver disease (NAFLD), is linked to an increased risk of obesity (Rinella et al., 2023). The key feature of MASLD, steatosis, develops when the rate of hepatic FA intake from plasma and *de novo* synthesis is higher than the rate of FA oxidation and export (as triglycerides within very low-density lipoproteins (VLDL)) (Polyzos et al., 2019). Massive lipid accumulation in the liver leads to an imbalance of lipid metabolism inducing protein unfolding and ER stress, mitochondrial dysfunction and, ultimately, cell death that subsequently causes chronic inflammation and extended liver damage (Parthasarathy et al., 2020; Powell et al., 2021).

In Spain, it is estimated that MASLD affects, at least, 25.8% of the population aged between 15 and 85 years. The risk of developing more advanced stages of MASLD increases for patients older than 45 years. Moreover, the societal costs of this epidemics are estimated between €3.625 and €5.571 million (Higado, 2021).

Patients with MASLD frequently eat large quantities of processed foods heavy in fat, refined sugars, and carbohydrates, lead sedentary lifestyles, and engage in little physical activity. However, in addition to these exogenous or environmental factors, numerous other factors frequently influence the progression of MASLD and end-stage carcinogenesis. For instance, 42% of MASLD patients develop steatotic liver disease (SLD) and only 2.4%–12.4% finally develop liver cancer (White et al., 2012). Overall, large variety in the predisposition to develop MASLD demonstrates that among risk factors, endogenous (i.e., genetic) factors are particularly important (Guo et al., 2021).

Recent research from our lab has demonstrated that transgenic mice, bearing overexpression of c-Myc only in hepatocytes (Alb-myc^{tg}) and fed a standard chow diet are predisposed to moderate obesity and aberrant hepatic lipid accumulation with ageing (Nevzorova et al., 2013; Guo et al., 2021).

Gene array analysis of the liver tissue of Alb-myc^{tg} mice consistently showed significant changes in FA metabolism. The overproduction of FA in c-Myc transgenic hepatocytes serves as a substrate and an inducer of P-450 (CYP)2E1 microsomal cytochrome FA oxidation systems (e.g., Cpt1, Adcam), which results in increased production of reactive oxygen species (ROS) and oxidative stress. The hepatic parenchyma becomes inflamed and infiltrated by immune cells triggered by ROS and lipid peroxidation products. Hepatic stellate cells (HSCs) are further activated by inflammatory cytokines released by immune cells (Arab et al., 2018). This prompts HSCs to produce collagen fibres and extracellular matrix (ECM) deposition in the hepatic parenchyma, which results in liver fibrosis (Nevzorova et al., 2013; Guo et al., 2021). Mechanistically, c-Myc overexpression in hepatocytes, caused by gene amplification or the inflammatory response to liver injury, initiates PDGF-B expression. The close proximity of dying PDGF-expressing hepatocytes pre-activates resident quiescent HSC, and encourages their transdifferentiation into myofibroblasts that produce collagen (Nevzorova et al., 2016; Zheng et al., 2017).

The excess FA produced by Alb-myc^{tg} liver is exported and transported to WAT for storage. This was linked to the enhanced deposition of VLDL particles high in triglycerides in the serum of middle-aged Alb-myc^{tg} animals (Alves-Bezerra and Cohen, 2017). As a result, compared to control littermates, c-Myc transgenic mice at 36 weeks of age gain significantly more weight, have higher BMIs, and have more WATs. Adiposity and low-grade WAT inflammation, which are demonstrated by the presence of macrophage crown-like structures (CLS), cause IR and hyperglycemia in transgenic mice (Bigornia et al., 2012; Zatterale et al., 2019). IR results in high level of blood glucose, and further contributes to metabolic disorders in the liver. Altogether, excessive c-Myc overexpression only in hepatocytes alters the body's metabolism and causes moderate obesity, spontaneous hyperlipidemia, glucose intolerance, and mild steatohepatitis/fibrosis (Guo et al., 2021). Additionally, in various mouse MASLD (Fang et al., 2023) and hepatocellular carcinoma (HCC) models, c-Myc-induced metabolic alterations further increase hepatocarcinogenesis (Ma et al., 2000). In fact, this closely resembles human MASLD, where a combination of endogenous (such as oncogenes) and external (such as dietary habits) factors work together to promote the development of HCC. As proof of clinical significance, c-Myc expression is elevated in MASLD patients (Younes et al., 2022) and MASLD-related HCC (Freimuth et al., 2010; Guo et al., 2021).

In agreement with several studies (Akinyeke et al., 2013; Shen et al., 2018; Wang et al., 2021b), we reported (Guo et al., 2021) c-Myc inhibition by metformin. We demonstrated that Alb-myc^{tg} mice on a chow diet rich in metformin were resistant to obesity, showed modest improvements in hyperglycemia and dyslipidemia, and had less liver steatosis and fibrosis. We found that metformin had a strong inhibitory effect on *de novo* lipogenesis and particularly on SREBP1 expression in a Alb-myc^{tg} animals. Our observation is also consistent with prior report that c-Myc orchestrates the induction of lipogenesis, activates its master regulators SREBP1 and they collaborate to activate FA synthesis, and drive FA chain elongation from glutamine and glucose. Importantly, after inhibition of FA synthesis c-Myc-induced tumorigenesis is blocked and tumors regress in both xenograft and primary transgenic mouse models, revealing the vulnerability of Myc-induced tumors to the inhibition of lipogenesis (Gouw et al., 2019).

However, in our experimental conditions despite a notable improvement in steatohepatitis in Alb-myc^{tg} mice treated with metformin, we were unable to find any significant alterations in c-Myc-induced hepatic proliferation (Guo et al., 2021). However, several studies indicate that metformin can lower the risk of cancer (including HCC) in people with T2DM in a dose-dependent manner (Hassan et al., 2010; Bo et al., 2012; Chen et al., 2013).

There is also evidence that statins might lower the frequency of HCC. In fact, statins have anti-inflammatory and immunomodulatory properties; they prevent the generation of cell growth mediators and encourage programmed cell death (Islam et al., 2020). It has been showed (Rao and Rao, 2021) that simvastatin, atorvastatin, and lovastatin prevent c-Myc activation, which in turn inhibits growth of cancer cells (Shachaf et al., 2004). MiR-33b, a specific inhibitor of c-Myc, is often missing in medulloblastomas. Its overexpression causes c-Myc downregulation. It has been demonstrated that lovastatin elevated mi-R-33b expression, which in turn inhibited cell proliferation (Takwi et al., 2012).

Tumour growth in orthotopically xenografted cells is also inhibited by lovastatin administration. The objective of statins as a pharmacological modulator of c-Myc via miRNA-based treatments may benefit from this research. This indicates that statins can be used as a pharmacological modulator of c-Myc via miRNAbased therapeutics (Di Bello et al., 2020).

Despite constant exposure to microbial-derived and food products from the gut, the liver is a crucial immune organ that is sterile and tolerogenic. One of the largest populations of T cells in liver are mucosal-associated invariant T (MAIT) cells, an innate T-cell that may quickly respond to stimulation, start proliferation, and produce cytokines and lytic molecules (Kurioka et al., 2016). MAIT cells are essential for the host's defence against bacterial and viral infections. c-Myc is required for the proliferation of MAIT cells. Upon activation, MAIT cells significantly upregulate c-Myc target proteins, regulating amino acid transport, glycolysis, and cell division. Obesity has been linked to impaired MAIT cell proliferation and reduced functional responses due to an impaired Myc-SLC7A5-glycolysis metabolic axis. Reduced MAIT cell proliferation in obese persons may increase host sensitivity to infection and malignancies (Kedia-Mehta et al., 2022).

Key points

- Middle-aged transgenic mice with c-Myc overexpression in hepatocytes (Alb-myc^{tg}) develop mild obesity and abnormal hepatic lipid accumulation upon standard chow feeding.
- Metformin partly attenuates the spontaneous obesity and MASLD in Alb-myc^{tg} mice.
- c-Myc overexpression is a hallmark of MASLD and MASLD-related HCC, highlighting the pivotal role it plays in the development of the disease.

- c-Myc is required for MAIT cells proliferation and is dysfunctional in obesity.

β-CELLS of the langerhans islets–the pancreatic player in obesity-linked T2DM

Obesity-linked T2DM is a disease of encompassing IR in combination with pancreatic β-cell dysfunction (Abdullah et al., 2010). The risk of T2DM is 93 times greater in patients with a BMI over 35 kg/m^2 (Barnes, 2011). Obesity is nowadays an epidemic of unforeseen proportions. In 2000, 9% of people in Spain had T2DM, while 15% of the population was obese. If the trend continues, 12% of the nation's population will have T2DM by 2030 (Huerta et al., 2013).

In early stages of obesity, β-cells increase their mass and function to compensate for peripheral IR. However, if the condition becomes more chronic and severe, the adaptability of β-cell declines, resulting in a reduction in β-cell mass. TD2M arises if the endocrine pancreas fails to secrete sufficient insulin to handle the metabolic demands caused by β-cell secretory disfunction and/or relative decreased β-cell mass (Chen et al., 2017). The fact that obesity-linked T2DM develops in only 25%–30% of obese individuals raises the possibility that a genetic predisposition plays a role in individual susceptibility (Lingohr et al., 2002).

A dynamic balance between cellular growth and death determines the number of β-cells required to maintain proper glucose homeostasis in mammals (Rhodes, 2005; Rosselot et al., 2021). Pancreatic β-cell mass is increased due to at least three mechanisms: i) β-cell neogenesis (differentiation from precursor cells); ii) β-cell proliferation; and iii) β-cell hypertrophy (increased cell size). In turn, β-cell death, primarily by apoptosis or β -cell atrophy (decreased cell size), reduces the number of β-cell (Bonner-Weir, 2000; Ackermann and Gannon, 2007; Saisho et al., 2013).

The signal transduction pathways controlling the proliferation and survival of β-cells hold particular significance (Lingohr et al., 2002). c-Myc seems to have an important physiological impact on these processes (Jonas et al., 2001). In β-cells, c-Myc is typically expressed at very low basic levels. However, in response to glucose, it may transiently and moderately rise, promoting the replication of β-cells (G1/S transition). The proliferative silence of β-cells can be successfully overcome by the ectopic expression of c-Myc. Even in the absence of replication, c-Myc plays a significant role in cell growth (size) (Collier et al., 2003). Therefore, c-Myc transiently and moderately increases during the growth of β-cells, acting as a metabolic regulator (Karslioglu et al., 2011).

Interestingly, plasma insulin does not induce c-Myc in pancreatic islets. Exogenous insulin added to primary rat β-cells failed to alter c-Myc expression, as demonstrated by numerous *in vitro* and *in vivo* experiments (Elouil et al., 2005). Additionally, the inhibitor clonidine reduces insulin release but does not stop the rise in c-Myc mRNA caused by glucose (Plant et al., 1991). Therefore, during hyperglycemia, glucose rather than insulin induces elevated c-Myc levels.

Chronic hyperglycemia, or high blood glucose levels, is the definition of T2DM. Consequently, β-cell exposed to high glucose concentrations in diabetic conditions. Moreover, pancreatic β-cells have substantially greater glucose concentrations than many other cell types because they are surrounded by a dense network of fenestrated capillaries that facilitates better blood glucose exchange (Veld and Marichal, 2010). Thus, c-Myc expression in β-cells *in vivo* is significantly impacted by hyperglycemia (Rosselot et al., 2021).

Short-term HFD feeding in young mice increases body weight, IR and glucose intolerance. After HFD feeding, c-Myc protein abundance in β-cell is increased and compensatory β-cell proliferation, expansion and cell function are induced. Mechanistically, c-Myc upregulation in pancreatic islets is mediated by a PKCζ, ERK1/2, mTOR, and PP2A pathway and target genes mediate cell cycle pathways (Rosselot et al., 2019). Consistently, glucose intolerance and hypoinsulinemia after short HFD feeding in mice with c-Myc deficiency in β-cells indicates that c-Myc is crucial for the adaptive response of islets to acute metabolic insults (Rosselot et al., 2021).

Due to restrictions in cell replication, adults' ability to increase their β-cell mass is limited. In contrast, the proliferation of neonatal functionally immature β-cells is robust. Juvenile β-cells undergo functional maturation in the early postnatal period and develop the glucose-responsive, insulin secretory phenotype. Importantly, c-Myc regulates β-cell proliferation and immaturity. Rodent juvenile islets have elevated levels of c-Myc, which promotes the rapid proliferation of neonatal β-cells. The number of proliferating cells in postnatal stages decreases when endogenous c-Myc in β-cells is deleted *in vivo* (Rosselot et al., 2021). Consistently, stabilisation of c-Myc not only encourages replication but also directs β-cells towards functionally immature phenotypes, simulating postnatal β-cell functionality. Ablation of c-Myc in neonatal β-cells consistently results in impaired cell cycle progression and proliferation, and reduced functional β-cell mass (Puri et al., 2018). *In vitro* studies using rodent and human cell lines, have revealed that the bidirectional shift between fully functional, mature, non-proliferative β-cells and proliferative, functionally immature β-cells is reversible (Scharfmann et al., 2014). Overall, the ability of β-cells to replicate impairs its function. However, if just a small percentage of cells replicate, as happens in adult islets, transitory loss of function in β-cells is adequate. When a larger fraction of β-cell divides, overall β-cell function deteriorates and the insulin processing and release are dysregulated (Liu and Hebrok, 2017; Puri et al., 2018). Consistently, the analysis of the active chromatin marks on human genomes confirms that c-Myc activity is increased at younger ages (Puri et al., 2018).

In both humans and rodents, the ability of β-cells to replicate decreases with age (Tschen et al., 2009). Ageing reduces both the adaptive responses to mitogens like HFD as well as the basic proliferative mitogenic response of β-cell. In contrast to young mice, older animals fed with HFD had diminished c-Myc action in their islets. Mechanistically, epigenetic-mediated c-Myc resistance restricts, at least partially, the adaptive proliferation of β-cell in the context of increased insulin demand during aging (Rosselot et al., 2021). "c-Myc resistance" in metabolically stressed aged β-cells can possibly explain why aging population are generally more prone to developing T2DM (Rosselot et al., 2021).

Overall, c-Myc is essential for the regeneration of for β-cells under basal or metabolically stressed conditions. From a therapeutic perspective, agents that promote human β-cell replication may be helpful if such activity is reversible. This, of course, provoke the

interest for c-Myc as potential therapeutic target in regenerative therapy for diabetic patients.

Mice with constitutive or inducible transgenic overexpression of c-Myc in β-cells were created by different groups (Pelengaris and Khan, 2001; Laybutt et al., 2002; Pelengaris et al., 2002; Cano et al., 2008; Murphy et al., 2008) to clarify whether c-Myc might be able to stimulate proliferation with therapeutic potential. Although remarkable β-cell proliferation was induced by c-Myc overexpression, this proliferation was very apparent, brief, and obviously carcinogenic, making these results disappointing. Moreover, β-cell proliferation was associated by immediate β-cell dedifferentiation and/or death, leading to diabetes. Indeed, in pIns-c-MycER^{TAM} mice upon tamoxifen stimulation β-cell destruction was so extensive that these transgenic mice were even used as a model of complete β-cell ablation (Cano et al., 2008). In islets from c-Myc-overexpressing mice, gene expression analysis revealed stabilisation of p53 and activation of the intrinsic apoptotic and DNA-damage checkpoint mechanisms (Cheung et al., 2010; Robson et al., 2011).

Overall, studies with transgenic mice show that high (estimated in the 20- to 50-fold range) and persistent overexpression of c-Myc in β-cells results in cell dysfunction and death. Certainly, c-Myc plays a critical role in glucotoxicity-induced β-cell death in chronic hyperglycemia and diabetes (Karslioglu et al., 2011).

Despite the fact that excessive c-Myc expression is harmful for β-cells, low physiological levels of Myc are necessary for normal β-cell functionality. Recent research has demonstrated that the usage of harmine (β-carboline alkaloid) mildly upregulates c-Myc expression and stimulates adult human β -cell cycle entry at rates that are in the physiological and potentially therapeutic range (Wang et al., 2015). In addition, harmine combined with GLP-1R agonists (Ackeifi et al., 2020) or TGFß inhibitors dramatically increases human β-cell proliferation (5%–8%), indicating that combination treatments targeting multiple signalling pathways may be more effective for islet regeneration in T2DM patients (Wang et al., 2019). Further approaches to optimize the use of harmine (Title et al., 2022). and the development of methods to specifically target β-cells, present an important translational challenge (Rosselot et al., 2021).

The great majority of research on β-cell proliferation was conducted on rodents, which has increased our understanding of murine rather than human β-cell replication. However, there are significant differences between human and rodent islets in terms of their function, composition, structure, and in proliferative capacity. These differences highlight the need to focus future research on human islets proliferation and partially explain why most substances that have been shown to increase β-cell proliferation in rodent islets have not been successful in humans (Wang et al., 2021a).

Key points

- Glucose rapidly stimulates c-Myc expression in β-cells.
- c-Myc is an inverse dual regulator of β-cell maturation and proliferation.
- Proliferation of β-cell is induced by mild physiologic upregulation of c-Myc.
- High and persistent c-Myc overexpression results in β-cells dysfunction and cell death.

Intestine—the gatekeeper of diet-induced obesity

While unhealthy diets and sedentary lifestyles synergistically with polygenetic risks represent major causes of obesity, a big plethora of data suggest that the intestine also plays a part as a crucial organ participating in glucose and lipid metabolism (Hur and Lee, 2015). In fact, the gastrointestinal tract is the first organ to be exposed to dietary components. Unhealthy diets interact with gut microbiota (GM) to promote early intestinal inflammation which favor obesity and IR. The altered epithelial permeability, bacterial products translocation, upregulation of proinflammatory cytokines and intestinal endocrine hormones are the main pathophysiological mechanisms (Ding and Lund, 2011).

Epithelium in the gastrointestinal tract has a precise architecture, formed by invaginations, or crypts, and finger-like lumenal protrusions, or villi. These "folds" create an enormous surface area, allowing efficient nutrient absorption from the intestinal space. The self-renewing intestinal stem cells (ISCs) are located in crypts and intervilli areas and continuously produce a population of rapidly proliferating progenitor cells that migrate towards the intestinal lumen. As they migrate, cells undergo cell cycle arrest and commit to different cell lineages by terminal differentiation (Marshman et al., 2002). In the small intestine and colon, cells develop into three functional cell types: 1. the predominant enterocyte; 2. the mucus-secreting Goblet cells and; 3. the peptide hormone secreting enteroendocrine cells. Moreover, cells that descend to the base of the crypt in the small intestine convert into the Paneth cells, the fourth cell type. Differentiated cells carry out their specific tasks and then after induction of apoptosis, discarded into the lumen (Allaire et al., 2019).

c-Myc plays an important role in regulating homeostasis, proliferation, differentiation, and transformation in the adult gut (Marshman et al., 2002; Sancho et al., 2003). All intestinal epithelial cells (IEC) of the crypt-villus unit, with the exception of Paneth cells, express c-Myc. Cell cycle arrest and the upregulation of the cell cycle inhibitor inhibitor p21$^{cip/waf}$ coincide with the differentiation of proliferative IEC, which is also accompanied by a decrease in c-Myc expression (Pinto et al., 2003). In gastric and colonic tissue c-Myc overexpression is associated with inflammation as well as with potentially neoplastic hyperproliferative states. Overall, c-Myc is crucial for maintaining control of intestinal crypt homeostasis and cellular proliferation. Wnt signalling pathway is a most likely upstream regulator that controls these processes (Bettess et al., 2005). Inhibition of the Wnt pathway in the intestinal mucosa of mice, via overexpression of Dkk1 inhibitor leads to diminished number of crypts, concomitant with a loss of cell proliferation (Kuhnert et al., 2004). In turn, the loss of c-Myc expression and a rise in p21$^{cip/waf}$ expression are linked to a reduction in proliferation (Pinto et al., 2003).

In adult mice, c-Myc is dispensable for homeostasis and IEC proliferation but essential for the development of intestinal crypts. Tamoxifen-inducible depletion of c-Myc in the mucosa of adult and juvenile mice at the onset of crypt morphogenesis causes the failure to form normal numbers of crypts in the small intestine. Yet, mice are able to recover from this insult and form and maintain a normal IEC and without compensation by n-Myc or l-Myc (Bettess et al., 2005). Knock-out mice of c-Myc specifically in IEC under the

control of a cre promoter (c-Myc$^{\Delta IE}$) die before adulthood. However, c-Myc$^{\Delta IE/+}$ heterozygous mice, with reduced c-Myc expression, are complete viable, metabolically fit and display normal intestinal morphology (Luo et al., 2021).

HFD overnutrition, induces IEC proliferation by stabilizing β-catenin. Activation of the β-catenin pathway stimulates the expression of downstream genes including cyclin D, that, in turn, prompts IEC proliferation, further contributing to the increased absorption of nutrient and obesity development (Petit et al., 2007; Mao et al., 2013). c-Myc is a β-catenin target gene and key TF regulating the cell cycle. Hence, a significant induction of intestinal c-Myc expression was shown in C57BL/6N mice fed with HFD (Luo et al., 2021). Higher c-Myc expression was also seen in the distal ileum biopsies of the obese patients, which is consistent with mouse results. Additionally, c-Myc expression had a positive correlation with BMI and ALT levels in serum (Luo et al., 2021).

Importantly, c-Myc$^{\Delta IE/+}$ heterozygous mice are protected against HFD-induced obesity, IR, hepatic steatosis and fibrosis. Mechanistically, reduced expression of c-Myc in the intestine increases ChREBP and GLUT2/SGLT1 expression, thus promoting glucagon-like peptide-1 (GLP-1) production and secretion. GLP-1 is one of the crucial gut-derived peptide hormones that stimulates insulin secretion and thereby controls glucose homeostasis (Andersen et al., 2018). Increased GLP-1 synthesis in c-Myc$^{\Delta IE/+}$ mice improves IR and boosts insulin release in response to glucose (Luo et al., 2021).

Furthermore, intestinal c-Myc enhances levels of ceramides by targeting Cers4, a crucial enzyme of *de novo* ceramides synthesis (Luo et al., 2021). Ceramides are bioactive lipids that have an impact on inflammation, apoptosis, oxidative and ER stress, IR, and energy metabolism. There are three different ways to synthesize ceramides: the *de novo* pathway, the sphingomyelinase pathway and the salvage pathway (Aburasayn et al., 2016). Through genetic or pharmacological modification of ceramide biosynthesis and catabolism in mouse models, a crucial role for ceramides in metabolic disorders was demonstrated (Chaurasia et al., 2016). Mice with decreased intestinal c-Myc expression are resistant to dietary-induced metabolic disorders, and this resistance has a strong correlation with lower blood ceramide levels (Luo et al., 2021).

Whether the c-Myc–GLP-1 pathway and the c-Myc–ceramide pathway in the intestine co-operate with each other is unknown and requires further investigation. Besides, the roles of intestinal cell-type-specific c-Myc in metabolic diseases are worth investigating thoroughly in the future.

Interestingly, oral administration of 10058-F4, a c-Myc-Max interaction inhibitor, to obese mice greatly reduces obesity, IR, steatosis, and liver fibrosis. The metabolic benefits are mostly mediated by changes in GLP-1 and ceramide levels. Taking into account the absence of the current therapy for MASLD, the intestinal c-Myc pathway may be an attractive new area of investigation. Given the lack of a current MASLD treatment, research into the intestinal c-Myc pathway would be an appealing new field (Luo et al., 2021).

The dynamic equilibrium between ISC self-renewal and differentiation is crucial for maintaining intestinal homeostasis. Infiltration of macrophages and other immune cells as well as a persistent low-grade inflammation are linked to obesity. Macrophages infiltrating in the colonic mucosa contribute directly to the production of colonic TNF-α. Additionally, TNF-α secreted by the immune cells in the adipose tissue is also found circulating in the colonic mucosa. TNF-α can induce the phosphorylation of GSK-3 and reduce the Apc complex's ability to phosphorylate and degrade β-catenin. In turn, this triggers the production of the Wnt target genes c-Myc and cyclin D1, which in turn promotes the growth of ISCs and the development of obesity-related colorectal cancer (Liu et al., 2012). Although the particular mechanisms causing the low-grade inflammation caused by obesity are not entirely understood, increased palm oil consumption may be one of the initial causes of gastrointestinal alterations (Ghezzal et al., 2020).

In addition to being a complex of various organs and systems, the human body also carries more than 500–1000 different species of microbes. Numerous studies have been lately done on the complexity and variety of the GM in relation to human health and disorders. Growing evidences have underlined the importance of GM dysbiosis for the development and progression of metabolic diseases and obesity-related carcinogenesis (Kobyliak et al., 2016).

A thinner mucous layer, uneven localization of tight junction proteins (TJP), an abnormal immunological response involving immunoglobulin A (IgA), and antimicrobial peptides like lipopolysaccharides (LPS) can all contribute to intestinal disbiosis in obese people. Collectively, these defects cause LPS leakage, which eventually leads to TLR4/MyD88 and NF-κB activation and inflammation (Singh et al., 2023).

Numerous tumorigenic pathways, including members of the STAT family (particularly STAT3), can be stimulated by inflammation. STAT3 enhances the expression of anti-apoptotic genes, which lead to cellular survival and growth by promoting cyclin D family members and c-Myc. Therefore, GM obesity-related alterations may accelerate the development of colorectal cancer (CRC) by triggering inflammatory pathways (Kolb et al., 2016; Singh et al., 2023).

The identification of specific microbial taxa associated with obesity and T2DM still remains difficult. However, specific bacteria may be essential in triggering metabolic inflammation during the course of a disease. For example, HFD results in the enrichment of the Enterobacteriaceae *family*, which is predominately represented by *Escherichia coli* (*E. coli*), and has a strong association with poor glucose homeostasis (Ju et al., 2023). Certain *E. coli* strains with the polyketone acid synthetase (pks) island have the ability to produce the colibactin toxin and cause a proliferative effect linked to colorectal cancer (CRC). c-Myc is activated in pks + *E. coli*-infected CRC cells, which causes miR-20a-5p upregulation. Upregulation of miR-20a-5p can subsequently cause the translational silencing of target SENP1. SENP1 is a crucial enzyme that prevents the modification of p53 patterns, which is a key regulator of cellular senescence. The senescence of IEC in pks + *E. coli*–infected CRC cells stimulates the secretion of growth factors, essential for the initiation of tumour growth (Xing et al., 2022).

The secretion of different metabolites plays a major role in mediating the beneficial effects of GM. Acetate, propionate, and butyrate are three small organic metabolites called short-chain fatty acids (SCFAs) that are formed when resistant starch and dietary fibres are fermented. SCFA showed a variety of beneficial effects on immunological responses, energy metabolism, and intestinal homeostasis. Obesity and metabolic disorders have been associated with an abnormal SCFAs production. Butyrate is one

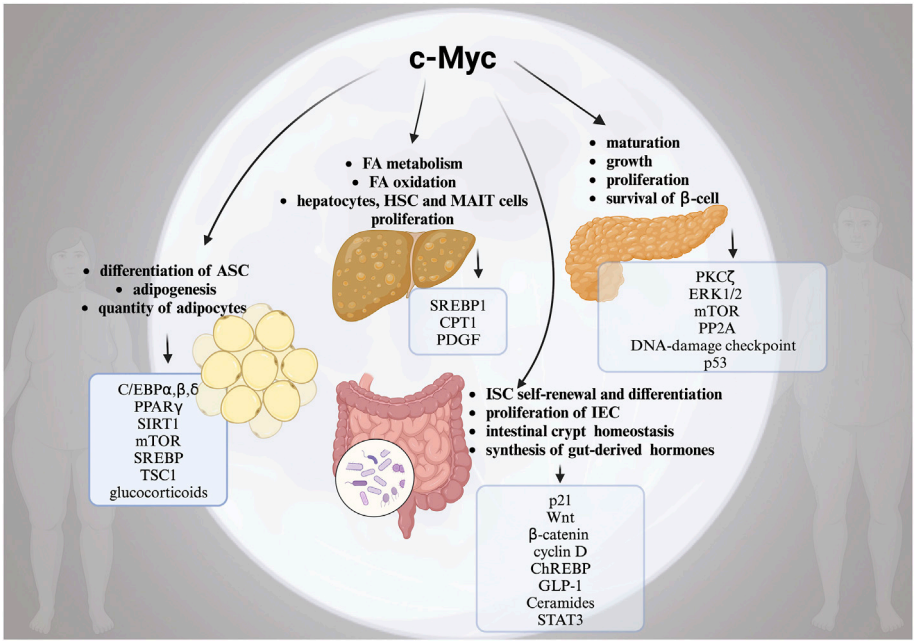

FIGURE 1
The complex role of moonlighting c-Myc for the development of obesity. Alterations in multiple c-Myc-related pathways in white adipose tissue (WAT), pancreas, liver and intestine in obesity. *Created with BioRender.*

of the SCFAs that has lately gained attention due to its ability to alleviate obesity and its associated comorbidities. Lower butyrate-producing microbial abundance in humans has been linked to a higher risk of metabolic disorders, demonstrating its potency in obesity prevention (Coppola et al., 2021). Interestingly, butyrate rapidly suppresses c-Myc levels in human CRC cells, which, in turn, reduces the levels of the miR-17–92 cluster miRNAs and decreases angiogenesis, metastasis, and cell proliferation (Hu et al., 2015). These indicate that butyrate may decrease the progression of CRC by altering the expression of tumour miRNAs, which causes changes in a number of critical signalling pathways, including c-Myc (Yuan and Subramanian, 2019).

Key points

- c-Myc is crucial for the control of homeostasis and the proliferation of IEC.
- Improvements in HFD-induced obesity, IR, and steatohepatitis are seen in mice with intestine-specific reduction of c-Myc.
- Obesity-associated changes of GM may activate c-Myc and cause progression of the colorectal cancer.

Conclusion and future perspectives

Nowadays, due to its alarming prevalence, obesity has emerged as the most dangerous nutritional disease and a significant health risk for people. In order to regulate the occurrence of this disease, it is necessary to control the nutritional habits and avoid sedentary life style. Yet the development of obesity is inseparable from epigenetics, which together with genetic factors play a pivotal role in its pathogenesis. Various TFs are critical participants in obesity and associated metabolic disorders such as T2DM and MASLD (Huang et al., 2018). In the present review, we show, that c-Myc is an important player in the multisystemic pathogenesis of obesity and its dysregulation is involved in inflammatory, metabolic, proliferative disorders in multiple organs. c-Myc is a typical moonlighting protein - a protein with a great number of functions that is unrelated and independent to each other. In WAT, liver, intestine, and pancreas, it controls the expression of genes involved in cell proliferation and growth, apoptosis, organogenesis, and metabolism. Additionally, it influences the nucleus' general structure, gene and microRNA expression, and genomic amplification (Lv and Lei, 2021).

Consequently, targeting c-Myc may open up novel strategies to combat obesity. However, the inactivation of a master regulator protein essential to normal cell proliferation and survival is thought to have substantial adverse effects, making c-Myc a dangerous therapeutic target (Dang et al., 2017). For instance, c-Myc is essential for potential regeneration strategies of the β-cells under baseline or metabolically stressed conditions. Furthermore, it is crucial for controlling intestinal cellular proliferation. All of this points to the urgent need for targeting c-Myc activity that is more cell-type specific, and taking into account the negative effects of its aberrant expression.

Over the last decades, several approaches have attempted to suppress c-Myc directly or indirectly at all levels of its regulation. Omomyc, for instance, has demonstrated promising properties in pre-clinical testing; it can induce apoptosis (Soucek et al., 2002) in cancer cells but not in normal cells, prevent proliferation and invasion (Beaulieu et al., 2019), stop the communication between the tumour and its microenvironment and recruit immune cells to the tumour site

(Demma et al., 2019; Madden et al., 2021). Omomyc is a 90 amino acid Myc mini-mutant that comprises the bHLH-LZ domain and competes with c-Myc, n-Myc and l-Myc for binding to DNA and preventing the transcription of the target genes (Soucek et al., 1998). *In vivo* Omomyc-mediated c-Myc inhibition resulted in sustained tumour regression and a strong anti-proliferative effect, with no negative effects on healthy tissue. Despite its short effective half-life, a phase I/II clinical trial started in 2021 making Omomyc (OMO-103) the first direct c-Myc inhibitor to reach clinical phase studies in patients with advanced solid tumors including non-small cell lung, colorectal and triple-negative breast cancer (Demma et al., 2019). Altogether, Omomyc taught us that c-Myc inhibition is a practicable approach and a safe and effective therapeutic strategy (Masso-Valles and Soucek, 2020). In a future, Omomyc and related polypeptide inhibitors of c-Myc function can potentially be a viable alternative therapeutic strategy for a wide variety of c-Myc-related disorders in obesity (Madden et al., 2021).

Another significant aspect is that obesity is a risk factor for a number of serious malignancies, such as CRC, HCC, and pancreatic cancer. In addition to altered FA metabolism, ECM remodelling, IR, GM dysbiosis, changed microenvironment, poor progenitor maturation, and chronic inflammation, the link between obesity and the development of cancer is not fully understood (Pati et al., 2023). As we summarized in this review, c-Myc actually plays a crucial part in each of these processes, contributing to multisystemic pathogenesis of obesity (Figure 1). Although the specific mechanisms for c-Myc and high risk of obesity and cancer are elusive, the correlation is definite. Hence, the evaluation of the molecular mechanisms underlying the dangerous liaisons between c-Myc, obesity, and obesity-associated cancers are of high priority for the identification of novel therapeutic targets. Importantly, c-Myc can be used as a diagnostic target to identify the "high risk" obese patients who require serious consideration for preventative measures like routine screening and personalized counselling.

Author contributions

YN: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing. FC: Writing–review and editing.

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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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Glossary

ASCs	Adipose stem cells
ATP	Adenosine triphosphate
Alb-myc^{tg}	transgenic mice with overexpression of c-Myc in hepatocytes under the control of albumin promoter
BMI	Body mass index
c-Myc$^{\Delta IE}$	Knockout mice of c-Myc specifically in intestinal epithelial cells under the control of a cre promoter
C/EBP	CCAAT/enhancer binding protein
CRC	Colorectal cancer
CLS	Crown-like structures
CDK	Cyclin-dependent kinase
NAFLD	Non-alcoholic fatty liver disease
ECM	Extracellular matrix
ER stress	Endoplasmic reticulum stress
FA	Fatty acids
GLP-1	Glucagon-like peptide-1
GM	Gut microbiota
HSCs	Hepatic stellate cells
HCC	Hepatocellular carcinoma
HFD	High fat diet
IR	Insulin resistance
IEC	Intestinal epithelial cells
ISCs	Intestinal stem cells
LPS	Lipopolysaccharides
mTORC	Mammalian target of rapamycin complex
MAIT	Mucosal-associated invariant T
MASLD	metabolic dysfunction-associated steatotic liver disease
NF-κB	Nuclear factor kappa b
PPARγ2	Peroxisome proliferator-activated receptor γ2
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
pIns-c-MycER^{TAM}	Tamoxifen inducible transgenic mice with overexpression of c-myc in pancreatic β cells under the control of an insulin promoter
Pks	Polyketone acid synthetase
ROS	Reactive oxygen species
SCFAs	Short-chain fatty acids
SREBP1	Sterol Regulated Element-Binding Protein
SIRT1	Sirtuin-1
SLD	Steatotic liver disease
TGF-β	Transforming growth factor beta
TJP	Tight junction proteins
TF	Transcription factor
TG	Triglycerides
TSC1	Tuberous sclerosis complex
T2DM	Type 2 diabetes mellitus
VLDL	Very-low-density lipoprotein
WNT	wingless-type MMTV integration site family
WAT	White adipose tissue
WHO	World Health Organization

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NOC1 is a direct MYC target, and its protein interactome dissects its activity in controlling nucleolar function

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The nucleolus is a subnuclear compartment critical in ribosome biogenesis and cellular stress responses. These mechanisms are governed by a complex interplay of proteins, including NOC1, a member of the NOC family of nucleolar proteins responsible for controlling rRNA processing and ribosomal maturation. This study reveals a novel relationship between NOC1 and MYC transcription factor, known for its crucial role in controlling ribosomal biogenesis, cell growth, and proliferation. Here, we demonstrate that NOC1 functions as a direct target of MYC, as it is transcriptionally induced through a functional MYC-binding E-box sequence in the NOC1 promoter region. Furthermore, protein interactome analysis reveals that NOC1-complex includes the nucleolar proteins NOC2 and NOC3 and other nucleolar components such as Nucleostemin1 Ns1 transporters of ribosomal subunits and components involved in rRNA processing and maturation. In response to MYC, NOC1 expression and localization within the nucleolus significantly increase, suggesting a direct functional link between MYC activity and NOC1 function. Notably, NOC1 over-expression leads to the formation of large nuclear granules and enlarged nucleoli, which co-localize with nucleolar fibrillarin and Ns1. Additionally, we demonstrate that NOC1 expression is necessary for Ns1 nucleolar localization, suggesting a role for NOC1 in maintaining nucleolar structure. Finally, the co-expression of NOC1 and MYC enhances nucleolus size and maintains their co-localization, outlining another aspect of the cooperation between NOC1 and MYC in nucleolar dynamics. This study also reveals an enrichment with NOC1 with few proteins involved in RNA processing, modification, and splicing. Moreover, proteins such as Ythdc1, Flacc, and splenito are known to mediate N6-methyladenosine (m6A) methylation of mRNAs in nuclear export, revealing NOC1's potential involvement in coordinating RNA splicing and nuclear mRNA export. In summary, we uncovered novel roles for NOC1 in nucleolar homeostasis and established its direct connection with MYC in the network governing nucleolar structure and

function. These findings also highlight NOC1's interaction with proteins relevant to specific RNA functions, suggesting a broader role in addition to its control of nucleolar homeostasis and providing new insight that can be further investigated.

KEYWORDS

NOC1, MYC, E-box, nucleolus, mass spectrometry, interactome

1 Introduction

MYC is a transcription factor crucial in the regulation of factors controlling ribosomal biogenesis and protein synthesis, which occurs primarily through its ability to regulate the transcription of genes required for ribosome assembly and function (van Riggelen et al., 2010; Campbell and White, 2014; Destefanis et al., 2020). MYC promotes the transcription of its target genes, such as ribosomal proteins and co-factors, by binding to specific DNA sequences known as E-boxes (5′-CACGTG-3′) within their promoter region (Fernandez et al., 2003; Orian et al., 2003; Hulf et al., 2005). MYC also promotes the transcription of ribosomal RNA (rRNA) genes, which are transcribed by RNA polymerase I to generate the precursor rRNA transcripts. Since ribosomes are central to protein synthesis and cell growth, MYC's role in promoting ribosomal biogenesis largely contributes to protein synthesis, necessary for cell growth and proliferation, a function that is conserved both in flies and vertebrates (Schlosser et al., 2003; Arabi et al., 2005; Grandori et al., 2005; Grewal et al., 2005; Van Riggelen et al., 2010; Destefanis et al., 2020).

NOC1 is a nucleolar protein that, together with NOC2 and NOC3, plays a critical role in the maturation of rRNA and the transport of the pre-ribosomal subunits (Sailer et al., 2022; Dorner et al., 2023). NOC1 in yeast works as a heterodimer with NOC2 during the initial maturation of the ribosomal RNA (rRNA) and in the transport of the pre-60S ribosomal subunit, a process that is completed by NOC2/NOC3 heterodimers (Milkereit et al., 2001). Studies on the distribution of affinity-tagged NOC1 and, more recently, proteomics and crosslinking coupled to mass spectrometry, confirmed the presence of NOC1 in the early pre-60S complex (Sailer et al., 2022; Dorner et al., 2023), while cryo-EM studies showed its role in the formation of heterodimers with NOC2, essential for the quality-control checkpoint of the maturation of the large ribosome subunit (Sanghai et al., 2023).

We recently characterized NOC1 function in flies and showed its role in controlling polysome abundance, rRNA maturation, protein synthesis, and cell survival (Destefanis et al., 2022). Furthermore, lowering NOC1 levels in different contexts, such as whole animals or specific organs, results in various developmental and functional impairments (Destefanis et al., 2022). Our initial transcriptomic analysis revealed NOC1 as a potential direct target of MYC (Hulf et al., 2005); thus, we further analyzed this critical function in the context of ribosomal biosynthesis directed by MYC.

Here, we show that NOC1 is a direct transcriptional target of MYC, and its activation is mediated by a functional E-box sequence located in the promoter region of the *NOC1* gene. We then used HA-NOC1 as bait to perform Mass Spectrometry (MS) analysis to determine the NOC1 interactome to characterize NOC1 function and connect its activity with biological processes, mainly focusing on components that control nucleolar homeostasis.

Bioinformatic analysis using the STRING database identified clusters of NOC1 protein interactors, and the most significant was on ribosome biogenesis. These data showed a significative enrichment of NOC2 and NOC3 ($p < 0.05$) strongly aligning with data published previously in yeast (Milkereit et al., 2001; Hierlmeier et al., 2013), and a significant cluster of nucleolar proteins, such as fibrillarin (fib) and nucleostemin 1 (Ns1), and others, like Novel nucleolar proteins (Non1 and Non3) and mushroom body miniature (mbm), involved in the 60S subunit biogenesis. Moreover, we found an enrichment of nucleolar and nuclear proteins, like Nnp (Hulf et al., 2005), and peter pan (ppan) (Migeon et al., 1999; Zielke et al., 2022), involved in pre-rRNAs production and RNA maturation, and modulo (mod) (Perrin et al., 2003), that were previously identified as direct targets of MYC, emphasizing the relation between NOC1 and MYC.

In addition, these studies also identified enrichment of the nuclear m^{6}A "reader" YTH domain RNA Binding Protein C1 (Ythdc1) (Roundtree et al., 2017), Flacc (Fl(2)d-associated protein), and spenito (nito) (Knuckles et al., 2018). Remarkably, these proteins are part of the complex that mediates the N6-Methyladenosine methylation of mRNAs for their nuclear export (Knuckles et al., 2018; Shi et al., 2021). We could outline a novel function for the MYC-NOC1 axis in regulating mRNA m^{6}A modification and transport.

Finally, the observation that NOC1 controls the nucleolar localization of Ns1, together with those indicating that MYC enhances NOC1-induced large granular structures in the nucleus, further sustains the functional relationship between MYC and NOC1 in maintaining nucleolar homeostasis.

In summary, these findings will provide significant insights into the role of NOC1 and its interactome that may contribute to the control of nucleolar functions, supporting the crucial role of MYC in regulating growth, proliferation, and protein synthesis.

2 Materials and methods

2.1 Fly stocks and husbandry

Fly cultures and crosses were raised at 25°C on a standard medium containing 9 g/L agar (ZN5 B and V), 75 g/L corn flour, 60 g/L white sugar, 30 g/L brewers' yeast (Fisher Scientific), 50 g/L fresh yeast and 50 mL/L molasses (Naturitas), along with nipagin and propionic acid (Fisher). The lines used were obtained by: *UAS-HA-MYC* (*Bellosta et al., 2005*); *NOC1-GFP* (*B51967*) *UAS-NOC1-HA* (Flyorf-CH) *NOC1-RNAi* (B25992). *UAS-Ns1-GFP* is a gift from Patrick J. Di Mario University of Louisiana, LA). *hsp70-Gal4* gift from Florenci Serras (University of Barcelona, Spain).

2.2 Cloning NOC1 E-box and molecular biology

Site-directed mutagenesis (SDM) was carried out using the following primers for the mutant E-box 5′ TTC GGC ACG AGT TTG AAT AGA ATT CCG AGT TGT TTC TAA CGC CG; 5' CGG CGT TAG AAA CAA CTC GGA ATT CTA TTC AAA CTC GTG CCG AA; following instructions from the SDM kit (Promega). Promoter elements used in luciferase reporter expression analyses were cloned into the pGL3-basic vector (Promega).

2.3 Cell culture and luciferase assays

S2 *Drosophila* cells were propagated in Schneider's *Drosophila* medium (Gibco), supplemented with 10% fetal bovine serum, at 24°C. S2 cell transfections were carried out using Cellfectin (Invitrogen). *NOC1* reporter constructs were added at 1 µg per 10^6 cells; tubulin- Renilla luciferase control DNA were co-transfected at 0.1 µg per 10^6 cells and incubated with a transfection mix for 12 h. Cells were harvested 24 or 60 h posttransfection. Relative gene expression was determined using the Dual-Luciferase Reporter assay system (Promega) on a luminometer.

2.4 RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from 8 whole larvae using the QIAGEN RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNAs were quantified using an ultraviolet (UV) spectrophotometer, and RNA integrity was confirmed with ethidium bromide staining. 1 µg total RNA from each genotype was reverse transcribed into cDNA using SuperScript IV MILO Master Mix (Invitrogen). The obtained cDNA was used as the template for quantitative real-time PCR (qRT-PCR) using qPCR Mastermix (Promega). mRNAs expression levels were normalized to *actin-5C mRNA* used as the internal control. The relative level for each gene was calculated using the 2-DDCt method (Hulf et al., 2005) and reported as arbitrary units. Three independent experiments were performed and cDNAs were used in triplicate. The following primers were used for qRT-PCR: *Actin5c:* 5′CAGATC ATGTTCGAGACCTTCAAC; 5′ACGACCGGAGGCGTACAG (Parisi et al., 2013).

Fibrillarin: 5′ACGACAGTCTCGCATGTGTC; 5′ATGCGG TACTTGTGTGGATG (this work).

MYC: 5′CATAACGTCGACTTGCGTG; 5′GAAGCTCCCTGC TGATTTGC (Parisi et al., 2013).

NOC1: 5′CTATACGCTCCACCGCACAT; 5′GTCGCTACC GAACTTGTCCA (Destefanis et al., 2022).

2.5 Protein extractions and Western blotting

Five larvae for each genotype were lysed in 200 µL of lysis buffer (50 mM Hepes/pH 7.4, 250 mM NaCl, 1 mM (EDTA), 1.5% Triton X-100 containing a cocktail of phosphatases inhibitors (PhosSTOP 04906837001, Merck Life Science) and proteases inhibitors (Roche, cOmplete Merck Life Science). Samples were sonicated three times for 10 s using a Branson Ultrasonic Sonifier 250 (Branson, Darbury, CA, United States) equipped with a microtip set at 25% power. Tissue and cell debris were removed by centrifugation at 100,00× *g* for 30 min at 4°C. Proteins in the crude extract were quantified by a bicinchoninic acid (BCA) Protein assay Reagent Kit (Pierce), following the manufacturer's instructions with bovine serum albumin as the standard protein. For SDS-PAGE, samples were incubated for 8 min at 100°C in standard reducing 1x loading buffer; 40 µg of total protein were run on an SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (GE-Healthcare, Fisher Scientific Italia) After blocking in 5% (*w*/*v*) non-fat milk in tris-buffered saline (TBS)-0.05% Tween (TBS-T), membranes were incubated overnight with primary antibodies: rat monoclonal anti-HA (1:1000, ROCHE), or Actin5c (1:200, #JL20) from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, United States. Appropriate secondary antibody was incubated for 2 h at room temperature, followed by washing. The signal was revealed with ChemiDoc Touch Imaging System (Bio-Rad Lab).

2.6 Immunoprecipitation

Hsp70 (hs)-Gal4> NOC1 larvae or control *hs-Gal4> w^{1118}* were heat-shocked at 37°C for 1 h and left to recover for 2 h at room temperature. 20 larvae from each genotype were washed in PBS and lysed with 750 µL of immunoprecipitation buffer (100 mM HEPES, 100 mM NaCl, 0.5% Triton, 10 mM MgCl) containing proteases and phosphatases inhibitors. Protein lysates were incubated for 20 min in ice and centrifuged at 13.000 rpm for 30 min a 4 °C. 500 µL of lysates were incubated with 50 µL of Sepharose-beads-Protein-G (Invitrogen) previously incubated with 4 µL anti-HA antibodies. Incubation was performed for 2 h at room temperature, and beads were washed extensively with ice cold lysing buffer. After centrifugation, bound proteins were eluted with 100 µL of SDS-loading buffer LDS Sample Buffer (Thermo Fisher Scientific) containing 5% Bolt Sample reducing agent (Thermo Fisher Scientific) at 80°C for 5 min 20 µL of the sample was run on a Western blot and 80 µL were used for the MS analysis. Experiments were repeated twice.

2.7 Mass spectrometry and proteomic interaction partners analysis

Immunoprecipitated samples were loaded on 10% SDS-PAGE and run for about 1 cm. Gels were then stained with Coomassie and the entire stained area was excised as one sample. Excised gel bands were cut into small plugs (~1 mm^3), rinsed with 50 mM ammonium bicarbonate and acetonitrile (ACN) solution, and vacuum dried. Dried gel pieces were then reduced using 10 mM DTT (56°C for 30 min) and alkylated using 55 mM iodoacetamide (room temperature for 30 min, in the dark). After sequential washing with 50 mM NH4HCO3 and ACN, gel pieces were dried and rehydrated with 12.5 ng/mL trypsin (Promega, Madison, WI) solution in 25 mM ammonium bicarbonate on ice for 30 min.

The digestion was continued at 37°C overnight. The tryptic peptides were sequentially extracted from the gels with 30% ACN/3% TFA and 100% ACN. All of the supernatants were combined and dried in a SpeedVac. The tryptic peptides were resuspended in 0.1% TFA, desalted on C18 stage tips, and resuspended in 20 µL of 0.1% formic acid buffer.

For LC-MS/MS analysis, the peptides were separated on an Easy-nLC 1200 UHPLC system (Thermo Fisher Scientific) using an 85-min gradient on a 25 cm long column (75 µm inner diameter) filled in-house with C18-AQ ReproSil-Pur material (3 µm particle size, Dr. Maisch, GmbH). The gradient was set as follows: from 5% to 25% in 52 min, from 25% to 40% in 8 min, and from 40% to 98% in 10 min, with a flow rate of 400 nL/min. The buffers were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The peptides were analyzed with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, United States) in data-dependent mode. Full scans were performed in the Orbitrap mass analyzer at a resolving power of 120,000 FWHM (at 200 m/z) in the mass range of 350–1,100 m/z, with a target value of 1×10^6 ions and a maximum injection time of 50 ms. Each full scan was followed by a series of MS/MS scans (collision-induced dissociation) over a cycle time of 3 s, with a maximum injection time of 150 ms (ion trap) and a target of 5×10^3 ions. The ion source voltage was set at +2,100 V and the ion transfer tube was warmed up to 275°C. Data was acquired using Xcalibur 4.3 and Tune 3.3 software (Thermo Fisher Scientific). QCloud was used for all acquisitions to control instrumental performance during the project, using quality control standards (Chiva et al., 2018).

For data and computational analysis, the raw files were searched in Proteome Discoverer version 2.2 software (Thermo Fisher Scientific). Peptide searches were performed using the UniProt *Drosophila melanogaster* (fruit-fly) database digested *in silico* (downloaded in July 2022) and a database containing common contaminants. Trypsin was chosen as the enzyme with 5 missed cleavages. The static modification of carbamidomethylation (C) was incorporated in the search, with variable modifications of oxidation (M) and acetylation (protein N-term). The MASCOT search engine (v.2.2 Matrix Science) was used to identify the proteins, using a precursor mass tolerance of 10 ppm and a product mass tolerance of 0.6 Da. False discovery rate was filtered for <0.01 at PSM, at peptide and protein levels. Results were filtered to exclude potential contaminants and proteins with less than two peptides.

MS downstream analysis was performed using the ProTN proteomics pipeline (www.github.com/TebaldiLab/ProTN and www.rdds.it/ProTN) (manuscript in preparation). Peptide intensities were log2 transformed, normalized (median normalization), and summarized into proteins (median sweeping) with functions in the DEqMS Bioconductor package (Zhu et al., 2020). Imputation of the missing intensities was executed by PhosR package (Kim et al., 2021). Differential analysis was performed with the DEqMS package, proteins with absolute log2 FC > 0.75 and *p*-value <0.05 were considered significant.

Protein-protein interaction network was constructed using STRING interaction database, version 12.0 (https://string-db.org/) (von Mering et al., 2003). Medium confidence interactions (score>0.4) were accepted as determined by the STRING database. The PPI network was grouped into relevant protein clusters using the Markov Cluster Algorithm (inflation parameter, 3) clustering option provided by STRING.

2.8 Immunostaining

Dissected tissues were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Science) in PBS for 30 min at room temperature. After permeabilization with 0.3% Triton/PBS, tissues were washed in Tween 0.04% in PBS, saturated with 1% BSA in PBS, and incubated overnight with anti-fibrillarin antibodies (1:100), anti-HA (1:100, ROCHE), anti-GFP (1:200, ThermoFisher A11122) and anti-MYC affinity-purified antibodies (1:1000) (Galletti et al., 2009; Destefanis et al., 2022). Relative secondary antibodies conjugated with Alexa555 and Alexa488 were used 1:2,000 (Invitrogen). After washing with PBST, samples were mounted on slides using Vectashield (Vector Laboratories) and fluorescence images were acquired using a Leica-TCS-SP8 confocal microscope.

