CELL FATE

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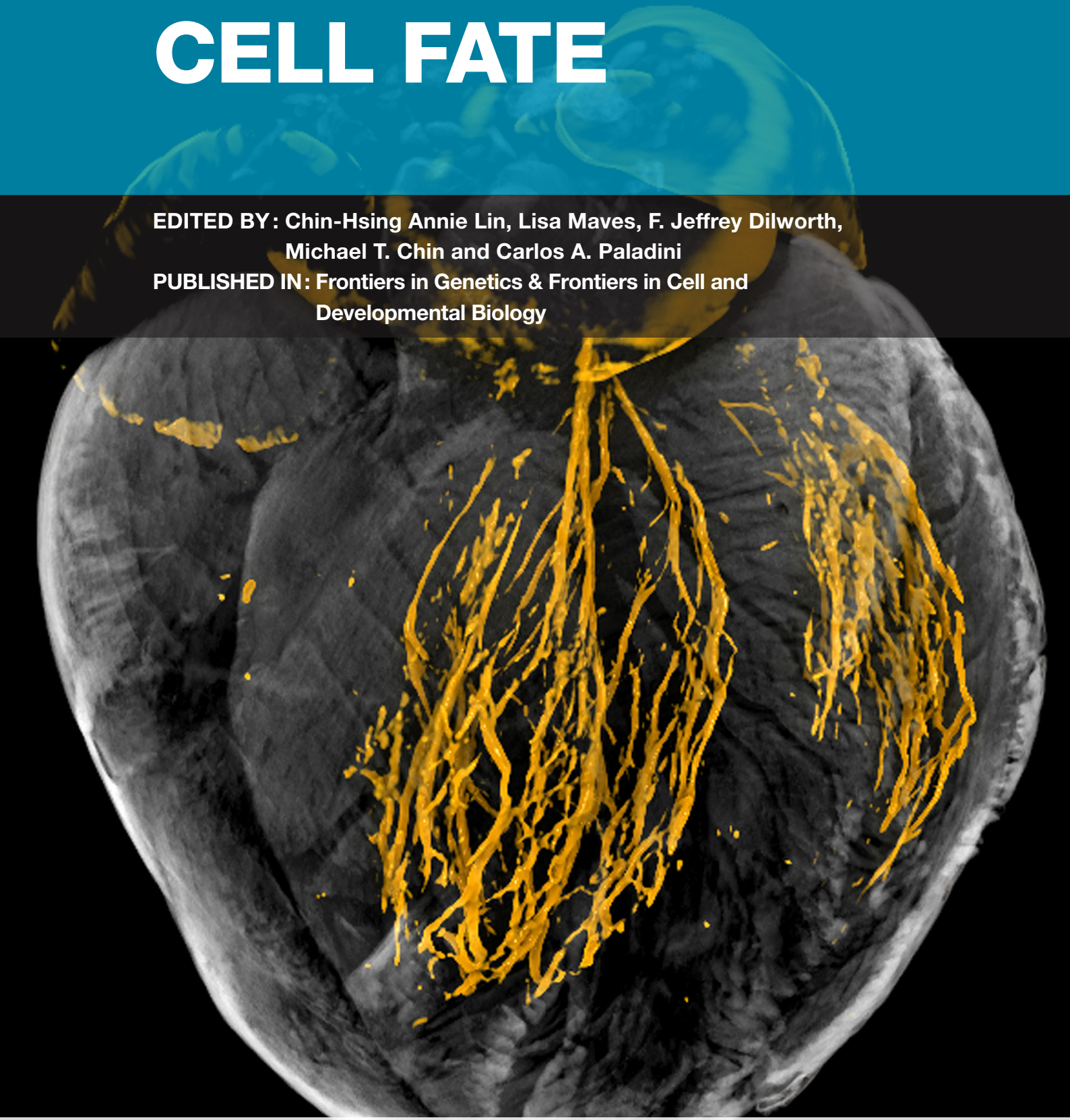

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CELL FATE

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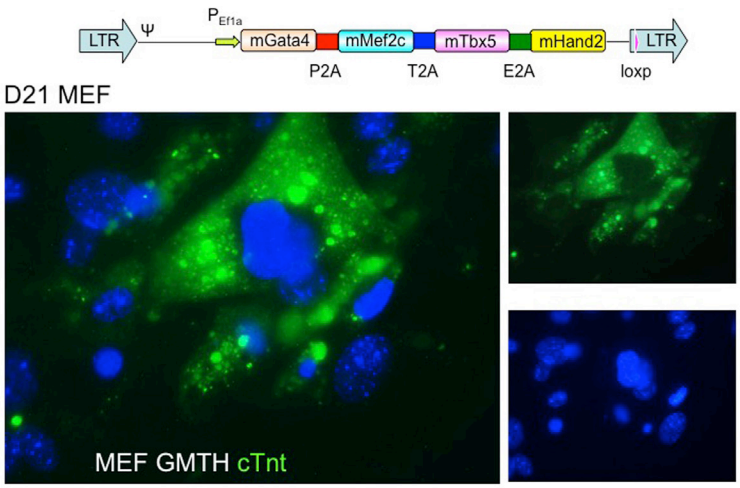

Fibroblasts can be directly reprogrammed to cardiomyocyte-like cells. In this figure, mouse embryo fibroblasts were infected with a lentivirus that expresses the transcription factors Gata4, Mef2c, Tbx5 and Hand2 and then were assessed for expression of cardiac-specific troponin T after 21 days in culture. From Wei-Ming Chien and Michael T. Chin, unpublished data.

Cover: As the heart develops, cardiomyocytes within the heart adopt specialized cell fates that contribute to overall organ function. In this image, specialized cardiomyocytes that form the cardiac conduction system are visualized using a cardiac conduction system specific beta galactosidase reporter and optical projection tomography. From Matthew E. Hartman and Michael T. Chin, unpublished data.

The fundamental question of how an undifferentiated progenitor cell adopts a more specialized cell fate that then contributes to the development of specialized tissues, organs, organ systems and ultimately a unique individual of a given species has intrigued cell and developmental biologists for many years. Advances in molecular and cell biology have enabled investigators to identify genetic and epigenetic factors that contribute to these processes with increasing detail and also to define the various molecular characteristics of each cell fate with greater precision. Understanding these processes have also provided greater insights into disorders in which the normal mechanisms of cell fate determination are altered, such as in cancer and inherited malformations. With these advances have come techniques that facilitate the manipulation of cell fate, which have the potential to revolutionize the field of medicine by facilitating

the repair and/or regeneration of diseased organs. Given the rapid advances that are occurring in the field, the articles in this eBook are both relevant and timely. These articles originally appeared online as part of the Research Topic "Cell Fate" overseen by my colleagues Dr. Lin, Dr. Buttitta, Dr. Maves, Dr. Dilworth, Dr. Paladini and myself and have been viewed extensively. Because of their popularity, they are now made available as an eBook, in a more easily downloadable form.

The opening editorial by Dr. Buttitta provides an excellent overview of the topics covered in this special issue. The online edition allows ordering of the articles by number of views, by article type or by date of publication, but Dr. Buttitta's editorial organizes them by subtopic, which will be the format for this eBook. These subtopics include "The Plasticity of Cell Fate," "Nuclear Architecture, the Cell Cycle, and Cell Fate" and "Technical Advances in Deciphering Cell Fate Regulation." The first subtopic begins with a minireview by myself that discusses current knowledge regarding direct reprogramming of adult cells from one cell fate to another, an area of major interest in both cell biology and regenerative medicine. The next article, by Robb MacLellan and coworkers, discusses how terminal differentiation of adult cardiomyocytes is associated with epigenetic and chromatin structural changes that both silence the expression of cell cycle genes associated with the G2/M transition and cytokinesis and activate cardiac-specific genes, thereby reinforcing the terminally differentiated phenotype. Understanding these mechanisms may lead to improved strategies for cardiac regeneration. The third article in this topic, by Maura Parker, discusses how aging leads to alterations in cell signaling and epigenetic marks in skeletal muscle satellite cells, thereby reducing their ability to self-renew and proliferate and promoting senescence. Manipulation of these processes may improve skeletal muscle aging.

The next subtopic, on nuclear architecture, opens with a minireview by Alyssa Lau and Gyorgyi Csankovski on dosage compensation in nematodes and describes how condensin reduces gene expression from X chromosomes in hermaphrodites during differentiation, by a mechanism related to mitotic chromosome compaction. The next article, by Laura Buttitta and coworkers, describes the interplay between cell cycle factors and chromatin architecture to influence cell fate, during both normal differentiation and during cell fate reprogramming. The third article, by Jessica Talamas and Maya Capelson, explores how dynamic changes in the nuclear envelope regulate the state of chromatin, tissue-specifc gene transcription and cell fate determination. The last article in this subtopic, by Lisa Julian and Alexandre Blais, discuss transcriptional control of stem cell fate by E2F transcription factors and their binding partners, pocket proteins. The role of these factors in cell cycle regulation is well known, but their role in fostering the development and differentiation of various progenitor cell types is less appreciated.

The last subtopic, on technical advances, opens with original research from Chin-Hsing Annie Lin and coworkers, in which they describe their technique for identifying repressive marks in neuronal stem cells from the subventricular zone that are relevant to controlling the timing of differentiation. They have developed a method for rapidly isolating the stem cells from this zone in baboon brains and quickly identifying repressive marks before they can change, as is often the case when these cells are cultured. The last article, by Kurtulus Kok and David Arnosti, expounds upon the link between transcriptional oscillations of HES genes and chromatin dynamics, and how they regulate timing of differentiation in neural progenitor cells.

Overall, this collection of articles underscores the complex interplay between transcription factors, gene expression, epigenetic modifications, cell cycle regulation, chromatin architecture, nuclear structure and cell fate determination. As a whole, they should not be viewed as a comprehensive reference, but rather as an introduction to the future of cell fate biology. On behalf of the other editors and authors, I hope that the articles contained in this eBook provide additional inspiration to students of molecular, cellular and developmental biology.

Michael T. Chin

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frontiers in Genetics

EDITORIAL
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Editorial: Cell Fate

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Keywords: differentiation, gene expression, stem cells, transcription factors, chromatin remodeling, epigenetics

The Editorial on the Research Topic

Cell Fate

INTRODUCTION

The complexity and plasticity of cell fate determination has intrigued cell and developmental biologists for decades. Cellular differentiation is the acquisition of specialized characteristics; which is intimately associated with changes in gene expression, alterations of chromatin, and changes in nuclear architecture. Differentiating tissues exhibit a progressive restriction of cellular plasticity. However, the regenerative ability of some organisms has revealed an amazing capacity for dramatic switches in cell fate, through trans-differentiation and de-differentiation (Sánchez Alvarado and Tsonis, 2006). Furthermore, the groundbreaking work on somatic cell nuclear reprogramming and induced pluripotency has revealed that commitment to cell fate can be far more flexible than previously thought (Lensch and Mummery, 2013).

In this research topic on cell fate we aimed to highlight new developments and outstanding questions in our understanding of how chromatin dynamics impact cell fate and cellular reprogramming. We include articles discussing cell fate decisions in a wide variety of contexts and model organisms. The contributions to this topic include review articles, mini-reviews, original research, and perspectives. The work described here encompasses organisms ranging from *C. elegans* to humans and deals with global cell fate issues of sex determination (Lau and Csankovszki), lineage choice (Chin), preventing premature differentiation (Foret et al.) cell fate and cell cycle regulation (Oyama et al.; Julian and Blais; Ma et al.; Parker), nuclear architecture (Talamas and Capelson) and how dynamic transcriptional repressors promote cell fate choices (Kok and Arnosti). We thank the authors, reviewers and editors for contributing to the stimulating discussion of the open questions in this rapidly changing field.

THE PLASTICITY OF CELL FATE

Despite the seemingly irreversible nature of cell fate decisions made during embryonic development, there is substantial literature on cellular reprogramming. This can include de-differentiation of cells to a naïve state, such as induced pluripotency, or it can mean direct reprogramming of cells between different fates. In a mini-review on reprogramming cell fate (Chin), Michael T. Chin summarizes advances made in the direct reprogramming of adult, differentiated cells from one cell fate to another, with a discussion of the impact of this research on strategies for regenerative medicine.

Terminally differentiated and postmitotic cells are at the opposite end of the spectrum from reprogramming in cell fate plasticity. How are cell fates properly maintained in the long-term in postmitotic tissues? In a review, Robb MacLellan and colleagues (Oyama et al.) discuss the specialized cell type of cardiac muscle, which undergoes a transition to a permanently postmitotic

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state coupled with terminal differentiation. They discuss recent work revealing a network of chromatin-associated factors that cooperate with tumor suppressors such as the Retinoblastoma protein to stably repress cell cycle genes and maintain the postmitotic state. How terminal differentiation and the repressive networks are coordinated remains to be deciphered, but whether they may be safely uncoupled is a question with huge potential impact on cardiovascular therapeutics and regeneration.

The proper maintenance of stem cells in aging tissues is a critical issue underlying age-related tissue decline. Maura Parker examines this issue in a review (Parker) on how signaling and epigenetic changes occur with age in satellite cells, the stem cells for skeletal muscle. She suggests that modulations of chromatin and the epigenetic memory of aging stem cells may be key to therapies aimed at "resetting the aging clock."

NUCLEAR ARCHITECTURE, THE CELL CYCLE, AND CELL FATE

Sexual determination occurs by a chromosome-based method in many organisms, which leads to an imbalance in gene dosage between the sexes. Dosage compensation acts to equalize X-linked gene expression between the sexes. In *Caenorhabditis elegans*, dosage compensation is achieved by a complex similar to the mitotic condensin complexes. Alyssa C. Lau and Györgyi Csankovszki discuss in a mini-review how dosage compensation in *C. elegans* shares features with condensed mitotic chromosomes (Lau and Csankovszki), and describe why examining condensins in dosage compensation provides unique insights into the relationship of chromatin compaction during interphase and modulation of gene expression.

There is detailed feedback between chromatin architecture, cell fate decisions and cell cycle regulators, as all three influence each other. We continue the theme of exploring chromatin changes associated with the cell cycle, and discuss directly how the mitotic cell cycle impacts chromatin architecture and cell fate (Ma et al.). We summarize new work in cellular reprogramming and nuclear transfer that addresses a provocative question; is there a cell cycling state or cell cycle phase that can increase cellular plasticity?

The discussion of nuclear architecture and cell fate continues in a review by Jessica Talamas and Maya Capelson, which discusses the nuclear envelope and genome interactions in cell fate decisions (Talamas and Capelson). This review describes the interconnected roles of nuclear compartments and asks whether nuclear envelope composition may serve as an unappreciated "cellular code" for directing cell type-specific gene expression programs through contacts with chromatin.

In a more specific focus on cell cycle regulators (Julian and Blais), Lisa M. Julian and Alexandre Blais discuss the transcription factor family, E2F, best known for its roles in regulating cell cycle genes with its repressive partners, the retinoblastoma family. However here, roles for the E2F family outside of the cell cycle are discussed. These are evolutionarily conserved functions in stem cell fate control in a number of lineages, that reveal pivotal roles for E2Fs in the execution of cell type-specific gene regulatory programs.

TECHNICAL ADVANCES IN DECIPHERING CELL FATE REGULATION

Original research by Chin-Hsing Annie Lin and colleagues describes a new technique for profiling chromatin marks and gene expression in specific cell types (Foret et al.). By exploring the adult neurogenic niche in the brain of a non-human primate, they reveal an enrichment of a repressive chromatin mark, suggesting transcriptional silencing protects against improper lineage differentiation in this critical zone.

Closing with the theme of transcriptional repression, in a Perspective piece Kurtulus Kok and David N. Arnosti ponder how repressive complexes on chromatin can display dynamic associations, leading to cycling expression of target genes (Kok and Arnosti). In several developmental contexts cyclic gene expression can impact cell fate decisions, and oscillations in gene expression are likely to be pervasive. Thus the oscillatory behavior and dynamic association of factors with chromatin will need to be considered more fully if we are to understand cell fate decisions.

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frontiers in
CELL AND DEVELOPMENTAL BIOLOGY

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Reprogramming cell fate: a changing story

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Direct reprogramming of adult, lineage-determined cells from one cell fate to another has long been an elusive goal in developmental biology. Recent studies have demonstrated that forced expression of lineage-specific transcription factors in various differentiated cell types can promote the adoption of different lineages. These seminal findings have the potential to revolutionize the field of regenerative medicine by providing replacement cells for various degenerative disorders. Current reprogramming protocols, however, are inefficient in that relatively few cells in a given population can be made to undergo reprogramming and the completeness and extent of reprogramming that occurs has been questioned. At present, the fundamental molecular mechanisms involved are still being elucidated. Although the potential clinical applications are extensive, these issues will need to be addressed before direct reprogramming may be used clinically. This review will give an overview of pioneering studies in the field, will describe what is known about direct reprogramming to specific lineage types, will summarize what is known about the molecular mechanisms involved in reprogramming and will discuss challenges for the future.

Keywords: direct reprogramming, transdifferentiation, lineage determination, regenerative medicine, cell fate

INTRODUCTION

A fundamental question in cell biology is whether the acquisition of a particular cell fate during embryonic development is reversible or changeable, and to what extent. From a practical standpoint, this question is also directly relevant to regenerative biology and its potential application to clinical medicine. For many years, the answer to this question has been a qualified affirmative, although progress has been mostly limited until the last decade. The first demonstration that somatic cell nuclei could be reprogrammed to direct enucleated oocytes to form mature fertile animals was achieved in amphibians (Gurdon et al., 1958). This technology was later used to clone mammals, nearly four decades later (Campbell et al., 1996; Wakayama et al., 1998). Although these studies demonstrated the feasibility of somatic nuclear reprogramming, the overall efficiency was low (1–2%) and worked better with nuclei from cells that were less differentiated, suggesting that epigenetic modifications are likely involved.

At the cellular level, early studies showing that 5-azacytidine treatment, which inhibits DNA methylation, could convert cultured fibroblast cell lines to myocytes, chondrocytes, and adipocytes suggested that differentiated cells could undergo transdifferentiation and that this process was under epigenetic control (Taylor and Jones, 1979). Subsequent studies on human amniocyte- mouse myocyte heterokaryons were able to demonstrate that the muscle phenotype was dominant and that cytoplasmic factors caused activation of muscle genes in the human nuclei (Blau et al., 1983). A single dominant acting bHLH transcription factor, MyoD, was later identified by its ability to transform cultured fibroblasts into myoblasts by activating muscle-specific genes (Lassar et al., 1986; Davis et al., 1987). In other terminally differentiated cell types, MyoD could activate muscle specific genes but could not suppress the starting cell phenotype, demonstrating that there are intrinsic cellular roadblocks to reprogramming (Weintraub et al., 1989). Nevertheless, this discovery prompted searches for other dominant acting transcription factors that could single handedly transform cells from one lineage to another, however, the results were largely disappointing. In general, cell fate switching seemed to occur more readily between related cell types, presumably due to similar epigenetic landscapes. Examples include conversion of primary B cells to macrophages by the transcription factor C/EBPa (Xie et al., 2004), activation of erythroid-megakaryocyte gene expression in monocytes by the transcription factor GATA1 (Visvader et al., 1992; Kulessa et al., 1995; Heyworth et al., 2002) and induction of myeloid gene expression in hematopoietic precursors by the transcription factor PU.1 (Nerlov and Graf, 1998).

REPROGRAMMING TO PLURIPOTENCY BY MULTIPLE TRANSCRIPTION FACTORS

The advent of technologies that facilitated global transcriptional profiling in cells and tissues allowed researchers to identify large numbers of genes that are differentially expressed in different cell types. Presumably, some of the factors that were differentially expressed in different cell lineages would contribute to the maintenance of the particular cell type. This presumption led to a pioneering study in which 24 candidate transcription factors identified in embryonic stem cells were expressed simultaneously in fibroblasts to determine whether they could confer a pluripotent phenotype, and were then gradually reduced in number to the minimum necessary to induce pluripotency, resulting in the breakthrough discovery of iPS cells. In this landmark study,

fibroblasts could be reprogrammed for the first time into pluripotent cells through the forced expression of four defined factors: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). These cells could be injected into blastocysts and contribute to all three germ layers of the developing organism, and thus can be used to generate a variety of cell types for tissue regeneration. The generation of iPS cells and their potential for use in research and therapy has discussed in several recent review articles and will not be discussed in detail (Hanna et al., 2010; Robinton and Daley, 2012). iPS cells and embryonic stem cells can be differentiated directly to a variety of cell types through a process known as "directed differentiation" using defined factors such as bone morphogenetic proteins (BMPs), Activin, Wnts, and Fibroblast Growth Factors (FGFs). Although the generation of iPS cells represents a major advancement in stem cell biology, the process is inefficient and time consuming, which will be compounded if the derived iPS cells will then be used for directed differentiation. These factors can limit their practical use in clinical settings.

DIRECT REPROGRAMMING OF CELL FATE FROM ONE TYPE TO ANOTHER

Direct reprogramming will theoretically facilitate the generation of clinically relevant cell types for organ repair from abundant, easy to obtain patient-derived cells such as fibroblasts, without the need for obtaining pluripotent stem cells. Generally this is accomplished through forced expression of lineage-specific transcription factors and has been used to promote reprogramming to a variety of cell types, such as skeletal muscle (Lassar et al., 1986; Davis et al., 1987; Weintraub et al., 1989), hepatocytes (Huang et al., 2011; Sekiya and Suzuki, 2011), neurons (Vierbuchen et al., 2010), pancreatic islet cells (Ferber et al., 2000; Zhou et al., 2008), endothelial cells (Ginsberg et al., 2012), smooth muscle cells (Cordes et al., 2009; Karamariti et al., 2013), and cardiac muscle (reviewed in Addis and Epstein, 2013). Direct reprogramming is conceptually attractive because in general it does not require reversion to a pluripotent state and represents a direct conversion from one cell lineage to another. It also provides the opportunity to directly convert cells *in situ*, which would be important in regenerative strategies. Several excellent reviews have been published recently on this subject (Vierbuchen and Wernig, 2012; Addis and Epstein, 2013; Morris and Daley, 2013). In general, reprogramming seems to work better when the starting cells share similar embryonic germ cell layer origins, but has been demonstrated to convert fibroblasts (mesoderm) to neurons (ectoderm), indicating that conversion across germ cell layers is possible (Vierbuchen et al., 2010). Although several different types of cells can undergo direct reprogramming to many different cell types (reviewed in Morris and Daley, 2013), we will focus primarily on what is known about direct reprogramming of fibroblasts, since they are generally ubiquitous, abundant and readily available for clinical use. Reports of direct fibroblast reprogramming are summarized in **Table 1**. We will also focus on directing cell fate conversion to neurons and cardiac myocytes, two cell types from organs that do not regenerate well, and are thus highly relevant to clinical regenerative medicine.

Table 1 | Reports of direct reprogramming of fibroblasts.

Reprogrammed cell type	References
Skeletal muscle	Lassar et al., 1986; Davis et al., 1987; Weintraub et al., 1989
Hepatocytes	Huang et al., 2011; Sekiya and Suzuki, 2011
Neurons	Vierbuchen et al., 2010; Ambasudhan et al., 2011; Caiazzo et al., 2011; Pang et al., 2011; Qiang et al., 2011; Son et al., 2011; Yoo et al., 2011; Lujan et al., 2012; Liu et al., 2013
Cardiomyocytes	Ieda et al., 2010; Efe et al., 2011; Pfisterer et al., 2011; Chen et al., 2012; Inagawa et al., 2012; Islas et al., 2012; Jayawardena et al., 2012; Protze et al., 2012; Qian et al., 2012; Song et al., 2012; Addis et al., 2013; Christoforou et al., 2013; Fu et al., 2013; Hirai et al., 2013; Nam et al., 2013; Wada et al., 2013; Hirai and Kikyo, 2014; Ifkovits et al., 2014; Muraoka et al., 2014
Smooth muscle cells	Cordes et al., 2009; Karamariti et al., 2013
Macrophages	Feng et al., 2008
Pancreatic islet cells	Lumelsky, 2014
Neural precursors	Mitchell et al., 2014b; Zhu et al., 2014

DIRECT REPROGRAMMING TO NEURONS

Direct reprogramming of fibroblasts to neuron-like cells was first achieved by overexpression of a pool of 19 virally expressed candidate genes that were known to be neuron-specific, play a role in neuronal differentiation or implicated in epigenetic reprogramming (Vierbuchen et al., 2010). By systematic removal of specific candidate genes and repeated transduction, these investigators were further able to demonstrate that a minimal combination of three transcription factors, Ascl1, Brn2, and Myt1l were able to rapidly reprogram embryonic and neonatal mouse fibroblasts to neuron-like cells that expressed multiple neuron-specific proteins, demonstrated spontaneous action potentials and were able to form functional synapses. The majority appeared to be cortical, glutamatergic excitatory neurons. Subsequent studies were able to demonstrate that the combination of Ascl1, Lmx1a, and Nurr1 can convert mouse fibroblasts to dopaminergic neurons (Caiazzo et al., 2011), the combination of Ascl1, Brn2, Myt1l, Lhx2, Hb9, Isl1, and Ngn2 can convert mouse fibroblasts to motor neurons (Son et al., 2011) and that the combination of Brn2, Sox2, and Foxg2 could convert mouse fibroblasts to neuronal precursor cells (Lujan et al., 2012). Ascl1, Brn2, and Myt1l have also been shown to directly convert striatal astrocytes into neurons *in vivo* (Torper et al., 2013). NeuroD has also been shown to directly reprogram reactive glial cells into functional neurons within the cerebral cortex after brain injury (Guo et al., 2014).

Parallel studies on human fibroblasts were able to show that various combinations of factors such as Ascl1, Brn2, Myt1l, and NeuroD1 (Pang et al., 2011); Ascl1, Myt1l, NeuroD2, miR-9/9, and miR-124 (Yoo et al., 2011); or Brn2, Myt1l, and miR-124 (Ambasudhan et al., 2011) could reprogram these cells to glutamatergic neurons. A group of five factors (Ascl1, Brn2, Myt1l, Olig2, and Zic1) could also reprogram human skin fibroblasts into glutamatergic neurons and was used to generate induced

neurons from patients with Alzheimer's Disease (Qiang et al., 2011). Similarly, the combination of Ascl1, Brn2, Myt1l, Lmx1a, and Foxa2 (Pfisterer et al., 2011) or the combination of Ascl1, Lmx1a, and Nurr1 (Caiazzo et al., 2011) could promote the formation of dopaminergic neurons from human fibroblasts. Human fibroblasts could also be directly reprogrammed into motor neurons by the combination of Ascl1, Brn2, Myt1l, Lhx2, Hb9, Isl1, and Ngn2 (Son et al., 2011).

DIRECT REPROGRAMMING OF FIBROBLASTS TO CARDIOMYOCYTES

The first demonstration that mouse fibroblasts could be directly reprogrammed to induced cardiac myocyte-like cells (iCMs) was achieved using an approach similar to that used to generate iPS cells and induced neuronal cells. A pool of 14 candidate factors was initially shown to induce cardiomyocyte-like cells and then the pool was narrowed down to the combination of Gata4, Mef2c, and Tbx5 (GMT) (Ieda et al., 2010). Only a small percentage of fibroblasts were directly reprogrammed, however, and although they had many features of cardiac myocytes, their transcriptional patterns were distinct from neonatal cardiomyocytes. In addition, only a small percentage of the cells could spontaneously contract. Another approach using a different strategy of transiently expressing the pluripotency factors Oct4, Sox2, Klf4, and c-Myc, then culturing the cells in defined media conditions commonly used in the stem cell field to promote cardiac differentiation, including the JAK inhibitor JI1, was also successful (Efe et al., 2011). Another group reported that the GMT factor combination was able to induce expression of cardiac genes, but did not produce any contracting cells (Chen et al., 2012), raising doubts about the efficacy and efficiency of the procedure. Two subsequent studies, however, were able to demonstrate that the retroviral expression of GMT transcription factors could directly reprogram fibroblasts at the site of myocardial injury and decrease infarct size, especially when given in conjunction with thymosin β4 (Inagawa et al., 2012; Qian et al., 2012). A different group reported that direct reprogramming of mouse fibroblasts was more efficient if the transcription factor Hand2 was added in conjunction with GMT, both *in vitro* and *in vivo* after myocardial injury (Song et al., 2012). A subsequent study evaluated the effect of three factor combinations from a pool of 10 candidate factors and determined that Tbx5, Mef2c, and Myocardin induced a broader spectrum of myocardial genes than Gata4, Mef2c, and Tbx5 (Protze et al., 2012). Another study investigated the potential for microRNAs to reprogram mouse fibroblasts to cardiac myocyte like cells and determined that the combination of miR-1, miR-133, miR-208, and miR-499, in conjunction with JAK inhibitor I was sufficient both *in vitro* and *in vivo* (Jayawardena et al., 2012). Others have tried to optimize the reprogramming further and have found that addition of Myocardin, SRF, Mesp1, and Smarcd2 to Gata4, Mef2c, and Tbx5 can enhance the process (Christoforou et al., 2013). To improve the likelihood of obtaining functional cardiac myocytes, another group used fibroblasts containing a calcium sensitive GFP reporter and found that the combination of Hand2, Nkx2-5, Gata4, Mef2c, and Tbx5 could reprogram adult mouse fibroblasts 50 fold more efficiently than GMT alone and that the induced cardiac myocytes demonstrated robust calcium oscillations and spontaneous beating (Addis et al., 2013). The efficiency of conversion by GMT to spontaneously contracting cardiomyocyte-like cells was also reportedly improved by the tethering of the MyoD activation domain to each of these transcription factors (Hirai et al., 2013). A follow up study showed that direct reprogramming with these factors was further enhanced by inhibition of repressive histone modifications (Hirai and Kikyo, 2014).

Direct reprogramming of human fibroblasts to cardiac myocyte-like cells has also been reported, but with different factor requirements. Forced expression of the transcription factors Ets2 and Mesp1 or recombinant ETS2 and MESP1 proteins modified with cell penetrating peptides were sufficient to convert human neonatal foreskin fibroblasts into cardiac progenitors (Islas et al., 2012). The transcription factors Gata4, Hand2, myocardin, and Tbx5 in conjunction with microRNAs miR-1 and miR-133 were sufficient to directly reprogram neonatal foreskin, adult cardiac and adult dermal fibroblasts to cardiomyocyte-like cells (Nam et al., 2013). The function of miR-133 in this context is reportedly to suppress Snai1 and fibroblast genes (Muraoka et al., 2014). The addition of Myocardin and Mesp1 to GMT was reported to reprogram human cardiac fibroblasts to cardiomyocyte-like cells that express a broad array of cardiac genes and exhibit calcium oscillations (Wada et al., 2013). GMT factors in conjunction with MESP1 and ESRRG have also been reported to directly reprogram several types of human fibroblasts to cardiomyocyte-like cells (Fu et al., 2013).

These studies in aggregate demonstrate that multiple transcription factors and microRNAs can contribute to direct reprogramming of fibroblasts. One potential contributor to the variation between these studies is the lack of consensus criteria for assessing the degree of reprogramming. The development and use of standardized criteria for evaluation of transdifferentiation to iCMs, in terms of gene expression, structural, and functional characteristics has been suggested for these types of experiments (Addis and Epstein, 2013).

MECHANISMS OF DIRECT REPROGRAMMING

The mechanisms of direct reprogramming are incompletely understood. While it is well established that transcription factors drive the process and that microRNAs can contribute, it is less clear how cells maintain lineage and in general prevent the development of inappropriate cell types. The process involves activation of target genes, which usually occurs within hours to days (Ieda et al., 2010; Vierbuchen et al., 2010), direct transition from one state to another, without the need to go through a pluripotent state (Zhou et al., 2008; Ieda et al., 2010), does not require cell division, in contrast to induction of pluripotency (Zhou et al., 2008; Hanna et al., 2009; Heinrich et al., 2010; Vierbuchen et al., 2010) and is stable after removal of reprogramming factors (Zhou et al., 2008; Huang et al., 2011; Sekiya and Suzuki, 2011). The interactions between the positive actions of transcription factors and the negative influences of chromatin architecture and epigenetic modifications are currently under investigation. It has long been known that the genome encodes many binding sites for a given transcription factor, but the local chromatin structure only allows certain sites to be accessible,

in a cell type-specific fashion. An example is the hematopoietic transcription factor Scl/Tal, which binds to different sites in different hematopoietic cell types (Wilson et al., 2010; Palii et al., 2011). Unneeded areas of the genome are packaged into heterochromatin and are generally not accessible to transcription factors (Beisel and Paro, 2011). To achieve reprogramming, not only must the reprogramming factors find appropriate binding sites, they must also remodel chromatin appropriately to allow ancillary factors to bind and activate a cell type-specific program. This challenge may explain the general requirement during direct reprogramming for multiple transcription factors that act cooperatively to remodel diverse areas throughout the genome. Another hypothesis being considered is that the reprogramming factors act as "pioneer" transcription factors that can bind to their cognate sites regardless of chromatin configuration (Zaret and Carroll, 2011). In this model, the pioneering factors can bind to their cognate sites and displace nucleosomes, thereby creating a permissive environment for other factors to bind. Given that some cell types are not amenable to direct reprogramming and that related cells are generally more amenable to reprogramming, it is likely that some degree of initial chromatin accessibility or "open access" is necessary even for factors that have "pioneer" capability. Studies on the muscle specific factor MyoD demonstrate that cells susceptible to reprogramming have accessible enhancer elements that allow MyoD binding despite being in an overall repressive state where gene transcription is turned off. Ectopic MyoD was able to quickly bind the enhancer element in the first 24 h, followed by acquisition of H3K4me marks by 48 h (Taberlay et al., 2011).