3 Results

3.1 NOC1 contains a functional E-box sequence in its promoter region and is transcriptionally induced by MYC

Our initial observation on the transcriptomic analysis of potential MYC target genes identified NOC1 as a predicted nucleolar gene that contains in its 5′promoter region the E-box sequence CACGTG typically within the first 100 bp from the initial translation initiation codon ATG (Figure 1A), and thus considered a *bona-fide* MYC binding region (Hulf et al., 2005). By qRT-PCR, we show that constitutive expression of MYC in whole *Drosophila* larvae (Figure 1B) using the *actin* promoter resulted in NOC1 transcriptional activation and also in the upregulation of *fibrillarin-mRNA* (Figure 1C), a known MYC target that contains functional E-boxes in its promoter region conserved both in flies and vertebrates (Orian et al., 2003; Hulf et al., 2005; Koh et al., 2011).

The 5′promoter region of NOC1 contains a putative TATA box sequence at about - 26 bp from the transcription start, a sequence identified as the Transcription Start Site (TSS), and the CACGTG sequence (E-box) at −82 bp from the ATG transcription start (Figure 1A). To investigate whether the CACGTG sequence responds to MYC activation, we cloned the 5′promoter region of *NOC1*, containing the wildtype CACGTG sequence or the scramble sequence GAATTC (Figure 1D), upstream of a plasmid expressing the Firefly luciferase ORF. The reporter plasmids were co-transfected into *Drosophila* S2-MT-MYC cells with a plasmid expressing the Renilla luciferase. MYC expression was induced by adding CuSO4 to the medium (Figure 1E). Firefly luciferase activity was measured in the cell lysates after 5 h of induction and normalized to the co-transfected Renilla luciferase expressed under the control of the constitutive tubulin promoter (Figure 1F). As shown upon MYC expression, cells expressing the *NOC1* promoter region with the mutated E-box have significantly reduced luciferase activity compared to that from cells expressing the wild-type *NOC1* promoter, indicating that the

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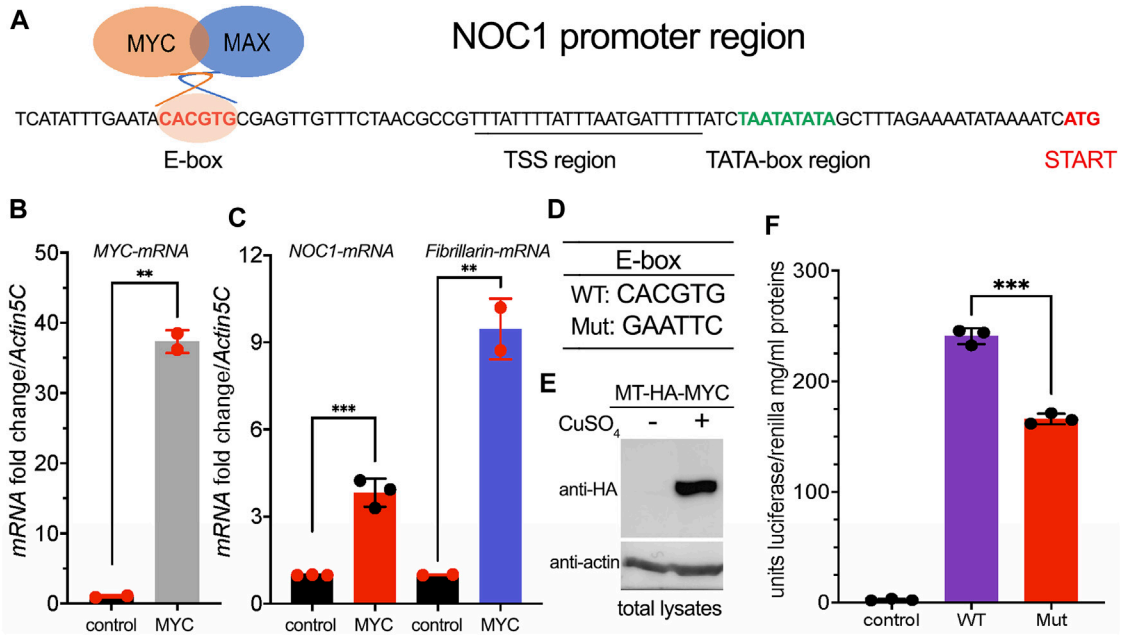

FIGURE 1
NOC1 contains in its promoter a functional MYC E-box sequence. **(A)** DNA promoter region of the *NOC1* gene showing the position of the E-box, the putative Transcription Start Sequence (TSS) with the TATA box, and the Initiation of Transcription point (START). **(B)** qRT-PCR from third instar whole larvae tissues showing the upregulation of *MYC-mRNA* **(B)** and of *NOC1* and *fibrillarin-mRNAs* **(C)** upon MYC induction. The expression of *UAS-MYC* was induced using the *actin-Gal4* promoter. **(D)** DNA sequences of WT and Mutant E-boxes. **(E)** Western blot from S2-MT-HA-MYC cells showing the expression level of the metallothionein HA-MYC upon induction for 5 h using $CuSO_4$. Actin was used as a control for loading. **(F)** Units of relative luciferase activity in lysates of S2-MT-HA-MYC cells treated for 5 h with $CuSO_4$ and transfected with Renilla plasmid alone (control), of with NOC1 promoter region containing WT (WT) or Mutant (Mut) E-box.

sequence CACGTG in the *NOC1* promoter functions as an enhancer of MYC activity.

3.2 Interactome analysis of NOC1 associates its expression with NOC2 and NOC3 proteins and other components of the nucleolus

To investigate how NOC1 might regulate nucleolus functions, we explored its binding partners by analyzing the total interactome through immunoprecipitation and tandem mass spectrometry analysis (Figure 2A). Third-instar larvae expressing *UAS-HA-NOC1* under the *actin-Gal4* were used first to test a few conditions to efficiently extract NOC1 protein from the cells (Figure 2B). As shown in the left panel, NOC1 is efficiently expressed in lysates from third-instar larvae as a 120 KDa protein detected by the anti-HA antibodies. We first tested three conditions for lysing the tissues to avoid high detergent and salt concentrations according to previous protocols for immunoprecipitation in whole larvae (Bellosta et al., 2005). The comparative analysis of the three lysis conditions led to selecting the buffer containing 0.5% Triton and 200 mM NaCl, which appears to balance mild stringency conditions and high recovery yield, making it suitable for extracting NOC1 protein in our experimental conditions (Figure 2; middle panel). Since we found NOC1 transcriptionally upregulated as early as 3 h upon MYC expression (Hulf et al., 2005) and (Figure 1C), we decided to use the inducible promoter *hsp70 (heat-shock)-Gal4* to ubiquitously express NOC1 to perform our analysis at a similar time point. *Hs-Gal4; UAS-HA-NOC1* larvae and control (*hs-Gal4; w^{1118}*) were heat-shocked for 1 hour and 37 °C. After 2 hours of recovery at room temperature, larvae were lysed to pursue the immunoprecipitation (IP) using anti-HA antibodies. Immunoblotting analysis showed enrichment of HA-NOC1 bands in the expected samples (Figure 2; left panel). While a weak band of 120 KDa is also visible in the control sample, the lower molecular weight bands characteristic of the NOC1 pattern are not present (Destefanis et al., 2022), confirming the specificity of the experiment.

To discover NOC1 protein partners, we used affinity purification coupled with label-free mass spectrometry (AP-MS). Specifically, we performed the co-immunoprecipitation of the tagged-NOC1 protein in *hs-Gal4; UAS-HA-NOC1* lysates, and the control tissues *hs-Gal4; w^{1118}*, respectively. Immunoprecipitates (IPs) were then analyzed by LC-MS/MS using an Easy-nLC 1200 UHPLC system coupled to an Orbitrap Fusion™ mass spectrometer. For protein identification and quantification, acquired raw data were imported into the Proteome Discoverer 2.2 (PD) platform and searched with MASCOT (v2.6 Matrix Science, London, United Kingdom) against the UniProtKB *Drosophila melanogaster* database. The quantitative output of PD was then further processed using the ProTN pipeline, enabling comprehensive quality control, statistical analysis, and interpretation of proteomic datasets. We identified a total of 239 proteins that were significantly ($p < 0.05$) enriched in HA-NOC1 immunoprecipitated (IPs) relative to control, representing

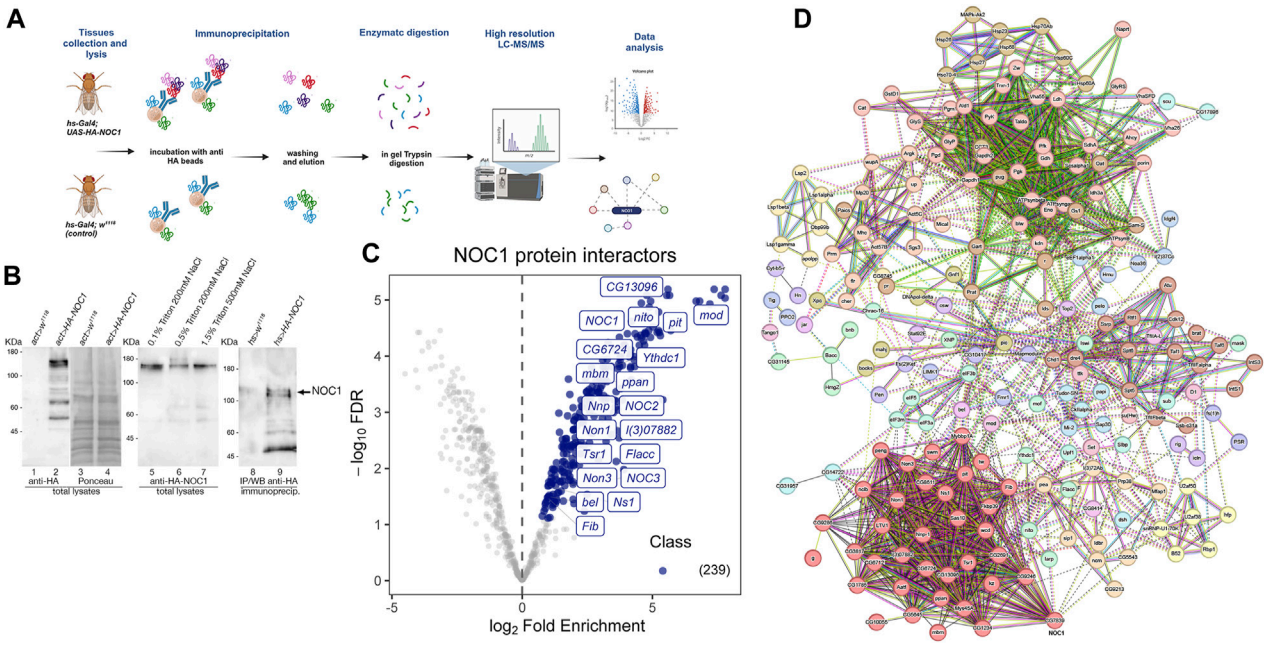

FIGURE 2
NOC1 is associated with components of the nucleolus. **(A)** Schematic representation of the workflow used to identify NOC1 interacting proteins. *hs-Gal4; UAS-HA-NOC1* larvae and control (*hs-Gal4; w*^{1118}) were lysed and subjected to immunoprecipitation using anti-HA conjugated beads. NOC1 immunoprecipitated proteins were eluted with Laemmli sample buffer and processed by *in-gel trypsin* digestion before MS/MS analysis. The figure is created with BioRender.com. **(B)** Western blot showing the expression of *UAS-HA-NOC1* in lysates from third instar larvae and the enrichment in the IPs using the *actin* > *Gal4* promoter. The left panel shows a band of about 120 KDa recognized by anti-HA antibodies and present only in the total lysates of control larvae (lane 1) or expressing HA-NOC1 (lane 2). In lanes 3 and 4 is shown the Ponceau staining relative to lanes 1 and 2. In the middle panel is shown the expression level of HA-NOC1 upon immunoblot with anti-HA antibodies from larvae lysate with buffer containing different concentrations of detergent and salt (lane 5–7). In the right panel is shown the immunoblot from the eluted material from the Seph-Prot-G conjugated with anti-HA antibody upon immunoprecipitation from lysates of larvae expressing HA-NOC1 using the *hsp70-heat-shock (hs)* inducible promoter after 1 h of heat-shock and 2 h of recovery. Lane 8 shows the immunoblot from lysates of control larvae *hs-w*^{1118,} while lane 9 shows the eluted from the immunoprecipitation from animals expressing *hs-HA-NOC1;* this represents 1/5 of the material used from the MS analysis. **(C)** Volcano plot highlighting all proteins enriched. The mean log2 ratio of hs-HA-NOC1 IPs versus control hs-w^{1118} IPs are plotted versus the corresponding *p*-values. 239 proteins significantly enriched (blue dots) with a *p*-value below 0.05 and log2-FC >1.5 thresholds were treated as putative NOC1 binding partners. The most representative interactors found for this analysis are indicated in the plot. **(D)** Schematic view of protein-protein interactions among NOC1 targets according to the STRING database (v.12). STRING protein-protein interaction analysis indicates the most prominent clusters with a medium confidence score of 0.4. Each node represents a protein, and each edge represents an interaction.

putative NOC1 binding partners (Supplementary List S1). The raw data are available via ProteomeXchange with identifier PXD047564. Results are illustrated by the volcano plot displaying the proteins significantly enriched in NOC1-IPs in light blue, with a fold change (FC) > 1.5 and *p*-value <0.05. To better characterize the NOC1 interactome, the list of putative interacting proteins was processed by STRING protein-protein interaction analysis, and clusters were identified in the resulting network using the Markov Clustering Algorithm (MCL) (Figure 2C). This analysis outlined a few interesting clusters of NOC1 interactors (Figure 2D). The most relevant is Cluster1, which includes NOC2 and NOC3 (Supplementary Table S1, and Volcano plot Figure 2C). The same cluster also includes nucleolar proteins such as Fibrillarin (Fib), an rRNA O-methyltransferase, and l(3)07882 required for the processing of the pre-rRNAs, Novel nucleolar proteins (Non1 and Non3) involved in the biogenesis of the 60S subunits and needed for the assembling of the mitotic spindle, like Nucleostemin 1 (Ns1), required for the release of the 60S ribosomal subunit, mushroom body miniature (mbm) involved in ribosome biogenesis. Others non nucleolar proteins, like the CG13096, a homolog of human Ribosomal L1 domain-containing protein (RSLD1), the CG6724, a putative homolog of WRD12 required for the maturation of rRNAs and the formation of the large ribosomal subunit, Nnp, and Tsr1 described for the processing of pre-rRNAs and the control of RNA maturation. Notably, we also found in the interactome the DEAD-box RNA helicases pitchoune (pit) (Zaffran et al., 1998) and bel, *Drosophila* homologs of MrDb (Grandori et al., 1996) and *DDX3* (Liao et al., 2019) respectively. Interestingly, few of these proteins, such as pit (Zaffran et al., 1998), modulo (mod) (Perrin et al., 2003), Nnp (Nnp1) (Hulf et al., 2005), and peter pan (ppan) (Zielke et al., 2022), have been previously identified as putative direct targets of MYC specifically in the context of controlling cell growth and proliferation.

This analysis also found a highly represented cluster containing Ythdc1 (YTH domain RNA Binding Protein C1), Flacc (Fl(2)d-associated protein), and splenito (nito). Ythdc1 is a conserved nuclear m^{6}A "reader" protein that mediates the incorporation of methylated mRNAs into the nuclear export pathway (Roundtree et al., 2017; Shi et al., 2021). Interestingly, Flacc was found to be associated with female lethal (Fl(2)d), a protein homolog of Wilms'-tumor-1-associated protein (WTAP) (Penn et al., 2008), that was

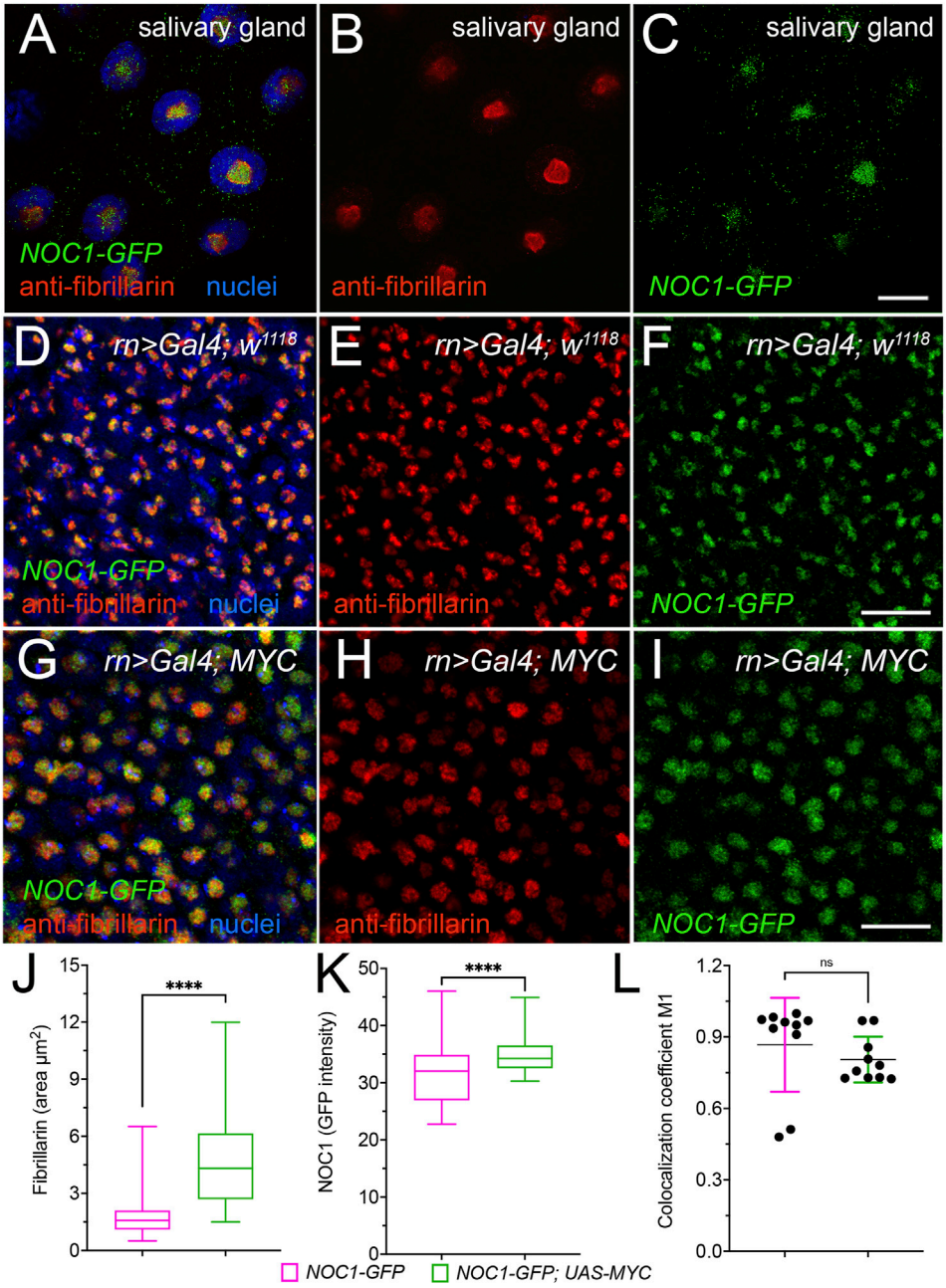

FIGURE 3
NOC1 nucleolar expression increases with MYC induction. **(A–C)** Confocal images of the cells from the salivary glands showing the endogenous fibrillarin expression in the nucleolus and its colocalization with NOC1-GFP, visualized with anti-fibrillarin **(B)** and anti-GFP **(C)** antibodies to visualize NOC1-GFP fusion protein (otherwise too low to detect directly), with nuclei stained in blue in A. **(D–F)** Cells of the wing imaginal discs showing endogenous fibrillarin **(E)** and NOC1-GFP expression **(F)**, and their colocalization in D. **(G–I)** Cells of the wing imaginal discs expressing *UAS-MYC,* using the *rotund-Gal4* promoter, stained for fibrillarin **(H)** and NOC1-GFP **(I)**. In **(G)**, they merged images with nuclei stained with Hoechst (blue). Note that the nucleolus size increases by MYC expression (see also Figure 6A for quantification). **(J)** Analysis of the fibrillarin area in cells of the wing imaginal disc of *NOC1-GFP; rn > w*^{1118} animals or expressing *NOC1-GFP; rn > UAS-MYC*. **(K)** Analysis of the GFP intensity relative to NOC1 expression in the nucleolus area in cells of the wing imaginal disc of *NOC1-GFP; rn > w*^{1118} animals or expressing *NOC1-GFP; rn > UAS-MYC*. **(L)** Coefficient of localization between NOC1 and fibrillarin in cells from control animals (*NOC1-GFP; rn > w*^{1118}) or expressing MYC (*NOC1-GFP; rn > UAS-MYC*). This analysis was performed using the Coloc2 plug-in of the Fiji software coloc2, and *p*-values calculated based on raw values that represent Manders' colocalization coefficient (with automatic Costes threshold). Scale bars in Figure C represent 20 µm, and in Figures F and D, 10 µm. The experiments were repeated at least three times, and the statistical analysis among the various genotypes was examined by Student's t-test, and *p* values are indicated with asterisks **** = $p < 0.0001$.

isolated in complexes with Snf (Penn et al., 2008), a component of U1 and U2 small nuclear ribonucleoproteins (snRNPs) that contained U2AF50, U2AF38, and U1-70K (small nuclear ribonucleoprotein 70K), which function in the regulation of the spliceosome. Notably, we observed an enrichment of the U2A proteins in our analysis (Supplementary Table S1), suggesting

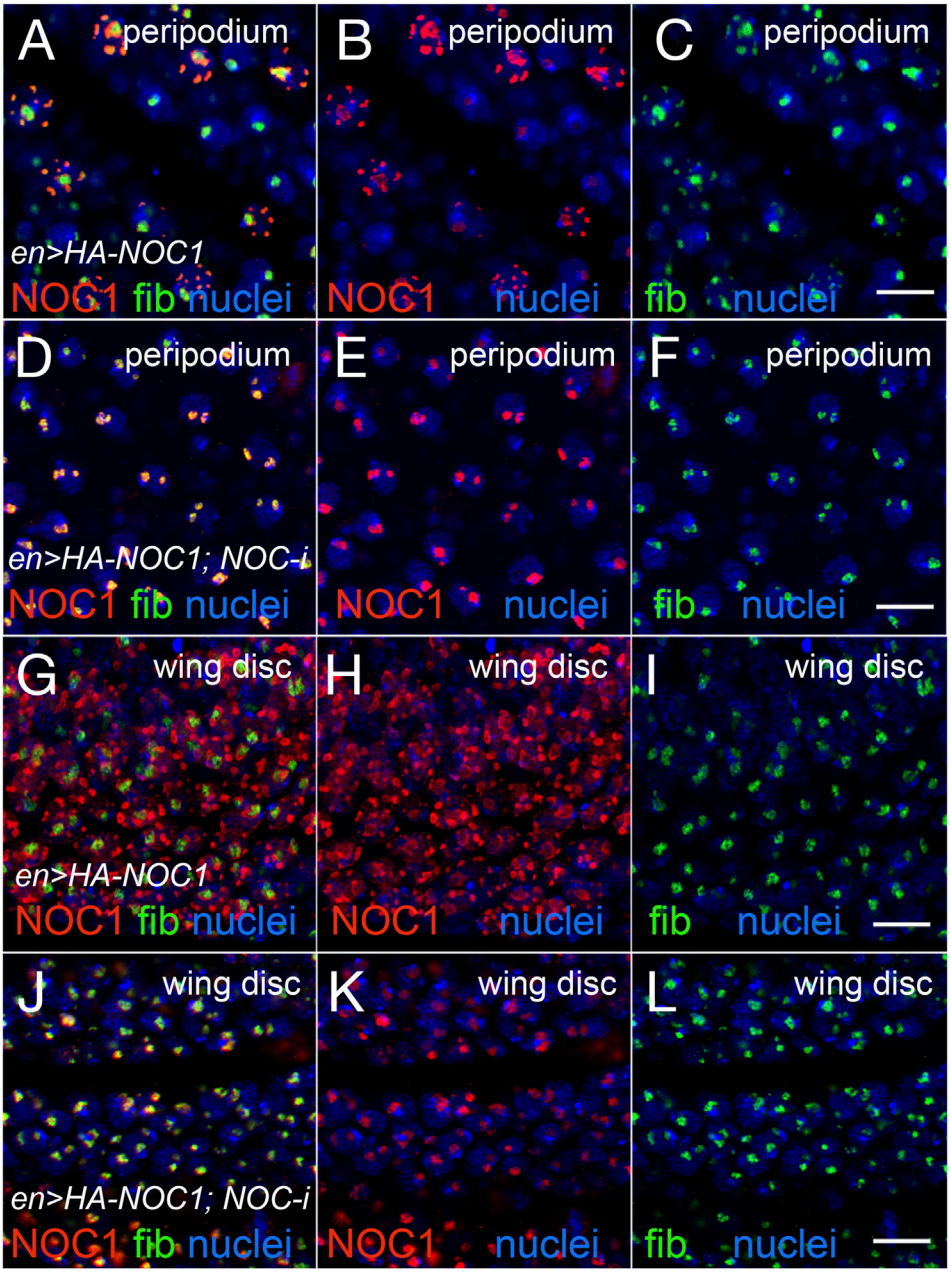

FIGURE 4
Expression of NOC1 induces extra nucleolar granules and enlargement of the nucleolus. **(A–C)** Confocal images of cells of the peripodium expressing HA-NOC1 alone or with NOC1-RNAi **(D–F)** using the *engrailed* promoter. **(G–L)** Images of cells from the imaginal disc expressing NOC1 alone **(G–I)** or with NOC1-RNAi (J–L). NOC1 and fibrillarin expression are visualized by immunofluorescence using anti-HA (red) and anti-fibrillarin (green) antibodies, respectively. Hoechst is used to visualize the nuclei. Scale bars represent 10 μm.

that NOC1 may play a key role in RNA splicing by linking the U1 snRNP particle to regulatory RNA-binding proteins and in the control of nuclear export via Ythdc1.

3.3 NOC1 expression in the nucleolus increases upon MYC induction

We previously showed that endogenous NOC1 colocalizes with fibrillarin in the nucleolus (Destefanis et al., 2022). Here, we confirm the co-localization of endogenous NOC1-GFP, expressed as GFP fusion protein (NOC1-GFP) under its endogenous promoter (Kudron et al., 2018) with fibrillarin. This is seen in the gigantic nucleolus of the salivary gland cells (Figures 3A–C) and the nucleolus of cells from the wing imaginal disc (Figures 3D–F). Furthermore, expression of MYC in cells of the wing imaginal disc, using *rotund-Gal4* promoter (Figures 3G–I), significantly increases the fibrillarin area in the nucleolus (Figure 3J) and also the fluorescence intensity of NOC1-GFP (Figure 3K), which are both direct transcriptional targets of MYC. However, statistical analysis

indicates that the coefficient of localization between NOC1 and fibrillarin does not change upon MYC expression, as shown from data in cells from control animals (*NOC1-GFP; rn > w*^{1118}) compared to that from cells expressing MYC (*NOC1-GFP; rn > UAS-MYC*) (Figure 3L), indicating that MYC promotes an increase in nucleolar size and of NOC1-GFP expression in the nucleolus.

3.4 NOC1 overexpression induces the formation of large nuclear granules and enlarged nucleoli that co-localize with fibrillarin

We previously reported that ectopic expression of NOC1 results in nucleolar morphology changes (Destefanis et al., 2022). To analyze how the ectopic expression of NOC1 could influence nucleolar morphology, we overexpressed the HA-tagged version of NOC1 in cells of the wing imaginal discs using the *engrailed-Gal4* promoter. *Engrailed* is expressed in both the columnar epithelium forming the wing imaginal disc and in the giant cells of the peripodium, a squamous epithelium adjacent to the columnar epithelium of the wing discs (Pallavi and Shashidhara, 2005; Smith-Bolton, 2016). Analysis of NOC1 expression in these cells, by immunostaining using an anti-HA antibody, revealed in the nucleus the presence of large granules containing HA-NOC1 and an enlargement of the size of the nucleolus, where NOC1 is visibly expressed. The granules are more easily distinct and visible in the peripodium because of the gigantic size of these cells (Figures 4A, B) and with a lower resolution also in cells of the wing imaginal discs (Figures 4G, H). HA-NOC1 expression colocalizes with fibrillarin mainly in the nucleolus (Figures 4A, D, G, J), while in the granules, its expression was very low but detectable, particularly in the cells of the peripodium (Figure 4A). Co-expression of NOC1 with NOC1-RNAi visibly reduced both HA-NOC1 and the formation of the abnormal enlarged structures expression in both types of cells (Figures 3E, K). At the same time, the levels of fibrillarin in the nucleolus did not significantly change upon expression of NOC1-RNAi (compare Figure 4C with Figures 4F, I with Figure 4L).

3.5 NOC1 colocalizes in the nucleolus with Nucleostemin1 (Ns1) and its reduction affects nucleolar localization of Ns1

In the analysis of proteins that can functionally interact with NOC1, we identified Nucleostemin 1 (Ns1) (Lo and Lu, 2010), a nucleolar protein necessary for the transport of the 60S subunit that shuttles between the nucleolus and the nucleoplasm, and essential for the nucleolar organization (Rosby et al., 2009). To investigate whether NOC1 interacts with Ns1, we first analyzed their co-localization in wt control *w*^{1118} animals. Ns1-GFP (*UAS-Ns1-GFP*) was ectopically expressed alone or in combination with NOC1-RNAi or with NOC1-HA overexpression using the *patched-Gal4* promoter (Vegh and Basler, 2003). These data showed that when Ns1-GFP is expressed alone, it is primarily nucleolar, with about 7% of cells showing NS1-GFP staining outside the nucleolar region (Figure 5B). When NOC1-RNAi was expressed instead we observed a significant alteration in the subcellular localization of Ns1-GFP, with a 30% increased of cells that showed NS1 localization in the nucleoplasm (Figure 5D). Analysis of NOC1 colocalization with Ns1, using anti HA immunostaining, showed the presence of both proteins in the nucleolus and also in the large granules (Figure 5F). These data together with MS results suggest that both Ns1 and NOC1 proteins may be part of a multi proteins complex that is necessary to keep nucleolar integrity (MODEL).

3.6 MYC cooperates with NOC1 to increase nucleolus size

We then analyzed if increasing the rate of protein synthesis by overexpressing MYC could have an effect on the size of the nucleolus or of the NOC1 granules, assuming that they might function as storage of ribosomal factors produced in excess by NOC1 overexpression. We examined and quantified the area of fibrillarin expression in the nucleolus in cells of the wing imaginal discs from control animals or expressing NOC1 or MYC alone, and a combination of both. These analyses confirmed that the expression of MYC or NOC1 alone significantly affects the nucleolar size (Figures 6A–C), with their co-expression that further increases the nucleolus size (Figures 6F–H). A more exhaustive analysis of the immunofluorescence images shows that NOC1-HA is found predominantly localized at the Dense Fibrillarin Center (DFC), that is, the external layer of the Fibrillarin Center (FC), while fibrillarin is in the center (Figures 6C–E). In the presence of MYC this effect of their localization is ever more pronounced (Figures 6F–H). From these experiments, we can also conclude that the granules are maintaining the structure with the core of fibrillarin (Red) with NOC1 surrounding the area (green), both in the condition of NOC expression alone or in combination with MYC (Figures 6E, F). In addition, we analyzed and found a high level of colocalization between NOC1 and *Drosophila* vito protein (Supplementary Figure S1). Nol12/vito is an RNA DNA binding protein homologous to human Nol12 and yeast Rrp17p (Scott et al., 2017). It was shown necessary for the processing of the 60S ribosomal subunits in yeast (Oeffinger et al., 2009), and required in flies for proper formation of nucleolar architecture in MYC-induced growth (Marinho et al., 2011). The two proteins colocalize in the nucleolus and the nuclear “granules” in cells of the wing imaginal disc. In these experiments, NOC1-HA localizes in the DFC of the nucleolus while Nol12-GFP is more present in the FC (S1 panel C); similarly, it was reported for human Hela cells, that Nol12 co-localizes with fibrillarin and was also expressed in the DFC (Scott et al., 2017). We should mention that the pattern of expression described for NOC1 in these experiments recapitulates the expression of nucleophosmin, which surrounds the core-shell architecture of fibrillarin in the center of the nucleoli (Lafontaine et al., 2021), further supporting the localization of NOC1 within the nucleolus.

4 Discussion

The nucleolus is a critical subcellular compartment involved in ribosome biogenesis, and proteins like NOC1 play essential roles in

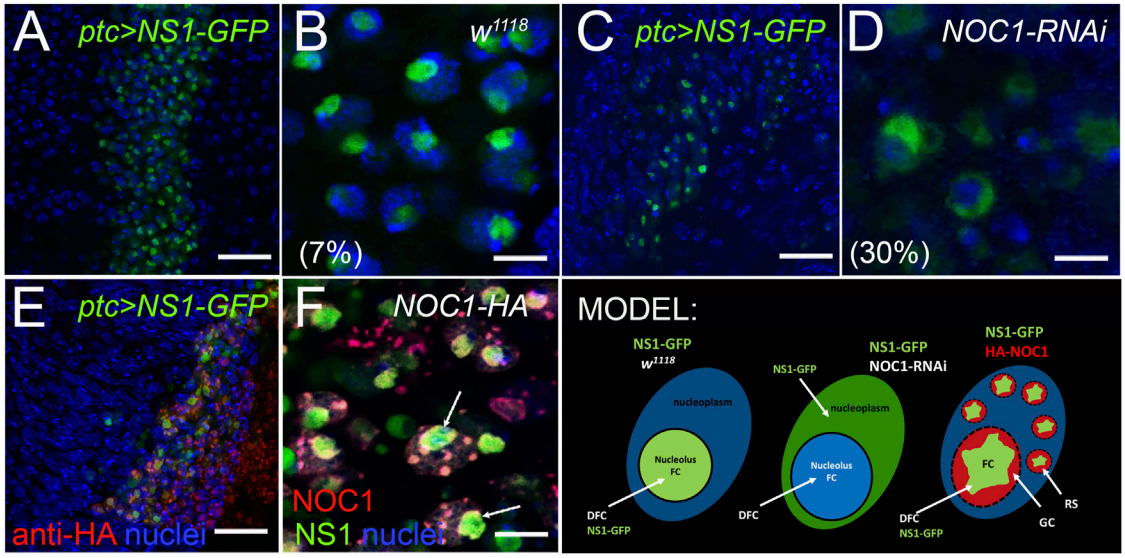

FIGURE 5
Reduction of NOC1 affects Nulceostemin1 (Ns1) nucleolar localization. Confocal images of cells from the wing imaginal disc expressing NOC1 (*UAS-HA-NOC1*) and Ns1 (*UAS-Ns1-GFP*) using the *patched-Gal4* promoter. **(A,B) (A)** shows a low-resolution image of the cells of the wing imaginal disc where patched is expressed as a stripe of cells along the Dorsal Ventral axis (Johnson et al., 1995). NS1-expressing cells are visible by GFP expression. **(B)** higher magnification of figure in panel A, showing expression of Ns1-GFP in the nucleolus. **(C)** low-resolution image of the cells expressing Ns1-GFP together with NOC1-RNAi. **(D)** higher magnification of the figure in panel **(C)**. In the parenthesis is reported the percentage of cells with NS1-GFP found perinucleolar or in the nucleoplasm (see also MODEL). **(E)** low-resolution image of the cells of the wing imaginal disc expressing Ns1-GFP together with NOC1-HA. **(F)** higher magnification of the figure in panel E shows HA-NOC1 that co-localizes with Ns1-GFP. This colocalization is visible in the nucleolus and (Arrows). also in the small granules characteristic of NOC1-HA overexpression. Scale bars in A-C and E represent 20 μm, and B-D and F represent 5 μm. MODEL suggesting the functional interaction of Ns1 with NOC1 in the nucleolus and describing the nucleolus organization as FC, Fibrillar Center; DFC, Dense Fibrillar Components; GC, Granular Center (Lam et al., 2005). GS, Granular Structures visualized by NOC1 overexpression.

this process. The conservation of NOC1 function across these diverse organisms, from yeast (*S.ce*) *Arabidopsis, Drosophila* (Milkereit et al., 2001; Li et al., 2009; de Bossoreille et al., 2018; Destefanis et al., 2022) and to some extent humans (Barbieri et al., 2017) (our unpublished data), indicates the fundamental role of this nucleolar factor in controlling basic and essential processes during ribosome biogenesis.

We have recently characterized the function of the sole nucleolar *NOC1* gene in *Drosophila* and show that it is necessary for proper rRNA processing and maturation, while its downregulation reduces protein synthesis and is detrimental to organ and animal growth (Destefanis et al., 2022). Here, we characterized NOC1 as a *bona fide* MYC target gene and demonstrated that NOC1 is transcriptionally induced through a functional MYC-binding E-box sequence in the NOC1 promoter region (Figure 1). We then analyzed NOC1 interactome by MS analysis (Supplementary List S1) to identify how NOC1 functions in controlling ribosomes and in relation to MYC. These data reveal that NOC1 is in a complex with the nucleolar proteins NOC2 and NOC3, confirming previous data in yeast, and probably forms functional heterodimers necessary for the transport of the large ribosomal subunit during ribosome maturation (Milkereit et al., 2001; Hierlmeier et al., 2013). Our data also evidence an enrichment in NOC1-IPs of other nucleolar proteins, many of them such as fib, mod, nnp1, have been previously characterized as direct MYC's targets (Perrin et al., 2003; Hulf et al., 2005). In support of this last observation, we also found that in response to MYC, NOC1 expression and localization within the nucleolus is significantly increased, suggesting a direct functional response between MYC and NOC1 activities in this organelle. Notably, NOC1 overexpression leads to the formation of large nuclear granules and enlarged nucleoli, which co-localizes with nucleolar fibrillarin and Ns1. Additionally, we demonstrate that NOC1 expression is helping to keep Ns1 nucleolar localization, suggesting a role for NOC1 in maintaining nucleolar structure. Finally, the co-expression of NOC1 and MYC enhances the size of the nucleolus and the formation of abnormal granular structures within the nucleus containing NOC1, outlining another aspect where NOC1 and MYC activities may cooperate or be additive in controlling nucleolar dynamics.

Furthermore, our study also highlights NOC1 interaction with proteins relevant for RNA processing, modification, and splicing. Indeed, we found highly represented Ythdc1 and Flacc (Fl(2)d-associated protein) and spenito (nito), the flies homolog of the nucleolar large ribosomal subunit (60S) assembly factor RBM28 (Bryant et al., 2021). Notably, all these proteins are part of the mechanism that mediates N6-methyladenosine (m6A) methylation of mRNAs (Shi et al., 2021; Deng et al., 2023). Ythdc1 is a conserved nuclear m^{6}A "reader" protein that mediates the incorporation of methylated mRNAs for their nuclear export (Roundtree et al., 2017; Shi et al., 2021). Flacc is a component of the complex that mediates N6-methyladenosine methylation of mRNAs essential for mRNA splicing efficiency of pre-mRNA targets and a key regulator of Sxl (Sex-lethal) pre-mRNA splicing (Knuckles et al., 2018). Flacc is in

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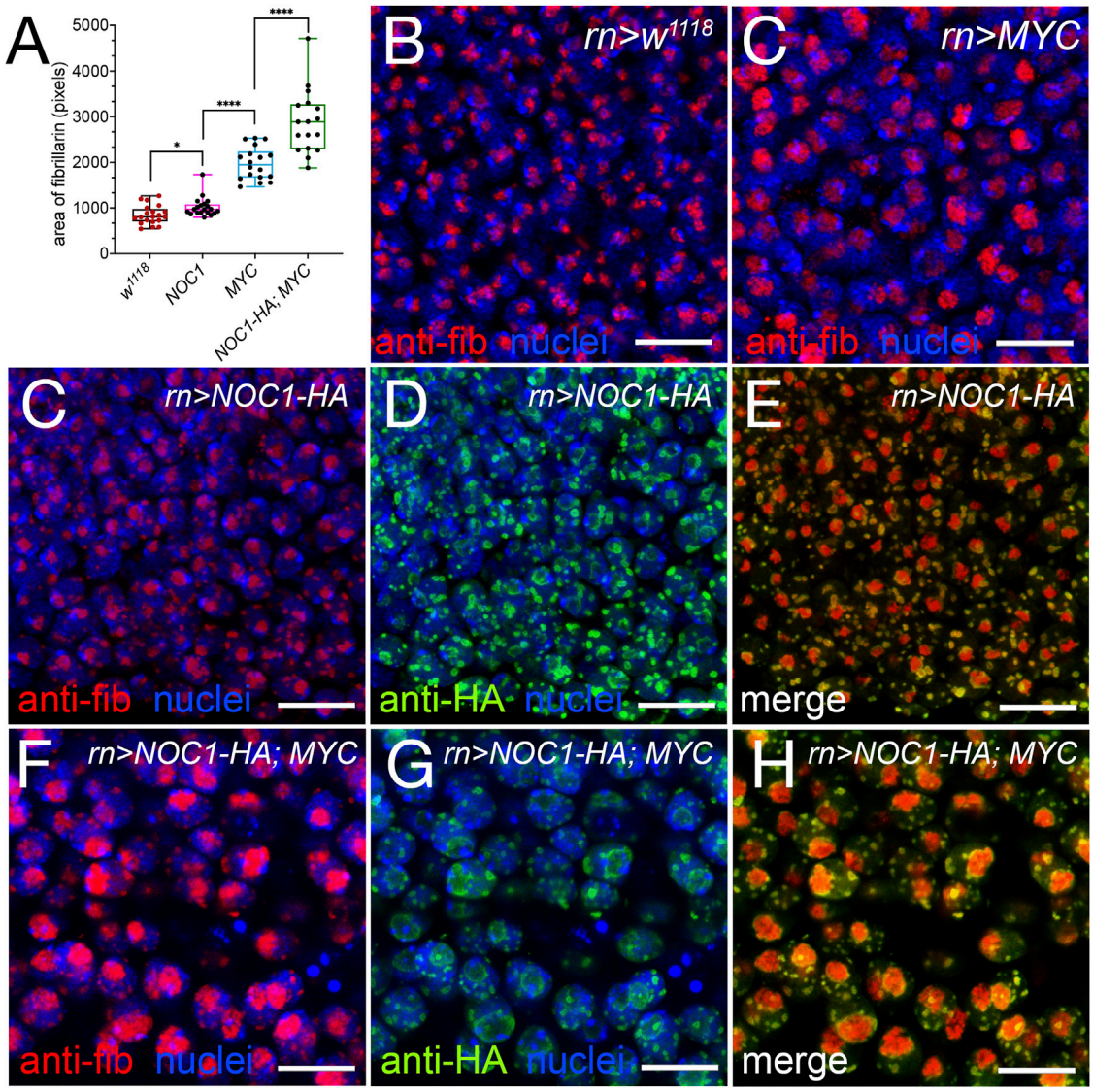

FIGURE 6
Expression of NOC1 and MYC enhances nucleolar size and morphology. **(A)** Graphic of the analysis of expression of fibrillarin in the nucleolus from cells of the wing imaginal discs, in animals expressing the indicated UAS-transgenes using the *rotund-Gal4* promoter. We considered the area stained by fibrillarin as a measurement of the nucleolar size and expressed it in pixels. These experiments were repeated at least twice, and the statistical analysis among the various genotypes was examined by Student's *t*-test, using the number of cells indicated in the graph. *p* values are indicated with asterisks * = $p < 0.05$, **** = $p < 0.0001$ respectively. **(B–H)** Confocal images of cells from the wing imaginal disc in control animals w^{1118} **(B)** and expressing MYC (*UAS-MYC*) **(C)**, NOC1 (*UAS-HA-NOC1*) **(C–E)**, or both NOC1 and MYC **(F–H)** using the *rotund-Gal4* promoter. Fibrillarin (red) and NOC1-HA (green) expression is visualized by immunofluorescence using anti-fibrillarin and anti-HA antibodies, respectively; nuclei are stained using Hoechst and visualized in blue. Scale bars represent 10 μm.

complex with female lethal (Fl(2)d), the *Drosophila* homolog of Wilms'-tumor-1-associated protein (WTAP) a component of human spliceosome (Zhou et al., 2002), and with Snf a component of U1/U2 small nuclear ribonucleoproteins (snRNPs) that contained U2AF50, U2AF38, and U1-70K necessary for splicing reaction of pre-mRNAs (Penn et al., 2008). Interestingly, we found an enrichment of these proteins in our analysis (Supplementary Table S1). Additionally, our data may suggest a potential link between NOC1 and snRNPs involved in regulating RNA-binding proteins and controlling mRNA nuclear export via m6A-dependent modifications by Ythdc1. This part highlights the complex and interconnected processes involved in gene expression regulation, from mRNA splicing to modifications. However, how NOC1 may control or be part of these mechanisms is still unclear.

Our previous analysis directly assessed the impact of NOC1 on pre-rRNA processing and cleavage and showed that its reduction induced an accumulation of pre-rRNA precursors (ITS1 and ITS2) (Destefanis et al., 2022). Similar data were found for the NOC1 homolog in yeast (Noc1p) using genetic screens and proteomic studies (Hierlmeier et al., 2013; Lebaron et al., 2013; Khoshnevis et al., 2019). However, we should comment on some crucial differences in the protein-interactome from our

experiments and those in yeast. Few reports in yeast annotated the Noc1p protein associated with Rrp5 (Ribosomal RNA Processing 5), a factor crucial for ribosome assembly that mediates the cleavage of the 35S pre-rRNA into the 18S rRNA, which is a critical step in the production of the small ribosomal subunit (Hierlmeier et al., 2013; Lebaron et al., 2013) and with Rcl1 (Ribosomal RNA Cleavage 1), another enzyme with a role in rRNA cleavage and processing (Khoshnevis et al., 2019). Both these proteins are conserved in flies. However, we did not find them in our NOC1-interactome analysis, even though Rrp5 was found in yeast bound to the pre-rRNA region of the ITS1 (Internal Transcriber Spacers-1) using protein crosslinking following by RNase treatment (Lebaron et al., 2013), and to interact with Noc1p and Noc2p (Hierlmeier et al., 2013) with a Noc1p-TAP purification system. We can explain these differences by hypothesizing that either the levels of Rrp5 and Rcl1 expressions are low in larvae compared to yeast or the use of different techniques and timing of purification of the protein used as bait in yeast compared to ours, i.e., during specific phases of RNA maturation and using Noc1p-TAP purification systems (Sailer et al., 2022). However, in the NOC1-interactome, we found NOC2 and NOC3, along with Nop53 (Rrp9) among others, described part of the Noc1p-yeast complex (Ohmayer et al., 2013), highlighting the significance of our preliminary studies in flies. It is important to acknowledge that studying the precise protein-interactome of NOC1 in *in vivo* can be challenging, and experimental conditions can limit the interpretation of results. In our case, conducting experiments at a single time point and under standard immunoprecipitation conditions may provide valuable insights into protein interactions but might not fully capture the dynamic and context-dependent nature of different NOC1's functions.

We found that NOC1 overexpression forms large granular structures containing NOC1, along with fibrillarin and Nucleostemin1 (Figure 4; Figure 5) and Nol12/viriato (Supplementary Figure S1). At the moment, we do not know the nature of these granules. We could hypothesize that these NOC1 granules work as dynamic and multifunctional structures regulating RNA metabolism and gene expression, including rRNA processing and transcription. These may include RNA stress granules formed during stress conditions to protect mRNAs from degradation or to control their translation (Putnam et al., 2023). This hypothesis is supported by our data that identify proteins of the DEAD-box RNA helicases family, such as pea/DXH8 and CG8611 pit, bel kurz, previously identified as components of RNA stress granules (Campos-Melo et al., 2021). This idea may also support the mechanism by which the abnormally large structures containing NOC1 and induced when MYC is overexpressed are the result of their synergistic effect in promoting cellular stress induced by a high protein synthesis or dysfunctions caused by the combination of MYC and NOC1 targets. Overexpression of MYC can lead to increased demand for ribosome biogenesis, and the presence of abnormal ribosomal intermediates due to NOC1 dysregulation can exacerbate this stress. This can result in nucleolar stress, activation of cellular stress responses, and potentially contribute to the insurgence of diseases.

Abnormal structures or extra nucleoli have significant implications in human diseases, particularly in cancer, where dysregulation of nucleolar functions is a hallmark of the disease (Orsolic et al., 2016; Penzo et al., 2019), and in ribosomopathies, a class of rare genetic diseases characterized by mutations in ribosomal proteins or components that impaired RNA translation associated with various clinical manifestations, including bone marrow failure, developmental disorders and an increased risk of cancer (Farley-Barnes et al., 2019; Kampen et al., 2020).

Finally, a few words about the human homolog of NOC1, called CEBPz (CCAAT/enhancer-binding protein zeta), a transcription factor so far associated with certain types of tumors. Notably, in acute myeloid leukemia (AML), CEBPz was shown to promote the m^6A modification of target mRNA transcripts, enhancing their translation (Barbieri et al., 2017; Hong et al., 2022). Thus, overexpression or downregulation of CEBPz in humans may also affect RNA processing, leading to defective translation. In support of this idea, the human gene rbm28, which we found in the NOC1 interactome, is responsible for the ribosomopathy-ane syndrome (Bryant et al., 2021), a rare genetic disorder caused by aberrant splicing in *RBM28* pre-mRNA. This, together with other indirect information on the potential role of NOC1/CEBPz in controlling alternative splicing, highlights the potential role of the human counterpart in the control of nucleolar processes that may cause genetic disorders.

Our research uses *Drosophila*, a simple and accessible model system, to identify novel conserved mechanisms to better understand MYC activity and its targets, including NOC1, in the context of RNA translation and ribosome biogenesis. The ultimate goal would be to identify specific targets within the translation machinery that small molecules or drugs can modulate for use in disease therapies.

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 in the article/Supplementary Material.

Author contributions

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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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c-Myc Drives inflammation of the maternal-fetal interface, and neonatal lung remodeling induced by intra-amniotic inflammation

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Background: Intra-amniotic inflammation (IAI) is associated with increased risk of preterm birth and bronchopulmonary dysplasia (BPD), but the mechanisms by which IAI leads to preterm birth and BPD are poorly understood, and there are no effective therapies for preterm birth and BPD. The transcription factor c-Myc regulates various biological processes like cell growth, apoptosis, and inflammation. We hypothesized that c-Myc modulates inflammation at the maternal-fetal interface, and neonatal lung remodeling. The objectives of our study were 1) to determine the kinetics of c-Myc in the placenta, fetal membranes and neonatal lungs exposed to IAI, and 2) to determine the role of c-Myc in modulating inflammation at the maternal-fetal interface, and neonatal lung remodeling induced by IAI.

Methods: Pregnant Sprague-Dawley rats were randomized into three groups: 1) Intra-amniotic saline injections only (control), 2) Intra-amniotic lipopolysaccharide (LPS) injections only, and 3) Intra-amniotic LPS injections with c-Myc inhibitor 10058-F4. c-Myc expression, markers of inflammation, angiogenesis, immunohistochemistry, and transcriptomic analyses were performed on placenta and fetal membranes, and neonatal lungs to determine kinetics of c-Myc expression in response to IAI, and effects of prenatal systemic c-Myc inhibition on lung remodeling at postnatal day 14.

Results: c-Myc was upregulated in the placenta, fetal membranes, and neonatal lungs exposed to IAI. IAI caused neutrophil infiltration and neutrophil extracellular trap (NET) formation in the placenta and fetal membranes, and neonatal lung remodeling with pulmonary hypertension consistent with a BPD phenotype. Prenatal inhibition of c-Myc with 10058-F4 in IAI decreased neutrophil

infiltration and NET formation, and improved neonatal lung remodeling induced by LPS, with improved alveolarization, increased angiogenesis, and decreased pulmonary vascular remodeling.

Discussion: In a rat model of IAI, c-Myc regulates neutrophil recruitment and NET formation in the placenta and fetal membranes. c-Myc also participates in neonatal lung remodeling induced by IAI. Further studies are needed to investigate c-Myc as a potential therapeutic target for IAI and IAI-associated BPD.