Direct reprogramming to different cell types occurs at varying efficiency but is usually low. In addition, successful reprogramming often requires high expression levels of reprogramming factors. Accordingly, another postulated mechanism of reprogramming involves transient accessibility to transcription factor binding sites during nucleosome turnover or other mechanisms in which DNA becomes accessible in a stochastic fashion, such as during different phases of the cell cycle (Egli et al., 2008; Vierbuchen and Wernig, 2012).

CURRENT LIMITATIONS AND CHALLENGES FOR THE FUTURE

In addition to low efficiency, another major limitation of direct reprogramming as a strategy to regenerate tissues is the presence of epigenetic memory. Epigenetic memory specific to the original cell type has been well documented in iPS cells (Kim et al., 2010, 2011; Polo et al., 2010). Despite induction of gene expression consistent with reprogramming to another cell type, in multiple cases, some residual gene expression specific to the cell type of origin persists (Feng et al., 2008; Marro et al., 2011). Induced neurons derived from hepatocytes still demonstrate some hepatocyte-specific gene expression (Marro et al., 2011), while induced macrophages derived from fibroblasts still express some fibroblast genes (Feng et al., 2008). In many reported cases of direct reprogramming, only a small set of target genes were assessed, and in cases where more thorough transcriptomic analysis has been performed, there is significant divergence in gene expression patterns from native cells (Ieda et al., 2010; Sekiya and Suzuki, 2011). Since epigenetic memory has also been shown to persist in embryos generated from somatic cell nuclei (Ng and Gurdon, 2005), this problem may be challenging to resolve.

A promising alternative approach has been to use pluripotency factors in the early stage of direct reprogramming followed by induction with cell-type specific factors to promote the differentiation of fibroblasts to cardiac myocytes (Efe et al., 2011). This method is thought to induce a transient state of plasticity more amenable to direct reprogramming without full induction of pluripotency, and reportedly is much more efficient than direct reprogramming. Oct4 in particular has been implicated to play an important role in this regard (Mitchell et al., 2014a,b). To date, however, this approach is limited by persistence of pluripotency markers in the reprogrammed cells and the resulting cells have properties of atrial cardiac myocytes, which may be less useful for regenerative purposes. In general, the phenotype of directly reprogrammed cells is often immature compared to fully differentiated native cells within the target organ of interest, and this may limit their utility in regenerative medicine. For cardiac cells in particular, incomplete differentiation may prevent proper electrical and mechanical coupling, leading to arrhythmias and possibly heart failure. Strategies to promote a state of differentiation comparable to that of target tissue will also be critical to facilitate the use of these cells in regenerative medicine. Exogenous, chemically defined components such as ascorbic acid, recombinant human albumin and other small molecules may be useful in this regard (Crescini et al., 2013; Burridge et al., 2014). The utility of small molecules and chemically defined conditions in promoting direct reprogramming is well established (Lin et al., 2013; Liu et al., 2013; Ifkovits et al., 2014; Lumelsky, 2014; Zhu et al., 2014).

Overall, the potential applications of direct reprogramming to regenerative medicine are extensive. More studies are needed, however, to characterize more fully the phenotype of reprogrammed cells, particularly the extent of epigenetic memory, residual gene expression specific to the original cell type and ability to achieve an appropriate differentiation state and function similarly to native cells. Further refinement of transcription factor combinations, the use of adjunct agents that promote chromatin accessibility, the use of small molecules and the potential utility of pluripotency factors are only a few of the possible approaches to enhance direct reprogramming that are expected to evolve in the future.

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Epigenetic regulation of cardiac myocyte differentiation†

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† ***eTOC Summary Statement:*** *Both Rb and p130 are required for the recruitment of heterochromatin proteins that mediate silencing of proliferation genes in adult cardiac myocytes.*

Cardiac myocytes (CMs) proliferate robustly during fetal life but withdraw permanently from the cell cycle soon after birth and undergo terminal differentiation. This cell cycle exit is associated with the upregulation of a host of adult cardiac-specific genes. The vast majority of adult CMs (ACMs) do not reenter cell cycle even if subjected to mitogenic stimuli. The basis for this irreversible cell cycle exit is related to the stable silencing of cell cycle genes specifically involved in the progression of G2/M transition and cytokinesis. Studies have begun to clarify the molecular basis for this stable gene repression and have identified epigenetic and chromatin structural changes in this process. In this review, we summarize the current understanding of epigenetic regulation of CM cell cycle and cardiac-specific gene expression with a focus on histone modifications and the role of retinoblastoma family members.

Keywords: cardiac myocyte, proliferation, differentiation, heterochromatin, histone modification, retinoblastoma protein

INTRODUCTION

The fetal heart increases in size throughout development via proliferation of CMs but switches to mainly hypertrophic growth of CMs with limited proliferation soon after birth, undergoing terminal differentiation which is associated with permanent cell cycle exit (Ahuja et al., 2007; Mollova et al., 2013; Naqvi et al., 2014). Terminal differentiation in ACMs is characterized by two distinct phenomena: the upregulation of a panel of cardiac-specific adult genes and the permanent withdrawal from cell cycle (Ahuja et al., 2007). The inability of ACMs to proliferate has been linked to the fact that E2F-dependent cell cycle genes specifically involved in regulating G2/M and cytokinesis are not re-expressed after growth stimuli in ACMs. Recent studies suggest the upregulation of adult cardiac-specific genes together with the silencing of cell cycle genes may be mediated by epigenetic mechanisms (Sdek et al., 2011).

Epigenetic mechanisms regulate chromatin structure (Li and Reinberg, 2011), which modulates gene expression and plays a crucial role in diverse biological events such as the specification and differentiation of various cell types (Chen and Dent, 2014). Epigenetic marks have traditionally been thought to be stable, however the recent identification of histone modification enzymes suggests that epigenetic regulation can be a dynamic and reversible process (Kooistra and Helin, 2012). This review focuses on epigenetic regulation in CMs and its role in cell cycle control and terminal differentiation with a focus on histone modifications and Rb family members.

EPIGENETIC REGULATION OF GENE EXPRESSION AND SILENCING

Epigenetics is typically defined as the regulatory mechanisms of gene activity that are not due to changes in DNA sequence. These include modifications of DNA and histone proteins, which affect chromatin structure, and microRNA. In the nuclei of eukaryotic cells, DNA is wrapped around an octamer of histone proteins that are packed into higher-order chromatin structures. Epigenetic regulation involves covalent modification of either of DNA (DNA methylation) or of nucleosomes, which is primarily through post-translational modification of histones (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ribosylation, deamination, and proline isomerization; Chen and Dent, 2014). These epigenetic post-translational modifications are tightly controlled by specific enzymes, for example HATs and HDACs as well as HMTs and HDMs. There are two fundamental types of chromatin: euchromatin and heterochromatin. Euchromatin is typically associated with transcriptionally active

Abbreviations: ACMs, adult cardiac myocytes; CBP, cAMP response element binding protein-binding-protein; CDK, cyclin dependent kinase; CGIs, CpG islands; CMs, cardiac myocytes; CpG, cytosine-guanine dinucleotides; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HDM, histone demethylases; HMT, histone methyltransferase; HP1, heterochromatin protein 1; jmj, jumonji; KO, knockout; Rb, retinoblastoma; TF, transcription factor; TSA, Tricostatin A; WHSC1, Wolf-Hirschhorn Syndrome Candidate 1.

genes because its looser structure is accessible to TFs. In contrast, heterochromatin has a high-density structure that prevents transcriptional machinery access and gene expression (Johnson et al., 2013).

Euchromatin formation is typically associated with histone acetylation, on the other hand, heterochromatin formation is associated with specific histone methylations. The effect of histone methylation is dependent on which amino acid residue of the histone is methylated, and whether the residue is mono, di, or trimethylated (me1, me2, and me3, respectively; Chen and Dent, 2014). For example, methylation of the lysine 4, 36, or 79 lysine residue of histone 3 (H3K4me, H3K36me, and H3K79me) at gene promoters is associated with transcription activation, while methylation of the 9th or 27th lysines (H3K9me, H3K27me) is linked to heterochromatin formation and gene repression (Chen and Dent, 2014). H3K9me3 is a potent inducer of stable heterochromatin by recruiting HP1s (Canzio et al., 2013). H3K27me3 is thought to be more dynamically regulated and mark repressed but poised genes (Rada-Iglesias et al., 2011; Lee et al., 2012). The addition of methyl groups on the lysine and arginine residues of histones is catalyzed by HMTs, while the removal of methyl groups is mediated by HDMs. For all the known lysine residues of HMT activity, counteracting HDMs have been identified, with the exception that the H3K79 HDM is not known, although evidence suggests methylations of this mark are reversible (Kooistra and Helin, 2012).

EPIGENETIC REGULATION OF CARDIAC-SPECIFIC GENE EXPRESSION

During cardiac differentiation, the epigenetic landscape changes dramatically, which is required for appropriate cardiac differentiation (Paige et al., 2012; Wamstad et al., 2012). The establishment and maintenance of a specific gene expression program includes activation of cardiac-specific genes and cell cycle inhibitors as well as stable repression of non-cardiac genes and cell cycle progression genes.

HATs, HDACs, AND HISTONE ACETYLATION

The HAT most studied in cardiac development is p300. p300 is highly expressed in embryonic myocardium but the level declines after birth (Schueler et al., 2012). 3,000–5,000 potential enhancers are associated with p300 in fetal and adult hearts (Blow et al., 2010; May et al., 2012), suggesting an important role in CM development. p300-deficient mice are embryonic lethal at E9-11.5 with heart malformations and reduced expression of cardiac-specific genes such as αMHC and αSA (Yao et al., 1998; Partanen et al., 1999). Knock-in experiments using an acetyltransferase activity-deficient p300 mutant demonstrated that p300 acetyltransferase activity is specifically required for cardiac development (Shikama et al., 2003). p300 interacts with GATA4, Nkx2.5, and Mef2c, which are key TFs regulating CM gene expression and differentiation, at promoters of their target genes (Sun et al., 2010; **Figure 1A**). In addition, expression of cardiac-specific genes such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) correlates with p300 occupancy and histone acetylation on their promoters (Hasegawa et al., 1997; Slepak et al., 2001; Mathiyalagan et al., 2010; Schlesinger et al., 2011; Schueler et al., 2012).

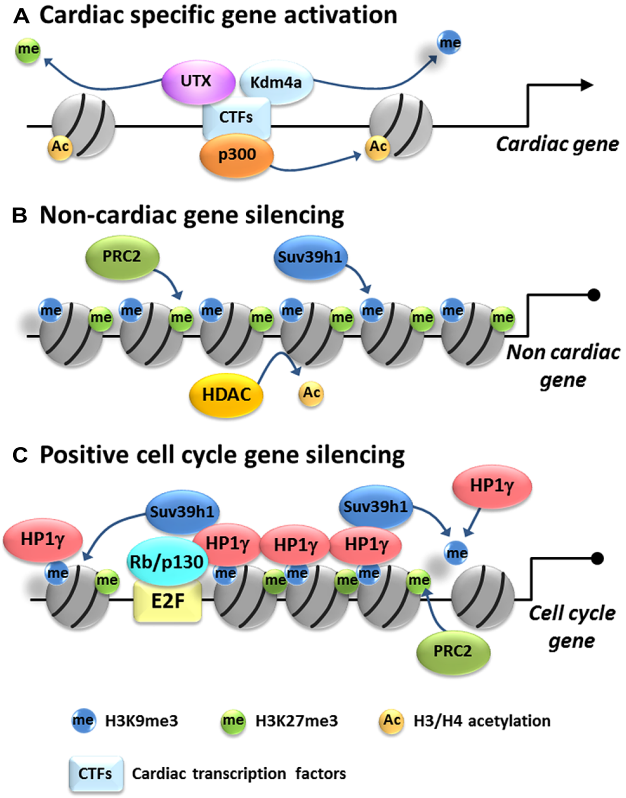

FIGURE 1 | Model of epigenetic gene regulation in cardiac myocytes. Cardiac differentiation is associated with activation of cardiac-specific genes and silencing of non-cardiac and cell cycle genes. **(A)** Cardiac-specific gene regulation: cardiac-specific transcription factors (CTFs) recruit histone acetyltransferases, such as p300, transferring acetyl groups to histone H3 and/or H4. Also they recruit histone demethylases such Kdm4a and UTX to remove silencing methyl marks from H3K9me3 and H3K27me3, resulting in activation of cardiac-specific genes. **(B)** Non-cardiac gene repression: HDACs remove acetyl groups from H3 and/or H4 and histone methyltransferases such as Suv39h1 and PRC2 put methyl groups on H3K9 and H3K27, respectively, promoting tighter histone packing and silencing non-cardiac genes. **(C)** Cell cycle gene silencing: Rb/E2F complex targets HP1γ on positive cell cycle gene promoters. HP1γ spreads H3K9me3 likely through recruitment of Suv39h1 and self-assembles to condensate chromatin, resulting in the packaging and silencing of positive cell cycle genes in heterochromatin. H3K27me3 is also enriched by an unknown mechanism but probably mediated by PRC2.

Consistent with this, inhibition of p300 resulted in decreased expression of cardiac-specific genes such as α-MHC and β-MHC and interestingly the expression of cardiac TFs such as Mef2c, Nkx2.5, and Hands were also decreased (Hasegawa et al., 1997; Lin et al., 1997; McFadden et al., 2000; Poizat et al., 2000; Dai et al., 2002). Cardiac TFs such as GATA4 and Mef2c can be directly acetylated by p300 and the acetylation potentiates DNA binding and transcriptional activity (Kawamura et al., 2005; Ma et al., 2005). CBP, a HAT structurally related to p300, is expressed in embryonic hearts, but CBP-deficient embryos don't show abnormal heart formation (Tanaka et al., 2000; Chen et al., 2009). Males absent on the first (MOF) protein, a HAT belonging to the MYST (MOZ, Ybf2/Sas3, Sas2, and TIP60) family member, is down-regulated in human failing hearts and mouse hypertrophic hearts (Qiao et al.,

2014). Cardiac-specific MOF overexpression ameliorated TAC-induced cardiac hypertrophy, however it was not determined if this protection was related to HAT activity or targeting of specific genes (Qiao et al., 2014). Other HATs such as Gcn5 have been implicated in cardiac differentiation *in vitro* but their relative importance is unknown (Li et al., 2010).

The effects of HATs are counteracted by HDACs, which typically repress gene activation. HDACi TSA promotes acetylation of H3 and H4 and CM differentiation *in vitro* (Kawamura et al., 2005; Karamboulas et al., 2006). Cardiac-specific deletion of either HDAC1 or HDAC2 singly does not evoke a phenotype; however, deletion of both genes results in neonatal lethality, accompanied by cardiac arrhythmias and dilated cardiomyopathy (Montgomery et al., 2007). Mice with cardiac-specific overexpression of HDAC3 show a decrease in global H4 acetylation and an increased thickness of myocardium which is due to cardiac hyperplasia without hypertrophy (Trivedi et al., 2008). The hyperplasia is related to suppression of Cdk inhibitors such as $p21^{cip1}$, $p27^{kip1}$, $p57^{kip2}$, $p18^{inc4c}$, and $p15^{inc4b}$. In contrast, mice with a cardiac-specific deletion of HDAC3 survived up to 4 months of age but demonstrated massive cardiac hypertrophy, myocardial lipid accumulation and elevated triglyceride levels (Montgomery et al., 2008). Indeed, ChIP assays show that HDAC3 co-occupies promoters of numerous genes involved in metabolic regulation with PPARα. It seems that HDAC3 is an important regulator of CM proliferation and energy metabolism during cardiac development. Interestingly global histone acetylation is unchanged in HDAC3 KO mice, suggesting that the effects of HDAC3 deficiency are very specific. HDAC4, a class II HDAC, has an anti-hypertrophic role through Mef2 suppression. Recent studies suggested that HDAC4 suppresses Mef2 in a histone deacetylation activity independent manner (Backs et al., 2011; Hohl et al., 2013). Indeed histone acetylation did not change on hypertrophic gene promoters when HDAC4 nuclear activity was reduced (Hohl et al., 2013). HDAC5 and HDAC9 are highly enriched in the heart and their functions are overlapping during cardiac development (Haberland et al., 2009). Single HDAC5 or HDAC9 KO mice are viable without apparent cardiac defects but mice lacking both HDAC5 and HDAC9 are embryonic or early postnatal lethal with ventricular septal defects, thin-walled myocardium and abnormality of CMs (Zhang et al., 2002; Chang et al., 2004). Since HDAC5 and HDAC9 interact with Mef2 to suppress its transcriptional activity (Zhang et al., 2002; Chang et al., 2004), the developmental cardiac defects in the double mutant mice are likely resulted from aberrant activation of Mef2. Interestingly it has been shown recently that HDAC can also be acetylated during cardiac hypertrophy, which alters their function (Eom et al., 2014). Numerous reports using inhibitors and gene manipulation techniques have revealed the importance of HAT/HDAC in cardiac development. However, the specific target genes and the histone acetylation-independent mechanism of each HAT/HDAC and their roles in cardiac development require further study.

HMTs, HDMs, AND HISTONE METHYLATION

There is also increasing evidence demonstrating the importance of histone methylation in regulating cardiac phenotypes (Gottlieb et al., 2002; Barski et al., 2007; Nimura et al., 2009; Fujii et al., 2011; Movassagh et al., 2011; Tao et al., 2011; He et al., 2012a; Lee et al., 2012). Recent exome sequencing analysis revealed that congenital heart disease cases show a marked excess of *de novo* mutations in genes involved in H3K4 and H3K27 modifications (Zaidi et al., 2013). ACMs with inducible, cardiac-specific KO of H3K4 HMT subunit, PAX interacting (with transcription-activation domain) protein 1 (PTIP), showed altered expression of genes involved in conduction, such as Kcnip2, but not genes involved in hypertrophy, such as β-MHC and ANP (Stein et al., 2011). Specific deletion of PTIP in ACMs led to dysregulated sodium and calcium handling, abnormal EKGs, and susceptibility to ventricular premature beats, but no abnormalities of cardiac growth. Smyd1 is a cardiac and skeletal muscle restricted chromatin remodeling protein that can also methylate H3K4 *in vitro*, suggesting it may function as a muscle-specific transcription activator (Sims et al., 2002; Tan et al., 2006; Sirinupong et al., 2010). Smyd1 deficient mice die *in utero* secondary to abnormal CMs maturation and right ventricular development (Gottlieb et al., 2002). Consistent with the right ventricular development defect, the expressions of Hand2 and Irx4 are downregulated in hearts lacking Smyd1 (Gottlieb et al., 2002; Park et al., 2010). Muscle-specific TF skNAC is a major partner for Smyd1 in the developing heart (Park et al., 2010; Sirinupong et al., 2010) and normal expression of Hand1 and Irx4 is dependent on Smyd1-skNAC interaction (Park et al., 2010). It is not clear if the defect in cardiac development and cardiac-specific gene expression in Symd1 deleted mouse is directly related to its HMTase activity (Tan et al., 2006; Just et al., 2011). Interestingly Symd1 interacts with sarcomere protein and potentially methylates myosin protein (Just et al., 2011; Li et al., 2013). Symd1 can also function as a transcriptional repressor by recruiting class I HDAC (Gottlieb et al., 2002; Costantini et al., 2005).

Another Smyd family member, Smyd2, is a H3K4 and H3K36 HMT that is highly expressed in neonatal CMs. A CM-specific KO of Smyd2 showed it is dispensable for normal cardiac development and had no effect on H3K4 and H3K36 methylation in mice, perhaps due to redundant HMTs that can compensate for the Smyd2 deficient (Diehl et al., 2010). Wolf-WHSC1 is a H3K36 HMT, which catalyzes mono-, di-, and tri-methylation. Deletion of WHSC1 is observed in all patients with Wolf–Hirschhorn Syndrome, which is associated with cardiac congenital defects (Bergemann et al., 2005). WHSC1 KO mice die perinatally with atrial and ventricular septal defects (Nimura et al., 2009). WHSC1 interacts with Nkx2.5 and occupies Nkx2.5 target genes to repress transcription presumably through H3K36me3 modification. Distinct H3K36me methylation patterns have been described for end-stage cardiomyopathic compared to age-matched normal human hearts (Movassagh et al., 2011), suggesting proper regulation of H3K36me may be important for cardiac development and maintaining physiological ACMs gene expression in humans as well.

Histone methyltransferases have also been implicated in cardiac health and disease. PRC2 is a HMT complex, which consists of four components: catalytic subunit enhancer of Zeste 1 (Ezh1)/Ezh2, suppressor of Zeste 12 (Suz12), embryonic ectoderm development (Eed), and RbAp46/48 (Margueron and Reinberg, 2011). PRC2 mediates the methylation of H3K27, which silences genes

and regulates tissue-specific differentiation by orchestrating the repression of unnecessary or stage-specific transcriptional programs (Boyer et al., 2006; Pasini et al., 2007; Shen et al., 2008). During CM development, H3K27me3 levels increase when cardiac progenitor cells are differentiating into CMs (Delgado-Olguin et al., 2012). In the heart, Ezh1 and Ezh2 are predominantly expressed in adult and embryonic stage, respectively (Sdek et al., 2011). The importance of PRC2 in cardiac development has been demonstrated using cardiac-specific deletion models (Chen et al., 2012; Delgado-Olguin et al., 2012; He et al., 2012a). Conditional inactivation of Ezh2 specifically in right ventricle progenitors by Mef2cAHF-Cre, which is active from E7.5, caused right ventricle hypertrophy (Delgado-Olguin et al., 2012). This hypertrophy was caused by derepression of Six1 gene which is stably silenced upon cardiac differentiation. Nkx2.5-Cre driven Ezh2 inactivation in early cardiac differentiation caused embryonic lethality with defects in heart development; however, inactivation of Ezh2 in differentiated CMs by TnT-Cre did not evoke a phenotype (He et al., 2012a). The developmental defect in Ezh2 deficient mice was associated with aberrant expression of non-cardiac and cell cycle inhibitor genes and ectopic expression of atrial-specific genes in ventricular myocytes. These findings indicate that Ezh2 and H3K27me3 promote and stabilize cardiac differentiation by silencing ectopic gene programs. Interestingly, Ezh2 directly binds to GATA4 and also methylates it, which attenuates its transcriptional activity by reducing its interaction with p300. This interact is important for suppression of αMHC expression in embryonic CMs (He et al., 2012b). G9a and GLP are major H3K9 mono- and dimethyltransferases and contribute to transcriptional silencing. Nkx2.5-Cre driven dual function loss of GLP and G9a (GLP-KO/G9a-KD) mice showed reduction of H3K9me2 level in CM and atrioventricular septal defects, but not in single either GLP or G9a function loss (Inagawa et al., 2013). Array analysis revealed expression of non-CM gene in GLP-KO/G9a-KD CM. Suv39h1 which mediates tri-methylation of H3K9 can regulate cell cycle exit in cardiac differentiation (Sdek et al., 2011). Silencing of Suv39h1 in ACMs by siRNA increased the expression of cell cycle progression genes and consistent with this silencing of HP1γ which binds H3K9me3, also induced cell cycle progression gene upregulation (Sdek et al., 2011). Thus deposition of suppressive histone marks such as H3K9me2/3 and H3K27me3 seems to be involved in non-cardiac gene silencing and cell cycle exit (**Figure 1B**).

Jumonji, coded by the Jarid2 gene, is a nuclear factor that plays an essential role in the development of multiple tissues, including the heart. Jmj has a DNA binding domain, ARID, and two conserved Jmj domains (JmjN and JmjC; Takeuchi et al., 2006). Based on homology to the Jmj domain, it is now recognized that this protein is part a family of proteins, most of which are associated with histone modifying activity. The JmjC domain is essential for the demethylase activity (Klose et al., 2006; Takeuchi et al., 2006). The identification that the Jmj family proteins have HDM activity, which can demethylate mono-, di and tri-methylation, suggested that histone methylation might be more dynamic than previously thought and regulate acute changes in gene expression (Bose et al., 2004; Tsukada et al., 2006; Lan et al., 2008; Lee et al., 2012). The Jmj family member Jmjd6 is a histone H3 and H4 arginine demethylase that is essential for cardiac development (Chang et al., 2007). Jmjd6-deficient mice die perinatally due to cardiac malformations with ventricular septal defect and double-outlet right centicle (Schneider et al., 2004). Kdm4a is a H3K9me3 and H3K36me3 HDM (Whetstine et al., 2006). In failing hearts ANP and BNP promoters have less enrichment of H3K9me3 (Hohl et al., 2013). Consistent with this Kdm4a is upregulated and enriched on these promoters (Zhang et al., 2011; Hohl et al., 2013). Although cardiac-specific Kdm4a deficient mice and transgenic mice which overexpress Kdm4a show no overt baseline phenotype (Zhang et al., 2011), when subjected to pressure overload, inactivation of Kdm4a attenuates hypertrophic response while Kdm4a overexpression enhances cardiac hypertrophy (Zhang et al., 2011). This Kdm4a mediated hypertrophy can be related to the expression of FHL1, a key component of the mechanotransducer machinery which is involved in hypertrophic development (Sheikh et al., 2008), via demethylation of H3K9me3 on FHL1 promoter. Recently another JmjC protein ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX), a H3K27 demethylase encoded on X chromosome, was shown to be essential for cardiogenesis (Agger et al., 2007; Hong et al., 2007; Lan et al., 2007). UTX is highly expressed in developing hearts and its deletion in female mice is embryonic lethal with severe cardiac malformation (Lee et al., 2012). UTX null embryonic stem cells (ESCs) also fail to develop spontaneous contractions and cardiac-specific gene expression (ANP, MLC2, and a-CA). UTX interacts with core cardiac TFs, Nkx2.5, Tbx5, GATA4, and serum response factor (SRF) as well as cardiac-specific enhancer of Brg1-associated factor Baf60c and potentiates their transcriptional activity to activate cardiac-specific genes (Lee et al., 2012). Interestingly, in addition to H3K27me3, UTX can also affect H3K4 methylation for activation of cardiac enhancers (Lee et al., 2012). This is likely to be an indirect effect of the loss of UTX due to the fact that UTX and MLL3/4 are in the same complex. Thus removal of silencing histone marks is important for cardiac-specific gene activation.

EPIGENETIC REGULATION OF CM TERMINAL DIFFERENTIATION

Terminal differentiation is not the only situation under which adult cells become postmitotic. Senescent cells also undergo an irreversible cell cycle arrest. In both situations, cells are unable to express the genes required for proliferation, even when stimulated with growth factors. At the molecular level, nuclei of senescent and terminally differentiated cells demonstrate accumulation of heterochromatin. This heterochromatin is a characteristic feature of the irreversible cell cycle exit of senescent and terminally differentiated cells (Narita et al., 2003; Brero et al., 2005; Sdek et al., 2011, 2013). Large-scale chromatin condensation and the reorganization of nuclear domains reduce the accessibility of transcription machinery within heterochromatic loci (Grewal and Jia, 2007). Localization of E2F target genes in heterochromatin regions is seen in both senescent and terminally differentiated cells (Narita et al., 2003; Sdek et al., 2011). Heterochromatic regions are characterized by histone hypoacetylation and enrichment of H3K9me3. Chromatin of proliferating embryonic CMs is hyperacetylated

(H3K9/14, H3K18, and H3K27), but following adult differentiation, acetylation deceases and histone methylation (H3K9me3 and H3K27me3) associated with transcriptional repression predominates (Sdek et al., 2011).

HISTONE ACETYLATION

Histone deacetylation mediated by HDACs is the initial step of heterochromatin assembly. Although little is known about the function of HDACs in CMs terminal differentiation, among the over 18 HDAC family members, HDAC1 plays critical role in regulation of proliferation in other cell types; however, the effects of HDAC1 on cell cycle are developmentally dependent. Deletion of HDAC1 results in embryonic lethality at E9.5 due to impaired cellular proliferation (Lagger et al., 2002). However, in cellular senescence, HDAC1 promotes irreversible silencing of proliferation related genes (Stadler et al., 2005; Bandyopadhyay et al., 2007; Willis-Martinez et al., 2010; Chuang and Hung, 2011). HDAC1 and hypo-phosphorylated Rb protein levels are elevated in senescent cells (Narita et al., 2003; Wang et al., 2008). HDAC1 forms a complex with Rb and E2F4 on the E2F-dependent promoters and is responsible for deacetylating histone H3 on E2F-dependent promoters (Wang et al., 2008). A critical role for HDAC1 in terminal differentiation has been revealed in several cell types (Stadler et al., 2005; Yamaguchi et al., 2005; Ye et al., 2009). HDAC1 is required for the switch from proliferation to differentiation by antagonizing Wnt and Notch signaling pathways to promote cell-cycle exit and the subsequent neurogenesis in zebrafish retina (Yamaguchi et al., 2005). Loss of HDAC1 in retina results in failure of differentiation, which correlated well with failure of precursor cells exit cell cycle and upregulation of proliferation promoting proteins (Stadler et al., 2005). An inducible-cardiac-specific model to delete HDACs at different stages in differentiating CMs might shed light on the specific roles of HDACs in CMs differentiation.

H3K9 METHYLATION

Di- and tri-H3K9me at promoters of growth-promoting genes is critical feature of cellular senescence and terminal differentiation associated with gene repression (Narita et al., 2003; Kotake et al., 2007; Sdek et al., 2011). However, it is not clear if H3K9me2 and H3K9me3 are equally important in gene silencing of postmitotic cells. Establishment of H3K9me2 and H3K9me3 requires different methyltransferases (G9a/GLP and Suv39h1/2 respectively), and the nuclear sublocalization of these two modifications in postmitotic cells is different. H3K9me2 is found in both euchromatin and heterochromatin regions while H3K9me3 is exclusively co-localized with heterochromatin (Sdek et al., 2011). This finding suggests H3K9me2 and H3K9me3 have slightly different roles in the repression of gene expression. Since Rb is intimately involved in targeting these methylations, knocking out Rb family member expression in specific cell types allows dissection of the roles of H3K9me2 and H3K9me3 in terminally differentiated cells (Sdek et al., 2011). Acute depletion of Rb alone does not trigger cell cycle reentry in ACMs although it dramatically reduces H3K9me2 levels, indicating H3K9me2 is dispensable for maintenance of the postmitotic state in ACMs (Sdek et al., 2011). In contrast, deleting both Rb and p130 disrupts heterochromatin and allows cell cycle reentry in ACMs; however, H3K9me3 levels were unchanged (Sdek et al., 2011), which suggests H3K9me3 is established and maintained by an Rb-independent pathway and the presence of H3K9me3 alone is not sufficient for heterochromatin formation in CMs. In contrast *in vitro* experiments have shown that knockdown of Suv39h1 resulted in de-suppression of cell cycle gene and terminal differentiation failure in myocytes, suggesting that H3K9me3 is, at least, necessary for establishing myocyte terminal differentiation (Ait-Si-Ali et al., 2004; Sdek et al., 2011).

H3K9me2/3 MEDIATOR HP1

The ability of Rb to stably repress transcription is related to its capacity to recruit HP1s to target gene promoters resulting in their incorporation into heterochromatin (Nielsen et al., 2001; Narita et al., 2003). HP1 is a family of proteins (HP1α, -β, and -γ) that play an important role in gene silencing in many organisms (James and Elgin, 1986; Kellum, 2003) by establishing and maintaining heterochromatin (Daniel et al., 2005). HP1 family members typically differ in their subcellular localization and interaction partners and thus likely have distinct cellular functions (Minc et al., 1999, 2001; Auth et al., 2006). A p130 and HP1α complex is recruited to E2F regulated promoters during neuronal differentiation to induce cell cycle exit (Panteleeva et al., 2007). Rb directly promotes permanent cell cycle exit in senescent cells by recruiting HP1γ to E2F responsive promoters that have undergone methylation of H3K9 (Narita et al., 2003). The role of HP1s in the heart largely remains unknown. All three HP1 family members are expressed in ACMs although their subnuclear localization differs. HP1γ in particular is essential for stably repressing proliferation-promoting genes in ACMs, and although HP1 family members share similar structure, HP1α and HP1β could not compensate for the loss of function of HP1γ in CMs (Sdek et al., 2011). HP1γ binds to G2/M and cytokinesis gene promoters in ACMs but disassociated from these promoters when Rb/p130 were acutely deleted, although H3K9me3 levels at G2/M and cytokinesis gene promoters remained intact. The dissociation of HP1γ at G2/M and cytokinesis gene promoters correlated with loss of heterochromatin in ACMs' nuclei, re-expression of G2/M and cytokinesis genes as well as cell cycle re-entry. Given the important role of HP1s in heterochromatin formation, the absence of HP1γ recruitment appeared to be the key factor in the disruption of heterochromatin and the reinduction of proliferation capacity in ACMs lacking Rb/p130. Thus, in CM terminal differentiation, Rb and p130 serve as a bridge to link histone modifications and heterochromatin formation through their interaction with HP1γ. Heterochromatin stably represses the expression of proliferation-promoting genes and maintains the postmitotic phenotype of ACMs (Sdek et al., 2011; **Figure 1C**).