KEYWORDS

intra-amniotic inflammation, preterm birth, bronchopulmonary dysplasia, pulmonary hypertension, placental inflammation, fetal inflammation

1 Introduction

Approximately 15 million infants are born preterm annually worldwide, and preterm birth is the leading cause of death in children under the age of 5 years (Blencowe et al., 2012; Liu et al., 2016). Survivors of preterm birth suffer a lifetime of disability and continue to require complex multidisciplinary medical care beyond the neonatal period and childhood years. Adult survivors of preterm birth are at increased risks for mortality, chronic multi-organ diseases, psychiatric disorders, and decreased quality of life (Crump et al., 2019; Markopoulou et al., 2019; Crump, 2020; Petrou et al., 2020). Intra-amniotic inflammation (IAI) is present in 70%–85% of preterm births that occur before 30 weeks of gestation and is the cause of up to 40% of preterm births (Romero et al., 1998; Romero et al., 2014). Current clinical strategies to manage IAI include the use of antibiotics and expectant management of labor, but IAI is most often sterile, and the poor clinical outcomes of preterm birth and neonatal morbidities are associated with the presence of inflammation itself, regardless of bacterial infection (Combs et al., 2014).

The presence of IAI is also associated with increased risk for bronchopulmonary dysplasia (BPD) in preterm infants, compounding the severe chronic morbidities that survivors of preterm birth already face (Villamor-Martinez et al., 2019). Moreover, the risk of BPD is inversely proportional to gestational age. With advances in neonatology over the past decade, more extremely low gestational age and extremely low birth weight infants are surviving, but the prevalence and burden of long-term impairment from prematurity and BPD have also increased. There is a lack of effective therapies for BPD, and BPD continues to be the most common long-term morbidity among preterm infants leading to lifelong respiratory impairment (Stoll et al., 2015). Survivors of preterm birth with BPD experience more childhood wheezing and respiratory illnesses and have more special care needs (Stoll et al., 2015; DeMauro, 2018). Adult survivors of BPD have altered lung structure, impaired lung function and exercise capacity, and decreased quality of life (Caskey et al., 2016). Preterm birth and BPD are major public health issues, hence there is a pressing need for effective targeted therapies to prevent preterm birth and BPD.

The pathogenesis of BPD is multifactorial and involves multiple pathways, posing a challenge to the development of new therapies (Mathew, 2020). c-Myc is an oncogene and key transcription factor that regulates multiple cellular functions including proliferation, differentiation, cell metabolism and apoptosis. c-Myc is downstream of multiple pathways that have been implicated in the pathophysiology of both preterm birth and BPD such as tumor necrosis factor-α (TNFα), Notch signaling, Wingless/Int-1 (Wnt) signaling, and Janus Kinase/Signal transducers and activators of transcription (JAK/STAT) signaling (Green and Arck, 2020; Mathew, 2020). c-Myc has a basic-helix-loop-helix-leucine zipper structure and heterodimerizes with a ubiquitous protein called Max to become transcriptionally active (Chen et al., 2018). In tracheal aspirates of preterm infants, the MYC/MAX complex was overrepresented in lung macrophages of infants prone to BPD (Sahoo et al., 2020). We hypothesize that c-Myc has a role in modulating inflammation of the placenta and fetal membranes in IAI, leading to fetal lung inflammation and neonatal lung remodeling. To test our hypothesis, our first objective was to determine the kinetics of c-Myc in the placenta, fetal membranes and lungs exposed to IAI in a pregnant rat model of IAI using ultrasound-guided intra-amniotic lipopolysaccharide (IA LPS) injections. We then prenatally treated pregnant rats with IAI induced by IA LPS with a small molecule c-Myc inhibitor 10058-F4 (Huang et al., 2006). We show that c-Myc inhibition in a rat model of IAI decreased inflammation in the placenta and fetal membranes, and attenuated lung parenchymal and vascular remodeling induced by IAI, demonstrating a potential role of c-Myc in modulating inflammation at the maternal-fetal interface, and neonatal lung remodeling induced by IAI.

2 Materials and methods

2.1 Animal model of IAI

To determine the kinetics of c-Myc in normal lung development and in IAI-exposed animals, time-mated Pregnant Sprague-Dawley rats received ultrasound-guided (Vevo 3100, VisualSonics) intra-amniotic injections of 10 μg lipopolysaccharide (*E. coli* O55:B5, cat. #L4525-5MG, Sigma-Aldrich, St Louis, MO) (IA LPS) or sterile phosphate buffered saline (PBS) at embryonic day 18 (Figure 1). A group of animals were delivered by cesarean sections 3 h after IA LPS injections for *in vivo* imaging of LPS distribution using a Cy5.5-tagged LPS (cat # LPS-S55-1, Nanocs, Boston, MA) and imaged on IVIS Spectrum *In Vivo* Imaging System (PerkinElmer Inc., Waltham, MA). For assessment of IAI, a group of animals were delivered by cesarean section at 24 h post-IA LPS for placenta and fetal membrane sampling. Another group of animals were allowed to deliver naturally around embryonic day (E) 21 and rat pup lungs were sampled at postnatal day 14. For assessment of c-Myc lung expression in postnatal development, a subgroup of animals was sacrificed at four timepoints: day of delivery (P0), and postnatal days (P) 3, 7 and 14.

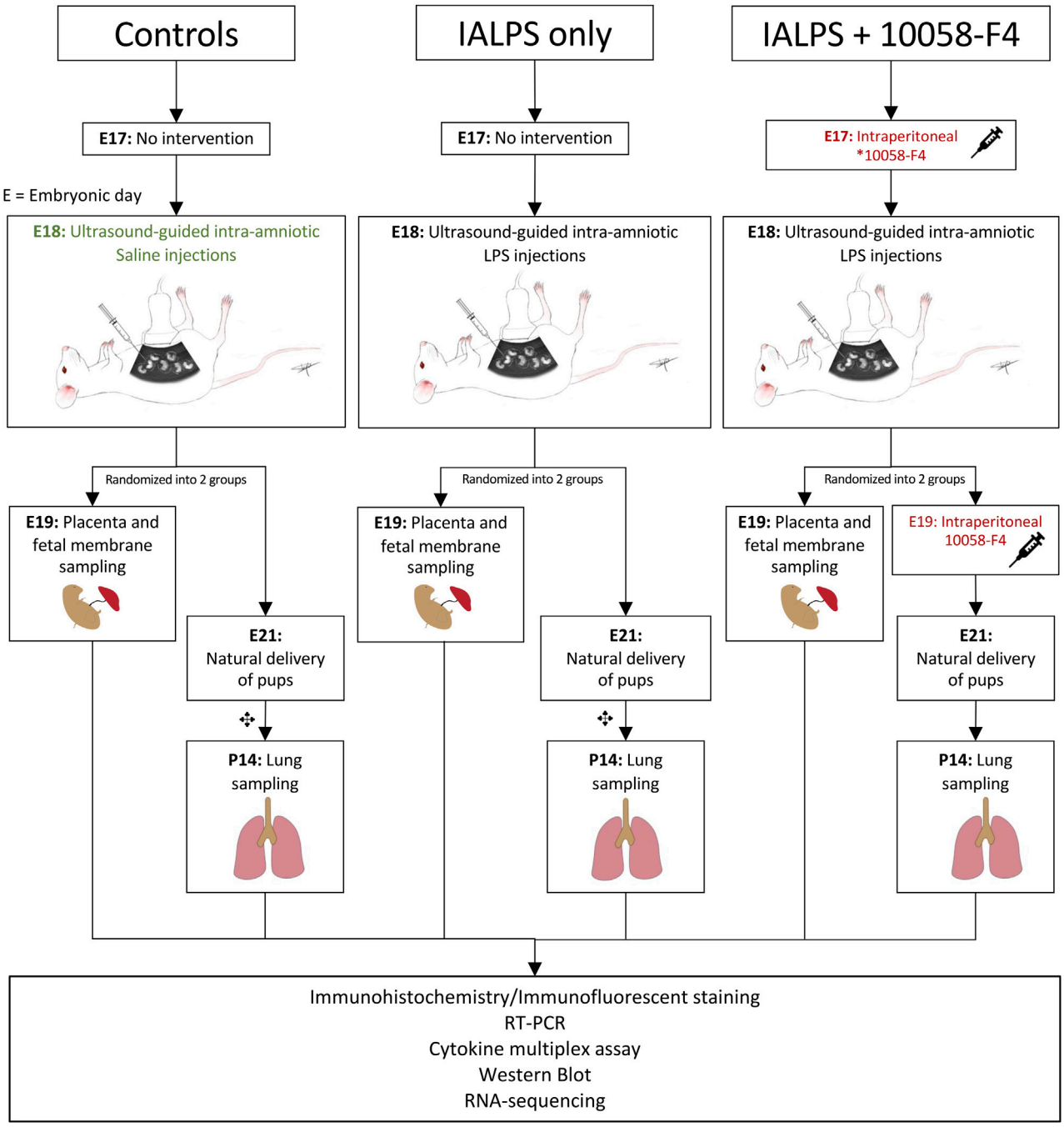

FIGURE 1
Experimental design. Pregnant time-mated Sprague-Dawley rats were randomized into 3 groups: 1) IA saline, 2) IALPS, or 3) IALPS +10058-F4. Intra-amniotic injections were performed on E18 under ultrasound guidance. Intraperitoneal c-Myc inhibitor injections were administered on E17 only for pregnant rats who underwent C-section for placenta and fetal membrane sampling on E19, and both E17 and E19 for pregnant rats undergoing natural delivery of pups for neonatal lung sampling. ✣subgroup of rat pups euthanized at P0, P3 and P7 for assessment of c-Myc expression over postnatal lung development. IALPS = Intra-amniotic lipopolysaccharide, E = Embryonic day. *10058-F4 = c-Myc inhibitor.

2.2 Treatment groups

For c-Myc inhibition experiments, time-mated pregnant Sprague-Dawley rats were randomized into three groups: IA saline injections (control); IA LPS injections only, or IA LPS injections with c-Myc inhibitor (IA LPS+10058-F4) (Figure 1). 10058-F4 (MedChemExpress, NJ, United States) was diluted per manufacturer's instructions in 10% dimethyl sulfoxide (DMSO) and 90% sesame oil to 10 mM. One group of pregnant dams received intraperitoneal injections of 20 mg/kg of 10058-F4 on E17. Placenta and fetal membranes were sampled and analyzed at 24 h post-IALPS injections after 1 dose of 10058-F4. The remaining group of

pregnant rats received a second dose of 10058-F4 on E19 and were allowed to naturally deliver. Rat pups were euthanized and neonatal lungs analyzed on P14.

2.3 Histological assessment

Whole placentas with fetal membranes and whole fetuses were fixed with 4% paraformaldehyde overnight. Following fixation, samples were embedded in paraffin and sectioned. Hematoxylin/eosin (H&E) staining was performed to assess for neutrophil infiltration in whole sections of placenta and fetal membranes. TUNEL assay was used to assess apoptosis in placenta and fetal membranes using a commercial kit (Click-iT Plus TUNEL Assay, cat #10617, ThermoFisher, Waltham, MA) according to the manufacturer's instructions. To assess differences in cell proliferation, placenta sections were stained with Ki67 antibody and total number of cells and number of Ki67-stained cells per high power field (hpf) were quantified using Zeiss Axio Observer Microscopy image processing software. Six distinct regions of two placenta sections per sample was analyzed. Analysis was performed by calculating ratio of cells stained with Ki67 to total number of cells per hpf.

Neonatal lungs were inflation-fixed with 4% paraformaldehyde at 30 cm H_2O via a tracheal cannula for 5 min and then fixed overnight. Following fixation, samples were embedded in paraffin and sectioned. To assess lung alveolarization in the peripheral parenchymal regions of lungs, lung sections were stained with H&E, and mean linear intercept (MLI) was performed on six distinct regions of one lung section per animal, avoiding large vessels and airways, and was calculated as previously described (Knudsen et al., 2010). Radial alveolar counts were performed on ten regions of one lung section per animal and calculated as previously described (Cooney and Thurlbeck, 1982). Pulmonary vascular muscularization was assessed by calculating ratio of small pulmonary vessels identified by endothelial cells staining with vWF that simultaneously stained positive for smooth muscle actin (SMA) antibody, as previously described (Ciuclan et al., 2011). Peripheral parenchymal regions of lungs were analyzed to avoid large vessels and airways. 4–5 animals were assessed per group. We performed immunohistochemistry on paraffin-embedded tissue sections with heat-assisted antigen retrieval with citrate buffer (pH 6.0). Primary antibodies (Supplementary Table S1A) were incubated overnight at 4°C followed by incubation with respective secondary antibodies for 1 h at room temperature. Stained sections were imaged on Zeiss AxioObserver microscope.

2.4 Cytokine/chemokine assay

Rat cytokine/chemokine concentrations in whole lung protein extract from 5 animals per group was determined by rat cytokine array/chemokines array-27 (Eve Technologies, Calgary, Canada). Flash frozen whole lung tissues were homogenized in RIPA buffer (Santa Cruz Biotechnology, catalog # sc-24948) and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was transferred to a new tube and protein concentration was measured by BCA protein assay (Thermo Scientific, catalog # 23228 and 1859078). Samples were then diluted for a target protein concentration of 3–4 mg/mL. Values for samples with signal outside the curve were calculated when feasible by the model.

2.5 Western blot analyses

Whole placentas with fetal membranes were sectioned into equal quarters and homogenized in RIPA lysis buffer. Homogenates were centrifuged at 18,000 × g for 5 min at 4°C and the supernatant collected for protein analysis. An aliquot of each sample was used for protein quantification by the Bradford method, using a commercial kit (Bio-Rad Protein Assay Dye Reagent, Bio-Rad Laboratories Inc., United States). For Western blot analysis, 40 µg of total protein from each sample were fractionated by SDS-PAGE on precast 4%–15% Tris-glycine gradient gels, and then transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc., United States). Total protein was stained using the Revert 700 Total Protein stain kit (LI-COR Biosciences, United States) followed by imaging. Subsequently, the membrane was blocked overnight at 4°C under gentle agitation in Phosphate Buffered Saline pH 7.4 with 0.1% Tween-20 (PBS-T) supplemented with 5% nonfat dry milk (Bio-Rad Laboratories Inc., United States). After blocking, the membrane was washed in PBS-T and incubated for 1 h with mouse monoclonal anti-c-Myc antibody (clone 9E10, Santa Cruz Biotechnology, Inc. United States) diluted 1:500 at room temperature under gentle agitation. The membrane was then washed and incubated with IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody (LI-COR Biosciences, United States). All images were collected using the Odyssey® Infrared Imaging System (LI-COR Biosciences, United States). Protein expression was estimated relative to total protein using Empiria Studio 2.3 software (LI-COR Biosciences, United States). Lung lysates were processed and analyzed in the same manner, with the exception that secondary antibody conjugated to horseradish peroxidase was used and proteins detected by chemiluminescence (Amersham, Piscataway, NJ, United States). Protein expression was estimated by densitometry analysis relative to actin expression using Quantity One software (Bio-Rad Laboratories Inc., United States). We analyzed 6–8 animals per group for placenta and fetal membrane analysis, and 4–7 rat pups per group for lung analysis.

2.6 RNA isolation and real-time qPCR

Total RNA was extracted from frozen placenta and lung tissues using the RNeasy universal Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two µg of total RNA from 4 to 7 animals per group was reverse-transcribed in a 20 µL reaction by using High-Capacity RNA-to-cDNA™ Kit according to supplier's protocol (Applied Biosystems, cat #43-874-06, Foster City, CA) The real-time q-PCR was performed on an ABI Fast 7500 System (Applied Biosystems, Foster City, CA). Each reaction included diluted first-strand cDNA, target gene primers, or 18S rRNA gene primers and master mix containing TaqMan probes according to the supplier's instruction (Supplementary Table S1B) (Applied Biosystems, Waltham,

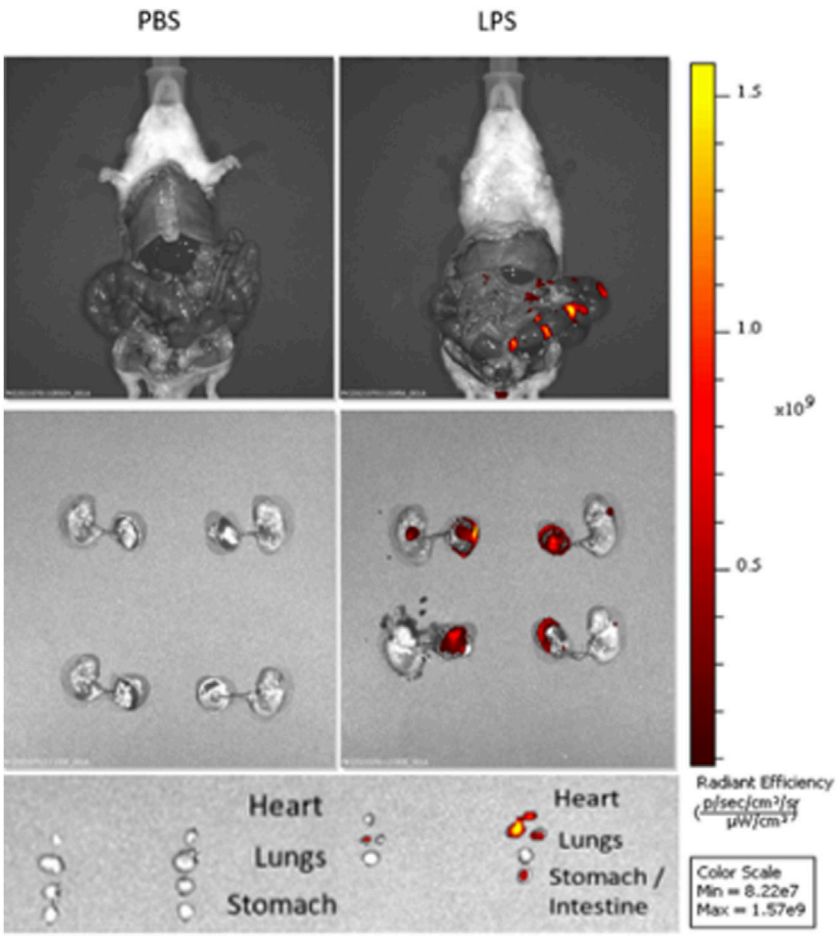

FIGURE 2
Intra-amniotic LPS localization and c-Myc expression in placenta. Distribution of LPS after intra-amniotic injections visualized by *in vivo* fluorescent imaging. Ultrasound-guided injections of LPS localized to amniotic cavity, fetal lungs and gastrointestinal tract.

MA). The expression levels of target genes were normalized to 18S rRNA.

2.7 RNA sequencing

RNA quality and integrity were verified using the Agilent 2,100 Bioanalyzer (Agilent Technologies). All samples had RNA integrity number >8. RNA-Seq was performed by BGI genomics with a read depth of 30 million reads per sample for 150 bp paired-end reads. The raw sequence reads in FASTQ format were aligned to the *Rattus norvegicus* genome build rno6.0 using kallisto (Bray et al., 2016) followed by gene summarization with tximport (Soneson et al., 2015). After checking data quality, differential expression analyses comparing treatment groups with control and between each other were performed using DESeq2 with false discovery adjustment (Love et al., 2014). Genes were considered differentially expressed based on their fold change relative to control (≥1.5), *p*-value (<0.05), and q value (<0.1). PCA analysis was performed with PCAtools (Blighe and Lun, 2023). Volcano plots were generated using the EnhancedVolcano package (Blighe et al., 2018) Heatmaps were generated with pheatmap (Kolde, 2019).

2.8 Functional enrichment and pathway analysis

Lists of differentially expressed genes were used for functional enrichment analysis of Gene Ontology (GO) and KEGG pathway terms using the ToppCluster web server (Kaimal et al., 2010). Only unique terms associated with either induced or suppressed genes and at least 2 genes are reported. Negative log *p* values represent terms associated with suppressed genes, and positive log *p* values are associated with induced genes.

3 Results

3.1 US-guided IA LPS injections accurately target the amniotic cavity

To verify localization of LPS after US-guided IA injections we performed cesarean sections 3 h post-injection of Cy5.5-tagged LPS. *In vivo* and *ex vivo* imaging showed that LPS localized to the uterus without signal from the maternal abdominal cavity or circulation (Figure 2). After dissection, LPS was noted to be present on the fetal

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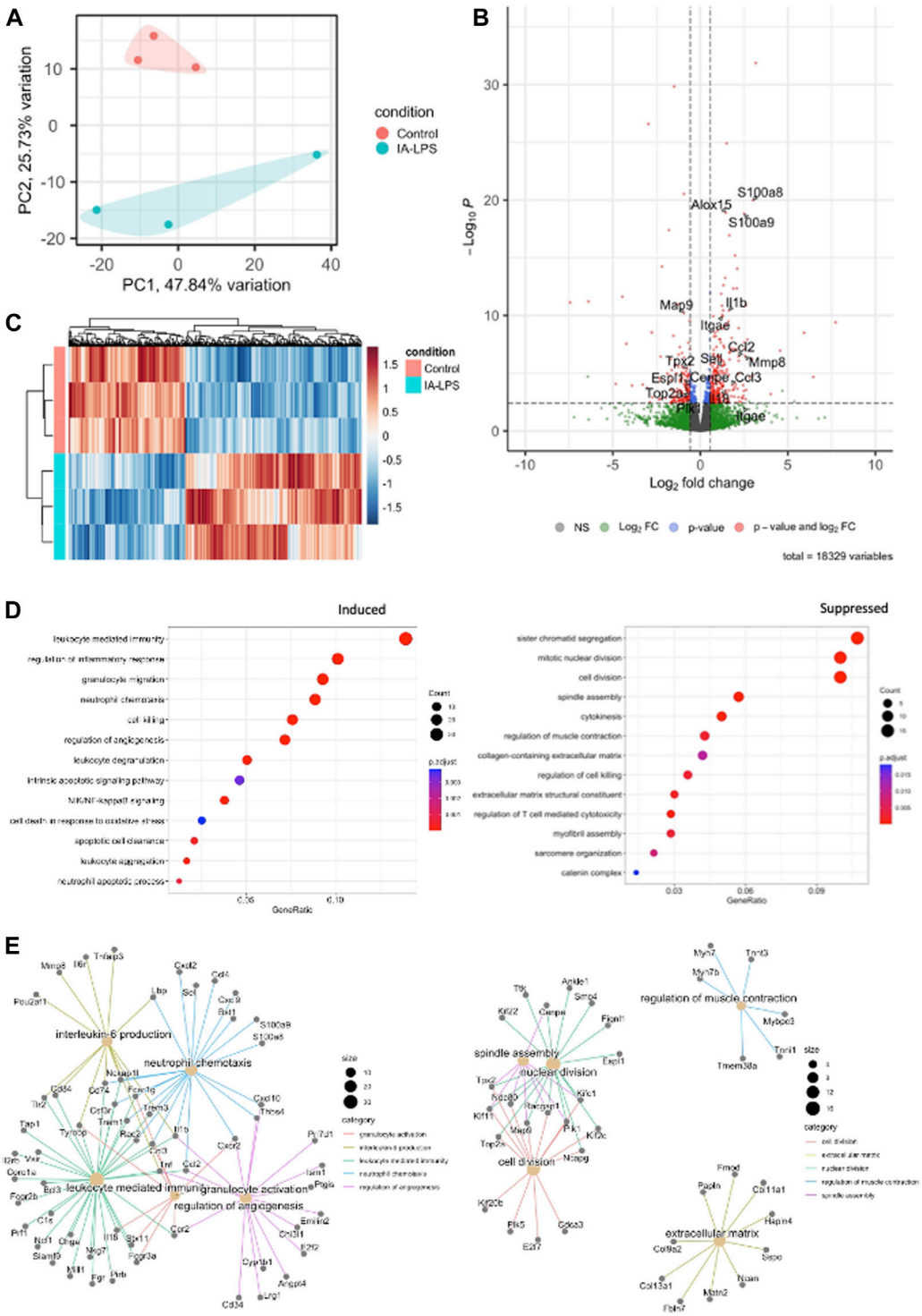

FIGURE 3
Intra-amniotic LPS changes the placental transcriptome. RNA-sequencing performed on placenta and fetal membranes sampled 24 h after LPS-exposure compared with controls showing: **(A)** Principal component analysis plot showing clear differentiation of gene expression between controls (pink) vs. LPS-exposed group (blue). **(B)** Heatmap of differentially expressed genes and **(C)** Volcano plot showing differential gene expression with genes of interest highlighted. Red–FDR<0.1 and fold change>1.5 **(D)** Dotplot showing related functions of genes that were differentially expressed between groups. LPS exposure induced genes associated with inflammation and leukocyte activation (*left*), and suppressed genes associated with cell proliferation (*right*). **(E)** Network plot of genes that were differentially induced (*left*) and suppressed (*right*) in LPS-exposed group compared to controls. *N* = 3 per group.

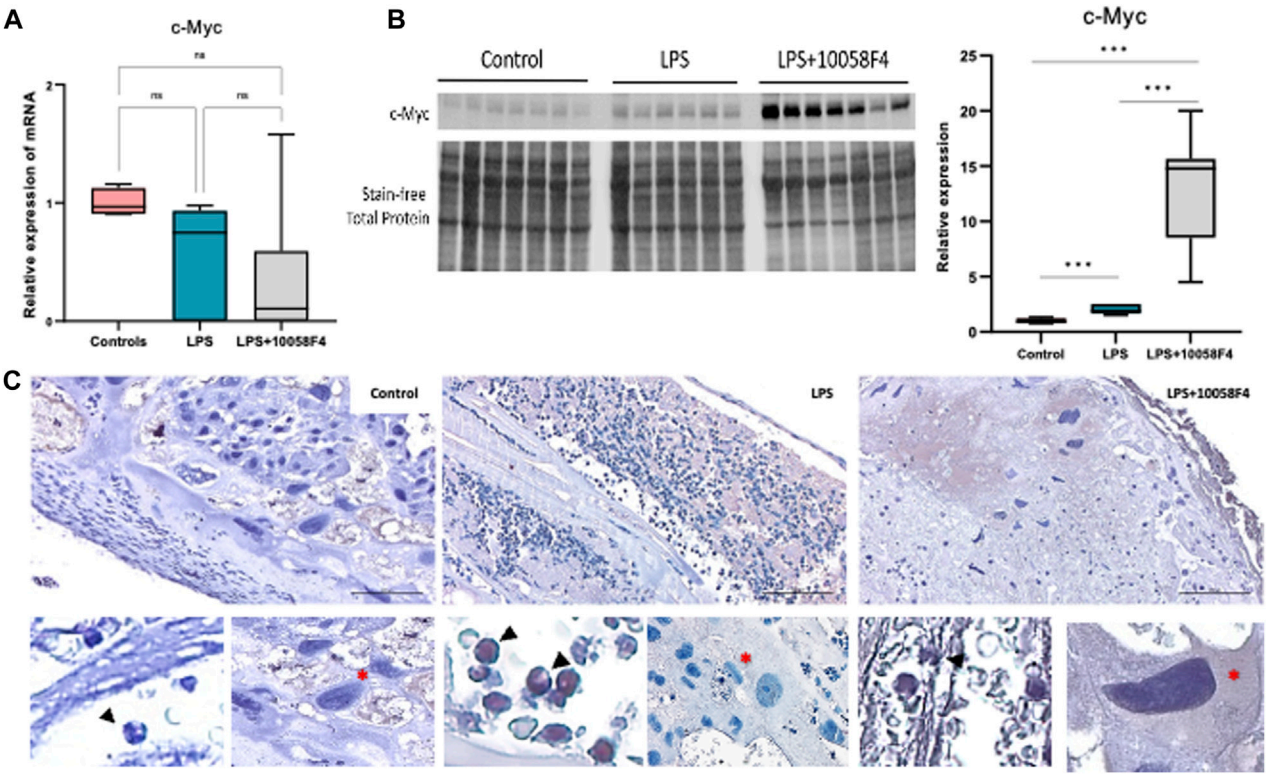

FIGURE 4
Prenatal c-Myc inhibition in IAI leads to increased c-Myc protein in the placenta and fetal membranes that are not neutrophil-driven. **(A)** RT-PCR performed on placenta and fetal membranes sampled at 24 h post-LPS exposure showed no significant changes. N = 4–8 per group. **(B)** Representative Western blot of placenta and fetal membrane lysates showing increased protein expression of c-Myc in LPS-exposed placenta and fetal membranes. With prenatal 10058-F4 c-Myc inhibitor treatment, c-Myc protein expression was further increased compared to both control and LPS groups. *N* = 6–8 per group **p < 0.05, ***p < 0.001, t-test, normalized to total protein.* **(C)** Immunostaining of placenta sections with c-Myc antibody showing c-Myc expression (brown staining) in neutrophils (cropped and magnified, bottom left picture in each group, marked by black arrows) in LPS-exposed placenta. Prenatal c-Myc inhibition with 10058-F4 decreased neutrophil infiltration in LPS-exposed placenta and c-Myc was not expressed in neutrophils, but is expressed in cytoplasm of maternal decidual cells (cropped and magnified, bottom right picture in each group, marked by red asterisks).

membranes and placenta as well as fetal lungs, heart, and gastrointestinal tract (Figure 2). These findings confirm the accuracy of US-guided injection in delivering LPS to the intra-amniotic cavity, simulating IAI.

3.2 IA LPS exposure modulates the transcriptome of placenta and fetal membranes

Bulk RNA-sequencing was performed on placenta and fetal membranes sampled 24 h post-LPS exposure and compared with controls of the same timepoint. We used PCA to identify global differences among samples on RNA-sequencing. PCA is a dimensionality reduction technique that allows quicker interpretation of the results while maintaining the maximum amount of information on each sample. There were large transcriptomic changes between groups with clear separation of LPS and control samples on PCA (Figure 3A). Differential expression analysis (Figures 3B, C, Supplementary Table S2) showed that IA LPS induced 271 and suppressed 165 genes relative to control. Overrepresentation analysis graphs show the *p*-value represented by the circle color, the number of differentially expressed genes belonging to each term represented by circle size, and the *x*-axis represents the gene ratio. Genes that were differentially expressed in LPS-exposed placenta and fetal membranes were associated with inflammation (*S100a8, S100a9, IL1b, Mmp8*), leukocyte activation (*Ccl2, Ccl3*), and decreased proliferation (*Map9, Tpx2*) (Figures 3C, D). Other differentially expressed genes were associated with regulation of angiogenesis, extracellular matrix organization and muscle contractility (Figure 3D). These findings confirm the induction of inflammation at the placenta in our model and suggest altered placental growth and development induced by IA LPS.

3.3 Prenatal c-Myc inhibition in IAI leads to increased c-Myc protein in the placenta and fetal membranes that are not neutrophil-driven

To assess c-Myc expression in our model of IAI, we performed RT-PCR, Western blot, and immunostaining for c-Myc in the placenta and fetal membranes. There were no significant changes in mRNA expression of c-Myc in the placenta and fetal membranes sampled at 24 h post-LPS

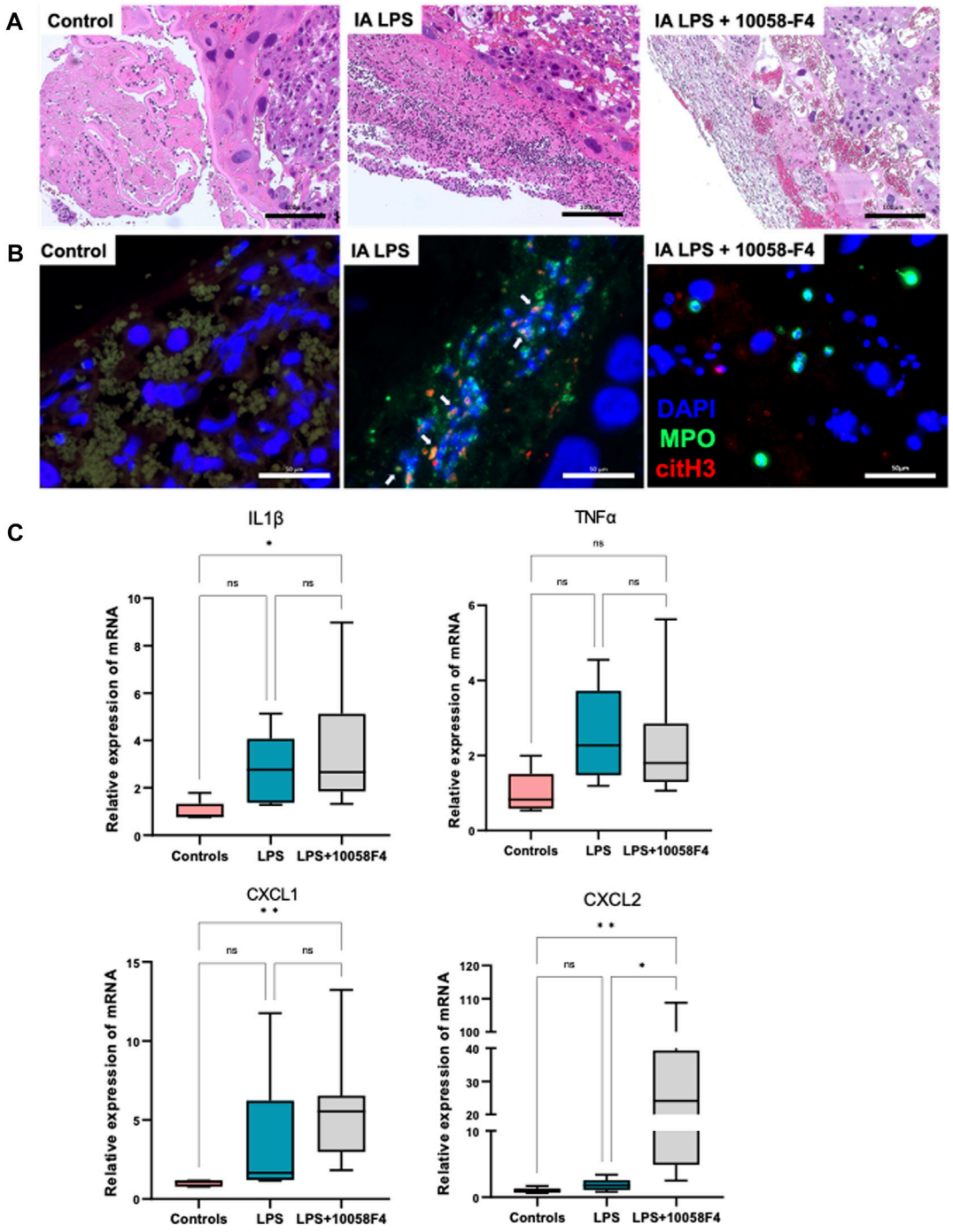

FIGURE 5
c-Myc inhibition decreases neutrophil infiltration, NET formation and modulates inflammation in placenta and fetal membranes. **(A)** Hematoxylin and eosin (H&E) staining of placenta sections showing increased neutrophil infiltration in placenta with LPS exposure when compared to controls, which was ameliorated with c-Myc inhibitor treatment. **(B)** Immunofluorescent staining of placenta sections with anti-myeloperoxidase (MPO) antibody (green) and anti-citrullinated histone (H3) antibody (red) showing increased NET formation in placenta with LPS exposure when compared to controls, which was ameliorated with c-Myc inhibitor treatment. **(C)** RT-PCR performed on placenta and fetal membranes sampled at 24 h post-LPS exposure. *N = 5–8 per group*. LPS exposure was not associated with significant changes in IL1β, TNFα, CXCL1 and CXCL2. Prenatal c-Myc inhibition in LPS-exposed pregnant rats induced IL1β and CXCL1 when compared to controls, and increased CXCL2 when compared to both control and LPS-exposed groups. *$p < 0.05$, **$p < 0.01$, Kruskal–Wallis test.

exposure in both IA LPS and IA LPS+10058-F4 treatment groups compared to controls (Figure 4A). The placenta and fetal membranes of IA LPS-exposed rats had increased c-Myc expression compared to controls by Western blot. Interestingly, prenatal c-Myc inhibitor treatment in IA LPS-exposed placenta and fetal membranes further induced c-Myc

protein expression compared to both controls and IALPS groups (Figure 4B). On immunostaining, c-Myc was intranuclear and localized to neutrophils present at the maternal-fetal interface in the IA LPS-exposed group. In the LPS+10058F4 treatment group, c-Myc was not expressed in scant neutrophils that were present in the placenta and fetal membranes, but was expressed in the cytoplasm of placental decidual cells (Figure 4C). These findings show that c-Myc expression is induced by LPS and is expressed in the neutrophils in IAI. However, prenatal systemic 10058-F4 inhibitor treatment did not suppress c-Myc mRNA expression in the placenta and fetal membranes, but led to an increase in c-Myc protein expression in the placenta and fetal membranes which were not neutrophil-driven.

3.4 Prenatal systemic 10058-F4 treatment ameliorates inflammation and NET formation in placenta and fetal membranes induced by IA LPS

IA LPS induced neutrophil infiltration of the placenta and fetal membranes, confirming presence of histologic IAI (Figure 5A). IA LPS also induced NET formation assessed by immunofluorescence staining with colocalization of myeloperoxidase (MPO) and citrullinated histone-3 (citH3) (Figure 5B). Furthermore, LPS-exposed pregnant rats prenatally treated with 10058-F4 had decreased neutrophil infiltration and NET formation compared to pregnant rats exposed to IA LPS only (Figures 5A, B). Real-time PCR performed on placenta and fetal membranes sampled at 24 h after IA LPS injection showed no significant changes in mRNA expression of pro-inflammatory mediators IL1β, TNFα, CXCL1 and CXCL2 (Figure 5C). Prenatal c-Myc inhibitor treatment in the LPS-exposed group induced increases in mRNA expression of IL1β and CXCL1 compared to controls, and a very variable but overall significant increase in CXCL2 compared to both controls and the LPS-exposed group (Figure 5C). These findings show that c-Myc modulates inflammation of the placenta and fetal membranes.

3.5 Prenatal systemic 10058-F4 treatment decreases apoptosis and attenuates arrest of proliferation in the placenta induced by IA LPS

IA LPS induced apoptosis in the placenta, assessed by TUNEL assay performed on placental sections (Figure 6A). When pregnant rats exposed to IA LPS were treated with c-Myc inhibitor 10058-F4, apoptosis was decreased compared to IA LPS only groups, similar to controls. Ki67 staining of placenta sections were used to assess cell proliferation, which were expressed in the villous cytotrophoblasts and most abundant in the control group (Figure 6B). Compared to controls, Ki67 staining was significantly decreased in placenta of pregnant rats that were exposed to IA LPS. However, when IA LPS-exposed pregnant rats were treated with 10058-F4, there was significantly increased number of Ki67+ cells per hpf compared to the IA LPS only group, and similar to controls.

3.6 IA LPS exposure modulates inflammation, collagen synthesis and extracellular matrix remodeling in the fetal lungs

Bulk RNA-sequencing was performed on lung tissue obtained at 24 h. after IA LPS to characterize transcriptional changes induced by IAI on lung inflammation and remodeling. IA LPS induced large transcriptional changes to the lung with clear separation of groups by PCA (Figure 7A). There were significant differences in gene expression between controls and LPS-exposed groups with 379 genes induced and 209 genes suppressed by IA LPS (Figure 7B, Supplementary Table S3). We used data from single-cell RNA-seq of fetal lungs from LungMAP (Du et al., 2015; Ardini-Poleske et al., 2017) to determine cell-type specific signature genes. Differentially expressed genes in IA LPS exposed lungs were mapped to the signature gene list for the different cell populations identified by single-cell RNA-seq. IA LPS exposure induced signature genes for airway and distal epithelium cells, proliferative mesenchymal progenitors, and matrix fibroblasts, and suppressed signature genes for myofibroblasts, suggesting maturation of alveolar epithelial cells and suppression of myofibroblasts, which play a role in alveolar septation (Figure 7C). Gene set enrichment analysis of differentially expressed genes showed that genes suppressed by IA LPS were associated with collagen synthesis (*Col9a2, Col9a1, Col11a2, Col11a1*) and extracellular matrix organization (*Cxcl1*). On the other hand, genes induced by IA LPS were associated with chemokines that drive leukocyte migration to the fetal lung (*Cxcl3, Ccl12, Cxcl1, Cxcl13, Itgam, Lyz2, Mr1, Lgals3, Tap2, Trem1*), cell killing (*Cxcl3*, *Trem1*), and surfactant homeostasis (*Sftpa1*, *Sftpb*). Other notable genes that were differentially expressed were related to cell proliferation (*Cebpa*), angiogenesis (*Bmp6*) and inflammation (*Apln*, *Pparg*) (Figures 7D–F).

3.7 c-Myc is expressed in lung macrophages and is upregulated in normal postnatal lung development. IAI further increases c-Myc expression in postnatal lung development

Lung sections at P14 stained with c-Myc antibody showed c-Myc expression in lung macrophages of pups exposed to IA LPS (Figure 8A). c-Myc was upregulated in normal postnatal lung development, and LPS exposure further increased c-Myc expression in postnatal lung development. Western blot analysis of c-Myc expression in lungs of controls at various neonatal timepoints showed upregulation of c-Myc expression over time in the neonatal period, suggesting a role of c-Myc in postnatal lung development. IA LPS exposure exacerbated the normal upregulation in c-Myc expression compared to controls, suggesting that IA LPS modulates neonatal lung c-Myc expression (Figure 8B).

3.8 Fetal lung inflammation induced by IA LPS is transient and is modulated by c-Myc

To determine the effect of prenatal c-Myc inhibition on fetal lung inflammation, we performed RT-PCR and cytokine

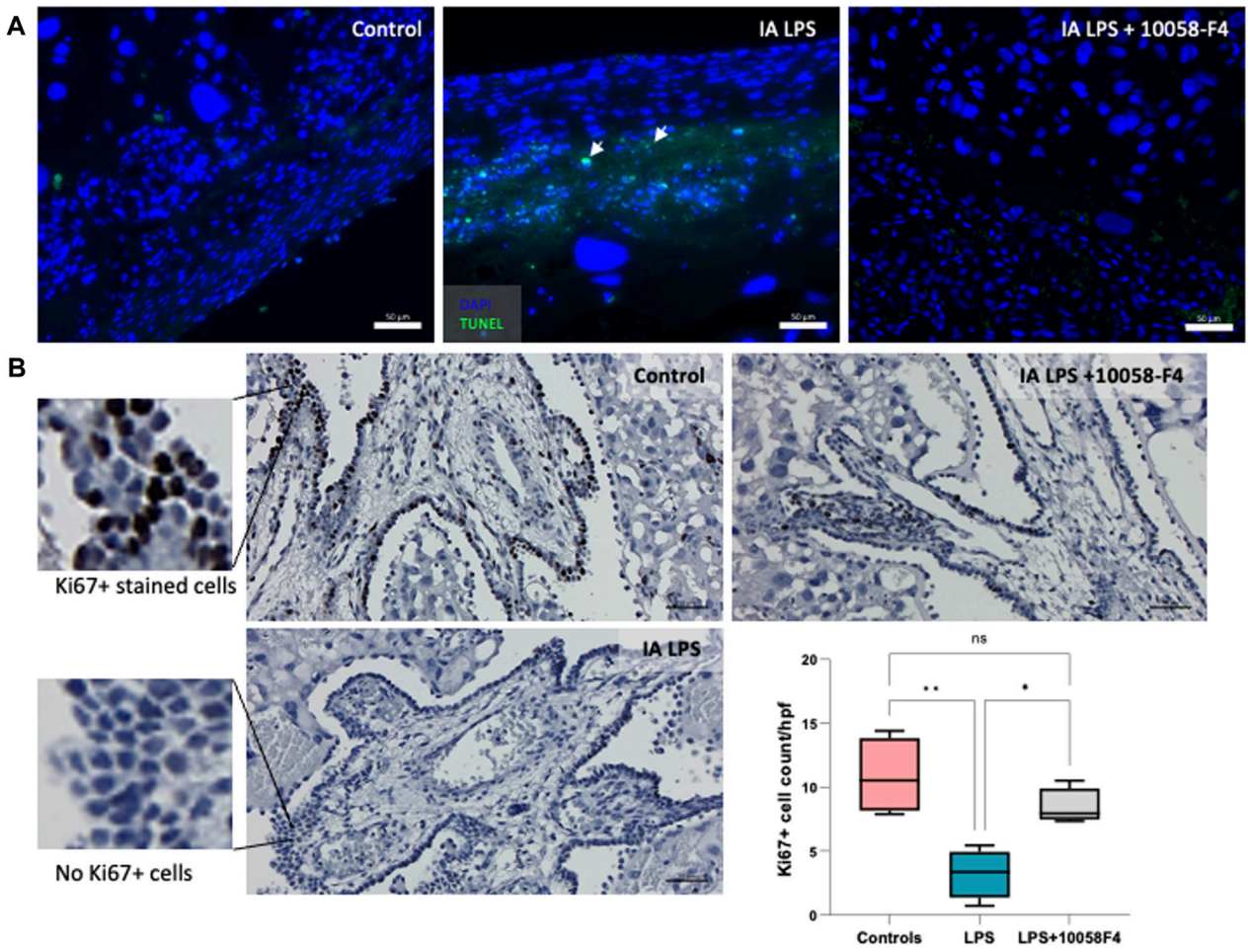

FIGURE 6
Prenatal systemic c-Myc inhibition decreases apoptosis and improves arrest of proliferation induced by LPS. **(A)** TUNEL assay performed on placenta sections showing increased apoptosis induced by LPS exposure when compared to controls, which was ameliorated with c-Myc inhibitor treatment. **(B)** Ki67 staining of representative placenta sections from each group showing decreased number of villous cytotrophoblasts positively stained with Ki67 (black stain) compared to controls, which improved with prenatal c-Myc inhibitor treatment. *$p < 0.05$, **$p < 0.01$, One-way Anova test. N = 4 per group.

multiplex assay. mRNA changes in IL1β, TNFα, CXCL1 and CXCL2 were not present at 24 h post-LPS exposure. Prenatal c-Myc inhibition in LPS-exposed fetal lungs suppressed IL1β and CXCL1 when compared to the LPS-exposed group, and suppressed TNFα when compared to both LPS and LPS+10058F4 treated groups. Similar to our findings in the placenta and fetal membranes, there was wide variability in mRNA expression of CXCL2 in response to LPS+10058F54 treatment, but these changes were not statistically significant when compared to control and LPS groups (Figure 9). On cytokine multiplex assay on lung lysates, LPS induced increased IL1β and CXCL10 at P0 only, and there were no significant changes in other pro-inflammatory mediators TNF□, IL6, IL10, CXCL1, CXCL2, CX3CL1 or CXCL5 on P0 or P3 (Figure 10). There were no significant changes in angiogenic factor VEGF induced by LPS. These findings demonstrate that fetal lung inflammation induced by IA LPS is transient and resolves before postnatal day 3.

3.9 Prenatal c-Myc inhibition improves LPS-induced changes associated with BPD

To assess alveolarization, lung sections obtained at P14 were stained with H&E and analyzed for the mean linear intercept (MLI) and radial alveolar count (RAC), which are measures of alveolar septation and quantification of lung airspaces. MLI was increased in LPS-exposed neonatal rats at P14, demonstrating alveolar simplification relative to controls. RAC was significantly decreased in LPS-exposed neonatal rats (Figure 11). To assess pulmonary vascular muscularization, lung sections were double-stained with von Willebrand factor (vWF) and smooth muscle actin (SMA) (Figure 12A), and the ratio of number of vessels stained with SMA-vWF to the number of vessels stained with vWF was calculated (Figures 12B, C). In IA LPS-exposed lungs at P14, there was an increased ratio of SMA/vWF-stained vessels, indicating increased pulmonary vascular remodeling, which occurs in pulmonary hypertension. Compared to pregnant rats exposed to LPS only,

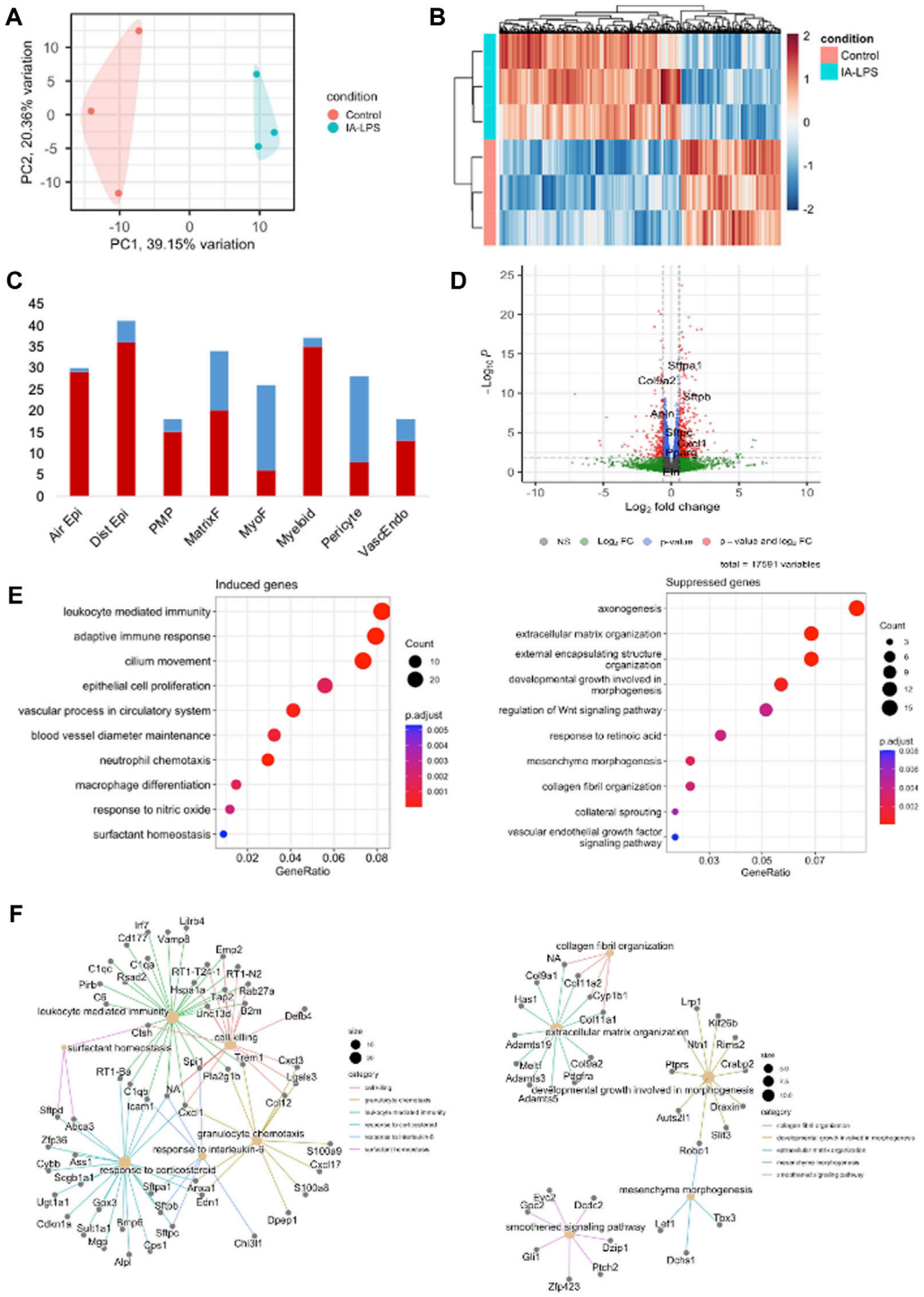

FIGURE 7
Intra-amniotic LPS induces fetal lung inflammation. Bulk RNA-sequencing of lungs at 24 h post-LPS exposure showing: **(A)** Principal component analysis showing clear differentiation of gene expression between groups. **(B)** Heatmap showing distinct differences in genes that were upregulated (*red*) and downregulated (*blue*) between control and LPS-exposed group. **(C)** Volcano plot showing differential gene expression with genes of interest highlighted. Red–significant. **(D)** Differential expression of genes by association with cell type. Red–induced. Blue–suppressed. *Air Epi–airway epithelium, Dist Epi–distal airway epithelium, PMP–proliferative mesenchymal progenitors, MatrixF–Matrix fibroflasts, VascEndo–Vascular endothelial.* **(E)** Chart showing related gene functions of genes that were differentially expressed between groups. Blue–Controls, orange–LPS-exposed. **(F)** Network plot of genes that were differentially induced (*left*) and suppressed (*right*) in LPS-exposed group compared to controls. N = 3 per group.

prenatal treatment of LPS-exposed pregnant rats with c-Myc inhibitor 10058-F4 led to improved alveolarization, increased angiogenesis and decreased pulmonary vascular muscularization induced by LPS (Figures 11, 12). These findings suggest that c-Myc has a role in modulating impaired lung development induced by IAI.