H3K27 METHYLATION

The role of H3K27me3 in terminal differentiation is not clear although the promoters of proliferation related genes displayed higher levels of H3K27me3 in ACMs and skeletal myotubes (Blais et al., 2007; Sdek et al., 2011). H3K27me3 is important for stable gene repression, including suppression of E2F-dependent

genes, in certain contexts (Blais et al., 2007). ACMs from mice where Rb and p130 were deleted embryonically did not undergo permanent cell cycle exit (MacLellan et al., 2005). H3K27me3 and heterochromatin formation in these CMs were dramatically impaired although global H3K9me3 levels were unchanged (Sdek et al., 2011). H3K27me3 is not exclusively enriched on heterochromatin, indicating H3K27me3 might relate to early stages of heterochromatin formation and repression of genes required for proliferation.

Rb REGULATION OF EPIGENETIC GENE SILENCING

Rb is the prototypical member of a gene family encoding three structurally and functionally similar proteins, Rb, p107, and p130 (Chen et al., 1996). Rb also plays critical roles in senescence and terminal differentiation associated with irreversible growth arrest including CMs (Narita et al., 2003; Sdek et al., 2011). Rb mediated inhibition of gene expression can be achieved by two mechanisms: direct inhibition of E2F or recruitment of epigenetic remodeling factors (Gonzalo and Blasco, 2005). These two mechanisms are selective: some promoters are repressed by the first mechanism, whereas other promoters, particularly cell cycle genes, are silenced by the second mechanism (Brehm et al., 1998; Luo et al., 1998; Angus et al., 2004). Rb family members associate with multiple chromatin remodeling factors, including HDACs (Luo et al., 1998; Magnaghi-Jaulin et al., 1998), Suv39h1 (Nielsen et al., 2001), HP1 (Nielsen et al., 2001), Ezh2 (Tonini et al., 2004), Pc2 (Dahiya et al., 2001), and DMNT1 (Robertson et al., 2000). Thus Rb affects a wide range of epigenetic regulation pathways, including histone acetylation, histone methylation and DNA methylation.

The major effect of Rb on the histone acetylation pathway is facilitating deacetylation of target gene promoters by recruiting HDACs (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Class I HDACs (HDAC1, −2, and −3) directly interact with Rb through the pocket domain (Lai et al., 1999). Similar to many Rb-interacting proteins, HDAC1 contain an leucine-X-cysteine-X-glutamic acid, X stans for any any amino acid (LXCXE) motif, which allows direct interaction with Rb (Magnaghi-Jaulin et al., 1998). Recruitment of HDAC1 to E2F-regulated promoters is important for Rb-mediated S phase repression (Brehm et al., 1998). Rb also regulates H3K9me3, which is important for gene silencing and heterochromatin formation. Histone methltransferase, Suv39h1/2, is specifically required for H3K9me3 establishment and maintenance (Schotta et al., 2004; Siddiqui et al., 2007). Rb physically interacts with Suv39h1 and it has been suggested that this interaction is critical for Suv39h1's gene suppression activity (Nielsen et al., 2001). Recent studies, however, have demonstrated that global levels of H3K9me3 is normal in fibroblasts that are triply deficient for Rb, p107 and p130 (Gonzalo et al., 2005; Siddiqui et al., 2007), indicating that Rb family members are dispensable for Suv39h1 imposed H3K9me3. H3K9me3 is specifically recognized by HP1 protein, and the binding of HP1 protein at H3K9me3 site is important to transmit its biological signals (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001). HP1 also recruits Suv39h1 and propagates H3K9me3 to adjacent chromatin (Yamamoto and Sonoda, 2003; Hathaway et al., 2012). Thus, heterochromatin formation is a self-assembling framework of "tethers" (H3K9me3) and "adaptors" (HP1) where the HP1 molecules bound to neighboring nucleosomes dimerize through their chromoshadow domains, leading to HP1-nucleosome complexes and chromatin condensation (Breitbart et al., 1985; Hathaway et al., 2012). HP1s contain an LXCXE (or LXCXD) motif which allows interaction with both Rb and p130 (Nielsen et al., 2001; Panteleeva et al., 2007); thus RB can directly target HP1 on cell cycle gene promoters (Nielsen et al., 2001; Panteleeva et al., 2007; Sdek et al., 2011), which seems to be a key step for the initiation of terminally differentiation and senescence. These findings are supported by fact that the absence of Rb results in loss of heterochromatin and disrupted H3K9me3 nuclear distribution, even though global H3K9me3 is intact (Narita et al., 2003; Gonzalo et al., 2005; Sdek et al., 2011; Zhang et al., 2013).

It has been demonstrated that Rb is also required for establishment and maintenance of H3K27me3 (Blais et al., 2007; Kotake et al., 2007). Rb physically interacts with PRC2, the major enzyme complex that methylates H3K27. In growing human and mouse primary cells, expression of p16, a prominent cell cycle inhibitor, is repressed by H3K27me3 at the p16 locus, which is established by PRC2. This recruitment of PRC2 is Rb dependent (Kotake et al., 2007). In contrast Rb also interacts with Pc2, the effector protein of the PCR1 complex that recognizes and binds to H3K27me3 (Dahiya et al., 2001; Cao et al., 2002); it has been reported that Pc2 cooperates with Rb to inhibit expression of cyclin A and cdc2 in senescence cells (Dahiya et al., 2001). The mechanism underlying this function switching of Rb remains to be elucidated.

Importance of Rb on CM terminal differentiation through epigenetic mechanism was demonstrated (Sdek et al., 2011). Although cardiac-specific information is limited, epigenetic role of Rb has been demonstrated in senescence and cell line model. Potentially the similar mechanisms discussed above are mediating CM terminal differentiation.

CONCLUSION

Commitment to a particular lineage requires both the repression of unnecessary genes while simultaneously up-regulating lineage-specific genes. High-throughput DNA sequencing technology has enable the search for the binding sites of cardiac TFs, enhancers, epigenetic marked histones and chromatin modifying factors on a genome-wide level by ChIP-sequencing (Blow et al., 2010; He et al., 2011; Paige et al., 2012; Wamstad et al., 2012; Papait et al., 2013). Core cardiac TFs such as Nkx2.5, Mef2c, GATA4, Tbx5, and SRF and cell cycle master regulator RB/p130 have been shown to form complexes with epigenetic modifying proteins and these complex multimers lead to modifications of histones at promoters of cardiac and cell cycle genes which locks in the ACM phenotype. This review has attempted to summarize the advances that have been made in our understanding of epigenetic regulation in cardiac differentiation and development. Although increasing evidence suggests crucial roles of epigenetic modifying proteins and epigenetic marks, their specific function in cardiac lineage commitment and differentiation as well as their orchestrating mechanisms still remain to be elucidated. Regardless, understanding this epigenetic regulation will undoubtable

uncover new insights into cardiovascular biology and potentially facilitate development of novel targets for cardiovascular therapeutics and regeneration.

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The altered fate of aging satellite cells is determined by signaling and epigenetic changes

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Skeletal muscle is a striated tissue composed of multinucleated fibers that contract under the control of the somatic nervous system to direct movement. The stem cells of skeletal muscle, known as satellite cells, are responsible for muscle fiber growth, turnover, and regeneration. Satellite cells are activated and proliferate in response to stimuli, and simplistically, have two main fates—to repopulate the satellite cell niche, or differentiate to regenerate or repair muscle fibers. However, the ability to regenerate muscle and replace lost myofibers declines with age. This loss of function may be a result of extrinsic changes in the niche, such as alterations in signaling or modifications to the extracellular matrix. However, intrinsic epigenetic changes within satellite cells may also affect cell fate and cause a decline in regenerative capacity. This review will describe the mechanisms that regulate cell fate decisions in adult skeletal muscle, and how changes during aging affect muscle fiber turnover and regeneration.

Keywords: skeletal muscle, satellite cells, niche, aging, quiescence, senescence

AGING, SARCOPENIA, AND THE SATELLITE CELL

Aging is characterized by a time-dependent accumulation of cellular damage. Cells suffer damage as a result of chronological aging, as well as replicative aging, which occurs as cells proliferate in response to various stimuli. Stem cells are thought to be protected from the effects of aging by quiescence, a state of cellular hibernation which provides cushioning from the stresses associated with cell proliferation, thereby limiting cellular damage. However, quiescent cells are not dormant. Instead, quiescent stem cells actively maintain their state; they are poised for action, waiting for signals that activate proliferation. Aging is associated with the inability to maintain stem cell quiescence, which increases the chances of stem cell damage, and results in a loss of stem cell self-renewal and regenerative capacity.

Aging is also associated with a gain of cellular senescence. Senescent cells are alive and metabolically active, but have lost the ability to divide. The primary purpose of senescence is to prevent propagation of damaged cells. Senescent cells are resistant to apoptosis, and are normally cleared by the immune system. Once cleared, tissue-specific repair mechanisms are activated and lost cells are replaced. During aging, senescent cells accumulate, which increases tissue inflammation, as senescent cells secrete pro-inflammatory cytokines, such as TNFα and IL-6. Thus, an increase in senescence, combined with a decrease in regenerative capacity, is predicted to result in a net loss of cells and/or tissue.

In skeletal muscle, aging is manifested as sarcopenia, the gradual loss of muscle mass and function in the absence of an attributable disease. Skeletal muscle is eventually replaced by fatty and fibrous tissue, which results in functional impairment of the muscle and physical disability. In the United States, sarcopenia occurs in approximately 45% of the population over the age of 60, and upward of 50% of muscle fibers are lost from limb muscles by the age of 80 (Janssen et al., 2004; Faulkner et al., 2007). However, sarcopenia can occur at any age as a result of disuse or malnutrition. In younger individuals, the loss of muscle mass is reversible, whereas in older or geriatric individuals, muscle loss appears to be irrecoverable.

The ability to generate skeletal muscle during post-natal growth and to regenerate skeletal muscle in adults is almost exclusively due to the action of Pax7-expressing satellite cells, the stem cells of skeletal muscle (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). During the process of muscle regeneration, satellite cells are activated and proliferate, and adopt one of two cell fates: differentiation to generate or repair muscle fibers, or a return to quiescence to repopulate the satellite cell niche.

Given the role of satellite cells in post-natal muscle growth and adult muscle regeneration, it is reasonable to hypothesize that satellite cells are responsible for maintaining muscle mass and myonuclei number through aging. If true, then sarcopenia is predicted to be the result of a loss of satellite cell number, or a failure of satellite cells to function in aged individuals. Indeed, many studies have shown an age-related decline in the number of satellite cells and/or an age-related loss of satellite cell function (Roth et al., 2000; Conboy et al., 2003; Shefer et al., 2006; Day et al., 2010; Chakkalakal et al., 2012; Sousa-Victor et al., 2014). Moreover, transplantation of young satellite cells into the muscle of progeroid mice extends lifespan and ameliorates degenerative changes in skeletal muscle (Lavasani et al., 2012).

A more recent study challenges the notion that loss of satellite cells or satellite cell function is responsible for age-related sarcopenia (Fry et al., 2015). In this study, young mice were

treated briefly with tamoxifen to deplete satellite cells by Pax7-dependent activation of diphtheria toxin A (DTA) expression, and then allowed to age naturally. The results clearly show that satellite cell depletion does not accelerate age-related sarcopenia, but does affect the ability of skeletal muscle to respond to acute injury. This study highlights the need to better understand myonuclear turnover in adult muscle, and raises the question of whether satellite cells play a role in adult muscle maintenance.

A small population of satellite cells remains after tamoxifen treatment, and appears to increase in number over time in the gastrocnemius, tibialis anterior, and extensor digitorum longus muscles. Radiation-induced depletion of functional satellite cells in mice also results in survival of a small sub-population of satellite cells, which maintain the ability to contribute to repair and regeneration of skeletal muscle (Heslop et al., 2000). Moreover, transplant of a very small number of satellite cells, associated with a single muscle fiber, has the ability to generate a significant number of donor-derived muscle fibers, and contribute to the recipient satellite cell niche (Collins et al., 2005). Therefore, it is possible that the small number of satellite cells that remain in tamoxifen-treated mice provides enough cells to maintain muscle mass until other age-related changes occur, at which point loss of satellite cell function manifests as sarcopenia.

Age-related changes can be satellite cell-intrinsic or extrinsic. Satellite cells exist within a niche that consists of surrounding cells and the extracellular matrix (ECM), which provide biochemical and biophysical signals that direct regeneration and self-renewal. Age-related changes to the niche have the potential to affect satellite cell fate by altering environmental cues, resulting in aged satellite cells failing to re-enter quiescence, aberrantly entering senescence and/or failing to prevent fibrosis.

Satellite cells from aged and young muscle appear to proliferate *ex vivo* with similar rates (George et al., 2010; Alsharidah et al., 2013; Verdijk et al., 2014). The inability of aged satellite cells to show the effects of aging in a culture dish suggests that the aged muscle environment is to blame for the decline in regenerative capacity. However, studies with human cells suggest that culturing with 20% fetal calf serum masks differences between young and aged satellite cells, and demonstrate that culturing with human sera of the same age reveals a delayed response to activating stimuli and reduced proliferation (Barberi et al., 2013). Moreover, reduced regeneration in adult mice transplanted with FACS sorted geriatric satellite cells as compared to adult mice transplanted with adult satellite cells suggests a cell-intrinsic change that affects aged satellite cell function (Sousa-Victor et al., 2014).

Together, these data this suggests that satellite cell-intrinsic changes, combined with satellite cell-extrinsic changes within the niche alter cell fate decisions, and manifest as inefficient skeletal muscle repair, resulting in sarcopenia. This review will examine how satellite cell-extrinsic and satellite cell-intrinsic changes during aging affect satellite cell fate decisions and implicate the loss of satellite cell function as causative in sarcopenia.

AGE-RELATED FIBROSIS AND SATELLITE CELL FATE

During the later stages of normal regeneration, a sub-population of macrophages in the muscle secrete TGFβ, which directs muscle-resident fibroblasts to secrete ECM proteins that reconstitute the basal lamina and the reticular lamina that surround muscle fibers. The ECM provides mechanical support and a scaffold to orient the fibers during regeneration (Sanes, 2003). Activation of TGFβ/activin signaling in cells specifically phosphorylates Smad2 and Smad3, stimulating nuclear localization and regulating gene expression. TGFβ-mediated phosphorylation of Smad3 is specifically required for expression of collagen and ECM components in fibroblasts, and for activation and proliferation in satellite cells (Ge et al., 2011, 2012).

During aging, skeletal muscle fibers are progressively replaced by adipose and fibrotic tissue, which is exacerbated by injury (Brack et al., 2007; Paliwal et al., 2012). The formation of excessive connective tissue, also known as fibrosis, is a characteristic feature of sarcopenia. A change in intensity and duration of the macrophage response in aged skeletal muscle results in a higher level of TGFβ signaling in skeletal muscle (Zacks and Sheff, 1982; Carlson et al., 2008). This extends the phase of protein deposition by skeletal muscle fibroblasts, resulting in an increased level of ECM proteins and the presence of atypical types of collagen (Marshall et al., 1989; Alexakis et al., 2007). Moreover, less collagen turnover and more collagen cross-linking results in a densely packed lamina that increases muscle stiffness and potentially limits skeletal muscle function.

Increased TGFβ signaling inhibits satellite cell activation and proliferation (Allen and Boxhorn, 1987, 1989; Rathbone et al., 2011). Sustained TGFβ signaling in aged muscle is expected to decrease satellite cell proliferation, stimulate proliferation of fibroblasts in skeletal muscle, and increase expression of ECM proteins. Specifically, loss of satellite cell-derived signaling to muscle-resident fibroblasts relieves repression of collagen Ia1, collagen IIIa1, collagen VIia2, and fibronectin expression (Fry et al., 2014). Therefore, satellite cells, in addition to participating in the generation and repair of muscle fibers, are also responsible regulating ECM production and preventing fibrosis.