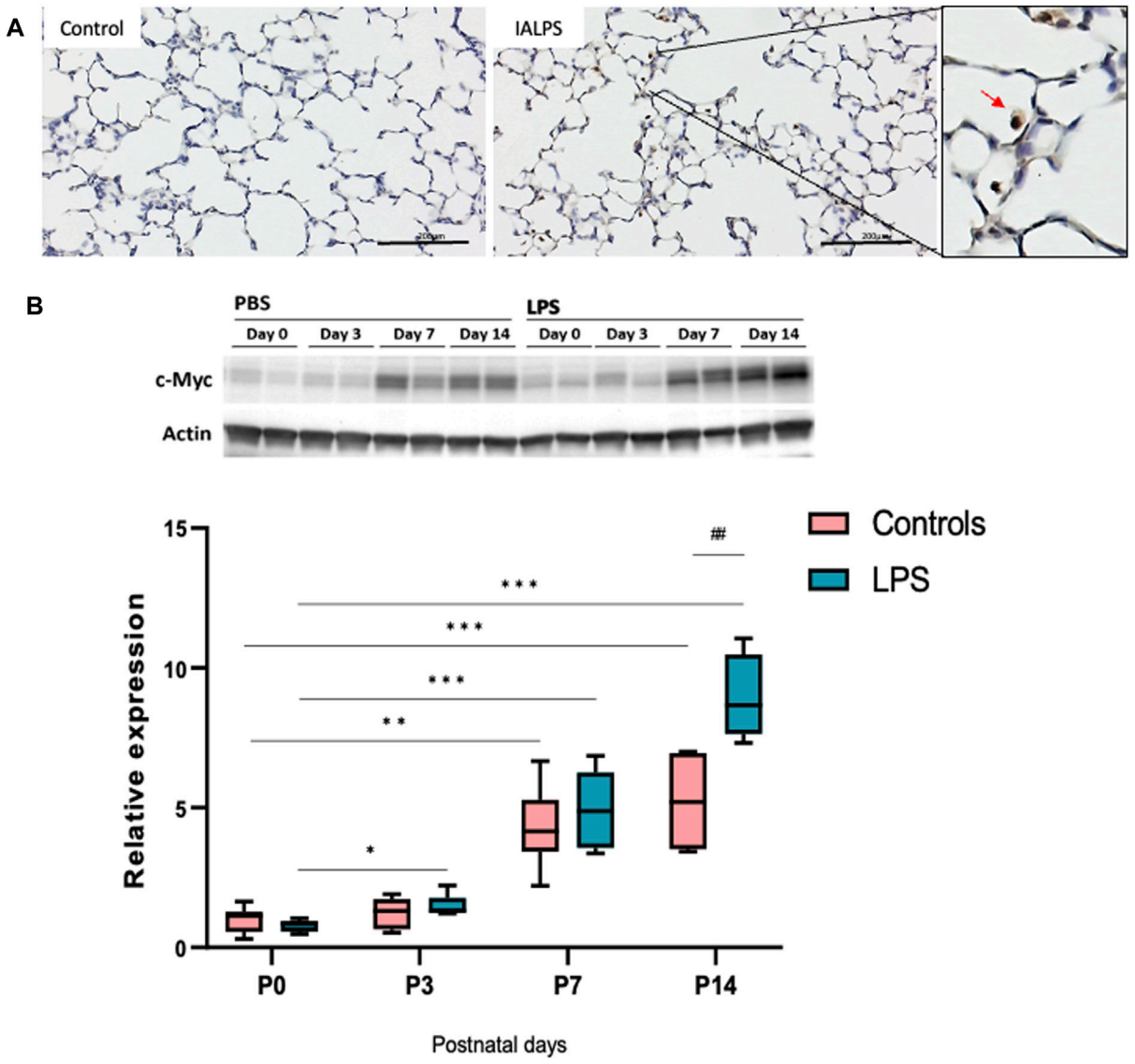

FIGURE 8
c-Myc is expressed in neonatal lung macrophages and is developmentally regulated. **(A)** Representative lung sections of rat pups at postnatal day 14 stained with c-Myc antibody showing localization of c-Myc expression to lung macrophages (brown-stained cells in magnified image on right). **(B)** Representative Western blot of lung tissue lysates showing expression of c-Myc over time (N = 4–7 per group). c-Myc was significantly upregulated during normal postnatal development, and this effect was more pronounced with exposure to LPS. *$p < 0.02$, **$p < 0.002$, ***$p < 0.0002$, ****$p < 0.00002$, ##$p < 0.002$.

4 Discussion

To understand the role of c-Myc on placental inflammation and BPD induced by IAI we used ultrasound-guided IA LPS injections and allowed natural delivery, eliminating confounding effects of stress and inflammation induced by maternal laparotomy. This rodent model of IAI has been validated to cause a subclinical syndrome of IAI, which is most clinically relevant, compared to intra-uterine or intraperitoneal injections (Gomez-Lopez et al., 2018). We further validated this model with demonstrated uptake of tagged LPS in the amnion, fetal lungs, and gut, confirming exposure of the fetus to intraamniotic inflammation through the fetal membranes, lungs, and gastrointestinal tract. These findings are consistent with large animal models of IAI using IA LPS (Kramer et al., 2010). Overall, our results suggest that c-Myc drives neutrophil infiltration in IAI, and that c-Myc has a role in modulating neonatal lung alveolar development and pulmonary vascular remodeling.

Bulk RNA-sequencing of placenta and fetal membranes showed transcriptional changes in genes associated with inflammation (*S100a8, S100a9, Alox15, Il1b, Mmp8*), leukocyte activation (*Ccl2, Ccl3*), and decreased proliferation (*Map9, Tpx2*) in response to LPS exposure. *S100a8*, *S100a9, Il1b,* and *Mmp8* have been strongly associated with fetal inflammatory response syndrome (FIRS), preterm labor, and chorioamnionitis in preterm infants (Kallapur et al., 2013; Holmstrom et al., 2019; Golubinskaya et al., 2020), further supporting our rat model using IA LPS injections to simulate IAI and a FIRS-like response. *Alox15* encodes for arachidonic acid through the lipoxygenase 15 (ALOX15) pathway, which participates

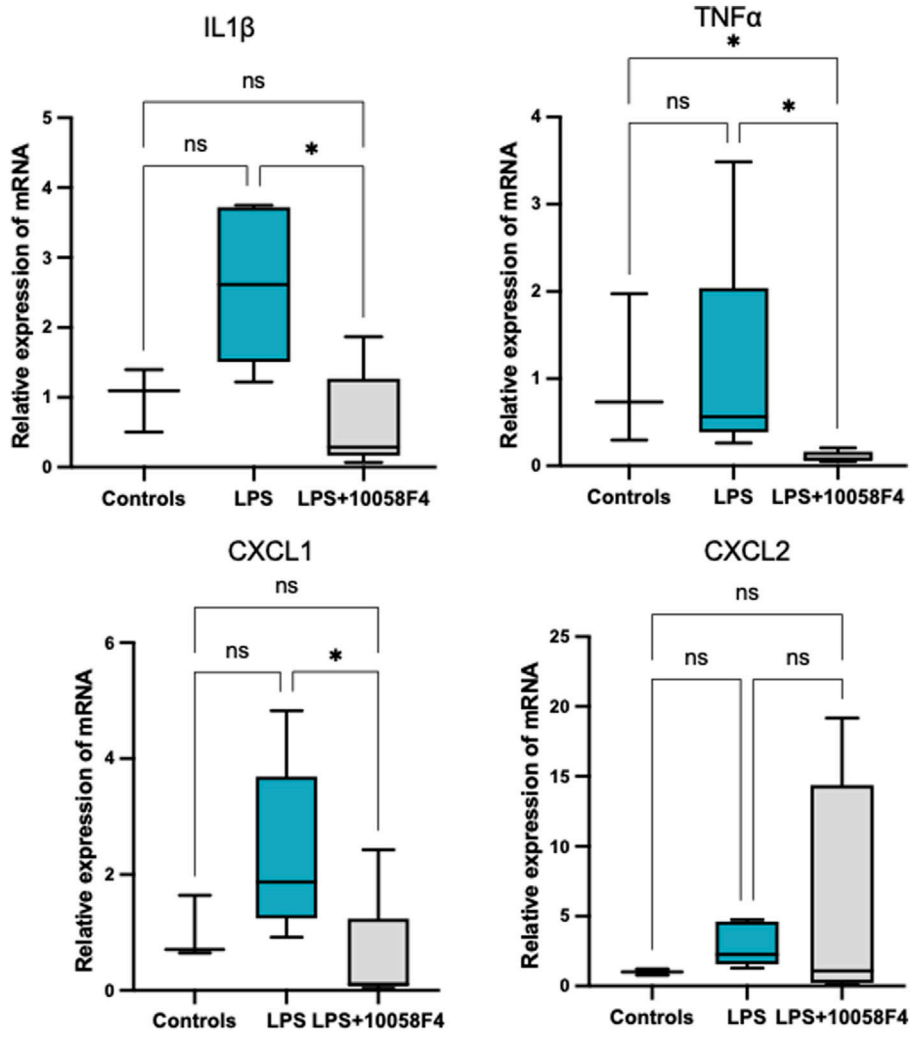

FIGURE 9
Fetal lung inflammation induced by IA LPS is transient and is modulated by c-Myc. RT-PCR of fetal lungs sampled at 24 h post-LPS exposure (N = 4–7 per group). There were no significant differences in mRNA expression of IL1β, TNFα., CXCL1 or CXCL2 with LPS exposure. Prenatal c-Myc inhibition in LPS-exposed fetal lungs suppressed IL1β and CXCL1 when compared to the LPS-exposed group and suppressed TNFα when compared to both LPS and LPS+10058F4 treated groups. There were no statistically significant changes in mRNA expression of CXCL2 with either LPS exposure alone or LPS+10058F54 treatment. *$p < 0.05$ Kruskal–Wallis test.

in glucocorticoid receptor response and modulates parturition through prostaglandin E2 synthesis pathway, suggesting a potential role in inflammation induced preterm labor (Zhang et al., 2023). *Ccl2* and *Ccl3* code for known inflammatory chemokines that are elevated in chorioamnionitis-exposed preterm infants (Stepanovich et al., 2023). LPS exposure suppressed *Map9* (Microtubule-Associated Protein 9) and *Tpx2* (Targeting protein Xklp2), which are protein-coding genes that are involved in cell growth and division. *Tpx2* abnormalities result in abnormal spindles and meiosis, and chromosome segregation errors in animal studies, which may be associated with birth defects and pregnancy loss (He et al., 2022; Zhang et al., 2022). These transcriptomic changes suggest a negative impact of placental growth and development induced by IA LPS.

LPS induces inflammation in the placenta and fetal membranes within 24 h of exposure, demonstrated by increased neutrophil infiltration and NET formation. Neutrophil infiltration is a hallmark of IAI, and neutrophil recruitment with NET formation are immune defense mechanisms against infections or danger signals (Gomez-Lopez et al., 2017). However, excessive neutrophil infiltration and NET formation in pathologic conditions may exacerbate tissue injury (Sorensen and Borregaard, 2016). In LPS-exposed placenta and fetal membranes, c-Myc is expressed in the nuclei of neutrophils. Prenatal systemic c-Myc inhibition with 10058F4 decreased LPS-induced neutrophil infiltration and NET formation in the placenta and fetal membranes, suggesting that c-Myc regulates inflammation in the placenta and fetal membranes in our rat model of IAI, and that c-Myc modulates NET formation in the placenta. Interestingly, c-Myc was not expressed in the neutrophils in LPS-exposed placenta treated with 10058F4 but is expressed in the cytoplasm of maternal decidual cells. The presence of c-Myc in the placenta has only been described in limited studies, in human choriocarcinoma and hydatidiform moles (Diebold et al., 1991; Cheung et al., 1993; Fulop et al., 1998). There is limited data on

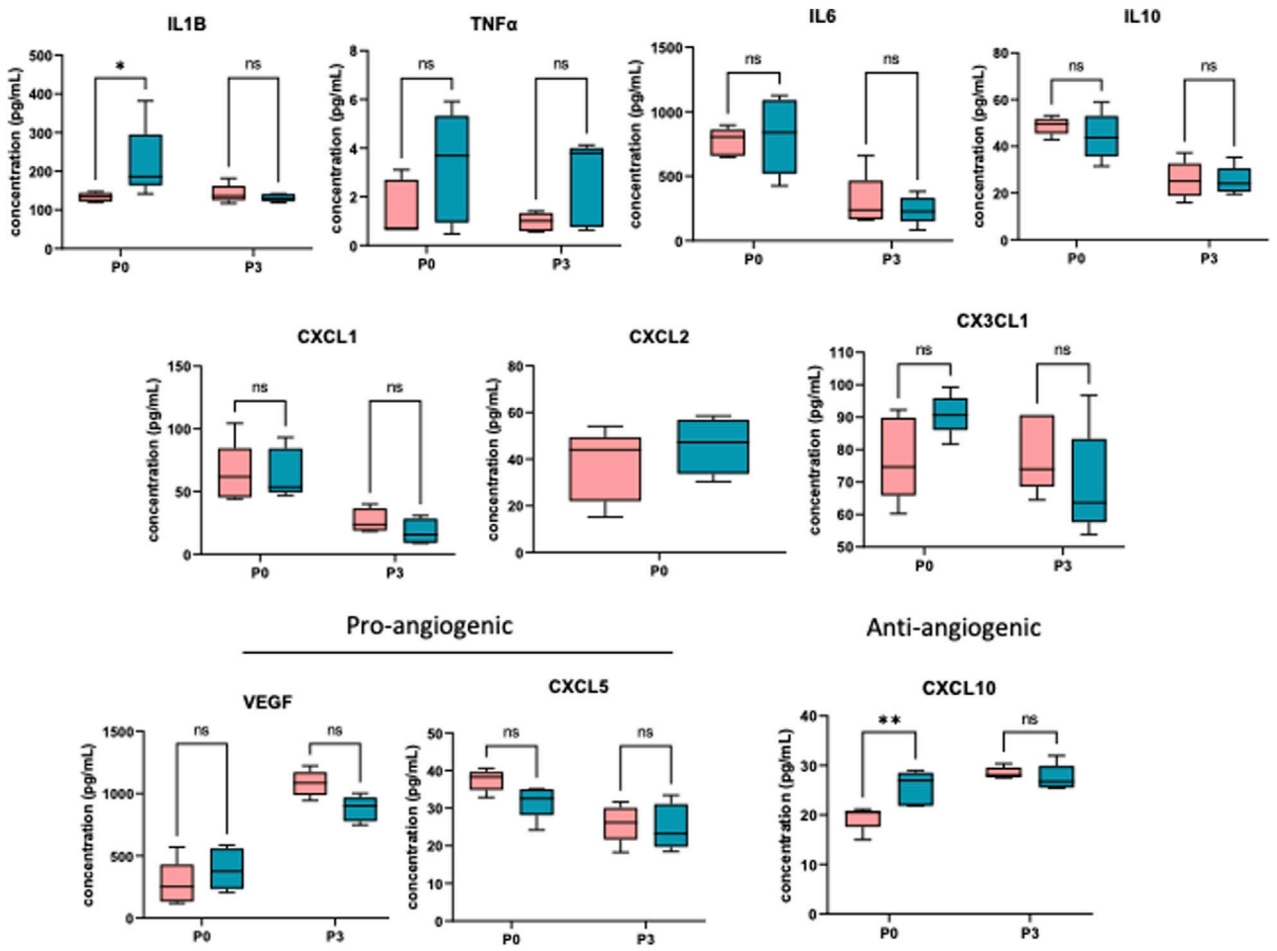

FIGURE 10
IA LPS induces fetal lung inflammation that resolves before postnatal day 3. Cytokine multiplex assay performed on neonatal lungs sampled on postnatal days (P) 0 and 3. In neonatal lungs at P0, LPS significantly induced IL1β and CXCL10. There were no statistically significant changes in TNFα, IL6, IL10, CXCL1, CXCL2, CX3CL1, CXCL5 and VEGF. N = 5 per group. *$p < 0.05$, **$p < 0.02$ Two-way Anova.

the role of c-Myc in the placenta in normal pregnancies and in IAI. On the other hand, the differences between intranuclear expression of c-Myc *versus* cytoplasmic c-Myc expression have been described, and the biological functions of c-Myc differ when expressed in nuclei or cytoplasm (Conacci-Sorrell et al., 2010). The expression of c-Myc in nuclei or cytoplasm has been used to risk stratify and prognosticate cancers (Geisler et al., 2004; Conacci-Sorrell et al., 2010; Gong et al., 2017).

We administered the c-Myc inhibitor 10058-F4 systemically, but it is not known whether 10058-F4 crosses the placenta. Since we performed ultrasound-guided intra-amniotic LPS injections to minimize systemic effects and to mimic subclinical chorioamnionitis, inflammation is localized to the amniotic sacs, placenta, and fetus. LPS is a toll-like receptor 4 (TLR4) agonist, and has been shown to prevent degradation of c-Myc via activation of the TLR/MyD88 pathway, but the exact mechanism by which LPS induces neutrophil-targeted c-Myc in the placenta are unknown (Wang et al., 2014). Our findings suggest that prenatal systemic 10058F4 treatment leads to inhibition of c-Myc in the maternal circulation, attenuating recruitment of neutrophils to the placenta and fetal membranes in IAI. However, prenatal 10058F4 did not inhibit c-Myc expression in the maternal decidual cells, which is likely constitutional in the placenta and fetal membranes and likely related to other functions regulated by c-Myc, such as cellular proliferation. The overall increase in c-Myc expression in the placenta and fetal membranes treated with 10058-F4 is not neutrophil-driven and may be a compensatory mechanism to overcome prenatal c-Myc suppression in cell types other than neutrophils, and possibly to protect the pregnancy and fetus. The role of c-Myc in placental and fetal development, and in IAI needs to be further explored.

We did not observe statistically significant changes in mRNA expression of specific inflammatory cytokines and chemokines (IL1β, TNF-α, CXCL1 and CXCL2) induced by LPS in the placenta and fetal membranes. This is likely due to lack of statistical power as there was an uptrend in IL1β and TNFα which corroborates with RNA-sequencing data. However, prenatal c-Myc inhibition in the LPS-exposed group significantly induced IL1β, CXCL1 and CXCL2 when compared to controls. CXCL1 and CXCL2 are members of the CXC chemokine subfamily that participate in wound healing, immunoregulation and neutrophil recruitment through activation of a CXCR2 receptor (Sawant et al., 2021). CXCR2 antagonism

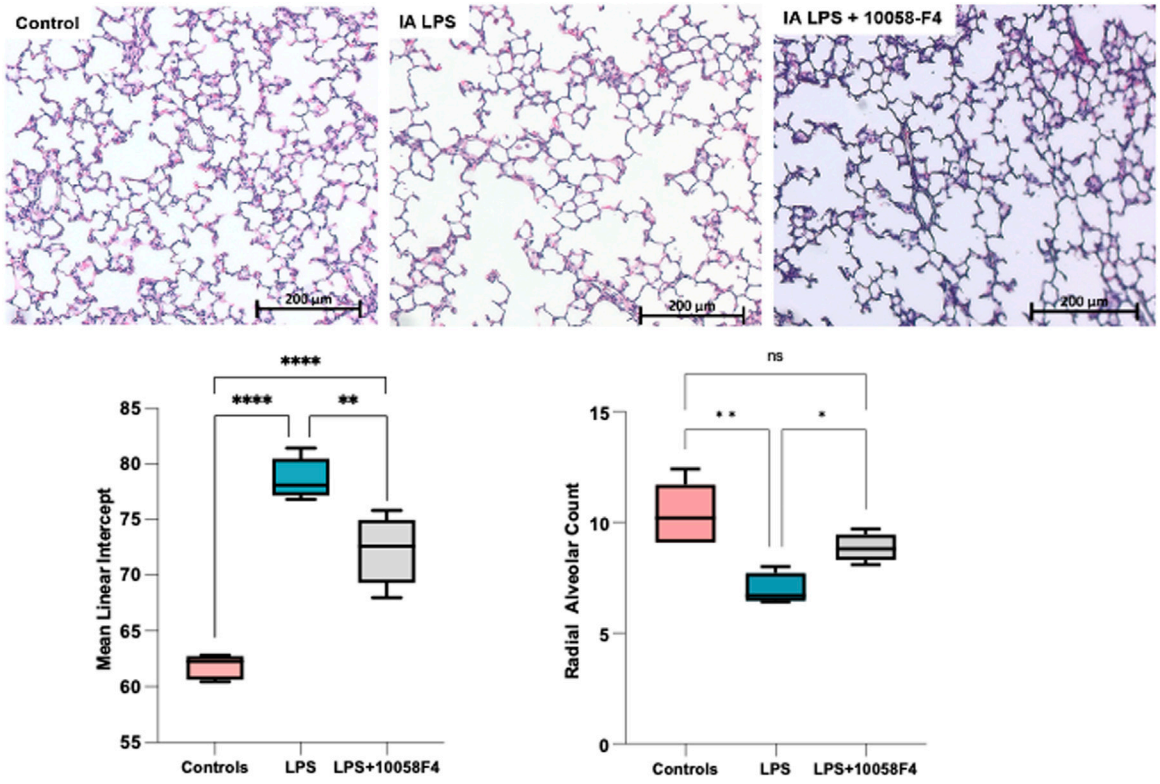

FIGURE 11
c-Myc inhibition improves alveolarization and pulmonary hypoplasia induced by IAI. Representative lung sections of rat pups at postnatal day 14 (n = 5 per group) stained with H&E to assess alveolarization. Mean linear intercept was significantly increased and radial alveolar counts were significantly decreased in IALPS-exposed lungs compared to controls at postnatal day 14, suggesting alveolar simplification and pulmonary hypoplasia. These effects were significantly decreased with c-Myc inhibitor treatment. *$p < 0.05$, **$p < 0.005$, ****$p < 0.0001$.

has been shown to decrease c-Myc expression in bone marrow of patients with chronic myeloid leukemia, through a CXCR2-mTOR-c-Myc cascade (Kim et al., 2021). c-Myc also regulates programmed cell death-ligand 1 (PD-L1), and c-Myc inhibition with 10058F4 in esophageal cancer cells downregulated PD-L1 (Liang et al., 2020). PD-L1 deficiency in neutrophils has been shown to lead to impaired secretion of CXCL1 and CXCL2 (Yu et al., 2022). In pregnancy, CXCL1 is produced in the placenta and participates in implantation, placentation and decidual angiogenesis (Korbecki et al., 2022). The dynamics between CXCL1, CXCL2 and CXCR2 receptor activation have been shown to be complex, and together, are vital in achieving homeostasis of inflammation and tissue healing (Sawant et al., 2021). CXCL1 and CXCL2 elevation in pregnant mice have been shown to be associated with massive decidual neutrophil infiltration and fetal loss (Mizugishi et al., 2015). The mechanisms by which c-Myc directly modulates CXCL1 and CXCL2, and their roles in IAI are unknown and need to be further explored. In our experiments, we observed that LPS exposure with prenatal c-Myc inhibition in a pregnant model induces an imbalance in CXCL1 and CXCL2 expression in the placenta and fetal membranes. Regardless, we observed reduction of neutrophil infiltration and NET formation with prenatal c-Myc inhibition, associated with improved neonatal lung remodeling. More studies are required to investigate the relationship between c-Myc, chemokine balance in pregnancy and effects on fetal development.

In fetal lungs, bulk RNA-sequencing showed that LPS exposure induced genes associated with chemokines that drive leukocyte migration to the fetal lung (*Cx3cr1, Cxcl3, Ccl12, Cxcl1, Cxcl13, Itgam, Lyz2, Mr1, Lgals3, Tap2, Trem1*), inflammation (*Angptl4, Chi3l1*), and surfactant homeostasis (*Sftpa1*, *Sftpb, Ctsh*). *Cx3cr1* encodes for the receptor of fractalkine/CX3CL1, which is a chemokine involved in adhesion and migration of leukocytes. CX3CL1-CX3CR1 axis is strongly associated with inflammatory lung diseases (Zhang and Patel, 2010). CX3CR1 is also a major receptor for respiratory syncytial virus infections and has been found to modulate airway inflammation and mucus production (Das et al., 2017), as well as LPS-induced lung injury through NFκB activation (Ding et al., 2016). *Cxcl1, Cxcl3, Cxcl13, Itgam, Lyz2, Lgals3* encode for cytokines that are known to be dysregulated in animal models of hyperoxia-induced BPD (Deng et al., 2000; Rudloff et al., 2017; Hurskainen et al., 2021; Dong et al., 2022), and *Trem1* is a protein-coding gene encoding for Triggering Receptor Expressed on Myeloid Cells 1 (TREM1) which is upregulated in preterm infants who developed BPD (Ambalavanan et al., 2009). *Angptl4* has been shown to be dysregulated in inflammation and may have protective anti-inflammatory and anti-angiogenic effects through modulation of NF-kBp65 and IL6 expression (Wang et al., 2013). *Angptl4* gene knockout in mice models of LPS-induced lung injury decreased inflammation and tissue damage, and improved recovery and mortality, suggesting a significant role of

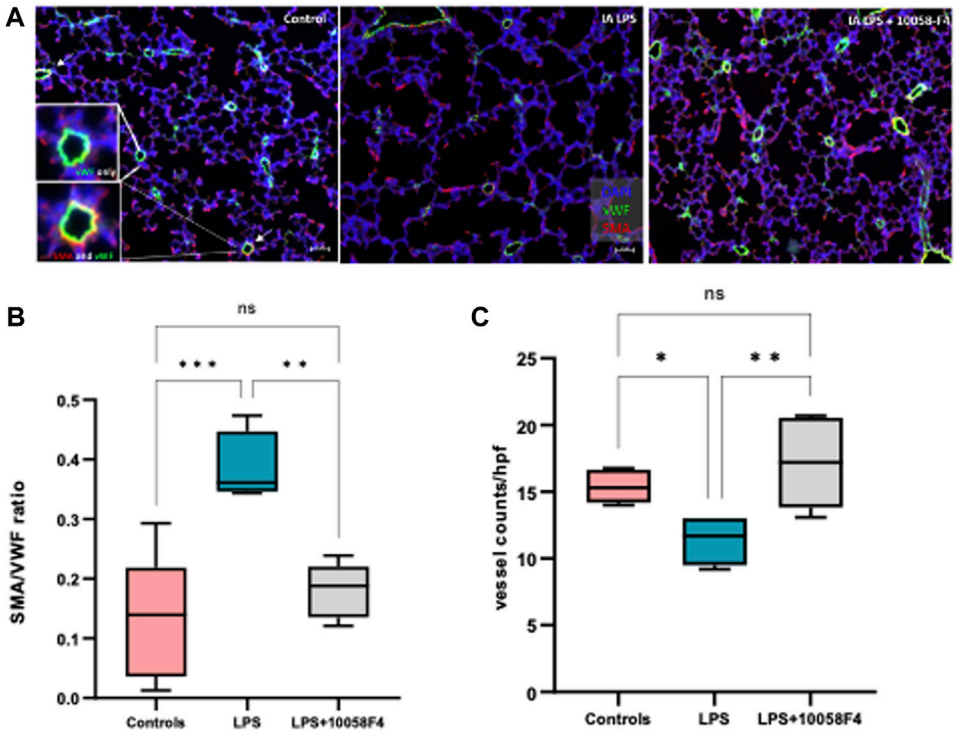

FIGURE 12
c-Myc inhibition increases angiogenesis and decreases pulmonary vascular remodeling in IAI at P14. **(A)** Immunostaining of lung sections sampled at postnatal day 14 with von Willebrand factor (vWF–green) and smooth muscle actin (SMA–red) (n = 4-8 per group) **(B)** Quantitative analysis of pulmonary vascular muscularization by calculating ratio of vessels stained with both SMA and vWF (demonstrated by white arrows and magnified images of vessels on far left), to number of vessels stained with vWF only (SMA/vWF ratio), and **(C)** quantitative analysis of angiogenesis using number of vessels stained with vWF per hpf. LPS exposure decreased angiogenesis and increased pulmonary vascular muscularization. Prenatal c-Myc inhibition with 10058-F4 treatment improved angiogenesis and decreased pulmonary vascular muscularization induced by LPS.

Angptl4 in the mechanisms of inflammation-induced lung injury (Guo et al., 2015; Li et al., 2015). *Chi3l1* has been implicated in pulmonary fibrosis and is expressed in lung alveolar macrophages and regulates inflammation, cell proliferation and apoptosis in connective tissue cells including fibroblasts (Recklies et al., 2002). *Ctsh* is involved in pulmonary surfactant protein B production and plays a vital role in lung development (Lu et al., 2007; Buhling et al., 2011). Similar to our findings in the placenta, RT-PCR of IL1β, TNF-α, CXCL1 and CXCL2 in the fetal lungs did not show statistically significant differences induced by LPS. However, in fetal lungs exposed to LPS+10058F4, we observed suppression in IL1β and CXCL1 compared to LPS-exposed group, and suppression in TNF-α compared to both controls and LPS-exposed group. IL1β, TNF-α and CXCL1 dysregulation are well-associated with IAI and BPD (Ambalavanan et al., 2009; Cappelletti et al., 2020; Heydarian et al., 2022).

Cytokine multiplex of neonatal lungs showed that IA LPS significantly induced IL1β and CXCL10 at P0. There were no statistically significant changes in TNFα, IL6, IL10, CXCL1, CXCL2, CX3CL1, CXCL5 and VEGF. IL1β is a well-known major modulator in IAI and bronchopulmonary dysplasia (Bry et al., 2007; Cappelletti et al., 2020). CXCL10 participates in inflammation, specifically macrophage infiltration, and modulates migration of vascular smooth muscular cells and endothelial cell permeability (Li et al., 2021). It is upregulated in tracheal aspirates of preterm infants with BPD and is strongly associated with idiopathic pulmonary arterial hypertension in adults (Aghai et al., 2013; Li et al., 2021; Cunningham et al., 2022). Inhibition of CXCL10 in animal models have been shown to improve pulmonary hypertension and LPS-induced lung injury (Lang et al., 2017; Cunningham et al., 2022). Collectively, our RNA-sequencing data showed that IA LPS induced inflammatory transcriptional changes in the fetal lungs which persisted through postnatal day 0, but these inflammatory changes appeared to be transient and resolved before postnatal day 3. However, prenatal systemic c-Myc inhibition suppressed mRNA expression of inflammatory cytokines in the fetal lungs.

At P14, rat pups exposed to LPS had alveolar simplification and pulmonary hypoplasia, decreased angiogenesis, and increased pulmonary vascular muscularization, consistent with a BPD-phenotype. Extracellular matrix (ECM) remodeling is another important component in the pathophysiology of BPD, and RNA sequencing of fetal lungs at 24 h after LPS exposure showed significant modulation of genes related to ECM remodeling (*Cxcl1*) and organization, collagen synthesis (*Col9a2, Col9a1, Col11a2, Col11a1*), proliferative mesenchymal progenitors and matrix fibroblasts. These results are consistent with previously reported data where extracellular matrix development and collagen protein synthesis was disrupted in lungs of preterm rhesus macaques exposed to IALPS (Schmidt et al., 2020). Targeted inhibition of extracellular matrix proteins provides partial protection from lung injury induced by

hyperoxia and anti-inflammatory treatment in animal models improved changes related to BPD through modulation of collagen and extracellular matrix protein expression and TGF-β1/Smads pathway, suggesting potential for targeted therapy in modifying abnormal extracellular matrix remodeling in IAI-induced BPD (Mizikova et al., 2018; Chen et al., 2020).

c-Myc expression in rat fetal lungs have been shown to be elevated during a period of growth, then decrease over increasing gestational age, coinciding with the time of cell differentiation and beginning of surfactant production (Kellogg et al., 1992). Interestingly, protein expression of c-Myc increases postnatally in neonatal lungs of control pups, demonstrating a role of c-Myc in normal postnatal lung development. c-Myc is an important regulator of cell proliferation, differentiation, and growth. When exposed to LPS, c-Myc is overexpressed during neonatal lung development, which is also associated with large transcriptomic differences in cell differentiation and proliferation, airway and alveolar epithelial cell differentiation, collagen and extracellular matrix organization, and surfactant proteins, predicting abnormal lung development, which is consistent with the lung parenchymal and vascular changes observed in our 14-day old pups. In LPS-exposed rat pups, prenatal c-Myc inhibition improved alveolarization, increased angiogenesis and decreased pulmonary vascular muscularization, demonstrating a role of c-Myc in IAI-associated BPD. The exact mechanisms by which c-Myc induces neonatal lung remodeling need to be further explored.

BPD with pulmonary hypertension is highly clinically relevant. A subset of preterm infants with moderate to severe BPD develop pulmonary hypertension (BPD-PH), which is associated with significantly increased morbidity and mortality (Hansmann et al., 2021). Compared to infants without pulmonary hypertension, infants with BPD-PH have higher rates of tracheostomy, need for tube feeds, poorer growth and neurodevelopmental outcomes, and hospital readmissions (Nakanishi et al., 2016; Al-Ghanem et al., 2017; Lagatta et al., 2018). Pulmonary hypertension is independently associated with exposure to IAI (Woldesenbet and Perlman, 2005; Yum et al., 2018). In cord blood of newborns exposed to IAI, there is an imbalance of angiogenic factors such as sFlt-1, VEGF, and endothelial progenitor cells. In preterm rhesus macaque fetuses, exposure to intra-amniotic LPS results in large transcriptional changes of genes regulating vascular development (Schmidt et al., 2020). In preterm lamb models, IA LPS exposure causes decreased pulmonary blood flow and increased pulmonary arterial pressures, suggesting a direct link between IAI and pulmonary hypertension (Polglase et al., 2010). The mechanisms by which pulmonary hypertension is associated with BPD and IAI are not completely understood, and given the significant burden of BPD-PH, there is a pressing need to investigate the mechanistic pathways and potential interventions to improve neonatal outcomes. Our results suggest that c-Myc regulate neonatal lung vascular remodeling in response to IAI.

We recognize limitations in our study. There is variance in RNA sequencing data within the control animals, which may be attributed to the fact that sterile PBS was injected in controls as a placebo under clean, but not sterile, conditions. Thus, the procedure by itself may induce an intra-uterine inflammatory response which may account for the data variance. Also, we do not have RNA-sequencing available for the LPS-exposed group treated with c-Myc inhibitor 10058-F4. Given the various perinatal interventions and treatment, there was rejection and death of pups in LPS-exposed and treated groups which could influence the results. Further animals treated with 10058-F4 were done in different days from control and IA LPS only animals, which could have introduced batch effect in our analysis. All animals were purchased from the same vendor and the same lot of LPS was used in all experiments.

Despite these limitations, we can conclude that the transcription factor c-Myc is dysregulated in the placenta, fetal membranes, and neonatal lungs in intra-amniotic inflammation and modulates inflammation of the placenta and fetal membranes in the rat model of IAI induced by IA LPS and modulates IAI-induced neonatal lung remodeling. Further studies are needed to explore the mechanisms by which c-Myc modulates NET formation, and to investigate c-Myc as a potential therapeutic target in IAI, and IAI-induced BPD. However, c-Myc has been challenging to target therapeutically due to its intranuclear nature and its highly disordered structure (Llombart and Mansour, 2022). Moreover, it is a ubiquitous transcription factor that is involved in multiple cell processes vital to physiologic functions. The ideal c-Myc inhibitor needs to be highly selective to diseased conditions and normalize c-Myc levels, rather than fully inhibiting all c-Myc-associated functions. Novel c-Myc inhibitors with such properties have since become available and should be the focus of future studies to investigate translational therapeutic potential of c-Myc inhibition to improve adverse neonatal outcomes induced by antenatal inflammation.

Data availability statement

The gene expression data have been deposited in NCBI's Gene Expression Omnibus (GEO: https://ncbi.nlm.gov/geo/) and are accessible through GEO Series accession numbers GSE237595 and GSE239349.

Ethics statement

The animal study was approved by University of Miami Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AT, KY, and AuS conceived and designed the study. AT, XT, SA-C, SK, PC, VN, RI, JD-B, MB, and SW performed the experiments or significantly contributed to the acquisition of data. AlS and AM prepared samples and perform analyses by Western blot. AT, KY, CR, and AuS analyzed the data, AT wrote the first draft of the manuscript. AuS critically reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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/fcell.2023.1245747/full#supplementary-material

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MYC: there is more to it than cancer

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MYC is a pleiotropic transcription factor involved in multiple cellular processes. While its mechanism of action and targets are not completely elucidated, it has a fundamental role in cellular proliferation, differentiation, metabolism, ribogenesis, and bone and vascular development. Over 4 decades of research and some 10,000 publications linking it to tumorigenesis (by searching PubMed for "MYC oncogene") have led to MYC becoming a most-wanted target for the treatment of cancer, where many of MYC's physiological functions become co-opted for tumour initiation and maintenance. In this context, an abundance of reviews describes strategies for potentially targeting MYC in the oncology field. However, its multiple roles in different aspects of cellular biology suggest that it may also play a role in many additional diseases, and other publications are indeed linking MYC to pathologies beyond cancer. Here, we review these physiological functions and the current literature linking MYC to non-oncological diseases. The intense efforts towards developing MYC inhibitors as a cancer therapy will potentially have huge implications for the treatment of other diseases. In addition, with a complementary approach, we discuss some diseases and conditions where MYC appears to play a protective role and hence its increased expression or activation could be therapeutic.

KEYWORDS

MYC, targeting, therapy, non-oncological diseases, transcription factor

1 Discovery and initial characterisation of MYC

The *c-MYC* gene encodes for a basic helix-loop-helix protein that acts as a pleiotropic transcription factor. It was discovered more than 40 years ago by the pioneering work to isolate and characterise avian retrovirus MC29, which showed its oncogenic potential, followed by the discovery of *c-MYC*, its cellular homolog identified from the chicken genome (Duesberg et al., 1977; Sheiness et al., 1978; Hu et al., 1979; Abrams et al., 1982; Vennstrom et al., 1982; Hann et al., 1983; Dang et al., 1989). Later studies discovered two human paralogs with overlapping roles and a more limited tissular expression: MYCN, or N-MYC, identified in Neuroblastoma cells, and MYCL, or L-MYC, found in Lung carcinoma cells, respectively, reviewed in (Massó-Vallés et al., 2020). c-MYC (from now on, MYC) and its paralogs share an N-terminal transactivation domain (TAD), capable of interacting with a plethora of proteins regulating chromatin remodelling, transcription and MYC stability, a central region, and a C-terminus basic helix-loop-helix (bHLH) domain (Beaulieu et al., 2020). The latter initially pointed to MYC as a protein capable of binding DNA, although it was not until 1990 that it was

discovered that MYC bound the sequence CACGTG (termed the E-box) (Prendergast and Ziff, 1991). Shortly after, a MYC dimerisation partner was identified: MYC-associated protein X, MAX a bHLH-Zip protein, specifically associated with c-MYC and its paralogs. Using a yeast model, DNA binding and transcriptional transactivation by MYC were found to be both dependent on this heterodimer (Blackwood and Eisenman, 1991; Amati et al., 1992), and a study in *Drosophila*, revealed that dMyc, dMax and the Max-binding protein dMNT could bind up to ~15% of the coding regions in the fly genome (Orian et al., 2003).

2 Physiological processes mediated by MYC

In this section we describe how MYC plays a key role in multiple aspects of the biology of cells and tissues. This is also summarised in Figure 1.

2.1 Proliferation

MYC's most established role under physiological conditions is to promote efficient proliferation (Jha et al., 2023). This has been studied in many model systems and organisms from mammalian tissues to flies. Although MYC is virtually undetectable in quiescent cells, upon mitogenic or serum stimulation, MYC levels are induced, and cells enter the G1 phase of the cell cycle through MYC-dependent upregulation and/or activation of key mediators of cell cycle progression, such as CCDN2, CDK4, and the CyclinE2-CDK2 complex, degradation of p27 (Kip1, encoded by CDKN1B), and repression of p21 and p15 (encoded by *CDKN1A* and *CDKN2B*, respectively) among others (Pelengaris and Khan, 2003). In contrast, MYC-dependent repression was described to be mediated by MYC-MAX interaction with Miz-1 (Staller et al., 2001). Expression of MYC is necessary, and in some cases sufficient, for inducing cell proliferation. In fact, ectopic expression of MYC locks cells in a continuously proliferating state, even in the absence of mitogens (Evan et al., 1994). This is probably the evolutionary basis for the tight regulation of MYC expression, which is in stark contrast to the ubiquitous expression of its binding partner MAX.

MYC's key role in proliferation and growth is highly conserved throughout the animal kingdom, with a presence in invertebrates such as *Drosophila,* where dMYC is the only paralog, whose loss impacts on cellular growth and size. Its overexpression promotes G1/S progression but not cell division, which is dependent on other players (Johnston et al., 1999). Interestingly, expression of dMYC is able to rescue the proliferation defects in mouse embryonic fibroblasts deficient for MYC, although it does not affect cell growth. Thus, MYC and dMYC have similar biological functions, but their outcomes depend on specific cell targets (Trumpp et al., 2001). Given this ancestral conservation, it is curious that MYC itself was lost during the evolution of *C.elegans,* which, instead, retains orthologous MAX and MLX networks (Yuan et al., 1998; Gallant, 2006; McFerrin and Atchley, 2011).

2.2 Differentiation

A key role for MYC in differentiation has been demonstrated in many tissues. One prominent example is found in the hematopoietic system, where MYC is involved in the expansion of committed progenitors by controlling the balance between self-renewal and differentiation through the modulation of stem cell migration and/or adhesion to the niche. MYC was described as necessary to induce the first differentiation steps in these murine stem cells, whereas in committed progenitors, MYC is required for proliferation and expansion (McFerrin and Atchley, 2011). Additionally, gene expression analyses using Krüppel-like factor 1 (KLF1), a master regulator of adult erythropoiesis (Perkins et al., 2016), and KLF2 knockout mice identified MYC as a central node in a network of genes controlled by both KLF1 and KLF2. Ablation of MYC in primitive proerythroblasts showed that its absence resulted in a block in the normal expansion of erythroid cells (Pang et al., 2012). In addition, the master regulator of haematopoiesis GATA-1 represses MYC transcriptional activity through binding to MYC's promoter or through activation of miR-144/451, inducing proliferative arrest, thus facilitating erythroid differentiation (Rylski et al., 2003). Conversely, depletion of miR-144/451 blocks erythroid differentiation through de-repression of MYC (Xu et al., 2020). This GATA-1-miR144/451-MYC axis controls normal erythroid differentiation.

Another example of MYC's role in differentiation can be found in murine embryonic stem cells (mESC), where MYC inhibits the expression of differentiation-specific genes through modulation of a set of miRNAs that attenuate their proliferation (Lin et al., 2009). Its inhibition or deletion strongly curbs transcription, splicing and protein synthesis, leading to a proliferative arrest, reminiscent of embryonic diapause. Remarkably, this arrest is reversible and does not compromise cell pluripotency (Scognamiglio et al., 2016). Additionally, MYC maintains the pluripotent transcriptome by amplifying the transcription of a large set of genes during the transition from mESC to the totipotent two-cell-like state (Fu et al., 2019), and is also important in metabolic and epigenetic regulation of mESCs during mouse embryonic development (Fan and Li, 2023).

In human adipose tissue, MYC was identified as a significant regulator of adipose stem cell differentiation, which is necessary for the maintenance and function of the tissue. MYC is induced by glucocorticoid in the early stages of differentiation and precedes the downregulation of key suppressor genes as well as the induction of functional effectors (Deisenroth et al., 2014).

In crypt development in the small intestine in the mouse, MYC signalling pathways are significantly enriched. Laser capture microdissection followed by functional genomics analysis of epithelial progenitors showed an enrichment, with respect to normal crypt base epithelium, of a series of transcripts encoding for proteins that regulate MYC transcription, protein stability, and transactivation of its target genes (Stappenbeck et al., 2003). Subsequent studies, however, showed that MYC is necessary for normal crypt formation, but does not affect cell proliferation or fate of already formed crypts (Bettess et al., 2005).

A role in differentiation is present in *Drosophila* too, where dMYC is required for intestinal stem cell maintenance, proliferation, and lineage differentiation during tissue homeostasis (Ren et al.,

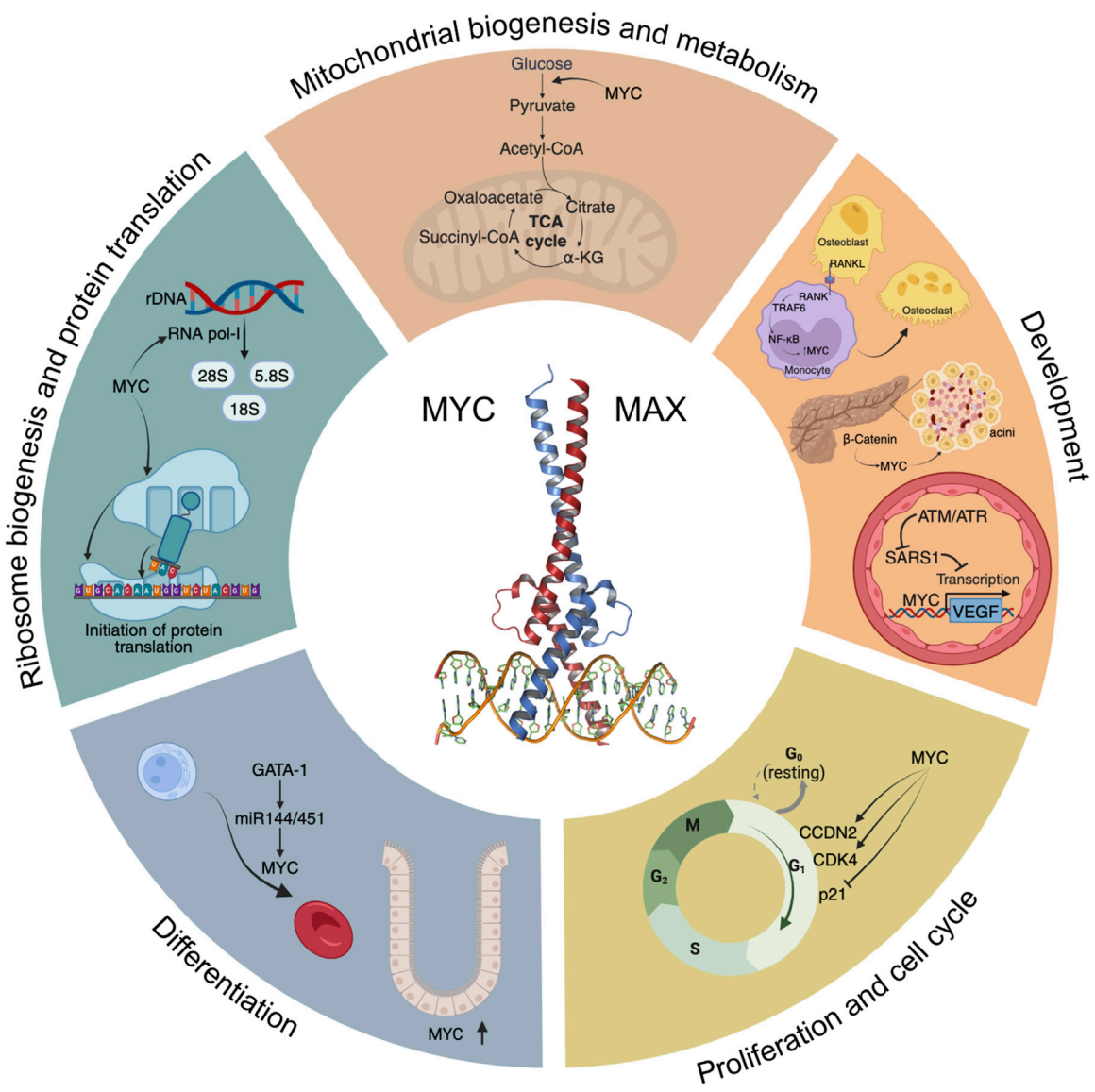

FIGURE 1
A "Hallmarks" style representation of MYC's physiological functions. Some of the main signalling pathways and processes modulated by MYC are depicted, including glycolysis and mitochondrial biogenesis, development, cell cycle progression, differentiation, ribosome biogenesis and initiation of protein translation.

2013). Also in *Drosophila*, IGF2BP stabilises *MYC* mRNA, increasing its protein levels, leading to larger neural stem cells and faster division rates (Samuels et al., 2020). In line with this, GSK3-α and -β differently regulate cortical development through MYC (Ma et al., 2017), while MYC inhibits the differentiation of neural progenitor cells into neurons (Wang et al., 2020).

2.3 Ribosome biogenesis and protein translation

Ribosome biogenesis involves the synthesis and processing of ribosomal RNA (rRNA) proteins, the assembly of ribosomal subunits and their export to the cytoplasm, and it requires the coordinated activities of the three nuclear RNA polymerases (RNA pol I, II and III). Not surprisingly, MYC regulates multiple stages of ribosomal biogenesis through RNA pol I-mediated transcription of rRNA, RNA pol II-dependent transcription of ribosomal protein genes and translation initiation factors, among others (reviewed in van Riggelen et al., 2010). Using Crispr-Cas9-based reverse genetics to dissect the transcriptional networks downstream of MYC *in vivo*, it was shown that MYC's ability to drive growth depends on its ability to upregulate ribosome biogenesis (Zielke et al., 2022). Consistent with this, inducible overexpression of MYC stimulates both ribosome biogenesis and protein synthesis (Mori et al., 2021).

Intimately related to ribosome biogenesis, protein translation is a critical process on which cell growth and division depend. It is regulated at different levels, although the key point of control seems to be the initiation of translation, which involves the translation initiation factor eIF4E binding to the 7-methyl guanosine cap at the

5′ end of mRNAs (Schmidt, 2004). Experiments carried out with *MYC* knockout rat fibroblasts showed that levels of protein translation, a mechanism that is under control of mammalian TOR complex 1 (mTORC1) (Ma and Blenis, 2009), are two-to-three fold higher in MYC wild-type when compared to $MYC^{-/-}$ cells (Mateyak et al., 1997). Microarray analysis of these cells showed that the largest category of MYC-induced genes was involved in protein translation, where an impressive 60% of the genes were upregulated by MYC (Guo et al., 2000). Additionally, a specific role in regulating eIF4E was confirmed after showing its expression correlated with and was regulated by MYC (Rosenwald et al., 1993). Indeed, MYC binds to two canonical E-boxes in the *eIF4E* promoter and is necessary for its expression. Importantly, inhibition of eIF4E was able to block MYC-induced transformation (Lynch et al., 2004).

The link between MYC and ribosomes is conserved in flies and even in the multicellular eukaryote *Nemostella* (Brown et al., 2008; Stine et al., 2015). Indeed, in *Drosophila,* expression of dMYC is necessary and sufficient to control rRNA synthesis and ribosome biogenesis (Grewal et al., 2005) and physiological dMYC targets, whose promoters are enriched in the E-box motif (frequently in the first 100 nucleotides following the transcription start site), play a role in nucleolar function and ribosome biogenesis (Hulf et al., 2005).

2.4 Metabolism and mitochondrial biogenesis

The first evidence of the *in vivo* induction of glycolysis by MYC was provided using transgenic mouse models where MYC was overexpressed in hepatocytes under the control of phosphoenolpyruvate carboxykinase. Transcriptional analyses of livers from these transgenic mice revealed increased expression of the glycolytic enzymes of glucokinase, PFKFB1, pyruvate-kinase L, and the glucose transporter GLUT2, which resulted in increased glycolysis compared to controls (Hulf et al., 2005). Later on, MYC was shown to induce a collection of glycolytic genes including ALDOA, ENO1, GAPDH, GPI, LDHA, HK2, PFKM, PGK1, PKM, and TPI1 (Kim et al., 2004), confirming the key role of MYC in controlling metabolism.

Importantly, MYC's effect on metabolism becomes more evident when MYC is absent. Even in the presence of adequate energy-generating substrates, *MYC*-knockout fibroblasts remain ATP-depleted and respond by activating AMPK, in an attempt to remedy this energy deficit. However, since AMPK activation leads to upregulation of glycolysis and oxidative phosphorylation, both dependent on MYC, the AMPK response fails and the cells, unable to correct the energy production, remain slowly proliferating (Edmunds et al., 2014).

A final well-established role for MYC is in the mitochondria biogenesis. Using a combination of *in vitro* and *in vivo* MYC-modulating models, a role was shown for MYC in regulating the expression of genes involved in mitochondrial structure, function, and biogenesis. These include TFAM, a key mitochondrial transcriptional factor and mtDNA replication factor. These results point to MYC's role as a master mitochondrial switch coupling metabolic needs to cell growth and proliferation (Li et al., 2005). In this line, further work suggested that mitochondrial structure, function, and subcellular localisation are regulated over time, responding more rapidly to inactivation of MYC than to its activation. The increased mitochondrial mass induced by MYC was associated with increased organelle turnover, involving both fission and fusion proteins (Graves et al., 2012). Overall, these results reinforce the notion that MYC links cellular energy generation and proliferative needs.

MYC's role in mitochondrial biogenesis is also conserved in *Drosophila*, where in the ovary, it stimulates gene expression, including that of many electron transport chain genes required for mtDNA replication and expression (Wang et al., 2019).

2.5 Development

An increasing number of studies point to a role for MYC in the development of multiple tissues and organs, including pancreas, bone, and blood vessels. Given the difference in phenotypes of the tissues, it is perhaps not surprising that the principal targets of MYC in each case are different. In fact, development of tissues is a phenotypic outcome of the physiological processes that MYC helps to control, so that MYC's regulation of proliferation, differentiation and metabolism results in different phenotypic outcomes depending on the cell-specific gene expression, tissue type and body location. This highlights a reason why the definition of a single critical list of MYC target genes across different tissue contexts has proven impossible.

2.5.1 Pancreas

The expansion of pancreatic acinar cells, the main components of pancreatic parenchyma, is promoted by β-catenin signalling, of which MYC is one key effector (Murtaugh et al., 2005). MYC's importance in pancreas is stressed by the evidence that pancreatic inactivation of both *MYC* alleles in a mouse model leads to death after birth. Already at a late embryonic stage, these mice show a severe pancreatic hypoplasia, with poorly branched pancreatic ducts, disruption of exocrine pancreas formation and severe reduction of acini, characterised by reduced MYC target CDK4 expression and proliferation (Nakhai et al., 2008). These results were confirmed in an independent study using a mouse model with a 60%–70% reduction in MYC expression, in which pancreata showed fewer proliferating progenitors at E12.5, leading to significantly reduced pancreatic weight in two-month-old mice. Both arborization of the exocrine tree and acinar development were impaired at birth, but partially recovered at 2 months. Overall, MYC inactivation impairs normal acinar development and maturation, leading to the formation of lipid vacuoles in acinar cells, acquiring an adipocyte phenotype with aging (Bonal et al., 2009; Zhang et al., 2010).

2.5.2 Bone

Bone remodelling results from the balance between two tightly regulated phenomena: osteoclastogenesis and osteogenesis. The osteoclast is a bone-resorbing cell with an origin in the monocyte-macrophage lineage (Yavropoulou and Yovos, 2008). As expected, MYC is involved in bone remodelling and is regulated by different signalling pathways. RANKL, a key cytokine expressed by osteoblasts, mediates osteoclastogenesis through a TRAF6-dependent NF-κB activation. Upon RANKL

binding to monocytes, this cascade results in the induction of MYC, which is necessary for osteoclast differentiation, since its inhibition by the MYC dominant negative In373-Myc almost completely inhibits osteoclastogenesis (Battaglino et al., 2002).

On the other hand, inhibition of FOXO1, whose expression decreases upon RANKL-induced osteoclastogenesis, promotes the expression of MYC, while 10058-F4, a small molecular inhibitor of MYC, abrogates the osteoclastogenic effect of FOXO1 inhibition in a dose-dependent manner (Tan et al., 2015). Additionally, RANKL-induced expression of osteoclastogenic marker genes is significantly reduced in MYC-deficient osteoclast progenitors *in vitro*, but rescued by ectopic expression of MYC (Bae et al., 2017).

Finally, RNA-seq analysis of *MYC* wild-type and deficient bone marrow cells revealed that MYC regulates mTORC1 signalling. mTORC1 is activated at the early phases after RANKL treatment and suppressed at later phases of osteoclastogenesis, and this biphasic regulation is dependent on MYC. While inhibition of mTORC1 by rapamycin prior to RANKL stimulation almost completely prevented osteoclast formation, osteoclasts showed enhanced resorbing activity when mTORC1 was inhibited three days post-RANKL. In parallel, unfolded protein response (UPR) genes were found downregulated by MYC deficiency. In line with this, the expression of GADD34, a factor that regulates UPR and negatively regulates mTOR signalling, is increased in wild-type cells upon RANKL stimulation but not in *MYC*-deficient cells, and its deficiency partially restores RANKL-induced mTORC1 inactivation and suppresses osteoclastogenesis. Taken together, these data suggest that mTORC1 is suppressed in osteoclasts through a MYC/GADD34 axis (Bae et al., 2022). This is in stark contrast to protein translation, where MYC and mTORC1 jointly contribute to its regulation.

2.5.3 Vascular development

MYC's role in vascular development was confirmed by modulation of MYC levels. In fact, *c-MYC* knockout mice are embryonic lethal and have under-developed vasculature, that can be partially rescued by transgenic VEGF expression (Baudino et al., 2002). On the other hand, overexpression of MYC is also embryonic lethal due to multiple haemorrhagic lesions and defects in the vasculature, with concomitant elevated VEGF levels (Kokai et al., 2009). Thus, MYC and VEGF levels must be precisely controlled during early development. One key control involves Seryl-TRNA synthetase 1 (SARS1), which competes directly with MYC to control VEGF expression levels, and thus enables proper vasculature development (Shi et al., 2014). In hypoxic conditions, SARS1 is phosphorylated by ATM/ATR, and this impairs its DNA binding capacity, allowing MYC to induce VEGF expression (Shi et al., 2020).

3 Physiological MYC functions hijacked by tumour cells

To become fully transformed and tumorigenic, normal cells must overcome several barriers imposed on cell-autonomous programs such as cell cycle progression, DNA replication, evasion of senescence and apoptosis, as well as cell non-autonomous processes such as angiogenesis and immune surveillance. These constitute many of the Hallmarks of Cancer (Hanahan, 2022) and, as MYC may impinge on all these programs, it is a common target for oncogenic activation. Indeed, although its expression is tightly regulated in normal cells, cancer cells are almost unavoidably characterised by deregulated MYC activity. This can be the result of many different processes such as gene amplification, translocation (Figure 2A), epistasis, epigenetic changes, upstream signalling, and increased protein stability (Figure 2B) (Dhanasekaran et al., 2022). Oncogenic MYC promotes tumorigenesis in different yet complementary ways, co-opting many of the physiological processes described above. Its deregulation is associated to uncontrolled proliferation, rewiring of cellular metabolism, increased ribosomal and protein biogenesis and chromosome instability. MYC also affects cell non-autonomous hallmarks including reshaping of the tumour microenvironment, angiogenesis, induction of immunosuppressive cytokine release, and upregulation of immune checkpoint inhibitor proteins (Whitfield and Soucek, 2012; Dhanasekaran et al., 2022).

However, the sole overexpression of MYC is not sufficient for tumorigenesis in most cellular contexts. Indeed, MYC activation usually induces DNA replication and S phase entry without cellular division, hence cells become polyploid, accumulate DNA damage, and undergo proliferative arrest, senescence, or apoptosis depending on the cellular context (Gabay et al., 2014). This is why genetic alterations that circumvent the hurdles imposed by cell cycle checkpoints or apoptosis/senescence usually synergise with MYC overexpression to induce tumorigenesis. This was shown in seminal studies with transgenic mice harbouring tissue-specific inducible forms of MYC. For instance, in $MycER^{TAM}$ mice that express switchable MYC in pancreatic β-cells, MYC activation is sufficient to drive the cells into cell cycle, but unfettered proliferation is constrained by subsequent apoptosis, which quickly results in β-cell loss and diabetes. However, solely by co-expression of the anti-apoptotic protein $Bcl\text{-}_{XL}$, MYC overexpression is then able to drive formation of pancreatic insulinomas (Pelengaris et al., 2002). Similarly, in adult mouse hepatocytes, conditionally expressed MYC leads to polyploidy in the absence of cell division, but concomitant reduction of p53 levels (by crossing with $TP53^{+/-}$ mice) resulted in increased tumorigenesis (Beer et al., 2004).

In this context, MYC expression levels seem an important determinant of the biological outcome. It was reported, for instance, that low levels of deregulated MYC can drive proliferation and oncogenesis by themselves, whereas apoptotic and p53 tumour suppressor pathways are only triggered above a certain MYC threshold (Murphy et al., 2008).

In summary, as observed in cancer but not limited to it, MYC's role in promoting multiple physiological processes means that its overexpression, deregulation, or insufficiency can lead to an array of human diseases and disorders.

4 MYC's involvement in diseases and conditions

4.1 Metabolic diseases

Metabolic dysfunction-Associated steatotic liver disease (MASLD), previously known as non-alcoholic fatty liver disease,

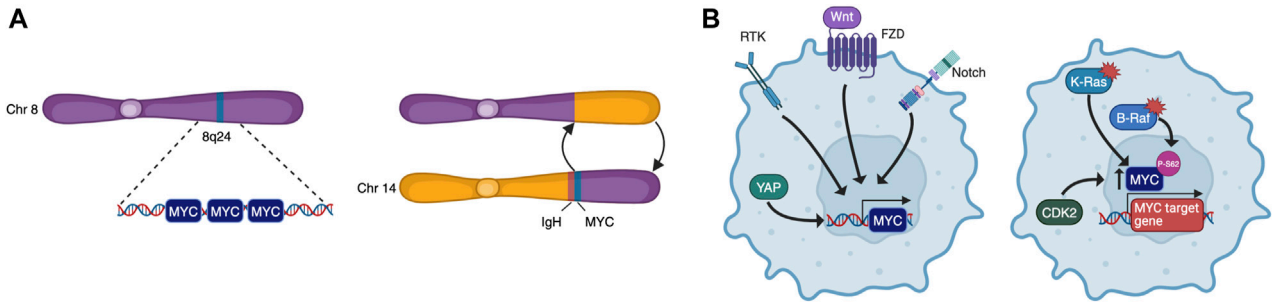

FIGURE 2
MYC is deregulated by multiple mechanisms. These include chromosomal rearrangements that lead to amplification or translocation **(A)**, and upstream signalling that causes increased transcription or protein stabilisation **(B)**.

is strongly associated with obesity and insulin resistance (Browning and Horton, 2004), as well as with increased mortality and cardiovascular disease burden (Kim et al., 2021). It begins with the aberrant accumulation of triglycerides in the liver (steatosis) and can proceed to a Metabolic Dysfunction-Associated Steatohepatitis (MASH), which in turn, can eventually give rise to cirrhosis and liver cancer.

Alb-myc^{tg} mice overexpress MYC in hepatocytes, and at 36 weeks, they spontaneously develop metabolic syndrome, characterised by obesity, hypertriglyceridemia, hypercholesterolemia, glucose intolerance and insulin resistance. The mouse livers show abnormal accumulation of lipids that leads to compensatory increased ß-oxidation that, in turn, generates oxidative stress. This results in, on the one hand, CD45^{+}, F4/80^{+} immune infiltration, and on the other, increased hepatocyte apoptosis and compensatory proliferation. Hence, hepatic overexpression of MYC affects metabolism and leads to the development of mild steatohepatitis/fibrosis that progress to liver tumours with long latency. Moreover, MYC overexpression provides a pro-fibrotic tissue environment characterised by moderate but chronic hepatocyte apoptosis, pre-activation of hepatic stellate cells (HSCs) and high basal collagen expression. These HSCs have a high potential to proliferate and to produce extracellular matrix, especially after a second hit. This link between MYC and hepatic fibrosis is reinforced by the fact that MYC mRNA expression was found to be upregulated in patients with liver cirrhosis (Nevzorova et al., 2013).

Alcohol-associated liver disease (ALD) includes a variety of hepatic conditions from steatosis to cirrhosis. In patients with advanced stages of ALD, MYC is strongly upregulated and correlates with the progression of liver fibrosis. In line with this, wild-type mice fed with a Lieber-DeCarli (EtOH) diet showed higher MYC expression at the initial stages of liver injury and MYC remained elevated during the early phase of ALD progression.

MYC overexpression and alcohol consumption were further studied with *Alb-myc*^{tg} mice. Following a 4-week Lieber-DeCarli diet, these mice presented deregulation of multiple disease-related pathways, and an increase in liver mass in the absence of proliferation, accompanied by hepatocyte hypertrophy, enhanced collagen deposition, increased mitochondrial oxygen radicals, and hepatic lipotoxicity. Mitochondrial and ER dysfunction caused metabolic effects involving glucose intolerance. Overall, MYC overexpression in the context of alcohol consumption led to impaired Akt-MDM2-p53 signalling that eventually may trigger ALD progression to fibrosis (Figure 3A) (Nevzorova et al., 2016). To our knowledge, studies to block MYC have not yet been performed in these models.

Intestinal MYC is also increased in humans and mice with obesity, likely due to activation of the ß-catenin pathway, of which MYC is a downstream target. In this case, its inhibition has been tested: intestinal-specific MYC disruption protected mice subjected to a high-fat diet against obesity, insulin resistance, hepatic steatosis and fibrosis (Luo et al., 2021). Overall, MYC plays multiple roles in obesity, including the maturation of progenitor cells, fatty acid metabolism and extracellular matrix remodelling. Of note, MYC modulates the inflammatory response, induces insulin resistance, and regulates intestinal dysbiosis (Nevzorova and Cubero, 2023).

Additionally, gerbils fed with a high-fat and high-cholesterol diet showed increased hepatic USP33 expression, whose modulation revealed a signal transduction pathway regulated by both this enzyme and MYC, which controls activation of HSCs, the main cells responsible for liver fibrosis (Ke et al., 2023). In this context, a potential drug treatment of MASH was recently identified: AZD3355, a GABA-B receptor agonist, proved to be anti-fibrotic, anti-inflammatory and hepatoprotective, and interestingly, MYC was identified as the top transcription factor regulated in HSCs treated *in vitro* with AZD3355 (Bhattacharya et al., 2021). All these data are all in line with a role for MYC in HSC activation and prompt the testing of MYC inhibitors in disease models.

Intriguingly, though, MYC expression in endothelial cells was shown to have a protective effect against diet-induced liver inflammation and fibrosis. *In vitro*, knockdown of MYC in human umbilical vein endothelial cells (HUVECs) induces cellular senescence accompanied by a proinflammatory senescence associated secretory phenotype (SASP) (Florea et al., 2013). *In vivo*, loss of endothelial MYC induced a significant increase in proinflammatory molecules CCL7 and osteopontin. Moreover, under a high fat diet, mice with *MYC*$^{-/-}$ endothelial cells showed transcriptional induction of inflammation-associated pathways characterised by an increase in neutrophil and macrophage infiltration and the secretion of chemo- and cytokines CCL11, CXCL1 and IL-17, all of which have a role in liver inflammation and MASH. Moreover, transcriptional analysis of endothelial cells from MYC knockout mice showed functions associated with liver

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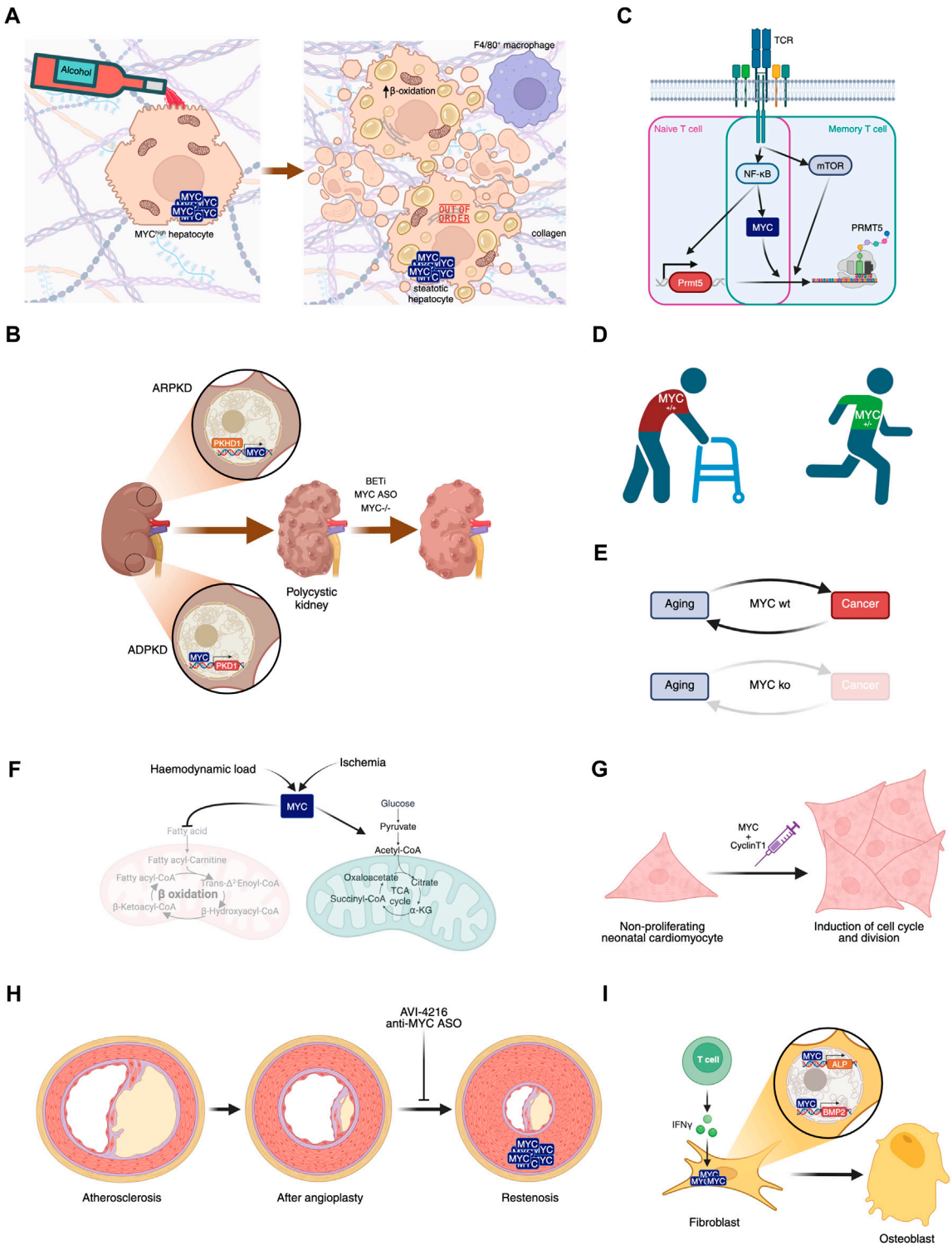

FIGURE 3
Involvement of MYC in diseases. **(A)** MYC overexpression in hepatocytes, in combination or not with alcohol consumption, leads to liver steatosis. **(B)** MYC's role in polycystic kidney disease and its potential inhibition leading to disease amelioration. **(C)** MYC plays a central role in naive and memory T cell activation in Multiple Sclerosis. **(D)** MYC haploinsufficiency prevents aged-related phenotypes. **(E)** MYC knockout after weaning leads to aging in the absence of cancer, disrupting this biunivocal relationship. **(F)** Haemodynamic load or ischemia lead to MYC-dependent metabolic rewiring in the heart. **(G)** Transient expression of MYC and Cyclin T1 could have regenerative therapeutic impact in the heart after myocardial infarction. **(H)** Proliferation of smooth muscle cells leading to restenosis can be prevented by MYC inhibition. **(I)** IFN-γ-dependent MYC induction of ALP and BMP2 contribute to Ankylosing spondylitis symptoms.

hyperplasia/hyperproliferation and hepatocellular carcinoma. These findings are in line with scRNA analyses showing that endothelial *MYC* expression was downregulated in male cirrhotic livers compared to those of healthy individuals (Qi et al., 2022). Whether endothelial MYC knockdown *in vivo* induces senescence, which is related to inflammation and cancer, was not evaluated.

4.2 Polycystic kidney disease

Polycystic kidney disease (PKD) is a group of genetic disorders characterised by the progressive development of renal cysts. It can be autosomal dominant (ADPKD) or autosomal recessive (ARPKD), and the dominant form affects some 1 in 500–1000 people. A role for MYC in the pathogenesis of PKD was suggested by work using the spontaneous congenital polycystic kidney $Cys1^{cpk/cpk}$ (*cpk*) mutant mouse that phenocopies human ARPKD. In this model, MYC overexpression was detected in polycystic kidneys, with only a minimal increase in proliferation, and also in collecting duct epithelial cells (Cowley et al., 1987; Harding et al., 1992). Notably, *in vivo* treatment with a *c-MYC* antisense oligomer (ASO) decreased *cpk* mouse kidney weight, improved their renal function and decreased the number of cysts, pointing to a therapeutic effect of MYC inhibition (Figure 3B) (Ricker et al., 2002).

Cystin, encoded by *Cys1*, is a lipid-microdomain associated protein found in the primary cilium of renal epithelia cells (Yoder et al., 2002) that binds to the *MYC* promoter and regulates its expression (Wu et al., 2013). While the loss of Cystin's proper function increases MYC expression, transgenic complementation with Cystin-GFP expression rescues the phenotype with concomitant normalisation of MYC levels (Yang et al., 2021). Also, MYC is overexpressed in kidneys from ARPKD. More in detail, fibrocystin/polyductin protein localise to the nucleus, binds to *MYC* promoter P1 and activates its expression (Figure 3B) (Harafuji et al., 2023).

Others have reported further links between MYC and PKD. The SBM transgenic mouse model, with an SV40 promoter and beta-globin enhancer that drives MYC overexpression in renal epithelial cells, bears similarities with human ADPKD, which is mainly caused by mutations in the gene *PKD1*, encoding for polycystin-1 (PC1). These mice show significant upregulation of PC1 and develop PKD with 100% penetrance that leads to fatal renal failure. Examination of the kidneys revealed higher levels of MYC expression in the epithelial lining of cystic and hyperplastic tubules (Trudel et al., 1991).

$SBPkd1_{TAG}$ mice overexpress *PKD1* mRNA leading to increased PC1 dosage in renal epithelial cells and exhibit a moderate rate of disease progression that leads to renal failure at five to six months. On the other hand, PC1 dosage-reduced *Pkd1*-cKO mice develop enlarged cystic kidneys that become very severe by P10 and leads to death due to renal failure. Puzzlingly, in both PC1 dosage-increased and -reduced mice, MYC expression (along with that of β-catenin) was found to be upregulated in renal cells with respect to wild-type mice. Moreover, MYC was found to be enriched in *PKD1* promoter regions in adult SBM mouse kidneys, while overexpression of MYC in HEK293 embryonic kidney cells increased the levels of PC1. These data suggest that *PKD1* expression is driven at least in part by MYC (Figure 3B) and unveils an inter-regulatory network involving MYC and PC1 that controls cystogenesis (Parrot et al., 2019).

While direct exogenous MYC inhibitors were not applied in these models, either genetic renal-specific ablation of MYC (Parrot et al., 2019), or treatment with inhibitors of BET bromodomain protein 4, an upstream regulator of MYC, reduced disease severity or delayed PKD progression (Zhou et al., 2015). Similarly, loss of MYC suppressed cystogenesis in a *Pkd1*-KO mouse model (Figure 3B) (Cai et al., 2018).

Combined, these data point to MYC as a causal cystogenic factor and a mediator of ADPKD. Its inhibition is therefore a potential therapy and further testing of inhibitors is highly warranted.

4.3 Multiple sclerosis

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterised by the self-reactive T cell-induced demyelination. T cells recognise antigens on myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein, and immunization against these antigens induces the MS-like experimental autoimmune encephalomyelitis disease (EAE) in mice. More than 10 years ago, genome-wide association studies in MS patients identified single nucleotide polymorphisms in the *MYC* gene (International Multiple Sclerosis Genetics Consortium et al., 2011). In recent years, a series of papers have linked MYC's transcriptional activity to T cell activation in MS.

First, MYC, together with NF-κB and mTOR, was found to be involved in the activation of memory Th and naïve T cells in EAE through the induction of PRMT5, an arginine methyltransferase that plays a crucial role in inflammatory T cell expansion and EAE disease (Webb et al., 2017; Webb et al., 2019). This constitutes another example of a positive interaction of MYC and mTOR, in contrast to UPR in osteoclastogenesis. Here, MYC's role was demonstrated using the small molecule inhibitor 10058-F4.

Second, *MYC* transcriptional activation through phospho-STAT3 and RelA/NF-κB mediates T cell receptor-independent downstream signalling from activated CD28 that leads to inflammatory T cell responses in MS (Figure 3C) (Kunkl et al., 2019).

Finally, bioinformatic analyses of protein-protein interaction networks in MS found common genes and biological pathways for disease susceptibility, among which *MYC* was found to be a central gene in peripheral blood mononuclear cells from MS patients (Safari-Alighiarloo et al., 2020). A similar study confirmed the role of MYC, along with HNF4α and SP1, as a master regulator of CNS autoimmunity (Colombo et al., 2023). In this case, MYC was inhibited using OTX015, an inhibitor of BET domain proteins that indirectly decreases MYC levels. This inhibitor has been tested in clinical trials (in oncological indications), although it is not specific for MYC only. Further preclinical validation of the role of MYC and the potential of MYC inhibition in MS seems warranted.

4.4 Aging

Many of the biological processes implicated in or associated with aging have also been linked to MYC and its deregulation. These include

the so-called hallmarks of aging (López-Otín et al., 2023): genomic instability, epigenetic alterations, stem cell exhaustion, energy production, protein translation, DNA damage, and inflammation. Transgenic mice have been used to explore the impact of systemic MYC level reduction, but so far there are contrasting results. Initially, MYC haploinsufficiency studies showed that *MYC*$^{+/-}$ mice had significantly extended lifespans with amelioration of aging phenotypes across a variety of pathophysiological processes when compared to wild-type littermates. These included healthier lipid and cholesterol metabolism, less fibrosis and cancer progression, higher metabolic rate and less immunosenescence. The exact mechanism(s) behind this have not yet been established, although they are expected to be multifactorial, through decreased expression of direct MYC targets, or indirectly through other transcription factors and/or miRNAs regulated by MYC. For instance, ribosomal *RPL* and *RPS* genes were found to be reduced in MYC haploinsufficient tissues with the concomitant reduction of *in vivo* translation, which is clearly associated with longer lifespan (Figure 3D) (Hofmann et al., 2015). Additionally, these mice showed decreased systemic levels of IGF1 through MYC-miR122 regulation. Reduced IGF1 has been linked to the development of age-related diseases such as osteoporosis, but female MYC haploinsufficient mice had a decreased incidence of osteoporosis, consistent with the finding that, in bone, IGF1 levels were unaltered (Petrashen et al., 2023).

The current understanding of aging considers it as a multifactorial process in which different signalling pathways converge on autophagy genes to regulate lifespan. WIPI1, and its *C. elegans* ortholog ATG-18, has been identified as one of the critical autophagic factors involved in extending lifespan (Tóth et al., 2008). In line with this and with MYC's supposed role in aging, it was found that the ABL-MYC axis represses WIPI1 gene expression. Interfering with this axis promotes autophagy and extends *C. elegans* lifespan (Sporbeck et al., 2023).

On the other hand and in contrast with the results above, transgenic mice engineered with near-complete elimination of MYC at weaning, named *MycKO* mice, aged prematurely yet lived longer with decreased cancer incidence. The phenotypic alterations were, as expected, copious and broad: bone marrow hypoplasia, peripheral cytopenia, alopecia, achromotrichia, glucose intolerance and mitochondrial dysfunction. Additionally, colonic epithelial flattening and villous atrophy were found, although there was no effect on body weight (Prochownik and Wang, 2023; Wang et al., 2023).

Hence, according to these latter results, aging appears to be associated with higher cancer incidence only in the presence of MYC (Figure 3E). It remains to be seen what effect chronic administration of MYC inhibitors could have on indicators of aging. Whether such chronic treatment could also be applied as a cancer prevention strategy (and not only to cancer treatment) is not clear beyond preclinical models.

4.5 Cardiac metabolism after pathological stress

Cellular oxygen concentrations are tightly regulated in eukaryotic organisms to maintain proper mitochondrial function and energy production. Mammalian cells adapt to oxygen deprivation by inducing protective mechanisms. For instance, a substantial decrease in protein biosynthesis is among the effects of hypoxic stress on cardiomyocytes, where transcription factor IIIB (TFIIIB) and TFIIIC-dependent RNA polymerase III (pol III) play a key role (Kraggerud et al., 1995; Schramm and Hernandez, 2002). *In vitro* experiments with neonatal rat myocytes at 1% O_2 revealed that HIF-1α induces the dissociation of MYC from TFIIIB, contributing to the decrease in pol III transcription (Ernens, 2006). Other pathological stressors such as haemodynamic load and ischemia divert metabolic pathways away from fatty acid oxidation (FAO) towards glucose metabolism (Stanley et al., 2005). In the adult heart, this metabolic rewiring in the myocardium is mediated by MYC, whose increased levels downregulate genes involved in FAO, while concomitantly upregulating genes mediating glucose oxidation, such as *ENO1*, *PFKM*, *LDHA* and SLC16A1 (Figure 3F). This was associated also with an increase in the number of functional mitochondria and represents MYC-dependent metabolic adaptation towards a better response to ischemic insults (Ahuja et al., 2010).

Cardiac progenitor cells (CPCs), however, become quiescent after ischemic hypoxia, limiting their self-renewal and vasculogenic properties, with the aim of preserving stem cell homeostasis (Guitart et al., 2010). Being a master regulator of the cell cycle and quiescence, it is no surprise that MYC, after *in vitro* hypoxia (0.5% O_2), is downregulated in mouse CPCs isolated from the myocardium, with a concomitant increase in the levels of the CDK inhibitor p21, a MYC target (Bellio et al., 2017).

Neonatal cardiac proliferative potential is lost after a week, coinciding with downregulation of multiple genes involved in cell cycle, including *MYC* (Walsh et al., 2010; Quaife-Ryan et al., 2017). Ectopic cardiac MYC-dependent transcription and cell cycle progression in the adult heart *in vivo* depends on the levels of P-TEFb, a protein complex consisting of CDK9 and Cyclin T1. In order to effectively drive cell division in the heart, MYC expression must be accompanied by higher levels of P-TEFb (Bywater et al., 2020). In line with this, transient expression of both MYC and Cyclin T1 by a single intramyocardial dose of a modified RNA coding for both genes was shown to be a potential regenerative therapeutic in the heart after myocardial infarction, inducing cell cycle and division of cardiomyocytes (Figure 3G) (Boikova et al., 2023).

Restoration of reperfusion is the most effective treatment for myocardial infarction. Paradoxically though, reperfusion leads to myocardial ischemia/reperfusion (MI/R) injury, which induces cardiomyocyte apoptosis through increased oxidative stress (Wang et al., 2017). Using an MI/R mouse model, MYC was found to be downregulated, with consequent oxidative stress and cardiomyocyte apoptosis (Wen et al., 2022). Notably, therefore, recovery after ischaemia using these regenerative or protective strategies represents one of the few conditions in which therapy would require MYC expression or activation.

Hypertension is one of the most common pathologies of the vascular system. It leads to overload, increasing the risk of myocardial infarction, among others. The myocardium of spontaneously hypertensive rats (SHRs) overexpresses MYC and its downstream target CYP2E1, whose overexpression leads to oxidative stress and other pathological processes. Long-term treatment with quercetin, a flavonoid with potential cardiovascular beneficial effects, resulted in a significant

TABLE 1 Additional diseases and conditions in which MYC has been implicated. In these cases, the evidence is more preliminary than for those described in the main text. We have indicated studies showing any links between MYC and the disease, and in particular, any data regarding MYC modulation, either by inhibition or overexpression.

Disease or condition	Studies linking it to MYC	MYC modulation experiments
Neurodegeneration 	• Phosphorylated c-MYC, c-MYC and N-MYC found in patient samples from various neurodegenerative diseases (Ferrer and Blanco, 2000; Ferrer et al., 2001)	• MYC inhibition by mithramycin (a non-selective inhibitor) was neuroprotective (Sleiman et al., 2011)
		• Downregulation of dMyc is neuroprotective in tauopathies in *Drosophila* (Chanu and Sarkar, 2017)
		•Expression of Myc induces ND in transgenic models (Lee et al., 2009)
Neuropathic pain 	• MYC expression in mouse models (Van et al., 2010; Jiang et al., 2022)	• MYC overexpression induces pain hypersensitivity while knockdown *in vivo* with siRNA alleviates neuropathic pain (Jiang et al., 2022)
Acute liver failure 	• MYC-dependent transcriptional program orchestrates cell activation during ALF (Kolodziejczyk et al., 2020)	• MYCi in mouse models using small molecule Kj-Pyr-9 attentuates ALF (Kobdziejczyk et al., 2020)
Diabetic nephropathy 	• Increased MYC expression in endothelial cells in response to glucose, and in DN patients and rats (Hou et al., 2022)	• MYC overexpression or siRNA knockdown impacts endothethial cell inflammation (Hou et al., 2022)
	• N-MYC stabilisation in models (Choi et al., 2023)	
	• MYC is one of the top Differentially Expressed Genes in glomerular samples of patients (Hojjati et al., 2023)	
Fanconi anemia 	• High levels of MYC mRNA in primary stem cells from patients and bone marrow, and nuclear MYC IHC in liver sections (Rodriguez et al., 2021)	• JQ1 inhibitor reduces MYC expression, and decreases clonogenic potential and genotoxic stress in stem cells from FA-mice (Rodriguez et al., 2021)
Diabetes	• MYC increased in mouse and rat diabetes models (Jonas et al., 1999; Jonas et al., 2001; Laybutt et al., 2003)	• MYC overexpression in beta-cells triggers diabetes in mouse models (Laybutt et al., 2002)
	• Upregulation of c-MYC/N-MYC networks in proteome and transcriptome analysis of non-obese diabetic mice (Gerling et al., 2006; Wu et al., 2013)	• MYC induction upon BCG vaccination improves glucose metabolism (KOhtreiber et al., 2020)
	• MYC gene network and protein increased in diabetic patients (Kaizer et al., 2007; Lee et al., 2008)	
	• c-Myc directly induces both impaired insulin secretion (Kaneto et al., 2002), potentially through PKC (Kaneto et al., 2002) and loss of 0-cell mass, independently of hyperglycemia (Cheung et al., 2010)	
Kefold scar	• MYC gene expressed increased in microarray analysis of patient skin (Chen et al., 2004)	• c-MYC overpexpression promotes the proliferation of keloid fibroblasts (Feng et al., 2023; Piao and Feng, 2023)
	• MYC protein expression increased in patient samples (Hu et al., 2002; Zhang et al., 2023)	
Developmental diseases 	• Cornelia de Lange syndrome and Roberts syndrome are linked to misregulation of MYC (Horsfield et al., 2012)	Not found
	• Feingold disease is caused by MYCN haploinsufficiency (van Bokhoven et al., 2005; Cognet et al., 2011)	
	• Achondroplasia linked to MYC downregulation (Zhou et al., 2011)	
Irritable Bowel Disease 	• MYC amplification in IBD-associated carcinoma patients (Hartman et al., 2018)	Not found
	• Some MYC IHC in IBD-associated low-grade dysplasias (Liang et al., 2023)	

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TABLE 1 (*Continued*) Additional diseases and conditions in which MYC has been implicated. In these cases, the evidence is more preliminary than for those described in the main text. We have indicated studies showing any links between MYC and the disease, and in particular, any data regarding MYC modulation, either by inhibition or overexpression.

Disease or condition	Studies linking it to MYC	MYC modulation experiments
Immune related diseases		
Systemic lupus erythematosus	Not found	MYCi by JQ1 abolishes the pathogenic response induced by functional Breg cells (Wang et al., 2022)
Uveitis	• MYC increased in experimental autoimmune uveitis (Chen et al., 2022)	• MYC knockdown reduces miR-181a-5p, involved in the pathogenic Th17 immune response (Chen et al., 2022)
Arthritis	• MYC expression increased in synovial tissue microarray analysis of OA patients (Zhang et al., 2023) • MYC gene expression is increased in RA patients (Harshan et al., 2022; Fan et al., 2023)	• Simultaneous inhibition of both c-Myc (with 10,074-G5) and HIF-la is efficacious for anti-inflammation *in vitro* and *in vivo* in RA model (Lee et al., 2020)
Pancreatitis	Not found	• MYCi by 10,058 reduces markers of acute pancreatitis in mouse models (Xu et al., 2020)
Asthma	• MYC gene and network upregulated in patients (Troy et al., 2016; Vargas et al., 2016; Salameh et al., 2022) • Higher MYC expression in inflammatory cells in allergic asthma mouse model (Shen et al., 2019) • MYC upregulation involvement in pathogenesis of ILC2 in asthma (Ye et al., 2020) • Wnt/p-catenin regulate asthma airway remodeling and upregulate c-MYC (Jia et al., 2019)	• iPSC-w/o-c-Myc transplantation had therapeutic effects in allergic airway hyperresponsiveness (Wang et al., 2013)
Coeliac disease	• MYC expression increased mouse models (Vaira et al., 2020) and patient samples (Ciclitira et al., 1987)	Not found
infections	• MYC as a hub gene in tuberculosis (Xiao et al., 2023) • Wnt6 increases MYC expression in granulomatous lesions of *Mycobacterium* in the lung (Schaale et al., 2013) • SARS-CoV-2 Orf7b protein upregulates MYC which mediates lung apoptosis and ferroptosis (Deshpande et al., 2024)	• MYC activation-deficient adenovirus impairs glutamine catabolism needed for viral replication and infection of primary cells (Thai et al., 2015) • MYC expression rescues *Chlamydia* persistence in cell lines (Vollmuth et al., 2022)

reduction of blood pressure with concomitant downregulation of MYC and CYP2E1, significantly improving the prooxidant-antioxidant profile (Maksymchuk et al., 2023). Whether downregulation of MYC alone would reduce blood pressure, CYP2E1 expression, and curb the oxidative stress, still remains to be seen.

4.6 Restenosis

The arterial wall response to pathophysiological stimuli, including atherosclerosis and angioplasty procedures, involves the proliferation of smooth muscle cells (SMC). Indeed, 25%–50% of patients undergoing angioplasty will develop recurrent stenosis, which is essentially a narrowing of the blood vessels, also called restenosis, that consists of the proliferation of medial SMC and their migration to the subintima. Because of this, considerable attention has been paid to the inhibition of SMC proliferation as a way of preventing restenosis. Initial studies involving antisense oligonucleotides (ASOs) targeted SMC *PCNA* *in vitro* with significant inhibition of proliferation (Speir and Epstein, 1992). Much later studies focused on the use of a phosphorodiamidate morpholino oligomer (PMO) antisense to the c-MYC translation initiation site, called AVI-4126 (Resten-NG®) (Figure 3H). It was successfully tested in a rabbit balloon injury model (Kipshidze et al., 2002) and porcine restenosis model (Kipshidze et al., 2003; Kipshidze et al., 2002) with promising results: significant reduction of the neointimal area with concomitant MYC inhibition. Although this was further validated in a Phase II trial with positive results (Philipp, 2012), the drug was not developed beyond this point.

4.7 Bone developmental disorders

Septic nonunion (SN) is a bone disorder caused by the failure of fracture healing. It is often caused by local inflammation. Expression of the lncRNA *RUNX2-AS1* was detected in SN biopsies, along with

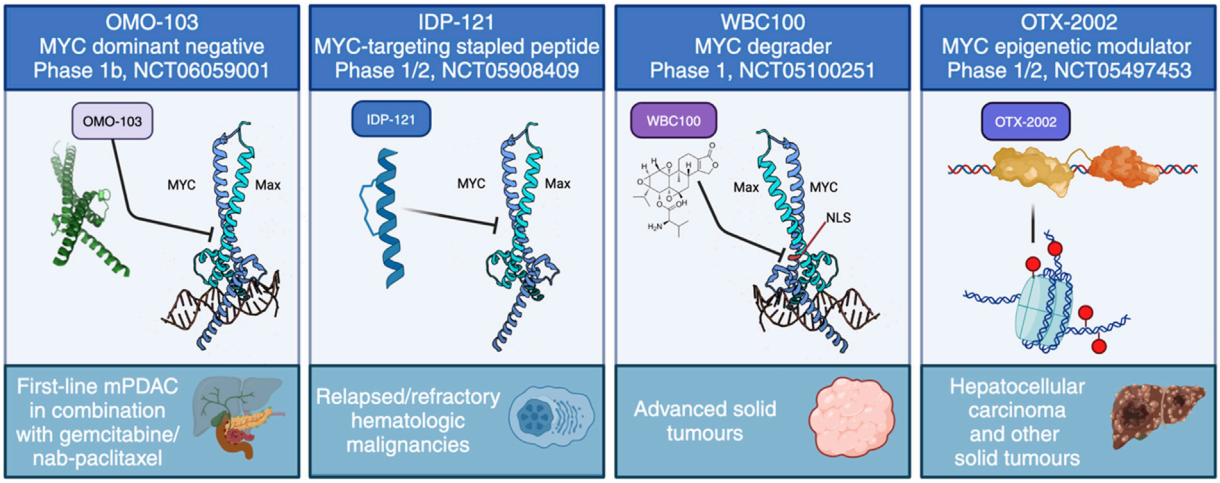

FIGURE 4
The current approaches to directly target MYC in clinical trials. These include four distinct strategies: MYC dominant negative OMO-103, anti-MYC stapled peptide IDP-121, MYC degrader WBC100 and MYC epigenetic modulator OTX-2002.

proinflammatory cytokines. *RUNX2-AS1* negatively regulates *RUNX2* expression and its downstream targets, which play an important role in bone differentiation and development. It was found that MYC associates with MAX, p300 and NCOA2 to induce RUNX2-AS1 expression, abrogating the expression of RUNX2 target genes, while LPS-induced inflammation induced the expression of NCOA2 and showed a dose-dependent increased association with MYC-MAX-p300. These results link the inflammatory microenvironment with the downregulation of RUNX2 and its target genes, which impairs bone differentiation and leads to nonunion (Li and Qian, 2022).

Ankylosing spondylitis (AS) is a heritable chronic inflammatory disease that affects the spine and pelvis, ultimately leading to joint ankylosis due to ectopic ossification and disability. Inflammation is an early characteristic of AS and inflammatory cytokines could promote ossification by modulating the osteoblasts (Li et al., 2020). MYC was found to be upregulated in AS ligament samples and in fibroblasts in an *in vitro* osteogenic model. In this model, two osteogenic genes were found to be dependent on MYC: alkaline phosphatase (*ALP*) and bone morphogenetic protein 2 (*BMP2*). Additionally, the inflammatory cytokines IL-23 and IFN-γ upregulate both *MYC* and *ALP in vitro*. In AS ligament samples, a higher proportion of IL-23 positive and IFN-γ positive cells were found with respect to osteoarthritis samples (Figure 3I) (Jin et al., 2023). Osteoporosis has also been linked to MYC. Based on bioinformatic analysis, a series of experiments showed that the MYC/ERRα axis regulates mitochondrial respiration in osteoclastogenesis, and their targeting protected mice of oestrogen deficiency-mediated bone loss after ovariectomy, pointing to MYC as a potential therapeutic target for osteoporosis (Bae et al., 2017).

4.8 Potential role of MYC in other diseases and conditions

Various studies link MYC to a range of other disorders, mainly through experiments to determine its expression in model systems or patients. In particular—and not surprisingly—there are strong suggestions of a role in endometriosis (Nothnick et al., 2023), mitochondrial diseases (Purhonen et al., 2023), immune-related, neurodegenerative and other metabolism-related diseases. These are summarised in Table 1 with some of the data hinting at a role for MYC. In general, more work is required to prove a clear link and determine whether MYC is playing a role in disease causation, or even whether modulation of its expression could be preventative.

5 Current state of MYC inhibition in the clinical setting

There are a huge number of reviews describing the search for MYC inhibitors and their application to cancer treatment. Here we will only briefly describe some targeting strategies, focusing on those reaching clinical testing, summarised in Figure 4, and refer the reader to a number of other much more in-depth reviews regarding MYC inhibitors, both from our group (Whitfield et al., 2017; Massó-Vallés and Soucek, 2020; Whitfield and Soucek, 2021; Martínez-Martín et al., 2023) and others (Ross et al., 2021; Karadkhelkar et al., 2023; Weber and Hartl, 2023).

Strategies employed fall into two main approaches: direct and indirect inhibitors. The latter include a much more expansive set of possibilities, since their target can be anything that interacts with MYC or controls its activity, expression, or localisation. These could also include synthetic lethal targets: here, any protein or signalling pathway that is essential for the survival of MYC-driven tumour cells can be targeted and many such targets are in clinical development. Direct inhibitors, on the other hand, impinge on MYC itself to control the expression or stability of the RNA or protein, or its interaction with DNA or dimeric partners.

Perhaps surprisingly, the earliest MYC inhibitors to be tested in clinical trials were applied to a non-oncological indication (Kipshidze et al., 2002). Antisense oligonucleotides (ASOs) were used for the

treatment of heart restenosis (NCT00244647, NCT00248066) (Kipshidze et al., 2007). These showed some positive effects in this coronary disease and were later tested against neoplasms, showing significant tissue accumulation in solid tumours (Devi et al., 2005), but to our knowledge no further development occurred.

Other more recent trials have also been discontinued, including: Quarfloxin (CX-3543) and APTO-253, G-quadruplex stabilisers thought to work by preventing *MYC* transcription (NCT00780663, NCT02267863); INX-3280, another ASO (Kutryk et al., 2002); and DCR-MYC, an siRNA to prevent *MYC* translation (NCT02110563, NCT02314052). These were all tested as cancer therapies (reviewed in Whitfield et al., 2017), but none was further pursued.

The first successful Phase I trial has recently concluded using OMO-103, a MYC dominant negative mutant based on the Omomyc mini-protein, delivered intravenously once per week. In line with its extensive preclinical validations, OMO-103 showed a good safety profile and some first hints at efficacy in all-comers solid tumours (Garralda et al., 2024). In addition, biomarkers were identified indicating MYC inhibition. A Phase Ib trial recently started in September 2023 in metastatic pancreatic cancer (NCT06059001).

To our knowledge, there are currently three other ongoing trials with a direct MYC inhibitor: one uses a MYC degrader called WBC100, in MYC-positive advanced solid tumours (NCT05100251), another is an epigenetic controller, OTX-2002, that downregulates MYC and is being tested in hepatocellular carcinoma (NCT05497453), while the third is with IDP-121, a stapled peptide MYC inhibitor being evaluated in patients with relapsed/refractory hematologic malignancies (NCT05908409). In addition, MYC-related indirect approaches have reached clinical trials. For instance, MYC-induced protein translation depends on GSPT1, and a molecular glue degrader of GSPT1 (MRT-2359) is currently being trialled for MYC-driven and other selected solid tumours (NCT05546268). Still, the focus remains firmly on testing in cancer.

As discussed already, any approved inhibitor could potentially be applied to other non-neoplastic conditions.

6 Possibilities to activate or express MYC

As explained in the previous section, the majority of examples of MYC involvement in diseases point to its inhibition as a therapeutic approach. However, MYC activation could be an option to favour regeneration in the heart after myocardial infarction or hypoxia. Further to such repair and regeneration approaches, a recent study highlighted the use of MYC activation by transgenic overexpression to stimulate *ex vivo* platelet production from induced pluripotent stem cells (Kayama and Eto, 2024). This could eventually provide improved transfusion systems. Additionally, an unexpected indirect approach could benefit Type 1 Diabetes (T1D) patients, in which the administration of BCG vaccine resulted in long-lasting blood sugar control with proper glucose metabolism (Li et al., 2018). A recent study found a gradual *MYC* mRNA upregulation in monocytes and CD4 T cells from T1D patients. This led to increased transcription of MYC-dependent glucose and glutamine metabolism genes (Kühtreiber et al., 2020).

7 Perspective

While MYC has long been considered an undruggable target, new therapeutic options against it are becoming clinically viable, as demonstrated by the completion of the first successful clinical trial of a direct inhibitor, OMO-103. Most of the trials and recent focus remains in the field of cancer treatment, and indeed the ongoing trials of direct MYC inhibitors are against PDAC, hepatocellular carcinoma, relapsed/refractory hematologic malignancies, and MYC-positive advanced solid tumours. As mentioned in this review, though, MYC's pleiotropic roles in multiple physiological processes suggest that its modulation could be applied to many other diseases. To date, there is preliminary data pointing to a role in a variety of diseases of different origins and clinical presentations such as neurodegeneration, diseases of the bone, digestive system and related organs, keloid scars, developmental and immune-related diseases (such as asthma, coeliac disease, and others), as well as the aging process. Further pre-clinical testing and even clinical trials seem merited in these cases.

In general, excess or over-active MYC is detrimental, so under physiological circumstances its levels are precisely controlled to keep the multiple downstream processes in check. In most diseases described so far, and as seen in cancer, where deregulation of MYC is frequent, such excessive MYC activity drives various processes that then lead to pathologies due to the unfettered proliferation, changes in differentiation and altered metabolism, among others. There is huge potential, therefore, for using MYC inhibitors that are currently being developed in the cancer field.

Diseases in which MYC activation may instead be desired include those where stimulation of cell proliferation and tissue regeneration is needed, such as after ischaemic damage in the heart, diabetes, and neuronal repair. It has been speculated that in neurodegeneration, MYC activation may be part of a failed neuroprotective response. Thus, extra MYC could help repair and regenerate neurons after cell death or damage. Of note, activation of MYC for such diseases will likely be required locally, in the affected tissues rather than systemic, to avoid the foreseeable massive and deleterious effects that body-wide activated MYC could have.

In summary, if we have learnt something from 40 years of literature about MYC, it is that we still have a lot to discover. Luckily, pharmacological tools for its modulation seem finally viable and hold promise for a better understanding of MYC biology, while also providing the basis for new therapeutics applicable to multiple indications in oncology and beyond.

Author contributions

Resources: LS. Conceptualisation: JW, MZ-F. Writing original draft: MZ-F, JW. Writing–review and editing: JW, MZ-F, LS. Funding acquisition: LS, JW, MZ-F.

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Conflict of interest

All authors are shareholders of Peptomyc SL; LS is CEO and founder of Peptomyc SL.

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.

Publisher's note

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Manipulating Myc for reparative regeneration

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The Myc family of proto-oncogenes is a key node for the signal transduction of external pro-proliferative signals to the cellular processes required for development, tissue homoeostasis maintenance, and regeneration across evolution. The tight regulation of Myc synthesis and activity is essential for restricting its oncogenic potential. In this review, we highlight the central role that Myc plays in regeneration across the animal kingdom (from Cnidaria to echinoderms to Chordata) and how Myc could be employed to unlock the regenerative potential of non-regenerative tissues in humans for therapeutic purposes. Mastering the fine balance of harnessing the ability of Myc to promote transcription without triggering oncogenesis may open the door to many exciting opportunities for therapeutic development across a wide array of diseases.

KEYWORDS

MYC, regeneration, cell cycle, proliferation, repair

1 Myc structure, function, and the proximal Myc network

Myc belongs to a class of proto-oncogenes (comprising *c-Myc*, *n-Myc*, and *l-Myc*), which are genes whose product induces cell proliferation in response to mitogenic stimuli and that can become oncogenic upon their mutation or deregulation (Vennstrom et al., 1982). While much Myc research has focused on its oncogenic properties, its activities as a proto-oncogenic transcription factor positions Myc as a key downstream factor in many signal transduction pathways important for development, tissue homoeostasis, and regeneration (such as WNT, RAS/RAF/MAPK, JAK/STAT, TGF-β, and NF-κB) (Dang, 2012). As such, it is part of the proximal Myc network (PMN), a system of transcription factors that consolidates signals from several distinct upstream pathways into the expression of thousands of target genes involved in many biological processes (Grandori et al., 2000; Conacci-Sorrell et al., 2014).