High levels of Wnt3a induce skeletal muscle fibrosis in mice, suggesting there may be a link between TGFβ and Wnt signaling in promoting fibrosis in aged muscle (Brack et al., 2007). Indeed, aged mice display an increase in the level of a serum factor that promotes Wnt activity, and this serum factor is postulated to promote excessive production of ECM proteins. This serum factor may be the complement protein, C1q, which can bind Fzd receptors and activate canonical Wnt signaling (Naito et al., 2012; Watanabe et al., 2014).

One study suggests that Wnt3a signaling stimulates canonical Wnt signaling and induces a change in cell fate, such that myogenic satellite cells are converted to the fibrogenic lineage (Brack et al., 2007). However, a separate study indicates that injection of a high level of Wnt3a into mouse skeletal muscle stimulates proliferation of a stromal cell population that produces collagen, resulting in replacement of adult skeletal muscle with fibrous tissue (Trensz et al., 2010). Importantly, both age- and disease-related fibrosis can be resolved by injection of DKK1, a Wnt signaling antagonist (Brack et al., 2007; Trensz et al., 2010).

The increase in fibrosis affects the ability of skeletal muscle to function. However, fibrosis also exacerbates the loss of satellite cell function by preventing satellite cell proliferation and

self-renewal. Laminin, a primary protein component of the ECM, specifically interacts with integrin receptors on the surface of satellite cells. Satellite cell proliferation depends on a properly organized network of laminin within the basal lamina of the ECM (Ross et al., 2012). Moreover, the laminin-integrin interaction induces cell-intrinsic polarity, which is essential for asymmetric cell division and satellite cell self-renewal (Kuang et al., 2007; Goulas et al., 2012). Therefore, persistent TGFβ and Wnt signaling during aging leads to accumulation of skeletal muscle fibrosis, which disrupts basal lamina architecture, and reduces satellite cell proliferation and self-renewal.

AGE-RELATED LOSS OF SATELLITE CELL SELF-RENEWAL

SIGNALING AND SATELLITE CELL SELF-RENEWAL

Impaired skeletal muscle regeneration in aged mice is due, in part, to loss of Notch signaling, and can be restored by forced activation of Notch in aged muscle, or parabiosis of aged mice with young mice (Conboy et al., 2003, 2005; Carlson et al., 2009). Similarly, skeletal muscle aging can be simulated in young mice by inhibition of Notch signaling after acute injury (Conboy and Rando, 2002). Satellite cell-specific deletion of RBP-J, the primary mediator of Notch signaling, in adult muscle results in loss of satellite cells and reduced regenerative capacity, as RBP-J-null satellite cells spontaneously enter the cell cycle and immediately progress through differentiation without self-renewing (Bjornson et al., 2012; Mourikis et al., 2012). Therefore, age-related loss of Notch signaling precludes satellite cell self-renewal, manifesting as a loss of satellite cells and impaired regenerative capacity.

Similarly, conditional deletion of RBP-J, in embryonic myogenic progenitors results in an absence of satellite cells in fetal muscle due to premature differentiation (Vasyutina et al., 2007). In a MyoD-null background, the loss of satellite cells is prevented, presumably due to a delay in myogenic differentiation (Brohl et al., 2012). Myogenic progenitor cells in RBP-J-null/MyoD-null mice are unable to home to the satellite cell niche, in part, due to loss of integrin α7 expression, which mediates the interaction between satellite cells and the basal lamina. Therefore, loss of Notch signaling in aging skeletal muscle, combined with alterations to basal lamina architecture as a result of fibrosis, disrupts satellite cell proliferation and self-renewal.

Diminished Notch activity in satellite cells is due, in part, to an age-related decrease in expression of the Notch receptor ligands, Jag1 and Dll1 (Conboy et al., 2003; Carey et al., 2007; Carlson et al., 2009). Reduced levels of Dll1 in mice results in severe muscle hypotrophy as a result of insufficient satellite cell proliferation (Schuster-Gossler et al., 2007). Jag1 is expressed in a subset of activated satellite cells, and generates asymmetry during cell division by activating Notch signaling in an adjacent receptor-expressing cell (Gnocchi et al., 2009). The Jag1 expressing cell expresses Numb, an antagonist of Notch signaling, and is fated to progress through commitment and differentiation; whereas the Numb-negative satellite cell responding to the Jag1 signal displays high Notch activity, and is fated for self-renewal (Conboy and Rando, 2002; Shinin et al., 2006). Loss of Jag1 expression in aging satellite cells prevents asymmetric Notch signaling, and therefore, prevents self-renewal.

Skeletal muscle regeneration has been postulated to be a balance between Notch and Wnt signaling, such that Notch is required for proliferation and self-renewal of satellite cells, and canonical Wnt signaling is required for induction of differentiation (Conboy and Rando, 2002; Conboy et al., 2003; Brack et al., 2007, 2008). However, tamoxifen-mediated deletion of β-catenin specifically in satellite cells suggests that canonical Wnt signaling is not required for differentiation during adult muscle regeneration (Murphy et al., 2014).

Wnts activate canonical and non-canonical pathways. In aging hematopoietic stem cells, a switch from canonical to non-canonical Wnt signaling causes a loss in stem-cell polarity and reduces regenerative potential (Florian et al., 2013). It is intriguing to hypothesize how a shift in which Wnt pathway is activated in aging could affect satellite cell fate and function. Given that non-canonical Wnt signaling in satellite cells specifically stimulates symmetric expansion, an age-related shift in Wnt signaling to the non-canonical pathway may impinge on satellite cell self-renewal, resulting in a loss of regenerative capacity (Le Grand et al., 2009). Therefore, persistent canonical Wnt signaling during aging may prevent satellite cell self-renewal, in addition to stimulating fibrosis.

Self-renewal of satellite cells requires asymmetric cell division and the ability of cells to re-enter and maintain quiescence. Increased expression of MyoD and Myf5 in aged muscle of rats and humans, in the absence of exercise or injury, suggests that satellite cells lose the ability to maintain quiescence during aging (Hameed et al., 2003; Edstrom and Ulfhake, 2005; Raue et al., 2006). Indeed, uninjured muscle from aged mice displays a greater percentage of actively proliferating MyoD-expressing satellite cells, as compared to the muscle of young mice (Chakkalakal et al., 2012). Reduced expression of p27, a cyclin-dependent kinase inhibitor (CDKi), and Sprouty1, a FGF signaling antagonist, is suggested to be the cause of spontaneous release from quiescence in aged satellite cells.

Spry1 expression is restricted to non-cycling satellite cells, and is required for satellite cells to return to and maintain quiescence during regeneration (Shea et al., 2010). Long-term deletion of Spry1 in adult skeletal muscle satellite cells decreases the percentage of label-retaining cells, consistent with the loss of satellite cell quiescence observed in aging. Furthermore, Spry1-null satellite cells are unable to contribute to the generation of myonuclei or the renewal of quiescent satellite cells during skeletal muscle repair. Therefore, loss of Spry1 expression in a subpopulation of aged satellite cells, combined with the increase in FGF2 expression in aged muscle fibers, drives satellite cell depletion by maintaining proliferation and preventing the return to quiescence. This is intriguing, as it suggests that the combination of cell autonomous changes (loss of Spry1 expression) and changes to the niche (increased FGF2 expression) are involved in the loss of satellite cell self-renewal.

The FGF2-induced signal in satellite cells is mediated by many downstream targets, including p38 MAPKs (Cuadrado and Nebreda, 2010). In turn, activated p38 MAPKs phosphorylate a broad range of targets, including MyoD, NF-κB, CREB, and STAT1/3. Conditional deletion of p38α expression, or inhibition p38α activity, promotes Pax7 expression and expansion of satellite

cells, and prevents differentiation (Palacios et al., 2010; Brien et al., 2013). Activated and proliferating satellite cells display asymmetric distribution of activated phospho-p38α/β (pp38α/β), such that cells expressing pp38α/β co-express MyoD and progress through myogenic differentiation, whereas, pp38α/β-negative cells revert to quiescence (Jones et al., 2005; Troy et al., 2012).

Aging satellite cells display elevated pp38α/β levels, combined with a loss of asymmetric distribution of pp38α/β, which correlates with a loss of self-renewal (Troy et al., 2012). Partial inhibition of p38α/β activity restores self-renewal of aged satellite cells *in vitro* and reestablishes engraftment potential (Bernet et al., 2014; Cosgrove et al., 2014). Analogously, expression of a ligand-independent constitutively active form of FGFR1 also drives asymmetric localization of pp38α/β in aged satellite cells, and permits satellite cell self-renewal (Bernet et al., 2014).

Like other signaling pathways, the p38α/β pathway does not operate independently. Par-3, an evolutionarily conserved regulator of polarity, colocalizes with pp38α/β in dividing satellite cells (Troy et al., 2012). In asymmetrically dividing radial glia, Par-3 is responsible for asymmetric localization of Mib to the apical daughter cell, which is fated for differentiation (Bultje et al., 2009; Dong et al., 2012). Mib is an ubiquitin ligase that regulates Notch ligand endocytosis in the apical cell, a process that is required for efficient activation of Notch signaling in the basal cell, which is destined for self-renewal. This is strikingly similar to satellite cells, in which high Notch activity marks satellite cells fated for self-renewal.

Therefore, aging disrupts satellite cell self-renewal. Specifically, age-related cell extrinsic changes in expression of signaling ligands, combined with satellite cell-intrinsic alterations in the ability to appropriately respond to signals, disrupt asymmetric cell division and limit satellite cell self-renewal. The inability to self-renew results in a progressive loss of satellite cells, which diminishes competence to respond to acute injury and maintain muscle mass.

EPIGENETICS AND SATELLITE CELL SELF-RENEWAL

Activation of p38α signaling directs satellite cells toward differentiation, and prevents self-renewal, by repressing expression of Pax7 and Notch1 through localized targeting of Ezh2, a histone methyltransferase, and DNMT3b, a DNA methyltransferase, to each gene (Acharyya et al., 2010; Palacios et al., 2010). Ezh2 is a component of the polycomb repressive complex (PRC2), which in combination with PRC1, establishes and stabilizes repression through post-translational modification of histones.

Post-translation modification of histones is an epigenetic change that marks genes as active or inactive. Methylation of lysine 4 (H3K4me3) of histone H3 is generally associated with active chromatin, while methylation of lysine 27 (H3K27me3) is linked with repressed chromatin (Dilworth and Blais, 2011). A bivalent state can exist in which histone H3 is methylated at both lysine 4 and lysine 27. Notably, the repressive H3K27me3 mark is dominant over the active H3K4me3 mark, and is heritably transmitted to daughter cells (Barski et al., 2007).

Genome-wide analysis of chromatin in young and aged quiescent satellite cells demonstrated that the level of H3K4me3 histone marking was comparable between young and old satellite cells; however, H3K27me3 accumulates and spreads with age in quiescent satellite cells (Liu et al., 2013). Notably, 30% of genes that acquire H3K27me3 were not expressed in either young or old quiescent satellite cells, and less than 0.5% of genes are marked solely by H3K27me3. It is difficult to correlate the global change in H3K27me3 in satellite cells with aging. However, this gain of H3K27me3 marks may reflect a loss of potential, as low levels of H3K27me3 is associated with pluripotency (Mikkelsen et al., 2007; Marks et al., 2012).

The increase in H3K27me3 in aging is thought to be linked to a redistribution of PRC1 and PRC2 complexes. Bivalent domains can be segregated into two types—those that are bound by PRC1 and PRC2, and those that are bound only by PRC2 (Ku et al., 2008). Binding of PRC1 more effectively retains the H3K27me3 mark, thereby maintaining repression. Specifically, PRC1 stabilizes bivalent domains, and reinforces the ability of stem cells and progenitor cells to retain cell fate choices, including self-renewal (Oguro et al., 2010). Therefore, loss of PRC1 is expected to drive satellite cells out of quiescence and prevent satellite cell self-renewal. Indeed, mice lacking Bmi1, a component of the PRC1 complex, show reduced numbers of $Pax7^{+}Myf5^{-}$ satellite stem cells, and an increase in $Pax7^{+}Myf5^{+}$ and $MyoD^{+}$ committed satellite cells, reminiscent of the age-related loss of quiescence (Robson et al., 2011). Moreover, Bmi1-null mice display a delay in regeneration upon injury. Therefore, cell-extrinsic changes to signaling and cell-intrinsic changes in signal response during aging can produce long-term and heritable results by inducing epigenetic changes.

AGE-RELATED INDUCTION OF SENESCENCE

Senescent cells are alive and metabolically active, but have lost the ability to divide. Senescence can be induced through several mechanisms, but is most closely associated with aging. The primary purpose of senescence is to prevent propagation of damaged cells. Senescent cells are cleared by the immune system, and lost cells are replaced by tissue-specific repair mechanisms. Age-related changes in the immune system, combined with an increase in the number of senescent cells, may result in the accumulation of senescent cells, which secrete cytokines and other molecules that induce inflammation and inhibit tissue regeneration (Kuilman et al., 2008; Rodier and Campisi, 2011).

Recent studies indicate that satellite cells enter senescence with advanced age. Sousa-Victor et al. (2014) compared adult (5–6 mo), old (20–24 mo), and geriatric (28–32 mo) mice, and showed that old and geriatric mice display a reduced number of satellite cells, but only satellite cells in geriatric mice display a reduced proliferative response. Moreover, skeletal muscle regeneration was marginally reduced in old mice, but is more markedly diminished in geriatric mice. Transplantation of FACS sorted cells from adult, old, and geriatric mice into young mice clearly showed a significant reduction in regenerative potential only in geriatric cells, indicating a cell-intrinsic loss of regenerative capacity with aging.

Mouse and human geriatric satellite cells express $p16^{INK4A}$, and display classic markers of senescence (Sousa-Victor et al., 2014). Silencing of $p16^{INK4A}$ expression in geriatric mouse satellite cells restores regeneration-induced activation of proliferation and reversible quiescence. In parallel experiments, ectopic

expression of $p16^{INK4A}$ prevents activation of satellite cells after injury of young muscle. These data suggest that expression of $p16^{INK4A}$ in geriatric satellite cells induces cellular senescence and is responsible for the aging phenotype in skeletal muscle. However, a mild and systemic increase in $p16^{INK4A}$ expression extends longevity in mice, suggesting the dosage of $p16^{INK4A}$ may be important for determining effect (Matheu et al., 2007, 2009).

In young cells, the combined action of PRC1 and PRC2 represses $p16^{INK4A}$ expression through maintenance of H3K27me3 marks, thereby preventing cellular senescence (Jacobs et al., 1999; Bracken et al., 2007; Margueron and Reinberg, 2011). Geriatric satellite cells display an increase in expression of genes normally regulated by PRC1 and PRC2, suggesting that satellite cells may exhibit age-related epigenetic changes. Bmi1, a component of the PRC1 complex, represses expression of $p16^{INK4A}$, and has been suggested to play an important role in delaying aging by preventing cellular senescence (Jacobs et al., 1999). Therefore, if Bmi1 expression or function is lost with aging, satellite cells would be expected to lose the ability to maintain quiescence and eventually senesce.

Notch signaling positively regulates expression of Bmi1, suggesting that loss of Notch signaling in aging satellite cells may reduce Bmi1 expression, de-repress the $p16^{INK4A}$ locus, resulting in satellite cell senescence (Fan et al., 2010; Schaller et al., 2010; Sousa-Victor et al., 2014). Indeed, deletion of Bmi1 in young satellite cells leads to de-repression of the $p16^{INK4A}$ locus, increased expression of $p16^{INK4A}$, which leads to a senescent-like state in young cells and prevents these cells from participating in regeneration (Robson et al., 2011). Moreover, reduced Notch signaling in aged satellite cells allows TGFβ-stimulated phosphorylation of Smad3 to activate expression of CDK inhibitors (CDKis; Beggs et al., 2004; Carlson et al., 2008). Therefore, persistent TGFβ signaling and loss of Notch signaling during skeletal muscle aging increases fibrosis, inhibits satellite cell proliferation, and induces satellite cell senescence.

CONCLUSIONS AND PERSPECTIVES

Age-related changes within satellite cells and to their niche limit cell fate and function. In the aged niche, satellite cells shift from a poised, quiescent state to the active state in the absence of a regenerative signal. Persistent TGFβ-and Wnt-dependent accumulation of skeletal muscle fibrosis disrupts basal lamina architecture. Dysregulation of Wnt, Notch, FGF, and p38α/β signaling results in a loss of cell polarity, and prevents asymmetric cell division. Age-related loss of Notch activity and persistence of TGFβ activity induce epigenetic changes that de-repress the *CDKN2A* locus and induce expression of $p16^{INK4A}$. These changes combine to drive satellite cells away from normal cell fate decisions—differentiation and self-renewal—toward age-realted senescence. In genome-wide association studies, the $p16^{INK4A}$ locus is genetically linked to the highest number of age-associated pathologies.