All members of the Myc family are dimerizing transcription factors that contain a basic helix–loop–helix leucine zipper (bHLH-LZ) domain (Figure 1). The heterodimers can interact with the DNA through recognition of an enhancer box (E-box, 5′-CACGTG-3′) via the bHLH-LZ; this drives the recruitment of co-activators/repressors, transcriptional regulation, and chromatin remodelling. The bHLH-LZ domain is present on the carboxyl-terminus (C-terminus) of Myc and has been shown to have helical conformation when unbound; it only assumes its full structure when bound to MAX and the DNA (Nair and Burley, 2003; Sammak et al., 2019). The amino-terminus (N-terminus) consists of a large unstructured intrinsically disordered region (IDR) containing multiple conserved domains called Myc boxes (MB). MBs are sites of interaction with regulators and interactors (transactivation domain—TAD, comprising MBI-MBII) and degron motifs central to Myc degradation (Sears et al., 1999; Sears et al., 2000; Sears, 2004). Importantly, Myc is unable to homodimerise and cannot bind DNA as a

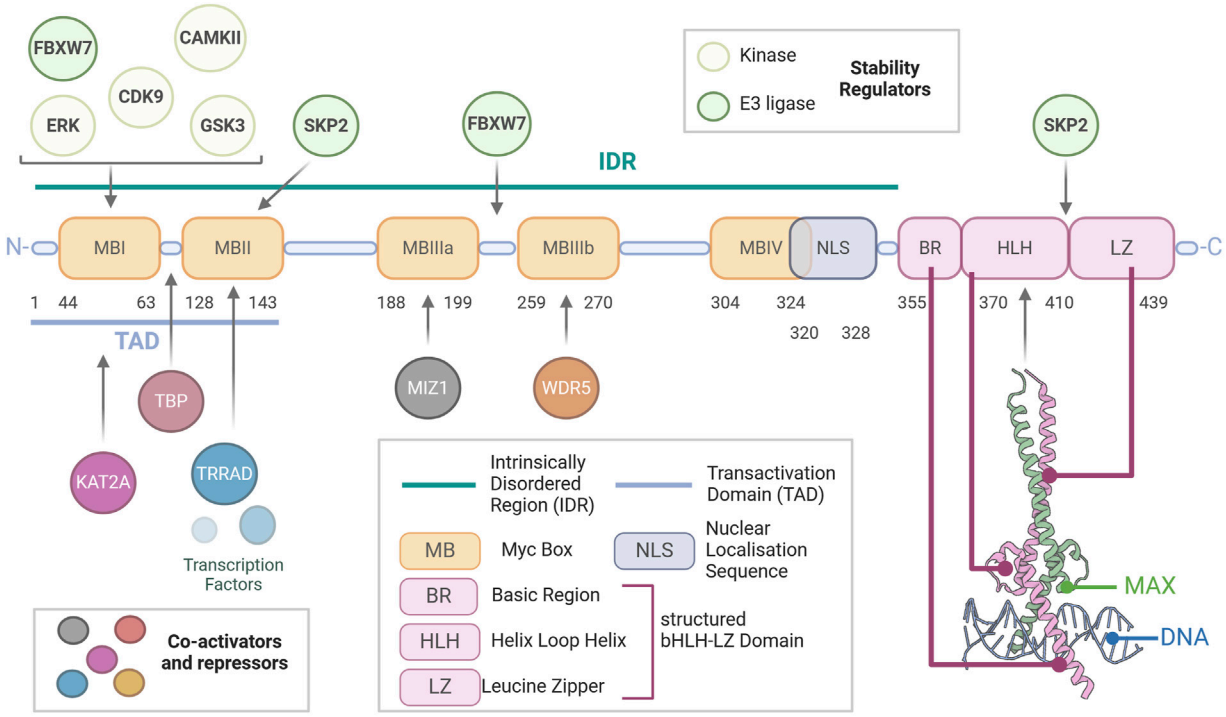

FIGURE 1
Structure of Myc. Schematic representation of the modular amino acid sequence of the Myc transcription factor. Myc is a largely unstructured protein with a vast intrinsically disordered region (IDR) extending from the N-terminus to the beginning of its basic region. The IDR contains the nuclear localisation sequence (NLS) and multiple disordered regions called Myc boxes (MB), which are conserved between Myc family members and are key to the function of Myc, being sites of essential protein–protein interactions. Most importantly, the transactivation domain (TAD), which contains MBI and MBII, allows for the binding of the co-activators and repressors of Myc activity. Within this, MBI is also a binding site for most regulators of Myc stability. Finally, essential for the ability of Myc to elicit transcription, the basic helix–loop–helix leucine zipper (bHLH-LZ) domain is essential for binding of Myc to MAX and the DNA, as shown in the crystal structure (PDBID:1NKP).

monomer, thus requiring its obligatory partner MAX (Amati et al., 1992, 1993). Due to its lack of functional domains, MAX does not possess direct transcriptional activity but rather forms transcriptionally inactive complexes in the form of MAX homodimers and heterodimers with MAX-binding proteins and dimerization proteins (e.g., MNT, MGA, MAD1-4, and MXI1), becoming functional antagonists to Myc-MAX dimers by competing for E-box binding. Therefore, MAX is the central node of the PMN, whereby changes in the balance between its heterodimerisation partners determine cell fate decisions and a switch from a proliferative or transformative state when Myc is abundant to differentiation or quiescence when abundant MADs outcompete Myc (Grandori et al., 2000; Conacci-Sorrell et al., 2014).

The overarching function of Myc in healthy tissues is to integrate multiple signals derived from different pathways to elicit global transcriptional change. The transcriptional activity of Myc hinges on its ability to recruit RNA polymerase II and members of histone acetylase complexes to Myc-binding sites, with Myc target sites presenting high histone acetylation. Specifically, the region between MBI and MBII binds the TATA-binding protein (TBP), a member of the transcription factor IID (TFIID) complex responsible for recruiting RNA polymerase II (RNAPII) at transcriptional start sites (Wei et al., 2019). Through MBI, Myc recruits the cyclin T1-CDK9 complex, which together comprise the positive transcription factor B (P-TEFb) that elicits phosphorylation of RNAPII and releases it from transcriptional pausing, thus initiating transcriptional elongation (Rahl et al., 2010). The abundance of P-TEFb is rate-limiting to Myc-driven hyper-transcription (Bywater et al., 2020). MBII mediates Myc's interaction with other regulators of transcriptional activity, including transformation/transcription domain-associated protein (TRRAP), an adaptor protein that forms complexes with lysine (K) acetyltransferase (KATs). MBIIIb interacts with WDR5 (WD repeat domain 5), an essential component of H3K4 methyltransferase complex (Couture et al., 2006; Thomas et al., 2015). Finally, Myc possesses transcriptional repressor activity, which MBIIIa mediates, specifically through interaction with MIZ-1, a transcriptional activator if not bound to Myc. MIZ-1's binding to co-activators p300 and NPM1 is impeded in the Myc-MIZ-1 bound form (Vousden, 2002; Möröy et al., 2011).

The result of Myc-driven transcription is the amplified expression of genes involved in various cellular programmes including proliferation, apoptosis (Evan et al., 1992; Kanazawa et al., 2003), metabolism (Stine et al., 2015), and senescence (Hydbring and Larsson, 2010; Singh et al., 2023). Myc-driven cell cycle progression results from its combined function as a transcriptional amplifier and repressor, with Myc mRNA and protein levels closely correlating with proliferation rates (Kelly et al., 1983; Dean et al., 1986; Waters et al., 1991; Bretones et al., 2015). Myc has been shown to directly bind components of the pre-

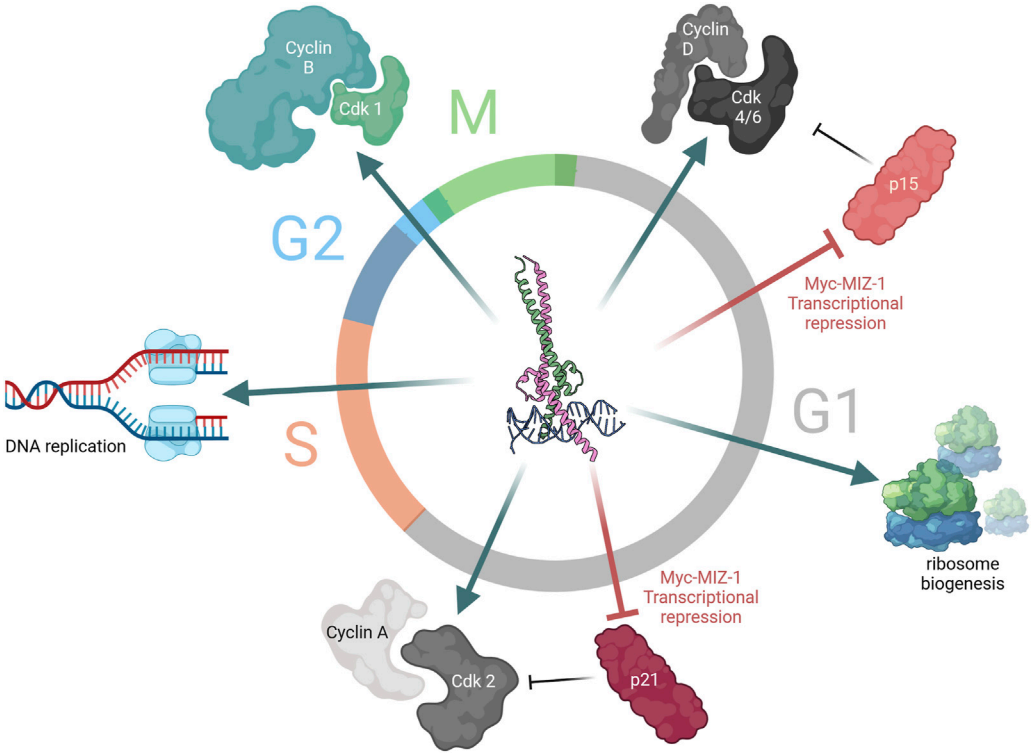

FIGURE 2
Myc is a key driver of cell cycle progression. Myc-driven cell cycle progression is ubiquitous throughout the different stages of the cell cycle. Early in G1, expression of cyclin D and cyclin-dependent kinase (Cdk) 4/6 is driven by Myc-MAX upon mitogenic sensing, concomitant with the repression of cyclin-dependent kinase inhibitor (CDKI) p15 by Myc-MIZ-1. Similarly, later in G1, the role of Myc as transcriptional activator and repressor continues to induce transcription of cyclin A and Cdk2 and repress CDKI p21 expression. Meanwhile, Myc also drives ribosome biogenesis through upregulation of RNA Pol III and tRNA expression, coupling cell cycle progression with increasing cellular size. In S-phase, Myc participates in DNA replication. Finally, at the G2/M transition, Myc induces the expression of the mitotic cyclin, cyclin B1.

replicative complex (Pre-RC), necessary for DNA replication; in early G1 phase, it binds the origin recognition complex, located at the origin of replication (Dominguez-Sola and Gautier, 2014). Activation of Pre-RCs to induce the functional initiation of transcription requires cyclin-dependent kinase (CDK) activity. Myc directly induces the expression of cyclins and cyclin-dependent kinases, specifically cyclins A, B, and D, as well as Cdk-4 and Cdk-6 (Bretones et al., 2015; García-Gutiérrez et al., 2019). As mentioned above, MIZ-1-bound Myc is capable of transcriptional repression, with two known targets of Myc-MIZ-1's transcriptional repression being p21Cip1 and p15Ink4b—two cyclin-dependent kinase inhibitors (CDKIs). For both genes, the Myc-MIZ-1 heterodimers bind the transcriptional start site, which does not affect the basal-level expression of these genes but, rather, their induction by anti-mitotic stimuli (Vousden, 2002; Wiese et al., 2013). Myc also prevents CDKI expression/activity through indirect mechanisms whereby it increases Cdk1 levels which phosphorylates p27, leading to degradation by E3 ligase Skp2 (García-Gutiérrez et al., 2019). Myc activity promotes ribosome biogenesis by regulating the expression of the core subunits of the RNA polymerase I apparatus and interacting directly to enhance pre-rRNA processing. Myc enhances the transcription of RNA polymerase III subunits, with which it cooperates to yield 5S RNA and tRNA production (Campbell and White, 2014). Furthermore, Myc-induced transcriptional amplification results in the upregulation of genes involved in nucleotide and miRNA synthesis, enzymes involved in RNA processing and capping, and eukaryotic translational initiation factor 4E (eIF4E) (Stine et al., 2015), allowing Myc to modulate cellular transcription. Myc is essential for sustained proliferation and rate-limiting for cell cycle progression, with cells which express high levels of Myc progressing to S-phase more rapidly than lowly expressing cells which present a longer G_0/G_1 (Liu et al., 2023). Furthermore, the inhibition of Myc expression in a panel of human cancerous and non-cancerous cell lines consistently results in cell cycle arrest (Wang et al., 2008). Interestingly, the cell cycle phase at which cell lines arrest varies according to their background, with healthy cells exiting the cell cycle at G_0/G_1, while most cancer cell lines displayed an arrest in later stages (S or G_2/M) (Wang et al., 2008). Altogether, Myc is essential for cell cycle progression where its contribution is three-fold: coupling cell growth with cell cycle progression, repressing cell cycle inhibitor proteins, and inducing DNA replication, transcription, and translation (Figure 2).

2 Control of Myc activity for safeguarding tissue integrity

To safeguard against the impact of the activation of the proto-oncogene on promoting cell proliferation, multiple processes

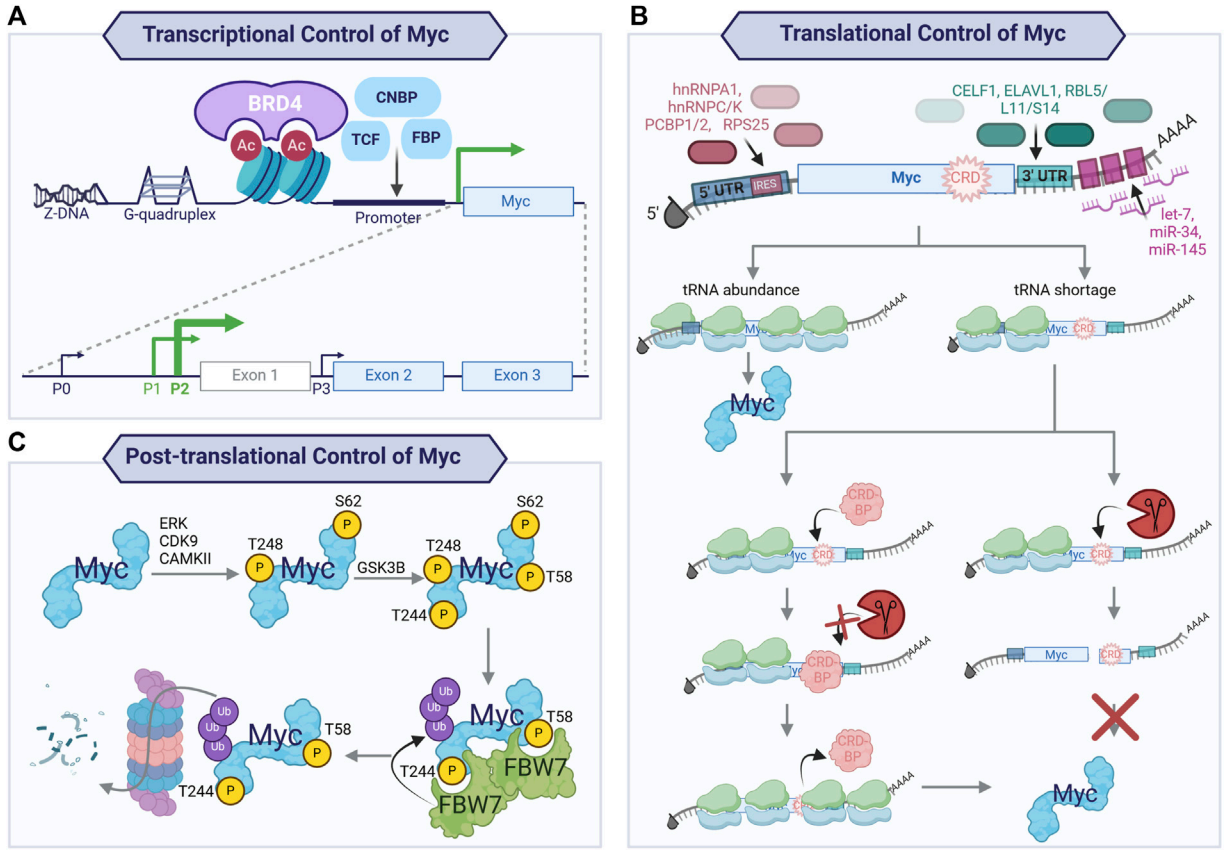

FIGURE 3
Transcriptional, translational, and post-translational control of Myc. Tight control of Myc expression, translation, and protein half-life is exacted to maintain physiological levels of Myc in regenerative tissues. **Transcriptional control of Myc (A)** is achieved through non-B DNA structures (Z-DNA and G-quadruplexes), binding of transcription factors (CNBP, TCF, FBP), and BET domain-containing transcriptional regulator, BRD4. This yields transcription preferentially from two of the four promoters (P0, P1, P2, and P3), with the majority of transcripts arising from P2 and, to a lesser extent, P1. The mRNA arising from P2 and P1 consists of three exons, with exons 2 and 3 encoding the main Myc protein isoform. The mRNA of the proto-oncogene is also subject to tight **translational regulation (B)**, resulting in a short-lived mRNA. The transcripts generated from P2 and P1 encode for a long 5′UTR which contains independent ribosome entry sites (IRES) providing binding sites for RNA-binding proteins (RBP; hnRNPA1, hnRNPC, hnRNPK, PCBP1, PCBP2, and RPS25). The coding sequence contains a coding region instability determinant (CRD) which, in the context of tRNA codon shortage, will cause ribosomal stalling and endonucleolytic attack by an endonuclease if not protected by a CRD-binding protein (CRD-BP). At the 3′UTR of Myc mRNA, CELF1 and ELAVL1 compete for binding to balance the transcriptional output, with CELF1 decreasing ELAVL1 association with the mRNA and, therefore, decreased transcriptional output. Additionally, the RNA-induced silencing complex (RISC) is recruited to Myc mRNA by ribosomal proteins (RB) L5, L11, S14, and miRNA binding at the 3′ UTR. Finally, the Myc protein is highly unstable with a short half-life, due to its many destabilising protein–protein interactions. Illustrated here **(C)** is the key mechanism for **Myc protein turnover** via a series of post-translational modifications. Myc is bound and phosphorylated by kinases (e.g., ERK, CDK9, and CAMKII) at S62 and likely T248, providing a priming phosphorylation that allows for GSK3β binding. This kinase phosphorylates T58 (and probably T244), generating phosphodegron sites for E3 ligase FBW7 binding. Once bound, dimers of FBW7 can ubiquitinate Myc, leading to its degradation by the ubiquitin proteasome pathway.

converge to restrain Myc levels and activity. Therefore, Myc is highly regulated at the transcriptional, translational, and post-translational levels (Figure 3). The *Myc* gene is located within an approximately 3-Mb area of chromosome 8q24 that lacks protein-coding genes. *Myc* expression is regulated by a wide array of transcription factors, including CNBP, FBP, and TCF (Levens, 2010), and by BRD4, a BET domain-containing transcriptional regulator (Delmore et al., 2011; Mertz et al., 2011). Additionally, non-B DNA structures are involved in regulating *Myc* expression: Z-DNA, single-strand bubbles, and G-quadruplexes, which are tertiary structures formed by guanine-rich sequences that are present in the NHEIII region of the Myc promoter (Brooks and Hurley, 2009; Brooks and Hurley, 2010; Levens, 2010). This region of chromosome 8 contains tissue-specific long-range enhancers and super-enhancers of *Myc* that contribute to modulating *Myc* expression (Lancho and Herranz, 2018).

The *Myc* gene contains three exons, with exons two and three encoding the protein and transcription arising predominantly from promoters P1 (25%) and P2 (75%) (Liu and Levens, 2006; Wierstra and Alves, 2008). Myc mRNA arises from different splicing of the three exons, and the resulting mRNA possesses a short half-life. Multiple microRNAs, such as let-7, miR-34, and miR-145, can target it for degradation (Sampson et al., 2007; Kim et al., 2009; Sachdeva et al., 2009; Cannell et al., 2010; Kress et al., 2011). Ribosomal proteins L5, L11, and S14 also bind *Myc* at the 3′ UTR, leading to its degradation by the RNA-induced silencing complex (RISC) via miR-24 (Liao et al., 2014; Spiniello et al., 2019) and miR-145 (Zhou et al., 2013). *Myc* mRNA contains a coding region

instability determinant (CRD) region with rare codons that cause destabilisation of the mRNA upon ribosomal stalling, thus hindering translation when not protected from endonucleolytic attack by a CRD-binding protein (CRD-BP, also known as insulin-like growth factor II mRNA-binding protein-1 (IGF2BP1)). Levels of CRD-BP are high in the foetus but decrease to low or absent in adult life (Leeds et al., 1997; Lemm and Ross, 2002; Weidensdorfer et al., 2009; Spiniello et al., 2019), allowing rapid *Myc* mRNA turnover in adult tissues. The untranslated regions (UTRs) of *Myc* mRNA are sites for regulation by RNA-binding proteins (RBPs). The long 5′ UTR arising from P1 and P2 promoters contain internal ribosomal entry sequences (IRESs) which interact with RBPs such as hnRNPC, hnRNPK, PCBP1, PCBP2, hnRNPA1, and RPS25 (Kim J. H. et al., 2003; Evans et al., 2003; Audic and Hartley, 2004). CELF1 and ELAV1 (also named HuR) compete to bind the 3' UTR, resulting in a balance of translational output, with CWLF1-binding resulting in decreased association of *Myc* mRNA with ELAVL1, thus reducing its translation (Liu et al., 2015; Spiniello et al., 2019). Many of these interactors were recently confirmed by HyPR-MS (hybridization purification of RNA–protein complexes followed by mass spectrometry—Spiniello et al., 2019). Concomitantly, novel RBPs ranging in function were identified, such as histone variant, transcription and translation factors, structural constituents of the spliceosome, nuclear ribonucleoproteins, and proteins involved in nuclear export mechanisms and mRNA metabolism (Spiniello et al., 2019); this demonstrates the complex regulation to which *Myc* mRNA is subject.

Once translated, the Myc protein is subject to tight post-translational control, resulting in a short-lived protein whose half-life is ~15–30 min. The most well-characterised pathway for Myc protein degradation results in the phosphorylation of phosphodegrons that allow the recognition by E3 ligase FBW7 (F-box/WD repeat-containing protein 7), a member of the SCF (SKP1, CUL1, and F-box proteins) complex. Specifically, the phosphorylation of serine 62 (S62) is involved in Myc stabilisation upon mitogen sensing and cell cycle re-entry and has been shown to be catalysed by ERK as part of the RAS/RAF/MAPK signalling cascade, amongst others (Sears et al., 1999; Sears et al., 2000; Sears, 2004). Phosphorylation at S62 is a pre-requisite for phosphorylation at threonine 58 (T58) by glycogen synthase kinase 3β (GSK3β). S62 and T58 phosphorylation occurs at different times of the cell cycle. Upon mitogen sensing and cell cycle entry, the RAS/RAF/MAPK signalling cascade is activated, leading to Myc phosphorylation and stabilisation, and inhibition of GSK3β via the activation of the PI(3)K/Akt signalling pathway, thus promoting early accumulation of pS62 Myc. Later in the G1 phase, Akt activity declines, leading to increased GSK3β activity, raising the levels of the double-phosphorylated form of Myc, and overall destabilising Myc, thus increasing its turnover. Recent evidence has shown that multiple kinases (ERK (Sears et al., 2000; Marampon et al., 2006; Hayes et al., 2016; Vaseva et al., 2018), CDK9 (Blake et al., 2019; Hashiguchi et al., 2019), and CAMKII (Gu et al., 2017)) phosphorylate Myc at S62 and pharmacological inhibition of such kinases can lead to decreased Myc protein stability. Subsequent to the phosphorylation of both S62 and T58, a series of interactions results in Myc with a single phosphorylated T58—the phosphodegron motif recognised by Myc's main E3 ligase, FBW7. A second phosphodegron site for FBW7 has recently been identified at T244 and T248 (Welcker et al., 2022). According to these findings, in the context of over-expressed Myc, FBW7 monomers can recognise either of the phosphodegron sites, leading to the ubiquitination and degradation of Myc. Indeed, ablation of phosphodegron at T58 via an alanine mutation (T58A), which had been previously reported as a version of Myc non-degradable by FBW7, was bound and degraded by FBW7 during the phosphorylation of T244 and T248. Conversely, in the context of endogenous Myc, both phosphodegrons are needed to allow FBW7 dimers to bind and degrade Myc. Other E3 ligases have also been shown to degrade Myc, especially Skp2, whose ubiquitination of Myc not only causes its degradation but also increases its transcriptional activity as it acts as a transcriptional co-activator (Kim S. Y. et al., 2003).

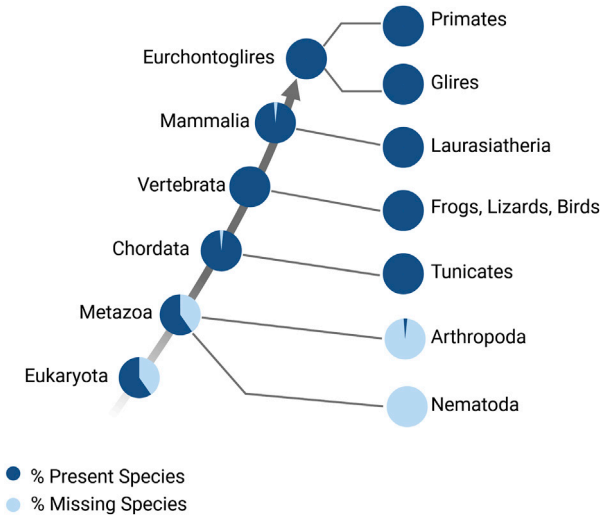

FIGURE 4
Myc family of genes over evolution. Percentage of species that have Myc family genes (Accession: TF106001), modified from http://www.treefam.org/ (Li, 2006; Li, 2006).

Finally, to safeguard against deregulated levels of Myc that bypass its transcriptional, translational, and post-translational control, Myc activity can trigger apoptosis in non-malignant cells (Wyllie et al., 1987; Evan et al., 1992; Murphy et al., 2008). The balance between the proliferative and proapoptotic activity of Myc depends on its transcriptional control and the cellular context in which it is activated as the proapoptotic response of Myc can be dependent on p53 activation (Zindy et al., 1998). It is important to highlight that Myc-induced proliferation and apoptosis are governed by distinct thresholds and are largely thought to be caused by Myc's ability to engage the same set of target genes, modulating the degree of target gene transcription in different cellular contexts. Therefore, in most cells, modest Myc activation can lead to increased proliferation and transformation and also to the low-level expression of proapoptotic genes. However, in cells already primed for apoptotic response and lacking other oncogenic lesions, Myc can trigger proliferation but will also amplify the proapoptotic response, leading to both p53-dependent and

TABLE 1 Role of Myc across regenerative species.

Species	Organ	Uninjured localisation of Myc	Injured localisation of Myc	Perturbation	Author
Hydra	Whole animal	Interstitial stem cells	No significant changes in expression	RNAi-mediated knockdown during injury results in abnormal tentacle morphogenesis	Hartl et al. (2010)
		Nematoblast nests			Lechable et al. (2023)
		Gland cells			Ambrosone et al. (2012)
Hothuria glaberrima (sea cucumber)	Digestive tube	Luminal epithelium of the intestine	Extensively in the mesothelial epithelial cells at 3 days post-injury	RNAi-mediated knockdown during injury results in reduced cell proliferation in intestinal explant	Mashanov et al. (2015a)
		Scattered individual cells in the mesothelium			Quispe-Parra et al. (2021a)
					Mashanov et al. (2015b)
	Radial nerve cord	Apical regions of the neuroepithelia	Apical regions of the neuroepithelia	RNAi-mediated knockdown during CNS injury results in failure of radial glial activation and dedifferentiation	Mashanov et al. (2015a)
		Scattered cell bodies in the neural parenchyma	Glial tubes		Quispe-Parra et al. (2021b)
			Radial nerve cords		Mashanov et al. (2015b)
Polyandrocarpa misakiensis (sea squirt)	Bud development	Proximal half of the developing bud		RNAi-mediated knockdown results in defects in gut formation	Fujiwara et al. (2011)
		Atrial epithelium			
		Branchial and gut primordia			
		Mesenchyme cells near the organ primordia			
Ambystoma mexicanum (Axolotl)	Limb	Little or undetected	Blastema at 3 h to 3 days		Stewart et al. (2013), Géraudie et al. (1989)
			Wound epidermis		
			Mesenchymatous-like cells		
Xenopus laevis froglet (African clawed frog)	Limb and tail	Low but present in the growing froglet limb	Wound epithelium		Géraudie et al. (1990)
			Limb regenerate		Lemaître et al. (1992), Christen et al. (2010)
			Mesenchymal cells in the blastema		
			Regenerating tail bud		
			Notochord		
			Neural tube		
Podarcis muralis (Wall lizard)	Tail	Sparse or undetected	Regenerative blastema		Alibardi (2017)
			Basal layers of the apical-lateral wound epidermis		Degan et al. (2021)
			Mesenchymal-like cells		
Danio rerio (zebrafish)	Retina	Low or undetected	Pan-retinal at 12 h post-injury	Morpholino knockdown or pharmacological Myc inhibitor blocks cell proliferation and Muller glia reprogramming in the retina	Mitra et al. (2019)
			Muller glia-derived progenitor cells and adjacent cells		
			Ganglion cell layer		

(Continued on following page)

TABLE 1 (*Continued*) Role of Myc across regenerative species.

Species	Organ	Uninjured localisation of Myc	Injured localisation of Myc	Perturbation	Author
	Neuromast hair cell	Undetected	Supporting cells within the boundary of mantle cells	Myc inhibition with small molecule or peptide reduces the number of regenerated hair cells	Lee et al. (2016)
	Fin		Pharmacological Myc inhibitor blocks fin regeneration	Mitra et al. (2019)	

-independent cell death (Murphy et al., 2008; McMahon, 2014; Jha et al., 2023).

3 Myc and regeneration across evolution

The ability of Myc to orchestrate cell cycle re-entry and proliferation makes Myc crucial for tissue regeneration. At its simplest level, regeneration is the regulation of transcription to drive proliferation and differentiation (cell fate changes) which lead to renewal or restoration of tissue function. The regenerative ability of tissues and organs varies widely across the animal kingdom, from whole-body regeneration in hydras to complex organ (e.g., limb, heart) regeneration in zebrafish and salamanders; more limited regeneration is observed in mammalian species, and regeneration is often limited to certain tissues at certain times (Yun, 2015). The Myc family of genes arose very early during evolution, before the diversification of metazoan evolution (Figure 4) (Young et al., 2011; Mahani et al., 2013); however, across species, the basic biochemical properties of Myc are very highly conserved. Therefore, the function of Myc as a master transcriptional regulator and its role in regeneration has been extensively studied across regenerative models.

Hydra live in fresh water and are members of the phylum Cnidaria. The species is one of the earliest to have evolved complex tissues in a defined body plan (Reddy et al., 2019), and they can regenerate their entire body following transverse and longitudinal dissection and dissociation. Four homologues of Myc (myc1, myc2, myc3, and myc4) and *Hydra*-max have been identified in Hydra; biochemically, myc and max complex and bind to E-Boxes. Once bound to DNA, the proteins transcriptionally regulate genes, such as cad, leading to cell cycle progression and ribosome biogenesis (Hartl et al., 2010; Young et al., 2011). *In situ* hybridization and single-cell RNA sequencing expression analysis have determined that the Hydra myc1 and myc2 genes are localised in all the proliferative cells of the animal, including the continuously proliferating interstitial cells, proliferating epithelial stem cells throughout the gastric region, and epithelial cells during gametogenesis. Myc3 lacks the N-terminal Myc boxes and is exclusively expressed in progenitor cells committed to nerve and gland cell differentiation (Hartl et al., 2010, 2014; Lechable et al., 2023). Myc expression is absent in all terminally differentiated cell types such as nerve cells and nematocytes (Young et al., 2011; Lechable et al., 2023). Myc has been shown to be crucial for controlling cell proliferation and differentiation processes in *Hydra*. It has been hypothesized that the myc1 and 2 homologues may compete with myc3 for max and E-boxes and regulate proliferation and differentiation, presumably by interacting with different protein partners, given the difference in the TADs (Lechable et al., 2023). Importantly, RNAi-mediated knockdown of myc1 during injury impairs the equilibrium between stem cell self-renewal and differentiation, leading to abnormal tentacle morphogenesis (Ambrosone et al., 2012). This suggests that Myc plays a key role in *Hydra* regenerative mechanisms (Table 1).

Another regenerative phylum is the invertebrate Echinoderms, which includes starfish, sea urchins, and sea cucumbers. Quite remarkably, the sea cucumber, *Holothuria glaberrima,* can regenerate most of its internal and external organs, following injury (García-Arrarás et al., 2018); even major parts of its central nervous system (CNS) can renew following severe injury. The sea cucumber homologue of Myc, like other species, contains a bHLH-LZ and TAD. Characterisation of *H. glaberrima* Myc expression in both the intestine and CNS immediately following injury demonstrates that Myc expression levels sharply increase, and both organs undergo extensive cell dedifferentiation (Mashanov et al., 2015a). This suggests that Myc is a critical transcription factor involved in the immediate regenerative response. Furthermore, a correlation is observed between increased Myc expression and the expression of genes involved in ribosomal biogenesis at the first and third days after intestinal injury (Quispe-Parra D. J. et al., 2021). The functional role of Myc in *H. glaberrima* regeneration has been determined by RNAi-mediated knockdown of Myc during injury. Knockdown of Myc during intestinal explant regeneration leads to reduced cell proliferation with no effect on dedifferentiation (Quispe-Parra D. et al., 2021). In the CNS, Myc denial at the same time as injury leads to a failure in radial glial activation, dedifferentiation, and a decrease in cellular apoptosis (Mashanov et al., 2015b). Together, these results indicate that Myc is a key gene controlling the immediate proliferative regenerative response in *H. glaberrima,* while the effect on dedifferentiation may be context-specific.

Ascidians or sea squirts are marine invertebrate sessile tunicates that belong to phylum Chordata. Ascidians present with a single Myc gene that contains a bHLH-LZ (Vanni et al., 2022). In ascidian species *Botryllus schlosseri*, *Ciona savignyi*, and *Polyandrocarpa misakiensis*, Myc is expressed in early development and disappears in adult tissues (Kobayashi et al., 2022; Vanni et al., 2022). Knockdown of Myc in embryonic/larval stages via morpholinos (modified antisense oligonucleotides), RNAi, or a dominant negative version of Myc suppresses mesenchymal and endodermal cell cycle and impairs organogenesis (Fujiwara et al., 2011; Kobayashi et al., 2022).

Amphibian species such as *Ambystoma mexicanum* (axolotl) and the African clawed frog, *Xenopus laevis*, have varying regenerative capacities. Axolotls remain in their juvenile stage throughout life and can regrow limbs and multiple internal organs, including the brain, spinal cord, liver, skeletal muscle, heart, and eyes. In contrast, *X. laevis* loses much of its regenerative ability when they metamorphose from tadpoles to adult frogs. There is surprisingly little research into the role of Myc in axolotl regenerative capacity; however, RNA sequencing data indicate that Myc is rapidly expressed at day 1 post-limb amputation and remains enriched for 10 days (Stewart et al., 2013). Proteomics data from limb amputation at days 1, 4, and 7 following injury highlight Myc as one of the most highly connected transcription factors (Jhamb et al., 2011); in agreement, regenerating axolotl limbs express Myc (Géraudie et al., 1989). There is a strong correlation in *X. laevis* between Myc expression and regeneration. In an undamaged setting, Myc expression in juvenile froglet limbs is low, but, following injury, Myc expression rapidly and significantly increases, together with the expression of proliferative marker PCNA. Myc expression then falls to a baseline by day 5 following resection (Géraudie et al., 1990; Lemaître et al., 1992; Christen et al., 2010). In reptiles (*Podarcis muralis*) after tail amputation, Myc expression has been studied by qRT-PCR, Western blotting, and immunohistochemical techniques and Myc is observed in the regenerating blastema in a similar location to the proliferating cells (Alibardi, 2017; Alibardi, 2022; Degan et al., 2021).

Zebrafish, *Danio rerio*, are teleosts (bony fish) which have been used as a regenerative model since the 1970s because of their incredible capacity to regenerate amputated fins, brain lesions, retinas, spinal cords, and hearts. Like mammals, the zebrafish Myc family consists of three family members—c-myc, N-myc, and L-myc—which complex with zebrafish max. The temporal and spatial expression patterns of Myc in zebrafish during development indicate that L-myc expression is limited to very early embryonic stages, whereas c-myc and N-myc are expressed during periods of growth and active cellular proliferation. N-myc expression is significantly downregulated in terminally differentiated adult tissues, whereas c-myc expression persists in some adult tissues such as gills and liver (Schreiber-Agus et al., 1993). The role of Myc has been studied across zebrafish regenerative organs, and several lines of evidence across cell types suggest that Myc is essential for an appropriate regenerative response. In the heart, the results from transgenic chemically induced cardiac injury and RNA sequencing have shown dramatic increases in Myc target gene expression, including genes involved in cell cycle and oxidative phosphorylation; this suggests a role for Myc in the induction of cardiomyocyte cell proliferation and mitochondrial biogenesis (Miklas et al., 2022). However, an alternative study using cardiac cryo-injury suggested that Myc target genes are downregulated at days 4 and 7 post injury, which is surprising given the observed increase in G2M checkpoint gene expression—which would normally overlap with Myc targets (Dicks et al., 2020). In the zebrafish retina, Myc expression is transiently upregulated following retinal injury, appearing 1 h post-injury, peaking at 24 h, and remaining increased for 7 days post-injury. Increased Myc expression coincides with elevated proliferative markers PCNA and BrdU and regulates the dedifferentiation of Muller glia to Muller glia-derived progenitor cells. Knockdown of Myc using morpholinos or the blockade of the Myc–Max interaction using the pharmacological inhibitor 10058-F4 abolishes proliferation and Muller glia reprogramming in the retina (Mitra et al., 2019). Another regenerative system in zebrafish is the sensory hair cells in the inner ear. During neuromast hair cell regeneration following damage, sensory hair cells display a rapid upregulation of Myc at 1 h that drops back to baseline levels by 18 h. The inhibition of Myc with 10058F4 or a cell-permeable Myc-specific peptide inhibitor suppresses cell cycle re-entry and hair cell regeneration (Lee et al., 2016). Furthermore, 10058-F4, abolishes fin regeneration (Mitra et al., 2019), demonstrating that Myc is essential to several regenerative processes in Zebrafish.

4 Myc and regeneration in mammals

Mammals have a more limited regenerative ability than amphibians and fish. In mammals, tissue regeneration processes are often classified into physiological regeneration and reparative regeneration. Ongoing physiological regeneration includes organs such as the intestinal gut lining, skin epidermis, red blood cells, and endometrium, whereby homoeostatic cell replacement involves stem cell differentiation or the replication of existing cells by proliferation or trans-differentiation (Iismaa et al., 2018). Reparative regeneration involves the restoration of damaged tissue or lost body parts and is therefore triggered by injury. Examples of organs that can partially or completely regenerate in adult mammals are the liver, spleen, bone, peripheral nerve, and urinary bladder (Mehta and Singh, 2019).

The role of Myc in maintaining tissue homoeostasis was first reported in pancreatic β-cells where it is activated in response to elevated levels of plasma glucose (Yamashita et al., 1988; Jonas et al., 2001), suggesting that it plays a role in β-cell proliferation and tissue maintenance under physiological conditions. Myc is also transiently expressed at days 1 and 2 during pancreatic regeneration after subtotal pancreatectomy in rats (Calvo et al., 1991). However, the function of Myc has best been characterised in the context of hyperglycemia, where Myc is shown to lead to altered secretory function and loss of differentiation of β-cells. Other reports of the role of Myc in β-cells showed that it is not necessary for the functioning of adult β-cells in physiological conditions but plays a key role in maintaining tissue homoeostasis in young mice under metabolic stress, whereby knockout of Myc in mouse β-cells resulted in β-cell dysfunction and impaired glucose tolerance. This protective function of Myc was shown to be lost in ageing mice, possibly through hypomethylation of the Myc response element (Rosselot et al., 2019). Interestingly, when Myc overexpression was explored as a therapeutic option to rescue dysfunctional β-cells, Myc induced cell death and differentiation (Laybutt et al., 2002; Pelengaris et al., 2002; Cheung et al., 2010). The observed cell death may be due to the overexpression methods chosen as a previous study demonstrated that the expression of Myc in β-cells from two different promoters resulted in β-cell proliferation or apoptosis, depending on the low- or high-expression system, respectively. Indeed, when the expression of Myc was driven from the locus that most accurately reproduced physiological levels of the proto-oncogene, Myc-induced apoptosis was only recorded in islets upon treatment with a sub-apoptotic dose of doxycycline (Murphy et al., 2008).

The involvement of Myc in the homoeostasis and wound healing of many epithelial tissues has been well-documented. Myc plays a vital role in the maintenance and regeneration of mammalian intestinal crypts. In a physiologically normal setting, Wnt-signalling in the rapidly proliferating progenitor and amplifying cells of the intestinal crypts drives Myc expression. Once the cells move out of the crypt niche and travel up the intestinal villi, they stop proliferating, Myc expression is lost, and the cells become terminally differentiated. The process of epithelia turnover takes around 5 days. Although Myc is not essential for the homoeostatic maintenance of juvenile and adult intestines (Bettess et al., 2005; Muncan et al., 2006; Konsavage et al., 2012), Myc null progenitor cells are smaller in cell size, have slowed cell cycle progression, reduced biosynthetic activity, and result in smaller daughter cells (Muncan et al., 2006). In a regenerating setting, following damage with gamma irradiation, Myc plays a vital role in the repair of intestine crypts. Wnt and c-Myc signalling is activated during intestinal regeneration (Muncan et al., 2006; Ashton et al., 2010) and where Myc is conditionally deleted, Myc-null crypts do not regenerate, and intestines become completely denuded of crypts. Therefore, Myc plays a central role in the regenerating intestine (Ashton et al., 2010).

The research surrounding the role of Myc in skin homoeostasis is complex and context dependent. Knockout of Myc in the basal cells within the epidermis of mice reveals that keratinocytes can continue to cycle, suggesting that Myc is not necessary for cell division, but animals display defects such as tight and fragile skin (Zanet et al., 2005). Conversely, others have shown that Myc is dispensable for epidermal homoeostasis and that mice show no defects in skin phenotypes (Oskarsson et al., 2006). Studies overexpressing Myc in epidermal cells have found that Myc can trigger proliferation and disrupt the differentiation of postmitotic keratinocytes (Pelengaris et al., 1999). However, others have determined that Myc overexpression can stimulate differentiation rather than drive proliferation (Gandarillas and Watt, 1997). To reconcile these opposing findings, it has been proposed that the level, duration, and timing of Myc may determine whether cells enter a proliferative or terminal differentiation state (Watt et al., 2008). In an injury setting following skin epidermal wounding, Myc levels significantly increase 7 days post-injury, co-localising with the proliferative marker BrdU. The levels of Myc then remain high during wound closure, decreasing to near baseline levels by day 30 when wound healing is complete (Shi et al., 2015). Denial of Myc during the wound healing process results in reduced proliferation in healing fronts and impaired healing with fewer layers of keratinocytes (Zanet et al., 2005). A recent eloquent study using lineage tracing and single-cell sequencing showed that wounding stimulates Myc-dependent dedifferentiation (Bernabé-Rubio et al., 2023).

Further evidence of the importance of Myc in epithelial regeneration in mammals comes from the oesophagus and lungs. The basal cells of the oesophageal epithelium express Myc relatively homogenously in an undamaged setting and require Myc for their self-renewal capacity. Upon conditional knockout of Myc (c-Myc and n-Myc), basal cells lose their undifferentiated state, leading to senescence (Hishida et al., 2022). In the lung, the conditional deletion of Myc in the epithelial club cells does not affect epithelial regeneration after naphthalene-induced injury, while the loss of Myc from the mesenchymal parabronchial smooth muscle cells causes reduced Fgf10 expression, decreased proliferation, and significantly impaired airway epithelial regeneration (Volckaert et al., 2013).

The role of Myc in reparative tissue regeneration has been extensively studied in the liver. Epithelial cell turnover in the liver is slow and the hepatocytes are mainly quiescent, with an estimated less than 1 in 10,000 hepatocytes in mitosis at any point in time (Kopp et al., 2016). The level of Myc in the homoeostatic liver is very low. During regeneration, after partial hepatectomy in rodents, over a third of hepatocytes can be seen proliferating within 24–26 h, and liver mass is restored to normal in around a week (Kopp et al., 2016; Michalopoulos and Bhushan, 2021). Following partial hepatectomy, Myc is rapidly induced within hours of damage, and Myc expression is followed by an increase in proliferation (Thompson et al., 1986; SOBCZAK et al., 1989; Morello et al., 1990), indicating its important role in driving hepatic regeneration. Similarly, following ectopic acute overexpression of Myc (low or high level) in the liver, rapid cell cycle progression and proliferation are observed (Murphy et al., 2008; Bywater et al., 2020).

Interestingly, Myc ablation studies have indicated that hepatocytes are still capable of entering the cell cycle in the absence of Myc (Baena et al., 2005; Sanders et al., 2012), suggesting that Myc is not essential for hepatocyte proliferation. However, the inhibition of Myc using antisense oligomers or the ablation of Myc in the regenerating rodent liver following partial hepatectomy leads to a reduction of proliferating cells (Arora et al., 2000; Baena et al., 2005; Rodríguez et al., 2006). More recently, knockout of Myc and Mlx (Max-like protein, the key node of the Mlx network and part of the Myc extended network) in mice has indicated that Myc denial leads to changes in the expression of mRNA translation and energy metabolism, ultimately impeding the regenerative potential of hepatocytes (Wang et al., 2022).

In general, data from regenerative species and regenerative tissues in mammals indicate that Myc is predominantly expressed in proliferating cells and that the expression of Myc drives key transcriptional programs, including ribosomal biogenesis, metabolism, and cell cycle progression. In normally quiescent but regenerative tissues following an insult, Myc expression can be observed as a short pulse, and its expression correlates with the pattern of proliferative cells. Myc appears to be non-essential to the homoeostatic regenerative processes of many organs, although, repair is attenuated when Myc is denied (Figure 5). Therefore, it is exciting to speculate whether Myc may have the potential to aid regeneration in tissues that do not normally have regenerative capacity.

5 Harnessing Myc in non-regenerative organs

Some adult mammalian tissues have strikingly little regenerative capacity, such as the heart and CNS. However, like *X. laevis*, some embryonic and neonatal mammal tissues have shown remarkable regenerative capacity. For instance, the adult mammalian heart cannot regenerate following injury, and loss of the contractile cardiomyocytes leads to adverse pathological remodelling that

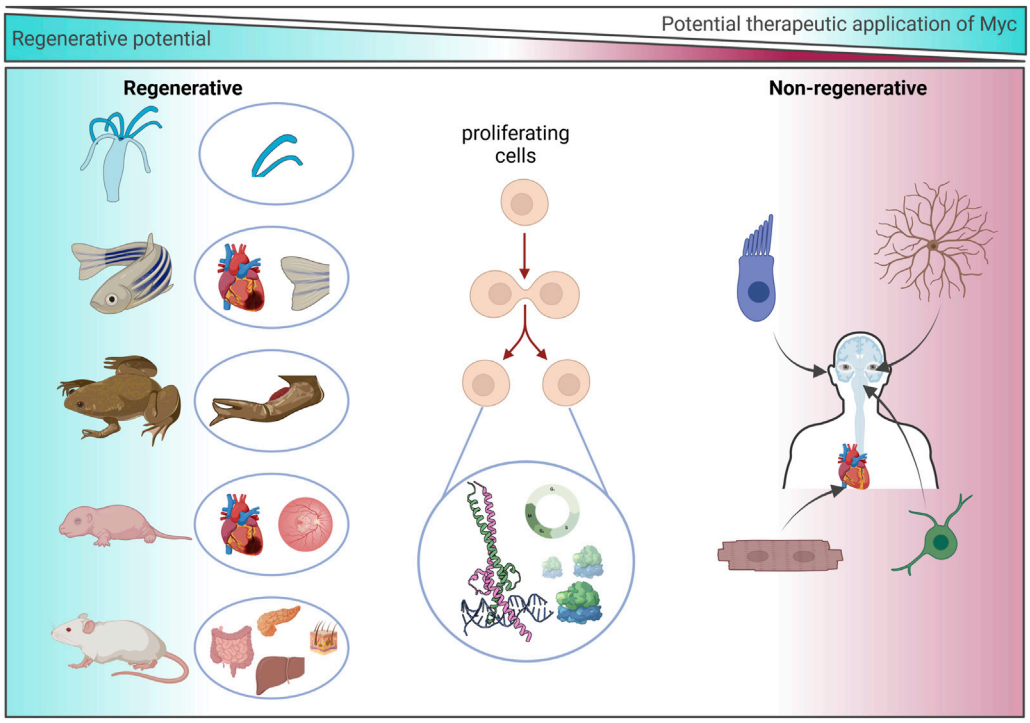

FIGURE 5
Regenerative potential of Myc. Representation of species/organs with an experimentally determined association or dependence upon Myc during regeneration (left) and non-regenerative human cell types in which the experimental use of Myc may be exploited to drive regeneration (right).

ultimately results in heart failure. Conversely, following resection of 15% of the myocardium at day 1 post-birth, the neonatal mouse heart can fully regenerate and regain normal cardiac function (Porrello et al., 2011). However, this regenerative ability is lost by day 7. Fate mapping has confirmed that regeneration occurs via cardiomyocyte proliferation, which is similar to the regenerative mechanism observed in the regenerating zebrafish heart (Porrello et al., 2011; Senyo et al., 2013). This short cardiac neonatal regenerative window has also been shown to exist in larger mammals such as pigs (Ye et al., 2018; Zhu et al., 2018), and there are some anecdotal case studies of newborn babies exhibiting a regenerative capacity briefly after birth (Haubner et al., 2016; Aly et al., 2021). Interestingly, the level of c-Myc and n-Myc in the mouse heart declines sharply at birth, and it is almost absent in the adult heart (Singh et al., 2018; Bywater et al., 2020). Transcriptional comparisons of the regenerating mouse neonatal heart to the adult non-regenerating heart indicate that adult cardiomyocytes do not express Myc and therefore fail to reactivate the neonatal transcriptional Myc programmes following injury (Quaife-Ryan et al., 2017; Singh et al., 2018). Even when Myc is specifically and ectopically activated in adult myocardium, the adult heart is refractory to proliferation (Xiao et al., 2001; Bywater et al., 2020; Chen et al., 2021). Myc instead induces hypertrophic growth and not hyperplasia, suggesting that Myc alone is insufficient for driving the cell cycle in cardiomyocytes (Xiao et al., 2001). However, global ChIP sequencing has established that Myc binds to largely overlapping promoter sites in proliferative (liver) and non-proliferative (heart) tissues that encode classic Myc programmes involved in ribosomal biogenesis and cell cycle, despite the difference in response to the activation of ectopic Myc. Interestingly, Myc-driven transcription in the heart is impeded by the limited availability of transcriptional machinery such as the P-TEFb complex, which allows efficient RNAPII-mediated transcriptional amplification (de Pretis et al., 2017; Bywater et al., 2020). Consequently, the Myc-driven transcriptional response is attenuated in cardiomyocytes, and, while many Myc target genes are seen to be marginally increased, hypertranscription is limited, leading to cell growth without division. Therefore, both Myc expression and Myc-driven transcription are limited in the adult mammalian heart. In agreement, Nox4 overexpression has been shown to prolong the postnatal period of cardiomyocyte proliferation via ERK1/2 activation and an increase in Myc phosphorylation. Stabilised Myc binds to and drives the expression of genes such as Cyclin D2, leading to cell cycle. However, Nox4 could not continue to drive cardiomyocyte proliferation in the adult heart, again indicating that Myc-driven proliferation is limited in an adult setting (Murray et al., 2015).

When Myc and the limiting transcriptional machinery, Cyclin T1, are ectopically expressed in cardiomyocytes, Myc-driven transcription is productive and can drive efficient cardiomyocyte proliferation with gene expression changes related to metabolism, cell proliferation, and division, and a reversion to the neonatal-like state (Singh et al., 2018; Bywater et al., 2020; Boikova et al., 2022). In an injury setting following experimental myocardial infarction, Myc together with Cyclin T1 overexpression, specifically in adult cardiomyocytes, can drive the functional repair of mouse hearts, so long as Myc expression is transient and localised to the injury site (Boikova et al., 2023). In an effort to develop a prototypical therapeutic to

TABLE 2 Evidence of mammalian regeneration by Myc.

Species	Organ/cells	Ectopic overexpression technology	Injury model	Findings	Authors
Mouse	Heart	Tamoxifen-inducible MycER	No injury	Ectopic Myc and Cyclin T1 in adult and juvenile cardiomyocytes results in cardiomyocyte proliferation	Bywater et al. (2020) and Boikova et al. (2022)
		Tamoxifen-inducible MycER and modified mRNA-encoding Myc and Cyclin T1	Myocardial infarction by occlusion of the left anterior descending coronary artery	Transient and local expression of Myc with cyclin T1 around the infarct results in functional cardiac recovery and reduced scar size	Boikova et al. (2023)
Guinea pig	Cochlea	Adenoviral vector encoding Myc	Acoustic trauma	Smaller auditory threshold shift at 7-day post-noise exposure. Reduction in outer hair cell stereocilia loss and cilia disarray	Han et al. (2009)
Mouse	Ear/explant organ culture of the utricle	Adenoviral vector encoding MycT58A	No injury	Supporting cells re-enter the cell cycle and proliferate. Small number of cells differentiate towards the hair cell lineage	Burns et al. (2012a)
Mouse	Ear/cochlea	Adenoviral vector encoding Myc	No injury	Combined transient ectopic Myc and Notch 1 intracelluar domain reprograms adult supporting cells to regenerate hair cell-like cells	Shu et al. (2019)
Mouse	Ear/cochlea	Cocktail of small molecules and siRNAs to activate Myc, Notch1, Wnt, and cAMP pathways	Kanamycin- and furosemide-induced hair cell loss	Reprograming of adult supporting cells to regenerate hair cell-like cells	Quan et al. (2023)
Mouse	Eye/optic nerve	Adeno-associated virus serotype 2 (AAV2) encoding Myc and tamoxifen-inducible MycER	Optic nerve injury by crushing	Increased survival of retinal ganglion cells and axonal regeneration following injury. Synergistic effects of ectopic Myc, PTEN, and SOC3 deletion	Belin et al. (2015)
Mouse	Eye/optic nerve	pEX4-c-Myc DNA plasmid	Optic nerve injury by crushing	Myc is both necessary and sufficient for sensory axon regeneration via the Myc-TERT-p53 signalling pathway	Ma et al. (2019)
Mouse	CNS/oligodendrocytes progenitor Cells (OPCs)	Dual-AAV system targeting *Pdgfra* endogenous locus resulting in ectopic Myc in all *Pdgfra*-expressing OPCs	No injury	Reprogramming of mature OPCs, increased OPC proliferation, and ability to differentiate into myelinating oligodendrocytes	Neumann et al. (2021)

drive endogenous regeneration in the adult mouse heart, Myc and Cyclin T1 have been delivered via mRNA to drive a transient short pulse of Myc and Cyclin T1 expression. Despite the short expression time of the mRNA of less than 24 h, functional improvement over 28 days was observed, suggesting that Myc and Cyclin T1 could be harnessed to drive regeneration of the heart (Table 2). A number of matters remain to be resolved. For instance, mRNA was injected directly into the heart and will be expressed in multiple cell types; therefore, the therapeutic would be greatly enhanced by the use of cell-specific expression techniques (Magadum et al., 2020a; Magadum et al., 2020b), and a catheter-based delivery system would expand the target patient population considerably. Furthermore, the relative functional benefit observed from Myc-Cyclin T1 mRNA was lower than that from the transgenic systems, so extending the expression time of Myc and Cyclin T1 may enable greater reparative success. Finally, while neoplasia following Myc-Cyclin T1 mRNA expression was not observed over the course of the experiment, careful consideration of the oncogenic potential of Myc must be considered. Interestingly, forced expression of the reprogramming factors Oct4, Sox2, Klf4, and Myc (OSKM) has also been shown to re-program and drive cardiomyocyte proliferation, ameliorate myocardial damage, and drive functional improvement, following infarction. In this model, prolonged expression of OSKM causes cardiac teratoma formation, although short-term OSKM-induced cardiomyocyte dedifferentiation was shown to be reversible (Chen et al., 2021). Therefore, a system such as mRNA—which allows a more physiologically normal pulse of Myc expression as observed following acute damage in regenerative systems—which can be

localized and has no issues surrounding genetic integration should be employed.