Restoring regenerative capacity to aged skeletal muscle could be as simple as replacing aged satellite cells with young satellite cells, and/or modifying signaling pathways to maintain reversible quiescence. However, it appears that the effects of aging culminate in epigenetics and expression of $p16^{INK4A}$. Global demethylation of DNA occurs after fertilization and is required for pluripotency (Guo et al., 2014; Smith et al., 2014). This suggests that skeletal muscle aging may be reversed simply by manipulating the epigenetic memory of satellite cells, and resetting the aging clock to zero.

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Condensin-mediated chromosome organization and gene regulation

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In many organisms sexual fate is determined by a chromosome-based method which entails a difference in sex chromosome-linked gene dosage. Consequently, a gene regulatory mechanism called dosage compensation equalizes X-linked gene expression between the sexes. Dosage compensation initiates as cells transition from pluripotency to differentiation. In *Caenorhabditis elegans*, dosage compensation is achieved by the dosage compensation complex (DCC) binding to both X chromosomes in hermaphrodites to downregulate gene expression by twofold. The DCC contains a subcomplex (condensin I^{DC}) similar to the evolutionarily conserved condensin complexes which play a fundamental role in chromosome dynamics during mitosis. Therefore, mechanisms related to mitotic chromosome condensation are hypothesized to mediate dosage compensation. Consistent with this hypothesis, monomethylation of histone H4 lysine 20 is increased, whereas acetylation of histone H4 lysine 16 is decreased, both on mitotic chromosomes and on interphase dosage compensated X chromosomes in worms. These observations suggest that interphase dosage compensated X chromosomes maintain some characteristics associated with condensed mitotic chromosome. This chromosome state is stably propagated from one cell generation to the next. In this review we will speculate on how the biochemical activities of condensin can achieve both mitotic chromosome compaction and gene repression.

Keywords: *Caenorhabditis elegans*, condensin, dosage compensation, gene expression, chromosome condensation, chromatin, interphase chromosome, epigenetics

INTRODUCTION

Dosage compensation occurs in many species with a difference in sex chromosome number between males (XY or XO) and females (XX). This mechanism equalizes gene expression between the sexes and balances X and autosomal gene expression (Ohno, 1967). Disrupting dosage compensation leads to lethality in the affected sex. Mammals, flies, and worms have distinct dosage compensation strategies. The fly, *Drosophila melanogaster*, upregulates the male X twofold to balance X, and autosomal expression and equalize male to female X-linked gene expression (Conrad and Akhtar, 2011; Ferrari et al., 2014). By contrast, mammals and the nematode, *Caenorhabditis elegans*, are hypothesized to upregulate X chromosome expression in both sexes (Gupta et al., 2006; Lin et al., 2007; Deng et al., 2011, 2013; Lin et al., 2011). This X upregulation balances male X and autosomal expression, but causes X overexpression in females/hermaphrodites. Therefore to compensate for this overexpression, mammals inactivate one X in XX females (Heard and Disteche, 2006; Payer and Lee, 2008; Barakat and Gribnau, 2012), while *C. elegans* downregulates both X chromosomes twofold in the XX hermaphrodites (Csankovszki et al., 2009b; Meyer, 2010).

In *C. elegans*, repression of gene expression is achieved by the dosage compensation complex (DCC), which binds the Xs in hermaphrodites to downregulate gene expression by half. The DCC contains five associated proteins and a subcomplex, condensin I^{DC}, which is similar to the evolutionarily conserved condensin complexes that promote chromosome condensation (Csankovszki et al., 2009a). This review focuses on our current understanding of condensins' biological functions and molecular mechanisms that enable them to achieve both mitotic chromosome compaction and gene repression.

CONDENSIN COMPLEXES

Condensin complexes are highly conserved five subunit complexes essential for chromosome compaction and segregation in mitosis and meiosis (Hirano, 2012). While yeast has one complex, higher eukaryotes have two, condensins I and II. They consist of a pair of SMC2 and SMC4 subunits belonging to the SMC (structural maintenance of chromosomes) family of chromosomal ATPases and three unique CAP (chromosome-associated polypeptide) proteins. Condensin I contains CAP-D2, CAP-G, and CAP-H, while condensin II contains CAP-D3, CAP-G2, and CAP-H2 (Ono et al., 2003, 2004; Hirota et al., 2004). Uniquely, *C. elegans* has three condensin complexes, condensins I, II, and an additional complex, condensin I^{DC}, which contributes exclusively to dosage compensation (Chuang et al., 1994; Lieb et al., 1996, 1998; Tsai et al., 2008; Csankovszki et al., 2009a; **Figure 1**). Interestingly, condensin I^{DC} differs from condensin I complex by only one subunit: DPY-27 replaces SMC-4 (Csankovszki et al., 2009a; Mets and Meyer, 2009). Unlike condensins I and II, which compact and segregate all mitotic and meiotic chromosomes, condensin I^{DC} is X-specific resulting in gene repression

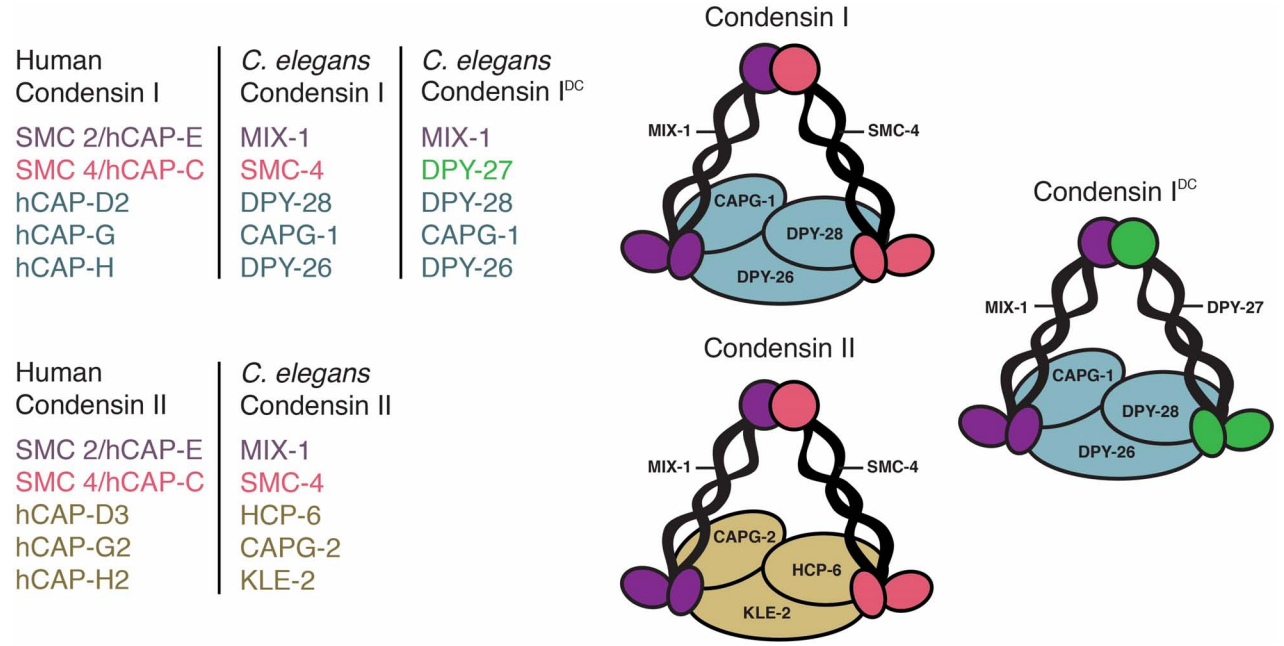

FIGURE 1 | Three condensin complexes. *Caenorhabditis elegans* condensin subunits and their human homologs. Condensins I and II share the same pair of MIX-1 and SMC-4 subunits and have three unique chromosome-associated polypeptide (CAP) proteins. Condensin I contains DPY-28, CAPG-1, and DPY-26, while condensin II contains HCP-6, CAPG-2, and KLE-2. In addition, *C. elegans* has a condensin I-like complex (condensin I^{DC}) that functions in dosage compensation. Condensin I^{DC} differs from the canonical condensin I by only one subunit: DPY-27 replaces SMC-4.

in hermaphrodites. Due to the similarity of condensin I and I^{DC}, similar mechanisms have long been hypothesized to mediate chromosome compaction and dosage compensation (Chuang et al., 1994). In this review we discuss the mitotic/meiotic and interphase defects caused by condensin mutations or knockdowns. Because condensin is depleted throughout the cell cycle in these experiments, is it is difficult to differentiate between mitotic and interphase functions of condensins. The effects of the activities of condensin in mitosis may persist in interphase and vice versa.

MITOTIC AND MEIOTIC DEFECTS IN CONDENSIN MUTANTS OR KNOCKDOWNS

In higher eukaryotes condensins I and II have different spatial and temporal localization patterns. Condensin I is cytoplasmic in interphase and accesses chromosomes only after nuclear envelope breakdown (NEBD) in prometaphase, while condensin II is predominantly nuclear and binds chromosomes as soon as condensation begins in prophase (Hirano and Mitchison, 1994; Ono et al., 2004; Gerlich et al., 2006; Collette et al., 2011; Shintomi and Hirano, 2011). This suggests that chromosome condensation may occur in two-steps, first with condensin II in prophase and then with condensin I after NEBD. An exception is mouse embryonic stem cells, where condensin I is nuclear during interphase (Fazzio and Panning, 2010). Furthermore, the global and regional localization of condensins I and II on mitotic chromosomes are different. In monocentric organisms, condensins I and II have non-overlapping distributions within the axis of each sister-chromatid arm, with condensin II enriched at the centromeres (Ono et al., 2003, 2004; Hirota et al., 2004). Similar differences were also found in *C. elegans*, a holocentric organism, in which microtubule attachment sites are scattered throughout the entire length of chromosomes. In *C. elegans*, condensin I associates with mitotic chromosomes in a diffuse discontinuous pattern, while condensin II is enriched at centromeres (Csankovszki et al., 2009a; Collette et al., 2011). Differences in spatial and temporal dynamics of condensins I and II are also present during meiosis (Collette et al., 2011; Lee et al., 2011) Recent studies explored the genome-wide distribution of condensin complexes at high resolution. These studies have uncovered both unique and similar binding sites of condensins I and II (Kim et al., 2013; Kranz et al., 2013; Van Bortle et al., 2014).

Although the two mitotic condensins are structurally similar, this difference in localization suggests that they may play distinct roles in chromosome organization. Consistent with this idea, the depletion of condensin I or II alone results in distinct chromosomal defects, while the depletion of both condensins leads to more severe defects (Ono et al., 2003, 2004; Hirota et al., 2004). Condensin I facilitates lateral compaction of mitotic chromosomes, whereas condensin II primarily contributes to axial compaction (Ono et al., 2003; Hirota et al., 2004; Shintomi and Hirano, 2011; Green et al., 2012). The roles the two condensins play in mitosis varies among different eukaryotic species. For example, in *Xenopus laevis*, *S. pombe*, and *S. cerevisiae*, condensin is required for mitotic chromosome condensation and mechanical stability (Hirano and Mitchison, 1994; Hirano et al., 1997; Sutani et al., 1999; Freeman et al., 2000; Gerlich et al.,

2006). Condensins also play critical roles in meiotic chromosome compaction and segregation (Chan et al., 2004; Lee et al., 2011). During *C. elegans* meiosis, depletion of condensin I or II leads to an expansion of chromosome axis (Mets and Meyer, 2009). A study using *Xenopus laevis* egg extracts showed that a critical determinant of chromatid shape is the relative ratio of condensins I and II (Shintomi and Hirano, 2011). In other organisms, such as mammals and worms, condensin II plays a primary role in prophase condensation (Hagstrom et al., 2002; Hirota et al., 2004; Csankovszki et al., 2009a). Interestingly, when both condensins are depleted in *Drosophila*, worms, mammals, and chicken DT40 cells, the primary defect appears to be anaphase chromatin bridging, rather than chromosome condensation (Hirano, 2012). This suggests that other factors may contribute to mitotic chromosome condensation in addition to condensin.

INTERPHASE DEFECTS IN CONDENSIN MUTANTS OR KNOCKDOWNS

Emerging evidence suggests that condensin complexes also contribute to a variety of interphase functions. It is believed that condensin II, rather than condensin I, plays a primary role in interphase, since in condensin II is nuclear throughout the cell cycle, while condensin I is cytoplasmic in interphase (Hirota et al., 2004; Ono et al., 2004; Gerlich et al., 2006; Collette et al., 2011; Shintomi and Hirano, 2011). In *Drosophila* ovarian nurse cells, condensin II disassembles polytene chromosomes into unpaired homologous chromosomes. This unpairing activity leads to interphase chromosome compaction (Hartl et al., 2008a,b; Joyce et al., 2012). In *Drosophila* cell lines, condensin-mediated interphase condensation is normally limited by the SCF^{Slimb} ubiquitin ligase. The condensin II subunit CAP-H2 is a Slimb target for ubiquitin-mediated degradation. Degradation of CAP-H2 inactivates condensin II, thereby preventing interphase chromatin reorganization. Inhibition of SCF^{Slimb} leads to CAP-H2 stabilization, resulting in chromosome unpairing and nuclear structural abnormalities (Buster et al., 2013). This suggests that in interphase, condensin II activity is suppressed in order to prevent chromosome condensation and changes in nuclear organization. In addition, condensin II also regulates chromosome territory formation in multiple cell types. This conclusion is based on the finding that CAP-H2 promotes axial compaction and proper compartmentalization of the interphase nucleus into chromosome territories in both nurse cells and salivary glands (Bauer et al., 2012). These findings suggest that the interphase function of condensin II is similar to its role in axial compaction of mitotic chromosomes.

Condensin subunits also play a role in regulation of cell-type specific gene expression. In mice, chromosome compaction by condensin II is required for T-cell development and maintenance of the quiescent state. Mutations in the condensin II subunit kleisin-β (CAP-H2) lead to open chromatin configuration and upregulation of normally silenced genes. After T-cell activation, chromatin decondenses and transcription is upregulated (Rawlings et al., 2011). Similarly, murine CAP-G2 represses transcription during erythroid cell differentiation. During erythroid cell maturation nuclei gradually condense, mediated by condensin (Xu et al., 2006). Condensin is also required for higher-order chromatin compaction and viability in ES cells. (Fazzio and Panning, 2010).

Yeast condensin has also been shown to play a role in interphase chromatin organization and RNA polymerase III-transcribed gene clustering. In budding and fission yeast, the three-dimensional organization of the genome is facilitated in part by condensin-mediated localization of RNA-polymerase III genes within the nucleus (Iwasaki et al., 2010). In budding yeast, tRNA genes are clustered at the nucleolus in a condensin-dependent manner. Mutations in yeast condensin subunits cause tRNA gene positioning defects and partially inhibit tRNA gene-mediated silencing (Haeusler et al., 2008), illustrating another connection between condensin-mediated genome organization and gene expression.

In the above examples, condensin either affected the entire genome, or a subset of genes scattered on different chromosomes. By contrast, in *C. elegans*, condensin I^{DC} causes chromosome-specific changes. Consistent with a role in chromosome condensation, *C. elegans* condensin I^{DC} mediates compaction of dosage compensated X chromosomes in interphase. Condensin I^{DC}-bound X chromosomes are more compact than expected by DNA content, whereas mutations or depletions of condensin I^{DC} result in decompaction of X chromosome territories (Lau et al., 2014). These results are consistent with the model that reduction of X-linked gene expression occurs as a result of condensin I^{DC}-mediated changes in chromatin structure. However, whether this condensation is a cause or consequence of transcriptional repression is unknown.