The inner ear sensory hair cells are essential in detecting sound from the external environment. However, they lack the regenerative capabilities to replace damaged cells upon acoustic trauma, leading to permanent hearing loss. In contrast, lower invertebrates retain the ability to regenerate damaged hair cells by driving the proliferation of supporting cells (Corwin, 1981; Ryals and Rubel, 1988; Janesick et al., 2022). This ability has been shown to exist in neonatal mice, where supporting cells can re-enter the cycle and transdifferentiate into hair cells (White et al., 2006). It has been postulated in mouse utricles that 51% of hair cells that have arisen after birth are from proliferating supporting cells that transdifferentiate into hair cells (Burns et al., 2012a). In zebrafish, Myc has been shown to be essential for hair cell regeneration as it drives the proliferation of hair cell precursors (supporting cells) and is upregulated during the regeneration process (Lee et al., 2016). Both c-Myc and n-Myc have been found to be expressed in the mammalian inner ear during development, and n-Myc plays an essential role in morphogenesis, patterning, and proliferation during development (Domínguez-Frutos et al., 2011; Kopecky et al., 2011). The expression of n-Myc and c-Myc declines postnatally and they are expressed at low levels in the adult inner ear (Domínguez-Frutos et al., 2011). Myc has been shown to play a protective role against noise damage. Guinea pigs inoculated with an adenoviral vector-encoding Myc prior to exposure to noise damage had a smaller auditory threshold shift 7-days post-noise exposure. Furthermore, morphological assessment of the cochlea indicated that Myc expression reduced the outer hair cell stereocilia loss and cilia disarray. These results indicate that the ectopic expression of Myc reduces the loss of hair cells, following acoustic trauma (Han et al., 2009).

The overexpression of Myc in a cultured adult mouse utricle can reverse the quiescent and post-mitotic state of the supporting cells and allow them to re-enter the cell cycle and drive proliferation. Some of these cells have acquired the ability to differentiate towards the hair cell lineage by expressing the hair cell marker myosin VIIA (Burns et al., 2012b). Furthermore, Myc and Cyclin A2 were reported to be downregulated during cochlear development and the overexpression of both genes was shown to enhance the proliferation of cochlear progenitor cells (Zhong et al., 2015). *In vivo*, the combined over-expression of Myc and Notch 1 intracellular domain in the adult mouse inner ear drives the proliferation of supporting and inner hair cells in the cochlea. Furthermore, when the Myc and Notch 1 intracellular domains were transiently activated for 3 days, the adult supporting cells were able to proliferate and then, following Myc downregulation, transdifferentiate into hair cell (HC)-like cells through the induction signal of Atoh1. Therefore, the transient nature of Myc and Notch and their subsequent downregulation are vital for the trans-differentiation process (Shu et al., 2019), further highlighting the need for transient Myc expression in regeneration systems. More recently, in an attempt to generate a clinically applicable regenerative therapeutic of Myc and Notch overexpression, Quan et al. (2023) used a cocktail composed of small molecules and siRNAs injected into the middle ear space, following injury and demonstrated regeneration of HC-like cells in response to Atoh1. However, the regeneration efficiency was attenuated compared to that achieved by ectopic Myc expression from a transgenic allele in the mouse and suggests that optimisation of Myc expression is required.

In the optic nerve, retinal ganglion cells (RGCs) are vital for the propagation of visual information from the eye to the brain through projections of their axons that run along the optic nerve. Unlike zebrafish, that can restore vision via the dedifferentiation and proliferation of Müller glia cells that generate all cell types required to regenerate, mammals lose the ability to regenerate their RGCs shortly after birth. Upon injury of the optic nerve, apoptosis of RGCs leads to an irreversible loss of vision (Boia et al., 2020; Soucy et al., 2023). Interestingly, single-cell RNA sequencing has shown that Myc is expressed in certain RGC subtypes (Rheaume et al., 2018) and that the expression of Myc mRNA is decreased in the optic nerve by 70%, following injury (Belin et al., 2015). Recently, it was found that Myc regulates axonal regeneration in the sensory optic nerve through the downstream target, telomerase reverse transcriptase (TERT), and p53. Both TERT and p53 are upregulated following an injury and decrease in expression when the sensory axons mature and lose the ability to grow. The functional inhibition of TERT and p53 or Myc resulted in impairment in axonal regeneration (Ma et al., 2019). Knockout of Myc significantly reduced the number of regenerating axons, whilst overexpression of Myc in the RGCs of mice orchestrated increased survival that drove regeneration of their axons, following optic nerve injury. Furthermore, a synergistic effect of AAV-mediated Myc overexpression combined with the co-deletion of PTEN and SOCS3 promoted neuronal survival and axon regeneration. Interestingly, delayed overexpression of Myc to day 1 following injury, which is more clinically relevant, continued to demonstrate that Myc could still rescue and improve the survival of injured neurons and induce axonal regeneration. These regenerated axons were also found to grow outside the injury site, highlighting an exciting prospect for neuronal regeneration (Belin et al., 2015).

Oligodendrocyte progenitor cells (OPCs) are a subtype of proliferating glia in the CNS that differentiate into myelinating oligodendrocytes which support and insulate axons. Myc expression is elevated in proliferating OPCs, and Myc plays a key role in their maintenance in a proliferative and undifferentiated state. The level of Myc in OPCs declines during the differentiation into oligodendrocytes in the developing white matter (Magri et al., 2014). The ability of OPCs to proliferate and differentiate into oligodendrocytes becomes impaired with ageing, and there is an age-related decline in the efficiency of re-myelination which can contribute to the progression of neurological diseases such as multiple sclerosis (Sim et al., 2002; Kuhlmann et al., 2008; Boyd et al., 2013; Neumann et al., 2019; Neumann et al., 2021). There is a correlation between the age-related decline of OPC function and Myc expression whereby Myc levels have been shown to dramatically reduce over time during OPC ageing, suggesting that Myc plays a role in maintaining the identity of OPCs (Neumann et al., 2021). In agreement with this hypothesis, the inhibition of Myc in neonatal OPCs leads to a quiescent state and aged-like OPC characteristics, loss of OPC self-renewal capacity, and the ability to differentiate (Neumann et al., 2021). Conversely, restoring the proliferative capacity of OPCs aids the differentiation potential of OPCs and enhances re-myelination efficiency (Foerster et al., 2020). Therefore, Myc overexpression has been examined in

aged OPCs, and ectopic Myc expression can revert OPCs to a more neonatal-like state characterised by increased proliferative potential whilst also increasing the ability to differentiate into myelinating oligodendrocytes. *In vivo*, the enhanced function of the aged OPCs by Myc showed an improvement in the myelin regeneration of the axons in aged animals where there is a poor re-myelination potential and efficiency due to their aged CNS. These results demonstrate that Myc can change the functional age of OPCs, highlighting a new strategy for treating neurological diseases such as multiple sclerosis, where the myelin sheath is damaged. However, in the case of OPCs, long-term Myc expression would likely be required to maintain OPCs in their proliferative, juvenile state, but given the oncogenic risk of gliomas it may be difficult to harness Myc directly (Neumann et al., 2021).

6 Concluding remarks

Myc was first discovered in the 1980s and has become one of the most extensively studied proteins, appearing in ~50,000 publications listed on PubMed (1980–2024). Myc is a highly conserved protein across the animal kingdom that regulates many critical processes within cells. The expression of Myc is highly synchronized, and its expression is typically kept at low levels or restricted to highly proliferative tissues. Myc expression has been shown to be crucial for sustaining pluripotency (Fagnocchi and Zippo, 2017; Fan and Li, 2023) and is one of the four factors essential to efficiently reprogram adult somatic cells to induce pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Araki et al., 2011). The overexpression or deregulation of Myc is seen in the vast majority of all human cancers, and cancer cells share many molecular characteristics with iPSCs. This review has concentrated on evidence that Myc may be central to regenerative processes across species. Complex tissue regeneration requires the coordination of a series of fundamental biological processes, including, wound sensing, barrier formation, cell cycle re-entry, migration, trans-differentiation, and remodelling. These processes are characterized by the altered expression of transcription factors, temporary de-differentiation, and the loss of cell fate markers. In regenerative species and organs, these expression changes are temporary and generally revert to baseline following resolution of the injury. Endogenous reparative regeneration is an emerging field that aims to restore organ function by harnessing and enhancing endogenous repair mechanisms. The Myc gene family is uniquely situated to synergize upstream pathways into downstream cell cycle control (Figure 5) and to correspondingly suppress differentiation-specific genes to allow for trans-differentiation. The data presented here highlight the need for controlled, transient, localized delivery of Myc. Careful consideration is therefore needed when selecting a possible therapeutic strategy to enhance Myc expression.

There are valid concerns that the ectopic expression of deregulated Myc may cause off-target effects or even neoplastic transformation. Any factor that is involved in cell growth and proliferation is, in essence, a proto-oncogene, and other factors capable of reactivating proliferation, such as the activation of Yap or Wnt signalling, are also potently oncogenic when deregulated. However, these are exactly the proteins that are required to drive efficient proliferation in non-regenerative tissues, and the need for these pro-proliferative factors highlights the importance for the systems that drive expression to be transient. Where reversible or transient expression systems are employed, de-differentiation is shown to be reversible (Bywater et al., 2020; Chen et al., 2021). The reversibility of deregulated Myc has also been observed in cancers where the deactivation of ectopic Myc in pancreatic and lung cancers leads to the complete regression of tumorigenesis and restoration of the normal tissue architecture (Kortlever et al., 2017; Sodir et al., 2020). The key, therefore, is the deactivation of Myc following immediate repair to aid re-differentiation and the later stages of the reparative regenerative program.

Similarly, where the long-term expression of pro-proliferative factors via AAV delivery systems has been pre-clinically employed, side effects from the continued proliferation such as the de-differentiation of cardiomyocytes and arrhythmic episodes have reduced their success (Gabisonia et al., 2019). From a safety perspective, the use of constitutive expression viral systems will probably be unsuccessful, so transient technologies with rapid kinetics are essential. A system such as mRNA that has no issues surrounding genetic integration and which allows a more "physiologically normal" pulse of Myc with its naturally short protein half-life, as observed following acute damage in regenerative systems, should be employed.

It must be noted that Myc may be harmful in some tissues; for instance, as well as the tumorigenic effect of Myc in the liver, Myc can induce liver fibrosis (Gabisonia et al., 2019). Therefore, in addition to transient or switchable technologies, cell-specific systems to restrict Myc expression to the cell types of interest are vital. Cell-specific systems for mRNA expression are beginning to emerge (Magadum et al., 2020a; Qian et al., 2022; Kaseniit et al., 2023) and hold much promise for application in endogenous regeneration. Furthermore, lipid nanoparticle cell targeting that impedes the accumulation of nucleic acid in hepatocytes is a future possibility (Kularatne et al., 2022).

Here, we have concentrated on the role of Myc in direct cell cycle regulation. However, Myc possesses the ability to mediate a plethora of processes resulting in microenvironment, immune (Kortlever et al., 2017), and metabolic (Stine et al., 2015) changes which are dependent on tissue. For instance, where Myc is specifically expressed in oncogenic KRas-driven lung epithelial tumour cells, Myc expression leads to a reversible influx of VEGF-expressing macrophages, an exclusion of T cells, and rapid onset of angiogenesis. In oncogenic KRas-driven pancreatic tumours, specific epithelial Myc activation leads to an influx of macrophages, an exclusion of T cells, but also an influx of neutrophils and an increase in activated fibroblasts and deposition of desmoplasia (Kortlever et al., 2017; Sodir et al., 2020). Therefore, Myc expression may not only lead to intrinsic cell number restoration but may be able to tap into the resident regenerative programs of tissues which may be vital for regeneration. Metabolic reprogramming is a key hallmark of cancer that is mostly directly regulated by Myc (Stine et al., 2015) and facilitates the generation of biomass for rapid cell proliferation. Likewise, cellular metabolism plays a key role during regeneration. In the heart, loss of mammalian cardiac regenerative capacity correlates with an increased metabolic state (a metabolic switch from glycolysis to fatty acid oxidation). Mimicking these changes in ES-cell derived cardiomyocytes can drive cells to become more mature and proliferate less (Mills et al., 2017). Conversely, metabolic reprogramming can allow for cardiomyocyte proliferation and cardiac regeneration *in vivo* (Magadum et al., 2020b; Bae et al.,

2021; Li et al., 2023). Therefore, during complex tissue regeneration, Myc may not only provide the stimulus for cell cycle but also the capability for the demands of growth and communication with the surrounding environment.

Author contributions

CA: visualization, writing–original draft, and writing–review and editing. RD: writing–original draft and writing–review and editing. CW: funding acquisition, supervision, visualization, writing–original draft, and writing–review and editing.

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Conflict of interest

The authors declare that this 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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Endothelial c-Myc knockout disrupts metabolic homeostasis and triggers the development of obesity

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Introduction: Obesity is a major risk factor associated with multiple pathological conditions including diabetes and cardiovascular disease. Endothelial dysfunction is an early predictor of obesity. However, little is known regarding how early endothelial changes trigger obesity. In the present work we report a novel endothelial-mediated mechanism essential for regulation of metabolic homeostasis, driven by c-Myc.

Methods: We used conditional knockout (EC-Myc KO) and overexpression (EC-Myc OE) mouse models to investigate the endothelial-specific role of c-Myc in metabolic homeostasis during aging and high-fat diet exposure. Body weight and metabolic parameters were collected over time and tissue samples collected at endpoint for biochemical, pathology and RNA-sequencing analysis. Animals exposed to high-fat diet were also evaluated for cardiac dysfunction.

Results: In the present study we demonstrate that EC-Myc KO triggers endothelial dysfunction, which precedes progressive increase in body weight during aging, under normal dietary conditions. At endpoint, EC-Myc KO animals showed significant increase in white adipose tissue mass relative to control littermates, which was associated with sex-specific changes in whole body metabolism and increase in systemic leptin. Overexpression of endothelial c-Myc attenuated diet-induced obesity and visceral fat accumulation and prevented the development of glucose intolerance and cardiac dysfunction. Transcriptome analysis of skeletal muscle suggests that the protective effects promoted by endothelial c-Myc overexpression are associated with the expression of genes known to increase weight loss, energy expenditure and glucose tolerance.

Conclusion: Our results show a novel important role for endothelial c-Myc in regulating metabolic homeostasis and suggests its potential targeting in preventing obesity and associated complications such as diabetes type-2 and cardiovascular dysfunction.

KEYWORDS

MYC, endothelial dysfunction, adiposity, metabolism, obesity, glucose intolerance

1 Introduction

Overweight and obesity are chronic conditions that result mostly from exposure to hypercaloric diet rich in saturated fat and sugar and lack of physical activity (Faruque et al., 2019; Elmaleh-Sachs et al., 2023). Other factors such as aging, stress, certain health conditions, medication, and genetics have also been associated with the development of overweight and obesity. Obese individuals have an increased risk of developing cardiovascular disease, liver disease, diabetes, and cancer (Scully et al., 2020; Powell-Wiley et al., 2021).

Endothelial cells play an essential role in tissue homeostasis by supporting regeneration through the regulation of surveillance and repair mechanisms (Reiterer and Branco, 2020). Endothelial dysfunction, which occurs with aging and exposure to environmental stress factors, has a detrimental impact on organ physiology, ultimately leading to multiple pathological conditions such as obesity, type-2 diabetes, and cardiovascular disease (Engin, 2017; Donato et al., 2018; Haybar et al., 2019; Kajikawa and Higashi, 2022). Most studies on obesity have focused on how exposure to a diet rich in fat triggers endothelial dysfunction. However, little is known regarding how early changes in the endothelium contribute to obesity.

The transcription factor c-Myc plays an important physiological role controlling multiple cellular functions (Hofmann et al., 2015; Zacarias-Fluck et al., 2024). Deregulated c-Myc expression has been associated with cancer, metabolic and inflammatory conditions (Zheng et al., 2017; Luo et al., 2021; Rosselot et al., 2021; Nevzorova and Cubero, 2023; Zacarias-Fluck et al., 2024). Several reports from our group and others have highlighted an essential role of the transcription factor c-Myc in endothelial cell function in development (Baudino et al., 2002; He et al., 2008; Rodrigues et al., 2008; Kokai et al., 2009) and disease (Riu et al., 2002; Riu et al., 2003; Hurley et al., 2010; Florea et al., 2013; Rosselot et al., 2019; Qi et al., 2022). In addition to regulating angiogenesis, we have shown that c-Myc controls endothelial self-renewal and inflammation (Florea et al., 2013; Qi et al., 2022). In the present work, we provide supporting evidence that endothelial c-Myc plays an essential role in regulating overweight and obesity, which extends to the prevention of insulin resistance and cardiovascular dysfunction.

2 Materials and methods

2.1 Animals

All animal experiments were approved by the University of Miami Animal Care and Use Committee according to the National Institutes of Health guidelines and conform to the Guide for the Care and Use of Laboratory Animals. A total of four transgenic mouse lines were used in this study to conditionally regulate c-Myc expression in endothelial cells. All animals were housed on a 12-h light/dark cycle with free access to food and water unless otherwise stated. Endothelial c-Myc knockout mice were generated by crossing c-Myc$^{flox/flox}$ (B6.129S6-*Myc*^{tm2Fwa}/Mmax, Strain #032046, Jackson Laboratory, Bar Harbor, ME, USA) and Cdh5(PAC)-CreERT2 (C57BL/6-Tg (Cdh5-cre/ERT2)1Rha), developed by Dr. Ralph Adams at Cancer Research UK (Cancertools.org reference number 151520) mouse lines. Knockout controls consisted of littermates carrying identical floxed genotypes, but lacking Cre (Cre-negative) or carrying only the Cre (Flox/Flox-negative). Males and females were used in the study. Induction of c-Myc knockout was performed between 4-6 weeks of age through daily intraperitoneal injections of 2 mg tamoxifen (#13258, Cayman Chemical, Ann Arbor, MI, USA) per animal for a total period of 5 days as previously described (Qi et al., 2022). Endothelial c-Myc overexpression mice were generated by crossing Tet-O-Myc (FVB/N-Tg(tetO-MYC)36aBop/J, Stanford University, Stanford, CA, USA) and Cdh5-tTA (FVB-Tg(Cdh5-tTA)D5Lbjn/J, Strain #013585 Jackson Laboratory, Bar Harbor, ME, USA) mouse lines in the presence of doxycycline diet (#TD.01306, Teklad, Indianapolis, IN, USA) to prevent the expression of the human c-Myc transgene. Overexpression controls consisted of littermates carrying the Tet-O-Myc genotype but lacking the transactivator (tTA-negative). Induction of c-Myc overexpression was performed between 4-6 weeks of age through withdrawal from the doxycycline diet. Only males were used in the study because the human c-Myc transgene is restricted to the Y-chromosome in this model. c-Myc knockout and overexpression in endothelial cells were confirmed by qPCR (Supplementary Figure S1).

For diet-induced obesity experiments, mice were exposed to control (#TD.08485) or high-fat diet (#TD.88137) (Teklad, Indianapolis, IN, USA) for a total period of 20 weeks.

2.2 Vasoactive response studies

Vasoactive response was assessed in mesenteric arteries harvested from control (CT) and endothelial c-Myc knockout (EC-Myc KO) mice using a pressure myograph system model 110p (Danish Myo Technology, Denmark) as previously described (Hernandez et al., 2019). Briefly, the mesenteric arcade was initially isolated and placed in physiological salt solution (PSS) for dissection of the second-order mesenteric arteries and preserve viability. Vessels were then mounted on two glass microcannulas and placed in the myograph chamber containing PSS solution at 37°C and under aeration with a special mix of 95% O_2 and 5% CO_2. Quality control was performed to confirm vessel viability prior to all

measurements as previously described (Coats and Hillier, 1999). After confirmation of viability, arteries were washed with PSS, and pre-contracted with norepinephrine followed by assessment of endothelium-dependent vasodilation through exposure to increasing doses of acetylcholine every 5 min. Pressure-outside diameter curves were recorded at an isobaric condition of 60 mmHg as evidence of vasoreactivity response for different acetylcholine concentrations.

2.3 Gross phenotypic analysis

We performed longitudinal analysis of experimental animals maintained under a standard control or a western style high-fat diet. Body weight was collected once a week (high-fat diet) or once a month (aging), and animals were macroscopically evaluated for any visible abnormalities. Food consumption was monitored in EC-Myc KO animals. Any signs of abnormalities, cancer development and sudden deaths were recorded.

2.4 Indirect calorimetry and body composition measurements

Whole-body energy metabolism was evaluated in CT and EC-Myc KO mice by indirect calorimetry using the Oxymax Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH, USA) as previously described (Fonseca et al., 2014). Chambers were maintained at 22°C and O_2 levels were calibrated against a standard gas mixture prior to use. Mice were individually housed under light/dark cycles of 12-h and allowed to acclimate to the chamber for 48 h prior to data collection. Food and water were available *ad libitum*. After acclimation, O_2 consumption, CO_2 production, respiratory exchange ratio (RER) and heat production were collected at 26-min intervals for a total period of 48 h.

2.5 Metabolic assessment of skeletal muscle slices

The redox state of skeletal muscle tissue harvested from CT and EC-Myc KO mice was estimated using a 3D fluorescence cryo-imaging system custom designed by the Biophotonics Laboratory at Florida Atlantic University (Ceyhan et al., 2023). Briefly, frozen tissues were embedded a day before imaging in a black absorbent medium. For imaging, the frozen tissue block was mounted to the sample stage, where its temperature was maintained at cryogenic temperatures (−10°C) for a higher quantum yield of fluorescence while retaining markers of metabolic state. A motor-driven stage and microtome blade allowed tissue slicing at 30 µm thickness. Images were acquired using a CCD camera (Retiga R6, Teledyne Photometrics, Tucson, AZ) with alternating filter wheels for nicotinamide adenine dinucleotide (NADH), and oxidized flavin adenine dinucleotide (FAD). The tissue was excited with a mercury arc lamp (200W lamp, Oriel, Irvine, CA). The broad light passes through excitation filters 350 ± 40 nm (UV Pass Blacklite, HD Dichroic, Los Angeles, CA) for the NADH channel and 437 ± 10 nm (440QV21, Omega Optical, Brattleboro, VT) for the FAD channel. All components of image acquisition operate with an automated virtual interface LabVIEW software (2022, National Instruments). The images of each slice were stacked in the *z*-direction to generate 3D images. Variables such as light intensity, illumination pattern, and dark current noise were considered for image processing. The redox ratio (RR = NADH/FAD) was calculated by dividing the fluorescence values of NADH over FAD images voxel by voxel. Representative images of NADH and FAD intensity are provided in Supplementary Figure S2.

2.6 Glucose tolerance test

Mice were fasted for 6-h with continuous access to water before the glucose load (i.p. bolus of 1.5 mg/kg body weight). Glucose levels were determined from a small drop of blood collected from the tail using a commercially available glucometer (AlphaTRAK®, Zoetis, United Kingdom). Samples were collected before and 15, 30, 60, 90 and 120 min after glucose administration.

2.7 Pathology and biochemical analysis

At endpoint, blood and major organs were collected for pathology analysis from all experimental groups. Tissues were macroscopically examined for visible signs of disease and organ weight was recorded. Blood was collected by cardiac puncture from fasted (approximately 6-h) and non-fasted animals. Plasma and serum were separated from blood for analysis of insulin and leptin levels using commercially available ELISA kits (#EZRMI and #RAB0334, Sigma-Aldrich Inc., St Louis, MO) per manufacture instructions.

2.8 Endothelial cell sorting and c-Myc expression analysis

Endothelial cells were magnetically sorted from hearts harvested from CT and EC-Myc OE mice based on CD31 expression using commercially available kits and instrument (Miltenyi Biotec Inc., Gaithersburg, MD). Briefly, harvested hearts were minced and dissociated with a mixture of enzymes (Multi Tissue Dissociation Kit 2, #130-110-203) using the gentleMACS Octo Dissociator system. After dissociation, cell suspension was cleared of debris (Debris Removal Solution, #130-109-398) and incubated with CD45 microbeads (#130-052-301) to exclude inflammatory cells, which may also express CD31. The CD45 depleted cell suspension was then incubated with CD31 microbeads (#130-097-418) for final sorting of endothelial cells. All steps followed manufacturer's instructions for each specific kit used. RNA was extracted from sorted endothelial cells for analysis of mouse and human c-Myc by qPCR using Taqman probes (#Mm00487804_m1 and #Hs99999003_m1, respectively) per manufacture instructions (Life Technologies Corp, Carlsbad, CA).

2.9 Gene expression analysis by RNA-sequencing

RNA was extracted from soleus and gastrocnemius muscle harvested from CT and EC-Myc OE mice using TRI-reagent

(#TR118, Molecular Resource Center Inc., Cincinnati, OH) per manufacturer's instructions. RNA concentration was determined using a NanoDrop spectrophotometer (Life Technologies Corp, Grand Island, NY) and samples were outsourced for library preparation, sequencing, and bioinformatics analysis (Novogene Corporation, Inc. Sacramento, CA). Prior to library preparation, RNA integrity was evaluated using an Agilent BioAnalyzer 2100 (Agilent Technologies), and messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection.

2.10 Statistical analysis

We used SigmaPlot (Inpixon) and Prism (GraphPad) Software for all graphs and statistical analysis. Sample numbers are indicated in all figure legends and p-values <0.05 were considered significant. For comparison between two groups, we performed Student's t-test and considered two-tailed p-values. When samples did not pass the normality and variance tests, Welch's t-test was used as an alternative. For comparison between four experimental groups, we performed two-way ANOVA. Holm-Sidak test was used for multiple comparison analysis. Tukey HSD tests were performed as a *post hoc* test to identify significant differences between individual groups. Two-way RM ANOVA was used for the interaction effect of the genotype with one continuous dependent variable. Individual animals are represented by dots in all graphs. Bars indicate the standard deviation unless otherwise stated.

3 Results

3.1 Endothelial c-Myc knockout impairs vasorelaxation

Endothelial dysfunction is an early predictor of multiple pathological conditions. Little is known regarding early mechanisms that trigger endothelial dysfunction. Previous studies from our group have shown that the transcription factor c-Myc plays an important role in endothelial homeostasis and regulation of inflammation (Florea et al., 2013; Qi et al., 2022). These findings propelled us to investigate if c-Myc contributes to endothelial dysfunction ultimately impacting overall animal health. To confirm the association between c-Myc deficiency and endothelial dysfunction, we performed vasoactive response studies as impaired vasorelaxation is one of the first signs of dysfunctional endothelium. Mesenteric arteries of control (CT) and endothelial c-Myc knockout (EC-Myc KO) mice were harvested and tested for acetylcholine-induced vasorelaxation after constriction with norepinephrin. Our results show that loss of endothelial c-Myc significantly impacted vasorelaxation in response to acetylcholine relative to control. At the highest dose of acetylcholine tested (10^{-5} M), the ability of EC-Myc KO mesenteric arteries to relax was reduced by 29% relative to CT mice (58.2% ± 12.5% vs. 81.9% ± 5.6%, p = 0.031) (Figure 1).

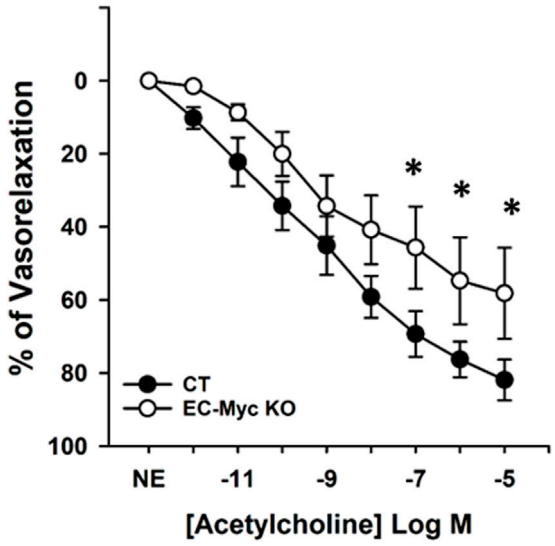

FIGURE 1
Vasoactive response of mesenteric artery from control and endothelial c-Myc knockout mice. Curves indicate vasorelaxation response to acetylcholine 1 month after induction of endothelial c-Myc knockout in male mice. Results are expressed as percentage of vasorelaxation and represent the mean ± standard error for individual concentrations of acetylcholine. Black and white circles represent control and knockout mice, respectively. CT, Control (n = 4); EC-Myc KO, Endothelial c-Myc Knockout (n = 4); NE, Norepinephrine. *p < 0.05.

3.2 Endothelial c-Myc knockout increases body weight and adiposity

We performed longitudinal analysis of CT and EC-Myc KO mice over a period of 16 months and found an expected increase in body weight over time, although findings were more pronounced in knockout animals relative to control (Figure 2A). At endpoint, body weight was significantly increased in EC-Myc KO females (31.67 ± 1.35 vs. 27.13 ± 0.61 g, p = 0.004) and males (44.2 ± 2.30 vs. 38.0 ± 1.03 g, p = 0.01) relative to CT (Figure 2B). When compared side by side, EC-Myc KO mice looked bigger in size than CT (Figure 2C).

Analysis of white adipose tissue (WAT) at endpoint in EC-Myc KO mice showed a significant increase by 76% in females (1.52 ± 0.20 vs. 0.86 ± 0.12, p = 0.007) and a trend increase by approximately 22% in males (2.74 ± 0.29 vs. 2.24 ± 0.22 g, not significant) (Figure 3A). Based on the observed increase in white adipose tissue (WAT) accumulation, in endothelial c-Myc deficient animals, we analyzed the circulating levels of leptin, which is expected to correlate with changes in adipocyte mass (Kiernan and MacIver, 2020). Our results showed a significant increase of approximately 47.98% in serum leptin in EC-Myc KO animals relative to CT (163.72 ± 21.48 vs. 110.64 ± 13.17 pg/mL) (Figure 3B). Representative images of fat deposits from CT and EC-Myc KO mice are shown in Figure 3C. Morphometric analysis of WAT from male and female EC-Myc KO animals revealed some sex-specific differences relative to CT (Supplementary Table S1). In EC-Myc KO females, we found a significant increase of 54% in the estimated number of adipocytes relative to CT (5.93 ± 0.75 vs. 3.85 ±

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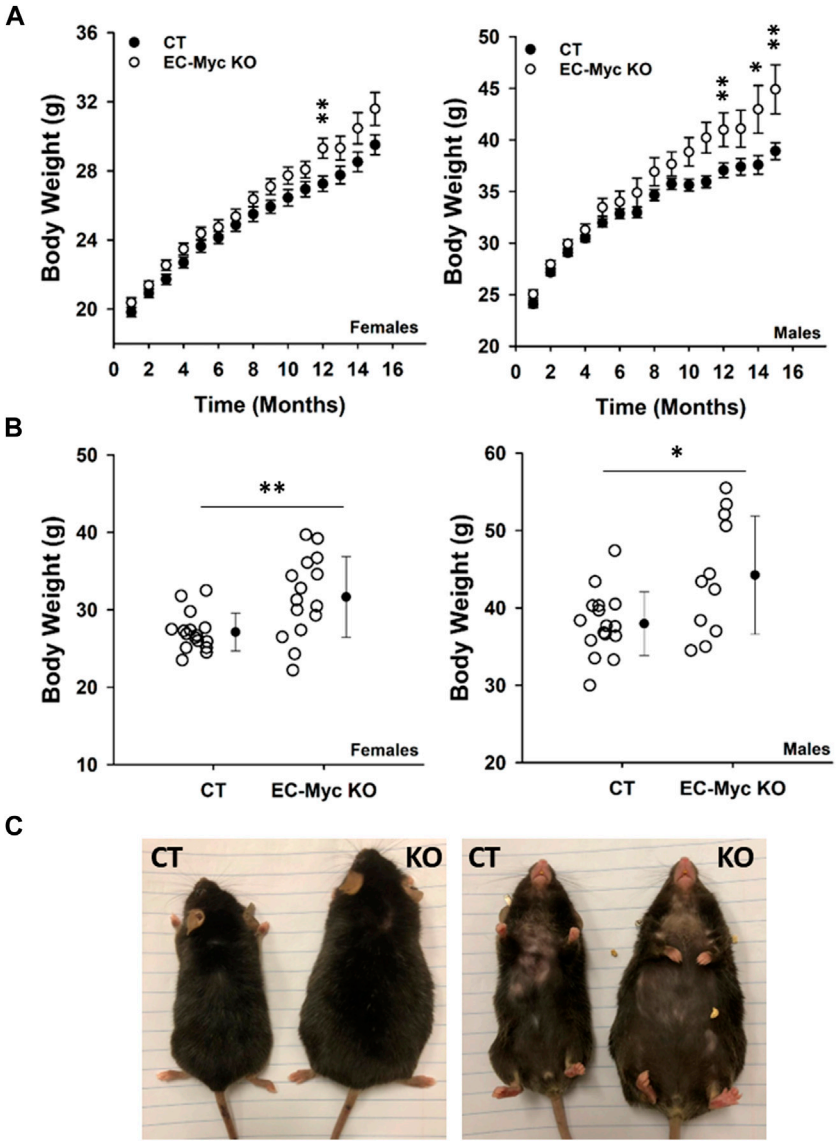

FIGURE 2
Body weight analysis of control and endothelial c-Myc knockout mice during aging. **(A)** Longitudinal analysis of body weight in male and female mice. Black and white circles represent control and knockout mice, respectively. Results represent the mean ± standard error **(B)** Analysis of body weight at 16-month endpoint. Dots represent individual animals and filled circles represent the mean ± standard deviation. **(C)** Representative dorsal and ventral images of males showing size differences between experimental groups. CT, Control (n = 19–65); EC-Myc KO, Endothelial c-Myc Knockout (n = 17–49). *p < 0.02, **p < 0.002.

0.40, p = 0.025), but no changes in other parameters (Figure 3D). In males, the only significant difference between CT and EC-Myc KO was in the frequency of adipocyte size. EC-Myc KO males showed a higher percentage of very large-sized adipocytes relative to small sizes, while no differences were found in CT (Figure 3E).

3.3 Endothelial c-Myc knockout reduces metabolic activity

Our observations described above suggest that loss of endothelial c-Myc causes an imbalance in energy metabolism. We analyzed food intake in CT and EC-Myc KO mice prior to the onset of weight gain and did not find any significant differences (data not shown). In the absence of alterations in calorie intake, one possible explanation for the observed increase in body weight in EC-Myc KO mice is a decrease in energy expenditure. Accordingly, we performed a series of metabolic studies with CT and EC-Myc KO mice by indirect calorimetry and found interesting sex-related differences between experimental groups. Similar results were found in dark and light periods (Supplementary Figure S3). A summary of all metabolic parameters is presented in Supplementary Table S2. In males, we found a significant increase in respiratory exchange ratio in EC-Myc KO relative to CT (0.91 ± 0.01 vs. 0.86 ± 0.02 RER, p = 0.015), without significant changes in other metabolic parameters

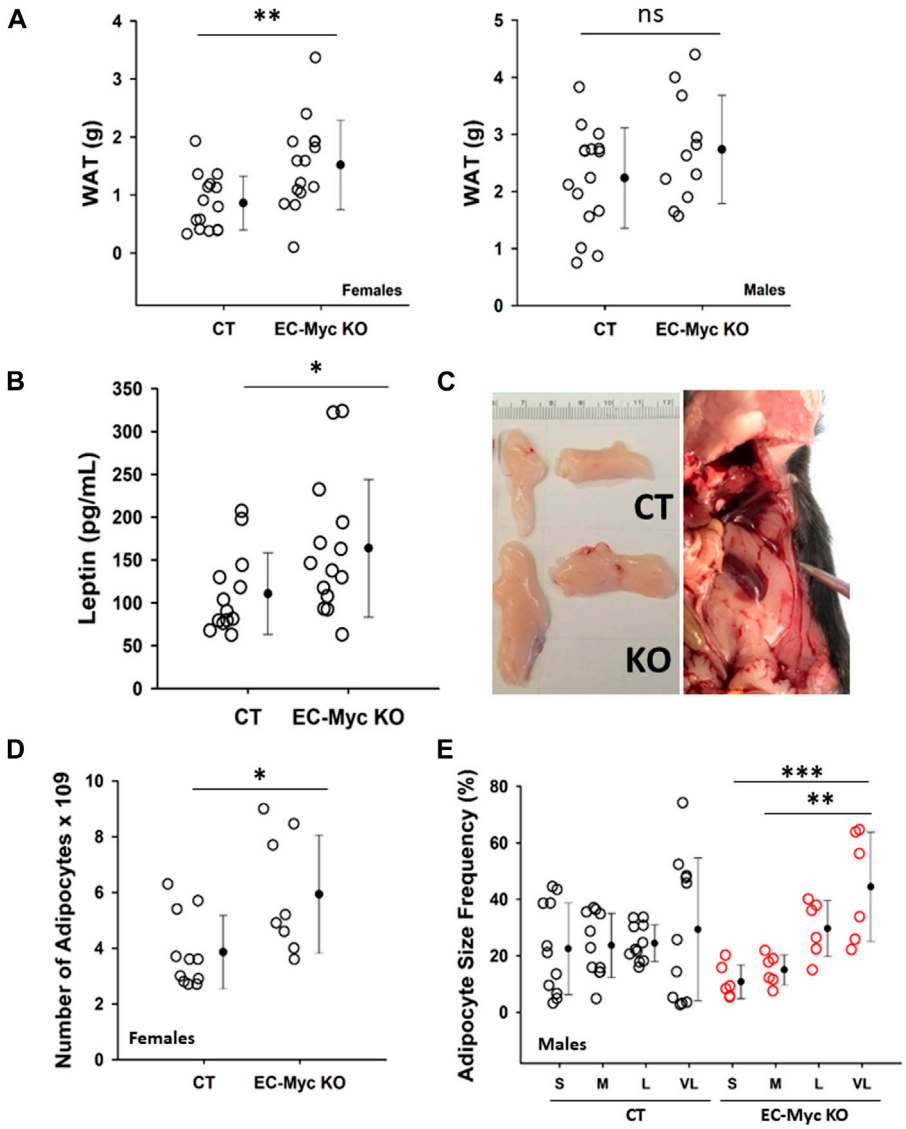

FIGURE 3
Analysis of white adipose tissue in control and endothelial c-Myc knockout mice. **(A)** Quantification of white adipose tissue mass. **(B)** Quantification of leptin levels. **(C)** Representative images of fat deposits. The image on the right corresponds to an extreme case of increased adiposity in knockout mice. **(D)** Morphometric analysis of white adipose tissue showing an increase in the number of adipocytes in female knockout mice. **(E)** Morphometric analysis of white adipose tissue showing significant difference in adipocyte size distribution in male knockout mice. Dots represent individual animals and filled circles represent the mean ± standard deviation. CT, Control (n = 10–16); EC-Myc KO, Endothelial c-Myc knockout (n = 5–15); S, small; M, medium; L, large; VL, very large; ns, non-significant. *p < 0.05, **p < 0.01, ***p < 0.005.

(Figure 4D). However, in females, we found a significant decrease in heat production (0.39 ± 0.01 vs. 0.44 ± 0.03 kcal/kg/h, dark period p = 0.04) (Figure 4A), which was accompanied by a decrease in VO_2 (3203 ± 61 vs. 3687 ± 187 mL/kg/h, p = 0.007) (Figure 4B) and CO_2 (2791 ± 62 vs. 3241 ± 199 mL/kg/h, p = 0.01) (Figure 4C). No significant differences in RER were found in females (Figure 4D).

To further explore the impact of endothelial c-Myc loss in energy metabolism, we performed analysis of redox ratio in skeletal muscle harvested from CT and EC-Myc KO animals. Our results indicated a significant reduction of 51% in the mitochondrial redox ratio in EC-Myc KO relative to CT animals (1.53 ± 0.17 vs. 2.32 ± 0.2), which suggests a decrease in cellular energy production (Figure 5).

3.4 Overexpression of c-Myc in endothelial cells attenuates visceral fat accumulation and prevents systemic leptin release induced by western diet exposure

Our results described above indicate an important role for endothelial c-Myc in the maintenance of metabolic homeostasis. As such, we hypothesized that overexpression of c-Myc in endothelial cells would protect animals from developing overweight and obesity, as well as associated complications such as glucose intolerance and cardiovascular disease. To test this hypothesis, we performed a series of experiments in which we challenged control (CT) and endothelial c-Myc overexpression (EC-Myc OE) mice with a western-style high-fat

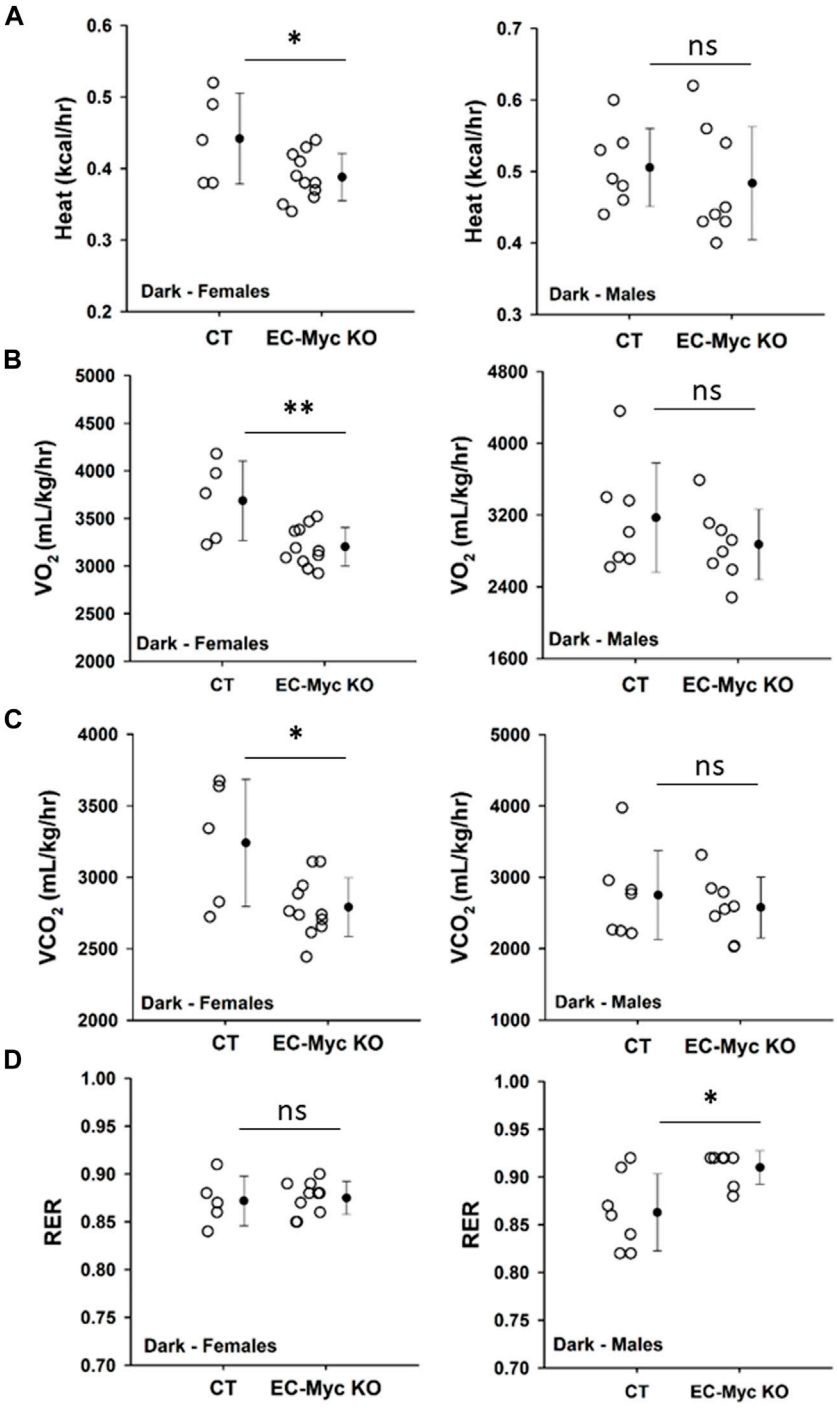

FIGURE 4
Metabolic phenotype of control and endothelial c-Myc knockout mice by indirect calorimetry. **(A)** Heat production. **(B)** Volume of oxygen consumed (VO_2). **(C)** Volume of carbon dioxide release (VCO_2). **(D)** Respiratory exchange ratio (RER). Dots represent individual animals and filled circles represent the mean ± standard deviation. CT, Control (n = 5–7); EC-Myc KO, Endothelial c-Myc Knockout (n = 7–11); ns, non-significant. *$p < 0.05$, **$p < 0.01$.

diet (WD) over a period of 20 weeks. Exposure to WD promoted a gradual increase in body weight in both CT and EC-Myc OE mice (Figure 6A). This effect was significantly attenuated around 5 weeks post-exposure in EC-Myc OE relative to CT (31.10 ± 0.55 vs. 33.03 ± 0.83 g, $p = 0.035$) (Figure 6B). At 10 weeks post-exposure, the difference in body weight between both groups was lost. However, analysis of fat deposits revealed significant attenuation by 22% in visceral adipose tissue accumulation in EC-Myc OE mice relative to CT (408 ± 26.3 vs. 522 ± 43.9 mg, $p = 0.028$) (Figure 6C). No significant differences were observed in epididymal and brown adipose tissue.

Quantification of systemic leptin levels showed a significant increase in CT animals under WD exposure relative to the control

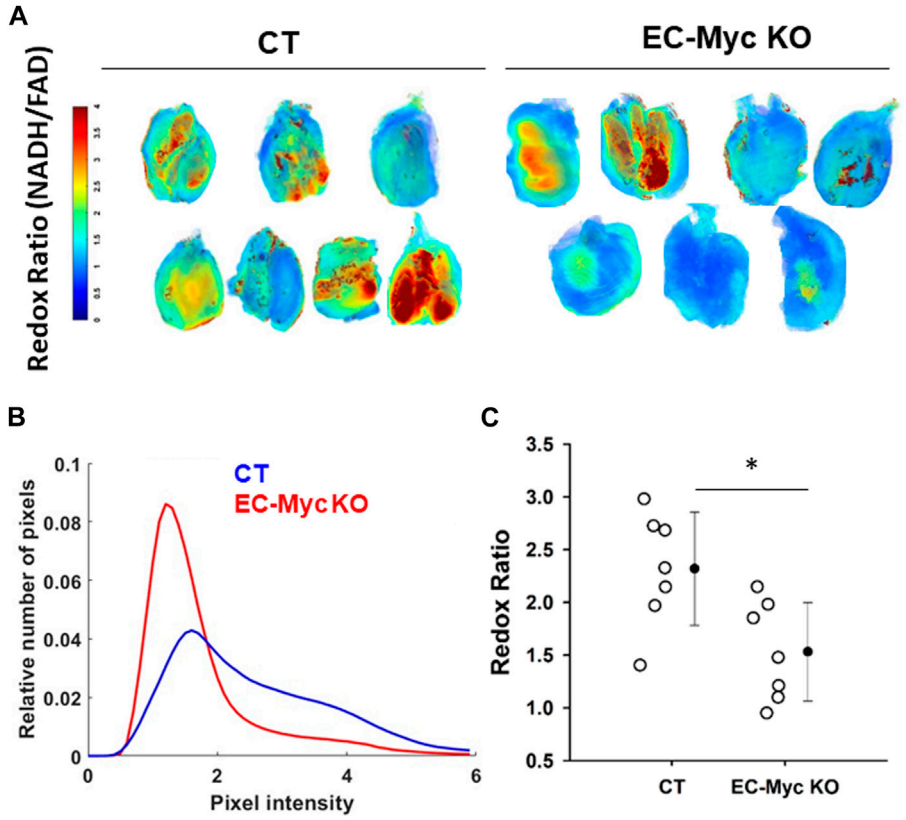

FIGURE 5
Analysis of redox ratio in control and endothelial c-Myc knockout skeletal muscle. **(A)** Normalized intensities of redox ratio. For each group, seven representative muscle images are presented. **(B)** Histogram plot showing decrease in redox ratio in skeletal muscle of endothelial c-Myc knockout mice relative to control. **(C)** Average redox ratio of individual animals. Dots represent individual animals and filled circles represent the mean ± standard deviation. CT, Control (n = 7); EC-Myc KO, Endothelial c-Myc knockout (n = 7). *p < 0.05.

diet (48.98 ± 7.85 vs. 5.39 ± 2.12 pg/mL, p < 0.001), while the response was prevented in EC-Myc OE mice (12.46 ± 3.13 vs. 2.47 ± 0.23 pg/mL, not significant). Our findings revealed significant differences between EC-Myc OE and CT under WD exposure (12.46 ± 3.13 vs. 48.98 ± 7.85 pg/mL, p < 0.001) (Figure 6D).

3.5 Overexpression of c-Myc in endothelial cells prevents the development of western diet-induced glucose intolerance

One of the major complications associated with obesity is the development of insulin resistance. CT and EC-Myc OE mice have similar fasting glucose levels under normal diet conditions. Exposure to WD for 5 weeks was sufficient to significantly raise basal glucose levels in both CT (164 ± 8 vs. 129 ± 13 mg/dL, p = 0.013) and EC-Myc OE (147 ± 8 vs. 121 ± 7 mg/dL, p = 0.048) mice relative to the control diet. However, after 10-weeks of exposure to WD, although basal glucose further increased in CT animals relative to control diet (184 ± 15 vs. 130 ± 5 mg/dL, p = 0.001), it remained the same in EC-Myc OE mice (148 ± 6 vs. 113 ± 8 mg/dL, p = 0.02). Our findings indicated significant differences in basal glucose between EC-Myc OE and CT mice in response to long-term exposure to WD (148 ± 6 vs. 184 ± 15 mg/dL, p = 0.014) (Figure 7A).

We performed a glucose tolerance test (GTT) in all experimental groups to account for the development of glucose intolerance. Under the WD diet, the time to glucose peak in CT was longer than in EC-Myc OE (60 vs. 30 min). After 120 min, the level of glucose in EC-Myc OE mice under WD was almost completely back to baseline (211.00 ± 92.25 vs. 161.11 ± 43.44 mg/dL, 30% above baseline), while in CT animals, it remained significantly elevated (402.88 ± 121.50 vs. 183.83 ± 42.64 mg/dL, 120% above baseline) (Figure 7B).

At the 10-weeks endpoint, we measured systemic insulin levels and found a significant increase in CT animals exposed to WD relative to control diet (2.51 ± 2.18 vs. 0.44 ± 0.26 ng/mL, p = 0.001), which was significantly attenuated in EC-Myc OE animals (1.12 ± 0.38 vs. 0.48 ± 0.18 ng/mL, not significant). Our findings indicated significant differences in insulin levels between EC-Myc OE and CT mice in response to WD (1.12 ± 0.13 vs. 2.51 ± 0.77 ng/mL, p = 0.019) (Figure 7C).

3.6 Transcriptome analysis of skeletal muscle revealed significant differences between control and endothelial c-Myc overexpression mice in response to western diet exposure

The skeletal muscle plays an important role in energy metabolism (Mengeste et al., 2021). Based on our findings

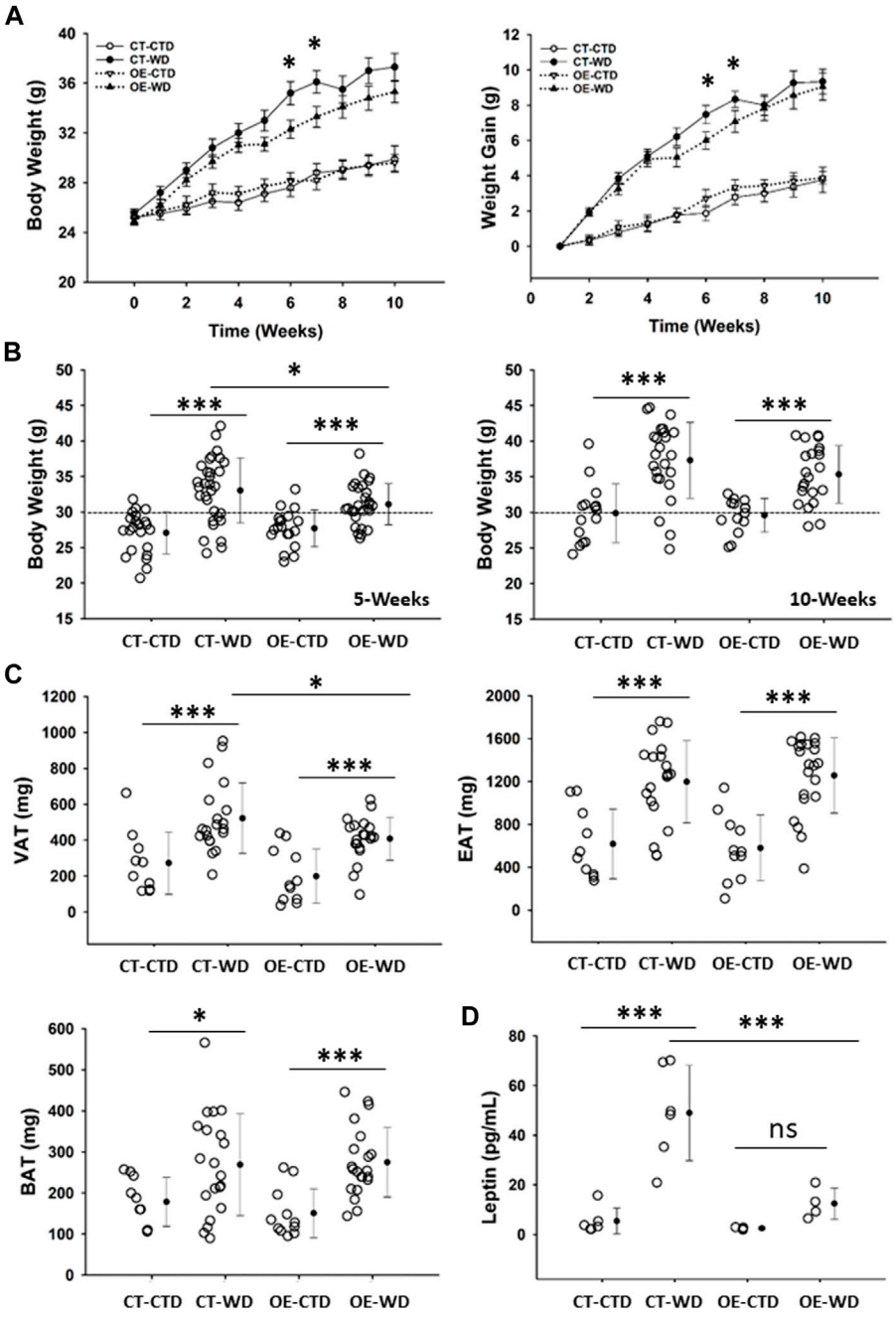

FIGURE 6
Gross phenotype analysis of control and endothelial c-Myc overexpression mice under exposure to western diet. **(A)** Longitudinal analysis of body weight and weight gain for a total period of 10 weeks. White and black symbols represent animals exposed to control and western diet, respectively. **(B)** Analysis of body weight at 5- and 10-weeks endpoints. **(C)** Quantification of visceral (VAT), epididymal (EAT) and brown (BAT) adipose tissue mass at 10-weeks endpoint. **(D)** Quantification of systemic leptin levels at 10-weeks endpoint. In A, results are represented as mean ± standard error. In all other graphs, dots represent individual animals, filled circles represent the mean ± standard deviation. CT, Control; OE, Endothelial c-Myc overexpression; CTD, control diet (n = 14–22); WD, western diet (n = 22–31); ns, non-significant. (*p < 0.05, ***p < 0.001).

suggesting that endothelial c-Myc overexpression attenuates visceral fat accumulation and insulin resistance, we performed transcriptome analysis of skeletal muscle harvested from CT and EC-Myc OE mice. Exposure to WD for 10 weeks had a significant impact on the gene expression profile of both CT (1016 genes altered >1.5-fold) and EC-Myc OE mice (666 genes altered >1.5-fold) relative to control diet. Venn diagram analysis showed that both experimental groups shared common targets altered by diet exposure (243 genes) and that each experimental group had their own exclusive list of altered genes. It was noticeable that the number of genes in CT was almost double of what was found in EC-Myc OE (709 vs. 422 genes) (Supplementary Figure S4).

Comparison of the transcriptome profiles of WD-treated groups showed a total of 207 genes (128-up and 79-down)

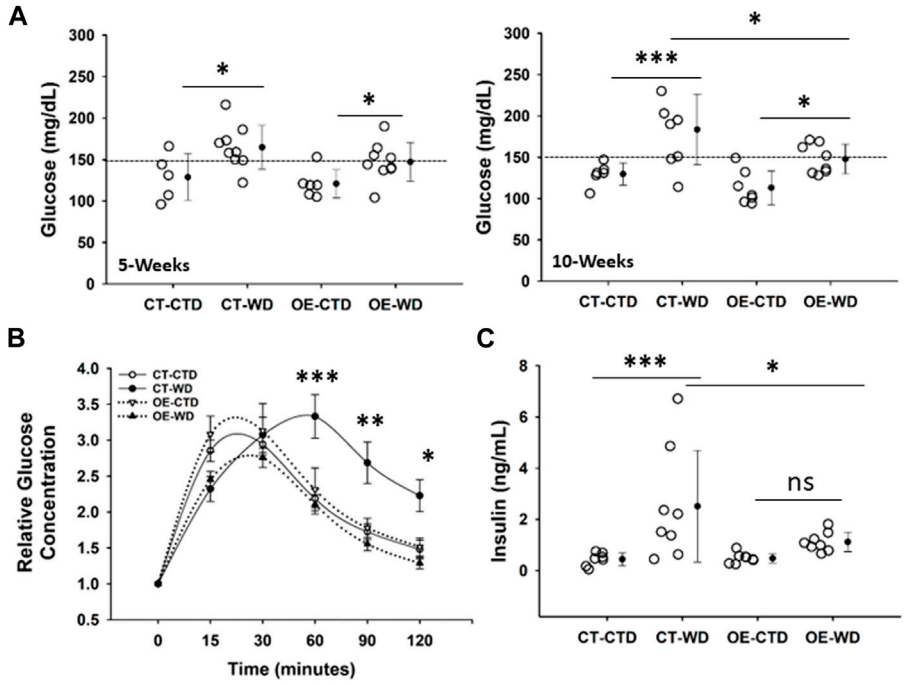

FIGURE 7
Analysis of glucose tolerance in control and endothelial c-Myc overexpression mice under exposure to western diet. **(A)** Basal glucose levels at 5- and 10-weeks. **(B)** Glucose tolerance test at 10-weeks. White and black symbols represent animals exposed to control and western diet, respectively. **(C)** Quantification of systemic insulin levels at 10-weeks endpoint. In B, results are expressed as fold change relative to baseline and represent the mean ± standard error. In all other graphs, dots represent individual animals and filled circles represent the mean ± standard deviation. CT, Control; OE, Endothelial c-Myc overexpression; CTD, control diet (n = 6–7); WD, western diet (8-9). *p < 0.05, **p < 0.005, ***p < 0.001.

significantly altered >1.5-fold in EC-Myc OE (OE-WD) relative to CT (CT-WD). Among the most significantly altered targets, we identified *Zbtb16* (2.35-fold, $p = 1.39 \times 10^{-19}$, *Acsl3* (1.56-fold, $p = 2.4 \times 10^{-10}$) and *Rabif* (2.04-fold, $p = 2.36 \times 10^{-07}$) as the top upregulated genes, and *Rrad* (−1.57-fold, $p = 1.61 \times 10^{-5}$) as the top downregulated. The top canonical pathways identified by Ingenuity software analysis that are affected by endothelial c-Myc expression are shown in Supplementary Figure S5. The S100 Family Signaling was among the top 5, comprising up- and downregulated genes. Some of the targets in this pathway include *S100a3* (6.9-fold, $p = 0.009$, *S100a14* (2.6-fold, $p = 0.043$) and *Wnt10a* (3.96-fold, $p = 0.002$).

We next analyzed the transcriptome profile associated with the exclusive response of EC-Myc OE and CT to WD relative to control diet (CTD). Pathway Analysis of upregulated genes differentially expressed showed significant increase in targets associated with extracellular matrix organization, collagen metabolism and organization for both groups. Analysis of downregulated genes revealed interesting differences between groups, including pathways associated with metabolism (Supplementary Figure S6). We focused our analysis on functions specifically related to metabolic disease based on our physiological and pathological findings. Both experimental groups showed changes in the expression of genes associated with diabetes, obesity, insulin resistance/sensitivity, glucose metabolism disorders, weight gain and energy homeostasis. However, the CT group showed a much higher number of genes altered under these function categories than EC-Myc OE (Figure 8). Interestingly, our analysis revealed other metabolic functions for EC-Myc OE that could account for the beneficial effects we observed. Some of the genes differentially expressed EC-Myc OE muscle have been related to weight loss, energy expenditure and glucose tolerance. Among the genes under these exclusive categories, we identified *Socs3* (−1.83-fold, $p = 0.009$) as a common target in multiple pathways.

3.7 Endothelial c-Myc overexpression prevents western diet-induced cardiovascular dysfunction and remodeling

Obesity is a major risk factor associated with the development of cardiovascular disease. Based on the protective effect of endothelial c-Myc overexpression described above, we performed cardiovascular assessment of animals exposed to control and WD for 18 weeks by echocardiography. It is noticeable from looking at our data that endothelial c-Myc overexpression prevents several functional and structural diet-induced alterations observed in controls. CT animals showed an increase in myocardial performance index (MPI) relative to those fed control diet (0.54 ± 0.08 vs. 0.39 ± 0.04, $p = 0.008$), which was related to an increase in isovolumetric contraction time (IVCT) (10.56 ± 1.73 vs. 6.25 ± 1.10 m, $p = 0.007$). No significant changes in isovolumetric contraction time (IVRT) were observed. In addition, CT mice showed an increase in E/A ratio (3.99 ± 1.15 vs. 1.31 ± 0.13, $p = 0.02$). Interestingly, no significant changes in functional parameters were found in EC-Myc OE mice exposed to WD. At structural level, WD induced a significant increase

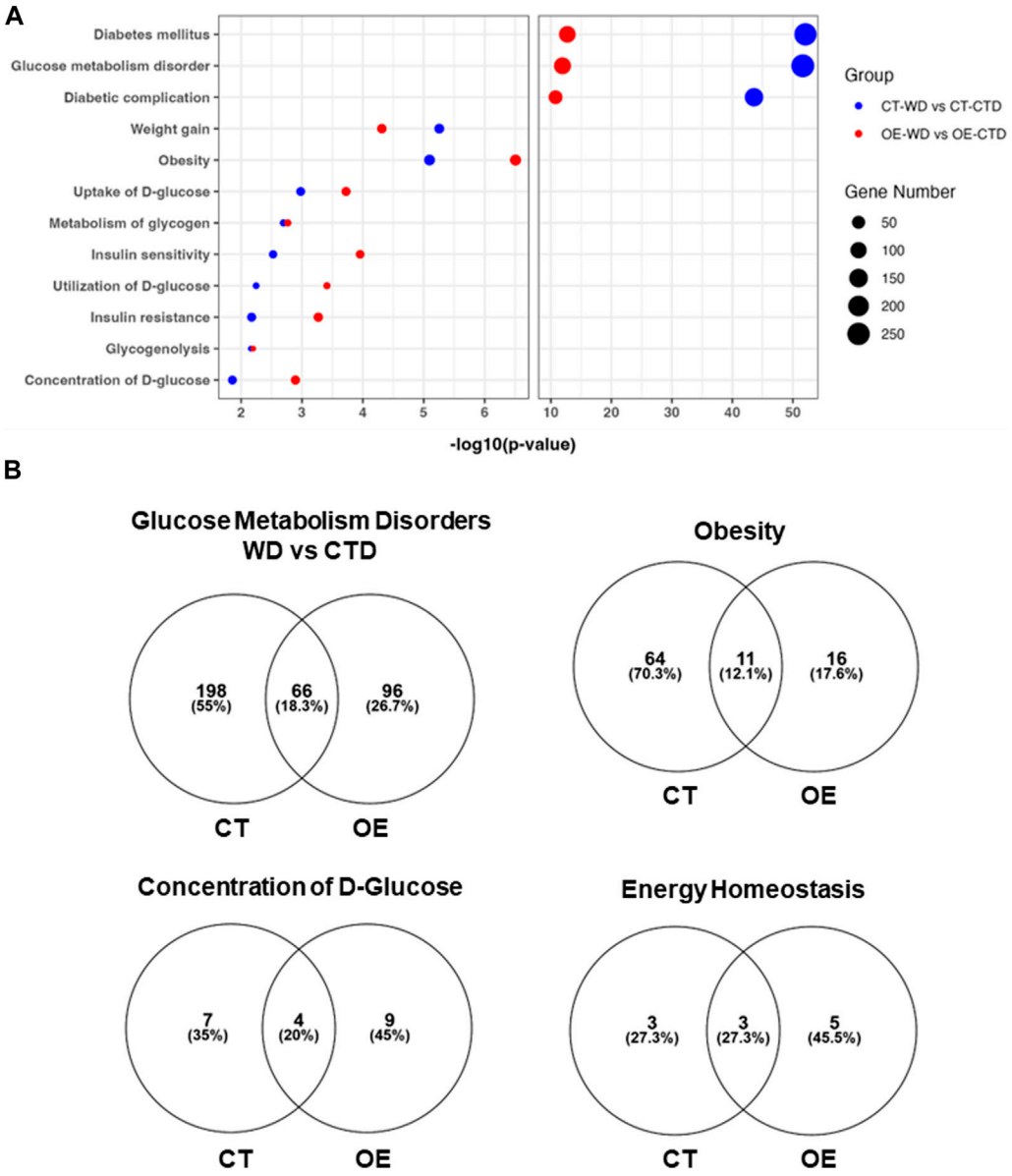

FIGURE 8
Transcriptome analysis of skeletal muscle from control and endothelial c-Myc overexpression mice. **(A)** Top metabolic-related diseases and functions affected by western diet exposure in control and endothelial c-Myc overexpression mice relative to control diet. **(B)** Venn diagram analysis showing the number of gene targets altered by western diet exposure in each metabolism-related disease or function. CT, Control; OE, Endothelial c-Myc overexpression; CTD, control diet (n = 3); WD, western diet (n = 4).

in CT left ventricular mass (154.71 ± 12.50 vs. 114.12 ± 13.07 mg, $p < 0.001$), whereas no changes were found in EC-Myc OE (Figure 9). However, significant changes in wall thickness were found in both CT and EC-Myc OE mice after exposure to WD. We observed that EC-Myc OE mice under normal diet showed some baseline changes relative to CT, such as an increased fractional shortening (41.82% ± 1.78% vs. 32.64% ± 4.16%, p = 0.008), a reduced systolic diameter (1.94 ± 0.15 vs. 2.56 ± 0.39, p = 0.025) and increased thickness of the left ventricle posterior wall during systole (1.73 ± 0.18 vs. 1.30 ± 0.28 mm, p = 0.014) and diastole (1.33 ± 0.12 vs. 0.91 ± 0.25 mm, p = 0.008). A summary of all cardiac parameters is presented in Supplementary Table S3).

4 Discussion

In this study, we aimed to elucidate the role of endothelial c-Myc in metabolic homeostasis. Our findings underscore a novel endothelial-mediated mechanism associated with the maintenance of metabolic and cardiovascular health regulated by c-Myc.

Aging and exposure to stress factors have been reported to cause endothelial dysfunction, which is an early predictor of multiple pathological conditions, including obesity, diabetes, and cardiovascular disease (Engin, 2017; Donato et al., 2018; Haybar et al., 2019; Kajikawa and Higashi, 2022). However, recognizing endothelial dysfunction as a cause or an effect in disease

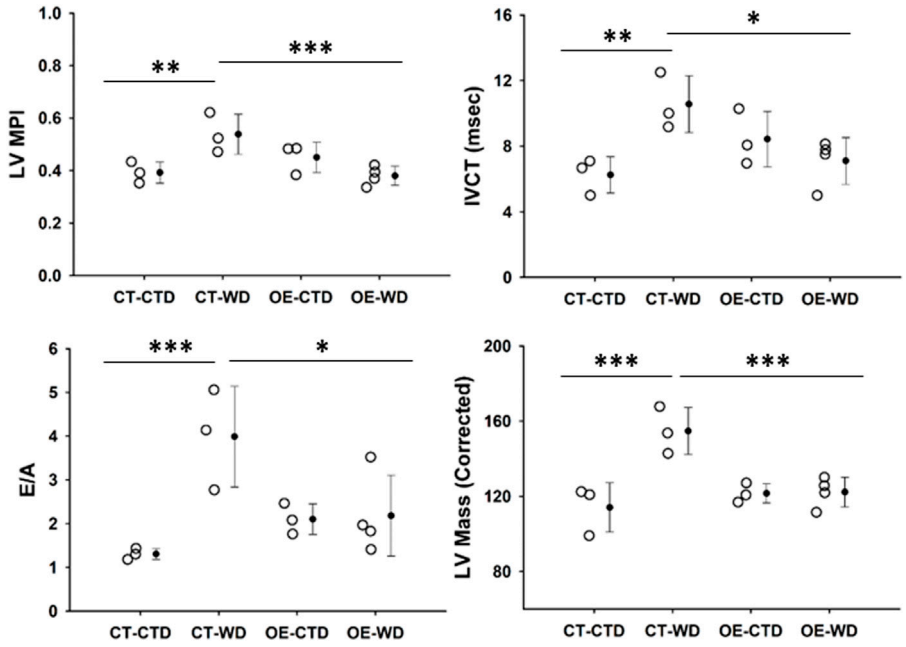

FIGURE 9
Transthoracic echocardiography analysis of control and endothelial c-Myc overexpression mice. Animals were analyzed after 18-weeks under control and western diet. **(A)** Myocardial performance index (MPI). **(B)** Isovolumetric contraction time (IVCT). **(C)** E/A ratio. **(D)** Left ventricular mass. Dots represent individual animals and filled circles represent the mean ± standard deviation. LV, Left ventricle; CT, Control; OE, Endothelial c-Myc overexpression; CTD, control diet (n = 3–4); WD, western diet (n = 3–4). *p < 0.05, **p < 0.01, ***p < 0.005.

conditions needs further investigation. Endothelial cells play a fundamental role in maintaining vascular tone, and impaired vasorelaxation is a primary sign of endothelial dysfunction (Donato et al., 2018). Our results show that depletion of c-Myc in endothelial cells is sufficient to decrease acetylcholine-induced vasorelaxation, supporting previous studies that this transcription factor is essential for maintenance of endothelial function (Baudino et al., 2002; He et al., 2008; Rodrigues et al., 2008; Kokai et al., 2009; Hurley et al., 2010; Florea et al., 2013; Qi et al., 2022).

A key finding of our study is the link between endothelial c-Myc loss and metabolic disturbances. We observed an age-dependent increase in body weight and adiposity with c-Myc depletion from the endothelium that was associated with significant decrease in metabolic parameters supporting a novel essential role for endothelial c-Myc in regulating energy metabolism. Importantly, these findings were backed up by our results on endothelial c-Myc overexpression in the context of obesity, which attenuates visceral fat accumulation and prevents insulin resistance and cardiac dysfunction. The relevance of c-Myc for metabolic homeostasis has been reported, but mostly on non-endothelial cell types. Contrarily to our findings, global c-Myc haploinsufficiency has been related to high metabolic rate without changes in adipose tissue mass relative to controls during aging (Hofmann et al., 2015). Exposure to high-fat diet leads to upregulation of c-Myc expression in adipose tissue and intestines (Liu et al., 2017; Luo et al., 2021). Overexpression of c-Myc in β-cells has been associated with the development of diabetes (Laybutt et al., 2002; Cheung et al., 2010), while reduction in c-Myc expression in intestinal cells was shown to improve high-fat-diet-induced obesity and insulin resistance (Luo et al., 2021). Despite these contradictory findings, which would be difficult to reconcile considering the differences in experimental models used, other work supports a protective role for c-Myc as we observed. Multiple lines of evidence suggest that some increment in c-Myc levels is likely beneficial. Recently, we have shown that loss of c-Myc endothelial cells leads to liver fibrosis (Qi et al., 2022). In hepatocytes, c-Myc is essential to drive proliferation during liver regeneration (Zhang et al., 2018; Wang et al., 2022), and overexpression of c-Myc in the liver has been shown to prevent obesity and insulin resistance (Riu et al., 2002; Riu et al., 2003). In pancreatic β-cells, c-Myc has been shown to promote proliferation as part of an adaptation response to glucose exposure (Puri et al., 2018; Rosselot et al., 2019). Treatment of β-cells the small molecule harmine promotes mitogenesis through a mild increase in c-Myc expression (Wang et al., 2015), suggesting the potential targeting of c-Myc in diabetes to improve insulin production.

Whole body metabolism involves crosstalk between multiple organ systems and endothelial cells serve as the interface of this communication, transmitting signals that will impact tissue response according to environmental cues (Castillo-Armengol et al., 2019; Katagiri, 2023). The most evident effect we observed upon knockout of endothelial c-Myc was an increase in adiposity. Our findings were associated with a raise in leptin levels, which is mostly produced by adipose tissue (Kiernan and MacIver, 2020). Although we observed a significant increase in adiposity in male and female EC-Myc-KO mice relative to CT, we found interesting sex-specific differences in the mechanisms associated with adipose tissue expansion. Sex-related differences in adipose tissue distribution have been reported and seem to play a role in the development of obesity and type-2 diabetes

(Tchoukalova et al., 2010a; Gavin and Bessesen, 2020). However, the mechanisms involved are not fully understood. The expansion of adipose tissue can be driven by the formation of new adipocytes (hyperplasia), increase in lipid storage (hypertrophy) and/or reduced lipid breakdown (Li and Spalding, 2022). In EC-Myc KO males, even though we did not observe changes in adipocyte number, we found significant alteration in adipocyte size frequency, with higher accumulation of very large adipocytes, suggesting a hypertrophic mechanism. On the other hand, EC-Myc KO females showed an increase in adipocyte numbers, suggesting a hyperplasia response. These findings are supported by previous studies in humans, indicating that adipose tissue in males involves adipocyte hypertrophy and in females, hyperplasia (Tchoukalova et al., 2008; Tchoukalova et al., 2010b). Our findings suggest a novel endothelial-mediated mechanism driven by c-Myc in adipose tissue morphogenesis.

The skeletal muscle plays an important role in energy metabolism and the crosstalk between endothelial and skeletal muscle cells is essential for energy balance, including insulin-dependent glucose metabolism (Mengeste et al., 2021; Pepe and Albrecht, 2023). One of the most remarkable results from our study was the prevention of glucose intolerance by endothelial c-Myc overexpression in response to high-fat diet exposure. Transcriptome analysis of skeletal muscle provided us with important clues regarding potential mechanisms by which endothelial c-Myc promotes metabolic homeostasis. Pathway analysis of transcriptome data showed that CT and EC-Myc OE share multiple biological and disease functions relevant for metabolic homeostasis, but significant differences were found in the number of genes altered and the identity of specific targets. Importantly, we found an enrichment for weight loss, energy expenditure and glucose tolerance functions exclusively in EC-Myc OE. Among potential downstream targets altered in skeletal muscle of EC-Myc OE that could account for protective results, we found *Socs3*, *Foxo1 and Angptl4*. *Socs3* was common to all the biological and disease functions enriched in our data and downregulated in EC-Myc KO skeletal muscle. Exposure to high-fat diet has been shown to induce SOCS3 expression in skeletal muscle and liver and proposed to act as negative regulator of insulin signaling (Ueki et al., 2004). Skeletal muscle specific deletion of SOCS3 protects mice from insulin resistance induced by high-fat diet exposure (Jorgensen et al., 2013), while overexpression impaired glucose homeostasis (Yang et al., 2012). The expression of *Foxo1* was downregulated in EC-Myc OE muscle. Previous studies have shown that overexpression of FoxO1 in skeletal muscle is associated with insulin resistance and glucose intolerance (Kamei et al., 2004; Teaney and Cyr, 2023). Inhibition of FoxO1 has shown positive effects on glucose homeostasis in experimental models of diabetes (Li et al., 2019; Mao et al., 2021) supporting its potential targeting. ANGPTL4, an adipokine mainly secreted by adipose tissue and liver is involved in lipid metabolism, glucose homeostasis, inflammation and angiogenesis (Xu et al., 2005; Cinkajzlova et al., 2018). Increase in *Angptl4* expression has been reported in skeletal muscle and associated with exercise and exposure to fatty acids, where it may be part of an adaptive response to physical activity (Staiger et al., 2009; Raschke and Eckel, 2013; Sabaratnam et al., 2018). We found the *Angptl4* expression was downregulated in EC-Myc OE muscle. This finding aligns with previous studies where genetic inactivation of ANGPTL4 was associated with improved insulin sensitivity and reduced risk of Type 2 diabetes (Gusarova et al., 2018).

5 Conclusion

Outside the domain of cancer, c-Myc plays a significant physiological and pathological role (Rosselot et al., 2021; Nevzorova and Cubero, 2023; Prochownik and Wang, 2023; Zacarias-Fluck et al., 2024). Although therapeutic interventions involving direct c-Myc manipulation may present challenges, the protective effects observed across various parameters suggest that its targeting may offer an approach to mitigate obesity-associated complications. Further mechanistic studies are necessary to unravel the precise molecular pathways by which c-Myc exerts its protective effects in whole body metabolism to establish its translational potential. By understanding the downstream targets of c-Myc and identifying key pathways influenced by its activity, we can advance novel strategies to counteract obesity-related complications and improve overall metabolic and cardiovascular health.

Data availability statement

The RNA-Seq data presented in the study are deposited in the Gene Expression Omnibus (GEO) repository under accession number GSE267493.

Ethics statement

The animal study was approved by University of Miami Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JM: Conceptualization, Formal analysis, Validation, Supervision, Investigation, Writing–original draft, Writing–review and editing. IA: Investigation, Writing–review and editing. YQ: Data curation, Writing–review and editing. AM: Investigation, Writing–review and editing. DS: Investigation, Writing–review and editing. DH: Investigation, Formal Analysis, Validation, Writing–review and editing. ND: Investigation, Formal Analysis, Writing–review and editing. AS: Investigation, Formal analysis, Writing–review and editing. MN: Investigation, Formal Analysis, Writing–review and editing. PN: Investigation, Formal analysis, Writing–review and editing. TC: Methodology, Data curation, Formal analysis, Writing–review and editing. JW-d-C: Formal analysis, Validation, Supervision, Writing–review and editing. MR: Formal analysis, Validation, Supervision, Writing–review and editing. FE: Formal analysis, Validation, Supervision, Writing–review and editing. RV-P: Formal analysis, Validation, Supervision, Writing–review and editing. EB-M: Supervision,

Writing–review and editing. CR: Conceptualization, Formal analysis, Validation, Supervision, Resources, Project administration, Writing–original draft, Writing–review and editing.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2024.1407097/full#supplementary-material

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Drosophila: a Tale of regeneration with MYC

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Regeneration is vital for many organisms, enabling them to repair injuries and adapt to environmental changes. The mechanisms underlying regeneration are complex and involve coordinated events at the cellular and molecular levels. Moreover, while some species exhibit remarkable regenerative capabilities, others, like mammals, have limited regenerative potential. Central to this process is the regulation of gene expression, and among the numerous genes involved, MYC emerges as a regulator of relevant processes during regeneration with roles conserved in several species, including *Drosophila*. This mini-review aims to provide valuable insights into the regeneration process in flies, focusing on significant organs where the role of MYC has been identified: from the imaginal discs, where MYC regulates cell growth, structure, and proliferation, to the gut, where it maintains the balance between renewal and differentiation of stem cells, and the central nervous system, where it influences the activities of neural stem cells and the interaction between glia and neuronal cells. By emphasizing the molecular mechanisms regulated by MYC, its significance in controlling regeneration mechanisms, and its conserved role in flies, we aim to offer valuable insights into the utility of *Drosophila* as a model for studying regeneration. Moreover, unraveling MYC's function in *Drosophila* during regeneration may help translate findings into the mechanisms underlying human tissue repair.

KEYWORDS

MYC, regeneration, imaginal discs, epithelial cells, gut, neurons and glia, *Drosophila*

1 Regeneration

The ability to regenerate and restore lost body parts after injury reflects key physiological pathways governed by developmental processes; regeneration capacity is widespread in animals and, in some species, has been lost during evolution, contributing to the variations in regenerative capacities across species (Losner et al., 2021). While remarkable abilities are observed in cnidarians, crustaceans, salamanders, and certain vertebrates, humans have limited regenerative potential (Wells and Watt, 2018), underscoring the need to understand molecular mechanisms of tissue and organ development for regenerative medicine.

Animal regeneration is categorized into five types: 1) structural regeneration, seen in the distal regrowth of appendages in vertebrates and arthropods; 2) organ regeneration, where damaged organs restore their mass; 3) tissue regeneration, responding to damaged epithelial or epidermis; 4) whole-body regeneration, involving the regrowth of an organism's central axis; and 5) cellular regeneration, such as the regrowth of severed nerve axons (Bely and Nyberg, 2010). Regeneration, depending on tissue and damage types, involves distinct steps,

including wound healing, the formation of a proliferative blastema, cellular differentiation, and tissue patterning. The blastema, comprised of progenitor cells responsible for the regeneration process, is formed temporarily at the injury site and undergoes morphogenesis through cell migration and proliferation to regenerate the missing organ (King and Newmark, 2012; Slack, 2017). Additionally, immune cells at the injury site play a crucial role in debris clearance and secretion of signaling molecules, initiating specific cellular proliferation and differentiation processes necessary for thriving tissue regeneration (Julier et al., 2017). Despite the progress made in understanding tissue regeneration, identifying novel signaling pathways that govern reprogramming mechanisms remains a significant challenge. Consequently, simple animal models are indispensable for gaining a deeper understanding of these intricate processes.

Although *Drosophila* does not possess the extensive regenerative abilities of some other species, its advanced genetic technology, previously used to uncover the complex genetic networks governing development, framework, which connects body parts and identity genes (such as the Hox genes), as well as pattern formation components (like Hedgehog, Decapentaplegic (Dpp), and Wingless (Wg) analogous to vertebrate Wnt), can now be utilized to investigate the molecular basis of regeneration (Fox et al., 2020). Here, we review the role of MYC in regeneration models such as wing imaginal discs, gut, and neuronal-glia cells, where processes like cell growth, division, and apoptosis may depend critically on MYC's function.

2 *Drosophila* MYC

The *MYC/MAX/MAD* network in *Drosophila* stands out for its lack of redundancy, as the *Drosophila* genome contains a single gene for each component (Gallant, 2006). Despite being only 26% identical to its human counterpart, the *Drosophila* MYC protein shares highly conserved functional domains such as Box I and II, the degron sequences, and the basic-helix-loop-helix leucine zipper (bHLH/LZ) domain, to mediate MYC: MAX heterodimers that bind the E-box sequences on target genes (Orian et al., 2003; Hulf et al., 2005). The discovery that *MYC* mutants, also called *diminutive*, are composed of smaller cells (Johnston et al., 1999) paved the way for genetic experiments that revealed MYC's role in controlling growth and ribosomal biogenesis. The similarity in phenotypes between *MYC* mutants and those of the *insulin* (*InR/IRS/chico*) (Bohni et al., 1999) and Target of Rapamycin (*TOR/S6K*) (Montagne et al., 1999) pathways has contributed to unveiling how growth pathways influence MYC activity in flies (Bellosta and Gallant, 2010; Parisi et al., 2011). These studies revealed the control of MYC protein stability by growth factors signaling through the phosphorylation of conserved domains (degrons) by Ras-ERK/MAPK and GSK3ß kinases, confirming this pathway of MYC protein degradation in flies (Galletti et al., 2009; Schwinkendorf and Gallant, 2009). Furthermore, MYC levels increase during starvation in the fat body, a metabolic tissue that parallels the function of vertebrate adipose tissue and the liver (Teleman et al., 2008; Parisi et al., 2013). Indeed, we showed that MYC increases metabolic processes like glycolysis and glutaminolysis during nutrient starvation (Parisi et al., 2013; de la Cova et al., 2014) and promotes the catabolic process autophagy in the fat cells, leading to survival (Nagy et al., 2013; Paiardi et al., 2017).

MYC's control over ribosome biogenesis is highlighted by its coordination of RNA polymerases I, II, and III activities. MYC facilitates the recruitment of RNA polymerase I to rDNA, ensuring proper rRNA synthesis with the transcription of ribosomal proteins (Destefanis et al., 2020). MYC's role in regulating ribosomal biogenesis led to the discovery of its role in cell competition; a physiological process initially observed in flies heterozygous for the *Minute* ribosomal proteins (Morata and Ripoll, 1975). In this process, cells with higher MYC levels outcompete unfit neighboring cells (with lower MYC), leading to their apoptosis (de la Cova et al., 2004; Moreno and Basler, 2004). This property of MYC was later demonstrated in the development of vertebrates (Claveria et al., 2013; Ellis et al., 2019), and it may underscore a role for MYC in mechanisms of tissue repair and regeneration across diverse organisms (Gogna et al., 2015; Yusupova and Fuchs, 2023).

3 Organ-specific regeneration: the wing imaginal discs, gut, and neural cells, three models to study regeneration

3.1 Wing imaginal discs

Imaginal discs in *Drosophila* larvae are sac-like structures of epithelial tissue (Figure 1A) and they are the precursors of adult organs. Due to their accessibility and the availability of a wide range of genetic tools, imaginal discs have become, in the last decade, an invaluable tissue for studying regeneration. They also provide an excellent platform for analyzing evolutionarily conserved pathways identified in the regeneration (Hariharan and Serras, 2017; Fox et al., 2020). Early studies on regeneration demonstrated that when imaginal wing discs were cut into small pieces and transplanted into either adult female abdomen, which served as natural culture chambers, or young larvae, they regenerated to their correct size and shape (Bergantinos et al., 2010b; Worley and Hariharan, 2022). This indicated the ability of the discs to resume proliferation and regenerate the missing part. These pioneering experiments demonstrated the regenerative potential of imaginal discs and unveiled their plasticity. In addition, fragments of discs cultured through prolonged transplantation cell-fate changes such as leg-to-wing, leading to the regeneration of alternative organs, in a phenomenon called transdetermination. This phenomenon demonstrates the capacity of *Drosophila* imaginal cells to be reprogrammed to various lineages (McClure and Schubiger, 2007). The refinement of surgical ablation of imaginal discs facilitated the exploration of regeneration during larval and pupal development. This technique revealed the critical role of cell division and the timing of ablation during development in shaping the regeneration timing (Diaz-Garcia and Baonza, 2013). More advanced technology was developed using genetic tools to induce apoptosis in specific domains of the disc and monitor tissue recovery, utilizing the binary UAS/Gal4 system (Brand and Perrimon, 1993). This widely used technique was adapted to study regeneration by temporally inducing the expression of

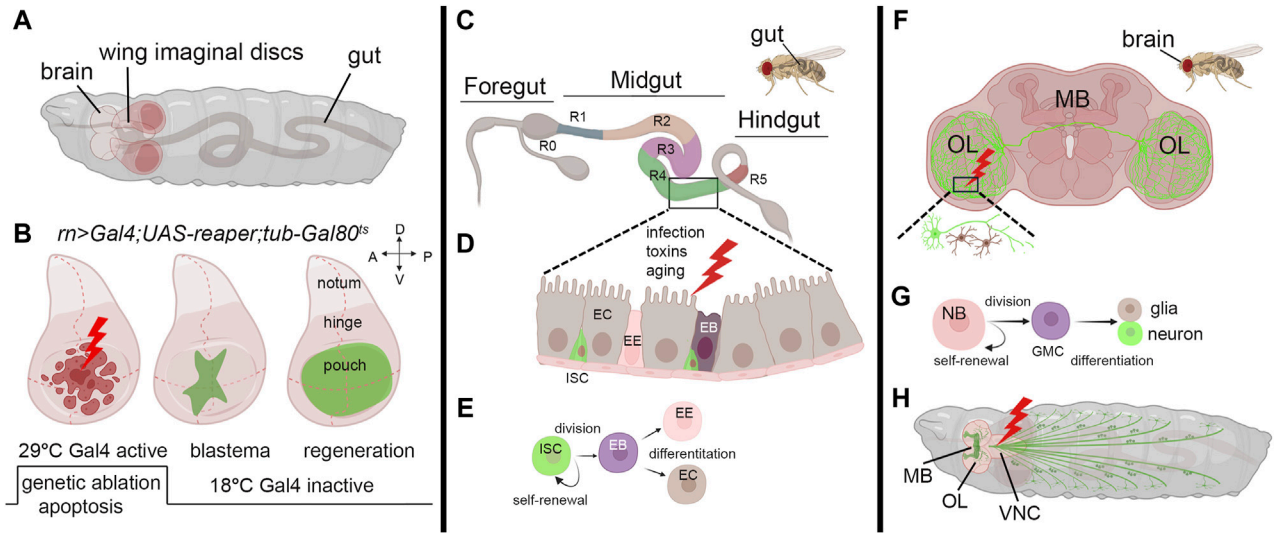

FIGURE 1
Models to study regeneration. **(A)** Schematic view of third instar larvae indicating the brain, wing imaginal discs, and the gut. **(B)** Third instar wing imaginal discs in which apoptosis is induced in the pouch using a specific Gal4-promoter. (Left) The induction of the apoptotic gene occurs through a controlled temperature switch. At 18°C, Gal80 binds to Gal4, repressing its activity and preventing its expression. However, when the temperature is switched to 29°C, Gal80 expression is suppressed, releasing Gal4 from inhibition and initiating the expression of the apoptotic gene and cell death (Hariharan and Serras, 2017). (Middle) After a few hours, animals are switched to the permissive temperature of 18°C to block apoptosis, allowing regeneration to occur with the formation of the blastema (green) that expands until a fully recovered pouch is obtained (Right). **(C)** Representation of the adult gut with the zone that characterizes its function (R0-5) (Buchon et al., 2013). **(D)** Model of the midgut epithelium where regeneration occurs upon injury. Cells are color-coded as in panel **(E)**, where the stem-cell niche is represented: ISC: Intestinal Stem Cell, EB: Enteroblast, EE: Enteroendocrine cell, EC: Enterocyte. **(F)** Schematic representation of the adult brain indicating the most common structures MB: mushroom body, OL: Optical Lobe. The inset represents a site of injury with neuron and glial cells represented in green and brown. **(G)** Representation of neuroblasts division. Neuroblasts (NB) divide asymmetrically, generating a ganglion mother cell (GMC), which then divides to produce a postmitotic neuron or glial cell (Homem and Knoblich, 2012). **(H)** Schematic representation of a third instar larva indicating the mushroom body (MB), the Optical lobe (OL) and the neurons (green). In red is a common site for injury in the Ventral Neural Cord (VNC). The figure was created using BioRender Premium, license (XV26VCD8GB), and further refined using Adobe Photoshop for its final appearance.

apoptotic genes in the wing disc, regulated by the temperature-sensitive allele Gal80^{ts}, an inhibitor of Gal4 (Figure 1B) (Smith-Bolton et al., 2009; Bergantinos et al., 2010a). Furthermore, the UAS/Gal4 system was combined with an engineered LexA-LexAop system, enabling precise temporal induction of cell death (Santabarbara-Ruiz et al., 2015). These methods allowed the identification of crucial genes involved in blastema formation, including Wg, a key regulator of regeneration in many species, and MYC (Smith-Bolton et al., 2009; Worley et al., 2012). Indeed, MYC was found to be upregulated in the proliferating cells surrounding the blastema, and its reduction partially impeded regeneration in the wing pouch (Smith-Bolton et al., 2009). Subsequent research demonstrated that MYC reduction, combined with *reaper* ablation, significantly hindered regeneration in the wing disc. Conversely, under the same conditions, MYC overexpression improved both the size and morphology of the adult wings, confirming its crucial role in the regeneration process (Harris et al., 2020). Additionally, MYC has been identified to regulate Yorkie (Yki), the unique *Drosophila* ortholog of YAP/TAZ, a component of the Hippo tumor suppressor pathway, in a feed-back mechanism that restrains the growth of the imaginal discs (Neto-Silva et al., 2010; Ziosi et al., 2010). In mammals, the Hippo-YAP/TAZ pathway regulates regeneration by controlling cell proliferation, apoptosis, and stem cell maintenance to ensure proper tissue growth and repair (Moya and Halder, 2019). Thus, MYC's regulation of Yorkie (Yki) could be crucial for balancing cell proliferation and tissue growth in response to damage. This coordination is vital for developmental processes and organ growth, where MYC and the Hippo pathway are key players (de la Cova et al., 2004; Pan, 2007). Cells at the regeneration site stimulate proliferation through non-autonomous mechanisms such as apoptosis-induced proliferation (AiP), compensating for the apoptotic zones by triggering cell proliferation (Fogarty and Bergmann, 2017). The mechanisms controlling AiP are still under investigation; however, one hypothesis is that the release of ROS by the dying cells activates the ROS-sensitive kinase 1 (Ask1), expressed during regeneration, and its signal attenuated by Akt1/PKB/InR in living cells surrounding the blastema modulates moderate JNK/p38 signaling, which is crucial for controlling apoptosis in the regenerative response (Santabarbara-Ruiz et al., 2019; Esteban-Collado et al., 2021). Recent single-cell transcriptomics analysis of blastema from wing imaginal discs identified Ets21C, a transcription factor that controls patterning and organ development. This factor is induced by cell damage and is essential for the expression of genes crucial for regeneration (Worley et al., 2022). Interestingly, our RNA sequencing data reveals that both Ets21C and MYC are upregulated in wing disc cells undergoing apoptosis induced by proteotoxic stress (not published), suggesting that their expression may share components in the stress response pathways still to be investigated.

Finally, we would like to briefly address the critical role of the steroid hormone ecdysone during regeneration and its relation with MYC. Ecdysone controls cellular and specific pathways that regulate

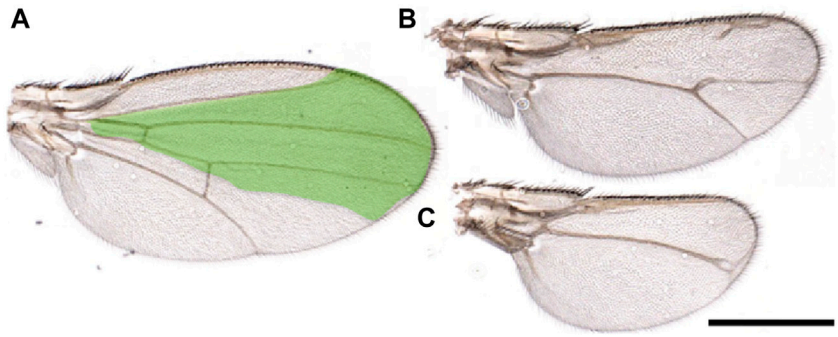

FIGURE 2
Starvation affects wing regeneration. **(A-C)** Wings from animals that underwent regeneration while subjected to amino acid starvation. Reaper was temporarily induced in the spalt domain (green), three days after egg laying in larvae of the genotype: *SpaltPE-Gal4/tub-Gal80^{ts}; UAS-rpr*. Animals were kept in a starvation medium (PBS/20% sucrose) until eclosion. **(A)** Wings from flies not expressing *reaper*. **(B, C)** or in which *reaper* was induced. These images highlight the morphological defects observed in the wings due to the incomplete regeneration process. Scale Bar 1 mm.

physiological organ growth and developmental timing (Andersen et al., 2013). Ecdysone is produced by the prothoracic gland (PG) at specific development times to regulate larval molting and metamorphosis (Edgar, 2006; Tennessen and Thummel, 2011). In the regeneration process, ecdysone levels determine the timing after which larvae terminate their window of regenerative potential by controlling the state of epithelial cell progenitors through regulating the transcription factors *chinmo* and *broad* (Narbonne-Reveau and Maurange, 2019; Karanja et al., 2022). Moreover, the release of ecdysone by the PG is indirectly controlled by the dying cells in the regenerating discs that secrete Dilp8, a peptide belonging to the insulin/relaxin-like growth factor family, which binds to the LGR3 receptor in the brain. This inhibits the release of ecdysone from the PG (Colombani et al., 2015; Vallejo et al., 2015) and slows down the development, allowing the damaged cells of the discs to complete their regeneration process (Blanco-Obregon et al., 2022; Karanja et al., 2022). Moreover, the physiological reduction of ecdysone at specific development points corresponds to an increase in MYC in the fat body (FB) (Delanoue et al., 2010). MYC in the FB favors the storage of nutrients (fat and sugars) and activates survival pathways such as autophagy to survive starvation (Parisi et al., 2013; Paiardi et al., 2017). It is known that regeneration in wing discs is affected by pathways regulated by nutrients (Esteban-Collado et al., 2021), and animals allowed to regenerate in starvation do not complete this process (Figure 2). The observation that animals in starvation have a reduced ability to regenerate suggests that non-autonomous signals from the FB are necessary to complete this process. Although MYC is upregulated in the FB of starved animals (Teleman et al., 2008; Parisi et al., 2013), the impaired regeneration observed under starvation conditions indicates that the upregulation of endogenous MYC activity in the FB is insufficient to sustain regeneration. Alternatively, starvation may prevent the storage of nutrients in the FB or hinder the production/secretion of factors necessary for regeneration.

3.2 Gut

Research on *Drosophila* gut regeneration offers valuable insights into repair mechanisms relevant to regenerative medicine, given the similarities in tissue composition, anatomy, and physiological functions with the human intestine. To investigate regeneration, various methods are employed to induce stress and cell damage, such as chemical exposure (e.g., Dextran Sulfate Disodium (DSS), bacterial infection, heat stress, oxidative stress (e.g., H_2O_2), and mechanical damage (Apidianakis and Rahme, 2011; Zhang and Edgar, 2022) *Drosophila* gut comprises an anterior, middle, and posterior hindgut (Figure 1C); however, regeneration primarily occurs in the midgut, where the Intestinal Stem Cells (ISCs) generate a niche initiated by Notch (Ohlstein and Spradling, 2007). These cells divide asymmetrically and give rise to a new ISC and an Enteroblast (EB) that will differentiate into Enterocytes (ECs) or Enteroendocrine cells (EE) in the absence of cell division (Figures 1D,E) (Mathur et al., 2010; Amcheslavsky et al., 2014). Wg is necessary to maintain ISCs self-renewal and is the balance between Notch and Wg signaling that controls the equilibrium between the proliferation and differentiation of ISCs (Zhang and Edgar, 2022). MYC plays a crucial role in mediating gut fitness both in ISCs and in ECs. MYC activity is essential for their differentiation and proliferation and acts downstream of stress-dependent and growth factor pathways such as JAK-STAT, Wg, Hippo, and EGFR (Ren et al., 2013). MYC is also crucial in maintaining gut health in response to different diet conditions. A nutrient-rich diet suppresses MYC in ECs, increasing cell death and gut permeability and shortening lifespan. Conversely, dietary restriction boosts MYC, enhancing EC fitness, gut integrity, and lifespan (Akagi et al., 2018). This may occur through MYC-inducing cell competition, which is crucial for maintaining the fitness of adult enterocytes (ECs), especially during dietary changes. Interestingly, this is similar to what was previously described in intestinal ISCs for *Minute* genes, many of which are MYC targets, where both ISC and differentiated *Minute*/+ cells were eliminated through cell competition to promote the proliferation and self-renewal of wild-type stem cells (Kolahgar et al., 2015). Recent evidence also reveals the role of MYC as a regulator of the amino acid transporter *arcus* (*acs*) in ECs (Socha et al., 2023). This signal is coordinated with the activation of the insulin pathway that favors aminoacidic absorption and ECs recovery after bacterial-mediated toxin damage, suggesting another active role for MYC in the gut to favor the regeneration of these cells.

3.3 Neuronal cells

Drosophila's neural stem cells (NSCs), or neuroblasts, are pivotal for brain development. They exhibit remarkable plasticity, transitioning between quiescent and active states in response to environmental cues or injury. This dynamic regulation underscores their importance in maintaining brain homeostasis and promoting tissue repair. Neuroblasts (NB) play a crucial role in larval development, undergoing asymmetric division to generate neuroblasts and smaller ganglion mother cells (GMC). These GMCs divide further to produce post-mitotic neurons or glial cells (Figure 1G) (Homem and Knoblich, 2012; Otsuki and Brand, 2020). The neurons establish identities via proneural and selector genes, resulting in four classes (I-IV) of dendritic arborization (da) sensory neurons. Class IV-ddaC neurons, known for their intricate dendritic arbors sensitive to mechanical stimuli, serve as models for dendrite repair and the study of neurodevelopmental disorders (Grueber et al., 2007; Liu et al., 2023).

Methods for investigating neuronal regeneration during development include gently crushing the larval segmental nerve to maintain larval viability or employing laser ablation (Figure 1H). This approach involves labeling specific axon patterns using GFP expressed by neuronal-specific promoters, facilitating the visualization of cells during regeneration events throughout larval development (Pfeiffer et al., 2008). In adult flies, few models exist for studying neuronal regeneration. Experimental stab lesions to either the optic lobes (OL) or the central brain result in local neurogenesis days after injury (Figure 1F). This response was attributed to dormant neural progenitor cells (qNPs) activation (Moreno et al., 2015; Crocker et al., 2021). Glial cells respond to nervous system damage by increasing their number and changing morphology after neuronal cell death. This process is conserved across regions of the peripheral nervous system and involves Dpp and Hh signaling, with the JNK pathway contributing to glial migration (Velarde et al., 2021). Glial cells exhibit an immune response like microglia, expressing the phagocytic receptor draper (drpr), crucial for axon regeneration and debris clearance. While macrophages aid central nervous system (CNS) regeneration in vertebrates, their role in *Drosophila* neural injury remains unclear (Losada-Perez et al., 2021).

Recent discoveries highlight the crucial role of NSCs in maintaining and regenerating adult brain tissues (Li and Hidalgo, 2020). In contrast to adult mammals, *Drosophila* NSCs can be activated by different diets or exercises initiated by larval hatching. However, the mechanisms by which NSCs transition between quiescence and activation remain elusive (Ding et al., 2020). Brain injuries in adult flies are thought to trigger the recruitment of quiescent neural progenitors (qNPs) near the injury site, facilitated by damage-responsive neuroglial clusters (DNGCs). These clusters stimulate the proliferation of distant qNPs, thereby expanding the zone of stem cell activation through the reactivation of dormant qNPs (Moreno et al., 2015; Crocker et al., 2021). Since previous research has shown that a ubiquitous pulse of MYC promotes qNP division (Fernandez-Hernandez et al., 2013), it is possible that MYC could induce growth factors in qNPs through injury-induced secretion, allowing these cells to survive and proliferate. MYC has also emerged as a non-autonomous regulator of metabolism in retinal ganglion glial cells, where using a model of reprogrammed glial cells that activate PI3K and EGFR pathways (RGCPE), MYC activity was shown relevant for the regeneration of neurons by mediating pro-regeneration metabolic pathways in glia (Li et al., 2020), including the glia-neuron lactate shuttle essential for neuronal survival (Volkenhoff et al., 2015). This highlights its important role in inducing nonautonomous signals that control axon regeneration.

4 Discussion

Studying regeneration in *Drosophila* has unveiled complex cellular and molecular mechanisms guiding tissue repair and organ regeneration across species. Although tissues display differing regenerative abilities, common pathways and principles govern regeneration. The pivotal role of MYC emphasizes its importance in regulating fundamental conserved processes, connecting metabolism and growth, influencing cell competition, and highlighting regeneration's complexity. Insights from *Drosophila* research hold potential for future advancements in regenerative medicine. Further exploring molecular mechanisms across organisms is fundamental to developing novel therapeutic strategies to enhance human tissue repair and organ regeneration.

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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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