CONDENSIN AND CHROMATIN MEDIATED CHROMOSOME COMPACTION

In addition to condensin-mediated condensation, histone modifications also influence chromatin compaction during mitosis and the structure of *C. elegans* dosage compensated X chromosomes. The similarity of chromatin modifications between mitotic chromosome and dosage compensated X chromosomes of *C. elegans* is consistent with X chromosome repression being mediated by mechanisms similar to mitotic chromosome condensation. On both mitotic chromosomes and interphase dosage compensated X chromosomes monomethylation of histone H4 lysine 20 (H4K20) is increased whereas acetylation of histone H4 lysine 16 (H4K16) is decreased (**Figure 2**; Rice et al., 2002; Oda et al., 2009; Vielle et al., 2012; Wells et al., 2012; Wilkins et al., 2014). During cell cycle progression the levels of both the H4K20 methyltransferase, PR-SET-7, and H4K20me1 increase in G2, remain high in mitosis, and decrease in G1 (Rice et al., 2002; Oda et al., 2009). Additionally, the depletion of PR-SET-7 leads to cell cycle defects, and mitotic and interphase chromosome decondensation (Oda et al., 2009), illustrating the importance of H4K20me1 in mitosis and chromosome compaction. By contrast, H4K16ac levels increase during S phase and decrease during mitosis (Rice et al., 2002; Wilkins et al., 2014). This data is consistent with findings that H4K20me1 antagonizes H4K16ac (Nishioka et al., 2002). In yeast, H4K16ac deacetylation in mitosis is achieved by Hst2 (Sir2 homolog), which is recruited by histone H3 phosphorylated on serine 10 (Wilkins et al., 2014). Deacetylation of H4K16ac leads

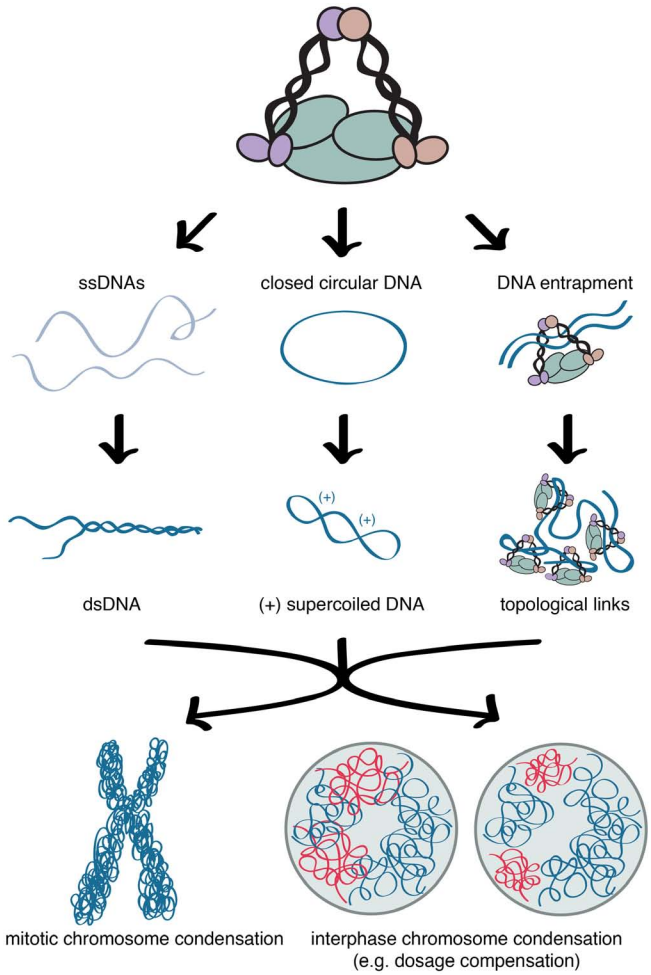

FIGURE 2 | Condensin and chromatin mediated chromosome compaction. Similar distributions of histone modifications and condensin in condensed mitotic chromosomes and interphase dosage compensated X chromosomes. Compaction is accompanied by enrichment of H4K20me1 and depletion of H4K16ac in both mitotic chromosome condensation and interphase dosage compensated X chromosomes.

to stronger interactions between H2A and H4 on neighboring nucleosomes, leading higher degree of condensation (Shogren-Knaak et al., 2006; Wilkins et al., 2014). In mitosis, this cascade of histone modifications is proposed to drive chromatin hypercondensation, independently from condensin (Wilkins et al., 2014). However, it has been shown that mitotic condensin II subunits CAP-D3 and CAP-G2 are capable of binding H4K20me1, suggesting H4K20me1 may play a role in condensin II loading (Liu et al., 2010).

Caenorhabditis elegans interphase dosage compensated X chromosomes show similar changes in histone modifications: H4K20me1 is increased, whereas H4K16ac is decreased on X. The enrichment of H4K20me1 is regulated not only by the DCC but also the H4K20 monomethylase, SET-1 (PR-SET7 homolog), and the H4K20 di- and trimethylase, SET-4 (SUV4-20 homolog; Vielle et al., 2012; Wells et al., 2012). The DCC also regulates SIR-2.1 (Sir2 homolog), which mediates the depletion of H4K16ac on X chromosomes (Wells et al., 2012). This cascade of histone modifications drives X chromatin condensation in a DCC- (therefore condensin-) dependent manner (Lau et al., 2014). By contrast, in mitosis, these histone modifications are proposed to act independently of condensin (Wilkins et al., 2014). These observations suggest that interphase dosage compensated X chromosomes maintain some characteristics associated with condensed mitotic chromosome.

MOLECULAR MECHANISMS OF CONDENSIN ACTIVITY

The mechanisms by which condensin generates and maintains chromosome condensation in interphase and mitosis are highly debated and poorly understood. The biochemical mechanisms discussed below have been proposed to contribute to chromosome condensation. However, whether these activities contribute to condensin's interphase or mitotic functions, or both, is unknown.

The two SMC proteins of condensin are able to hydrolyze ATP and this activity is believed to be essential for regulating higher-order chromatin structure (Kimura and Hirano, 1997; Hirano, 2012). The SMC proteins also have the ability to reanneal complementary ssDNAs into dsDNAs (Sakai et al., 2003), perhaps as

a preparatory step for the formation of mitotic chromosomes (**Figure 3**). The best-characterized mechanism of mitotic condensin, detected in many eukaryotic species is the ability to introduce ATP-dependent positive supercoils into DNA *in vitro* (Kimura and Hirano, 1997, 2000; Kimura et al., 2001; Hagstrom et al., 2002; St-Pierre et al., 2009). Using closed circular DNA and in the presence of topoisomerase I, mitotic condensin I is able to supercoil the DNA with its DNA-stimulated ATPase activity (Kimura and Hirano, 1997). This activity requires the entire five-unit complex. The SMC proteins alone do not have ATPase activity and cannot bind chromatin *in vitro* (Kimura and Hirano, 2000). Positive supercoiling is proposed to facilitate topoisomerase II-mediated decatenation of the sister chromatids and lead to the formation of chiral loops. Higher order assemblies by condensin–condensin interactions can then compact the chromatin fiber (**Figure 3**; Kimura et al., 1999; Baxter et al., 2011).

Phosphorylation of condensin's CAP subunits by the kinase CDK1 (cyclin-dependent kinase 1) is required to supercoil DNA and initiate mitotic chromosome condensation *in vitro* (Kimura et al., 1998; Takemoto et al., 2006). By contrast, the supercoiling activity is not detected when the interphase form of condensin is incubated with circular DNA in the presence of ATP and topoisomerase I (Kimura et al., 1998). In fact, phosphorylation of condensin I by a different kinase, CK2 (casein kinase 2), suppresses supercoiling activity during interphase (Takemoto et al., 2006). This suggests that condensin I-mediated DNA supercoiling may not be involved in chromosome compaction during interphase. However, it is not known whether condensin II-mediated supercoiling or additional molecular mechanisms drive interphase chromatin organization.

Alternatively, or in addition to supercoiling, condensin is proposed to entrap the chromatin fibers in a ring-like structure (Cuylen et al., 2011). This hypothesis is based on condensin's resemblance to cohesin, both containing a pair of SMC proteins, forming a V-shape, and additional non-SMC proteins, proposed to close the ring (**Figure 3**). Cohesin is believed to hold pairs of the sister chromatids together by entrapping DNA from each chromatid within its ring-like structure (Haering et al., 2008). A recent study on yeast minichromosomes provided evidence that condensin forms similar topological links by encircling DNA. Linearization of the minichromosome DNA or opening the condensin ring eliminated the association between the DNA and condensin (Cuylen et al., 2011). While cohesion is believed to hold sister chromatids together, condensin is proposed to entrap different sections of the same DNA molecule, to facilitate condensation.

Condensin's ability to shape chromosomes is further illustrated by its localization to topologically associating domain (TAD) boundaries in interphase chromosomes. A TAD is a contiguous chromosomal region with high frequency of interactions between sequences within the TAD, but few interactions with sequences outside the TAD. In interphase *Drosophila*, mouse, and human ES cells, condensin II has been found to localize at high occupancy architectural protein binding sites (APBSs) located at the borders of TADs (Van Bortle et al., 2014). Localization of condensin II at TAD boundaries, together with its ability to entrap DNA, suggests a possible mechanisms for regulating interphase chromatin organization. Unlike interphase chromatin, which is partitioned into small sub-megabase TADs and large multi-megabase compartments (Dekker and Mirny, 2013), mitotic chromosomes do not exhibit chromosome compartments and TADs (Naumova et al., 2013). Instead it is believed that chromatin is linearly compacted into consecutive loops, potentially by SMC complexes, and then homogeneous axial compression leads to the formation of dense mitotic chromosomes (Naumova et al., 2013). Thus, there may be unique and overlapping mechanisms involved in condensin-mediated chromosome compaction in interphase and mitosis.

Which of these biochemical activities, if any, contribute to *C. elegans* dosage compensation is unknown. Mutations in the ATPase domains of DPY-27 and MIX-1 lead to dosage

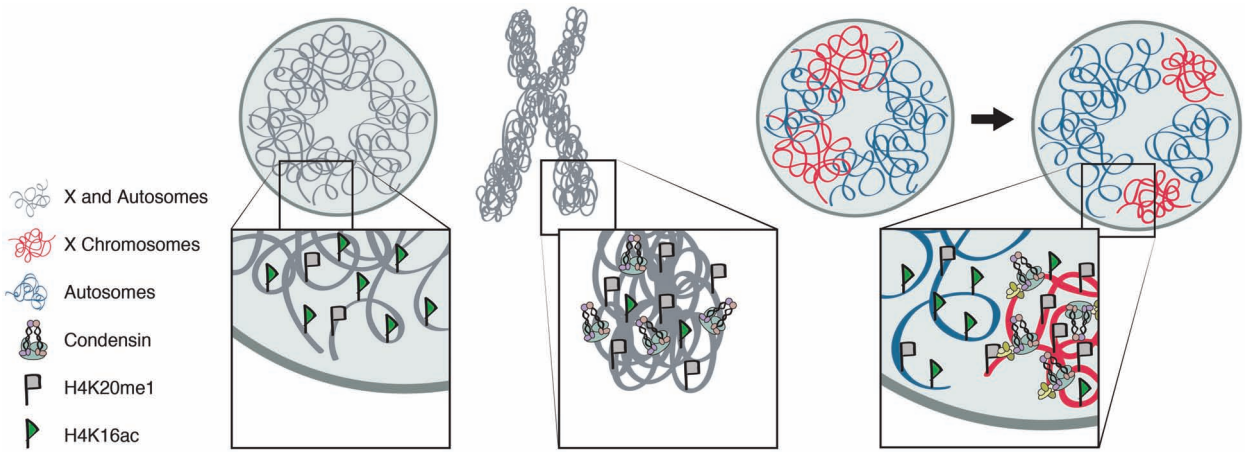

FIGURE 3 | Molecular mechanisms of condensin activity. The proposed mechanisms by which condensin generates and maintains chromosome condensation in interphase and mitosis. Condensin's structural maintenance of chromosomes (SMC) proteins can reanneal complementary ssDNAs into dsDNAs, in preparation for subsequent coiling steps. Condensin can also introduce ATP-dependent positive supercoils into DNA *in vitro*. Alternatively, or in addition, condensin is proposed to entrap the chromatin fibers in its ring-like structure.

compensation defects (Chuang et al., 1994; Lieb et al., 1998), suggesting that the ATPase activity is required for the mechanisms that mediate dosage compensation. Whether condensin I^{DC} is able to reanneal single stranded DNA, supercoil DNA, or entrap chromatin fibers has not been investigated. Future studies of condensin's biochemical activities will reveal how condensin is able to achieve both mitotic chromosome compaction and gene repression.

CONCLUSION

Condensin complexes emerged as important regulators of chromatin organization throughout the cell cycle. Recent studies revealed that in addition to their role in mitotic chromosome condensation and segregation, condensins function in diverse interphase processes. Emerging evidence connects mitotic condensin-mediated condensation with epigenetic control of gene expression. Although there is increasing understanding of the biological functions of condensins in mitosis, meiosis, and interphase, the molecular mechanisms of condensin activity are still poorly understood. Since most of our knowledge of these molecular mechanisms comes from analysis of condensin I in mitosis, it will be important to examine the mechanistic similarities and differences between the activities of condensins I and II, both in mitosis and interphase. Studying *C. elegans* condensin I^{DC}'s function in dosage compensation will shed further light on how condensin affects interphase chromosome organization and how the activities involved differ from condensin's function in mitosis.

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How the cell cycle impacts chromatin architecture and influences cell fate

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Since the earliest observations of cells undergoing mitosis, it has been clear that there is an intimate relationship between the cell cycle and nuclear chromatin architecture. The nuclear envelope and chromatin undergo robust assembly and disassembly during the cell cycle, and transcriptional and post-transcriptional regulation of histone biogenesis and chromatin modification is controlled in a cell cycle-dependent manner. Chromatin binding proteins and chromatin modifications in turn influence the expression of critical cell cycle regulators, the accessibility of origins for DNA replication, DNA repair, and cell fate. In this review we aim to provide an integrated discussion of how the cell cycle machinery impacts nuclear architecture and vice-versa. We highlight recent advances in understanding cell cycle-dependent histone biogenesis and histone modification deposition, how cell cycle regulators control histone modifier activities, the contribution of chromatin modifications to origin firing for DNA replication, and newly identified roles for nucleoporins in regulating cell cycle gene expression, gene expression memory and differentiation. We close with a discussion of how cell cycle status may impact chromatin to influence cell fate decisions, under normal contexts of differentiation as well as in instances of cell fate reprogramming.

Keywords: cell cycle, chromatin, histones, nucleoporins, mitosis

INTRODUCTION

Chromatin serves as a platform for numerous cellular signals to influence gene expression. Post-translational modifications (PTMs) of histone proteins or covalent modifications of nucleotides influence a cell's transcriptional program, which ultimately impacts cellular behavior and cell fate. Chromatin modifications are converted into transcriptional instructions by the interplay of modification "writers," "erasers" and "readers" residing, often together, in a multitude of chromatin remodeling complexes that interact directly or indirectly with transcription factor complexes (Jenuwein and Allis, 2001). Like transcription factor complexes, the components of chromatin remodeling complexes may change with the differentiation status or fate of cells. For example lineage-specific chromatin remodeling complexes have been identified, as well as stem-cell specific complexes with functions in maintaining pluripotency (reviewed in Hargreaves and Crabtree, 2011).

Work by many groups over the past 10 years, including the extensive chromatin modification and accessibility mapping performed through the Encyclopedia of DNA Elements (ENCODE) and model systems-based ModENCODE projects have clarified that: chromatin accessibility and chromatin modifications are predictive of gene expression, DNA replication timing is correlated with an accessible chromatin structure, and chromatin is dynamic during fate acquisition and cellular reprogramming to pluripotency (for example, Ding and MacAlpine, 2011; Orkin and Hochedlinger, 2011; Thurman et al., 2012; Ho et al., 2014). However, with the exception of a few studies on replication timing, much of the mapping in these projects has used either asynchronously dividing cell lines, whole animals of various developmental stages, or tissues containing mixed cell lineages with differing cell cycle dynamics. How exactly the cell cycle status of a cell influences its chromatin state and how this impacts cell fate and cell fate plasticity remains a largely unaddressed question.

Chromatin in proliferating cells is highly dynamic. Two important events occur during the cell cycle that allow for global chromatin restructuring. First, the incorporation of new histones onto nascent DNA occurs during S-phase and creates a requirement for the re-establishment of histone PTMs. Second, many chromatin remodeling complexes and transcriptional complexes are dissociated from chromatin during mitosis and the nuclear architecture, including chromatin domains or associations with the nuclear interior vs. periphery breaks down (**Figure 1**). This raises the question of how the cell maintains its transcriptional identity and fate through S-phase and mitosis. This question intersects with the field of *epigenetics*, which for the purposes of this review—is defined to encompass mechanisms that provide a cellular memory of gene expression, inheritable through the mitotic cell cycle (Berger et al., 2009). We define *cell fate* as a gene expression program that drives the acquisition of cell type-specific characteristics. Our goal in this review is to summarize recent findings that provide insight into how cell cycle status can influence chromatin and nuclear architecture to impact cell fate decisions. Also, we consider how developmental programs and acquisition of cell fate can feedback onto the expression of cell cycle regulators and cell cycle processes.

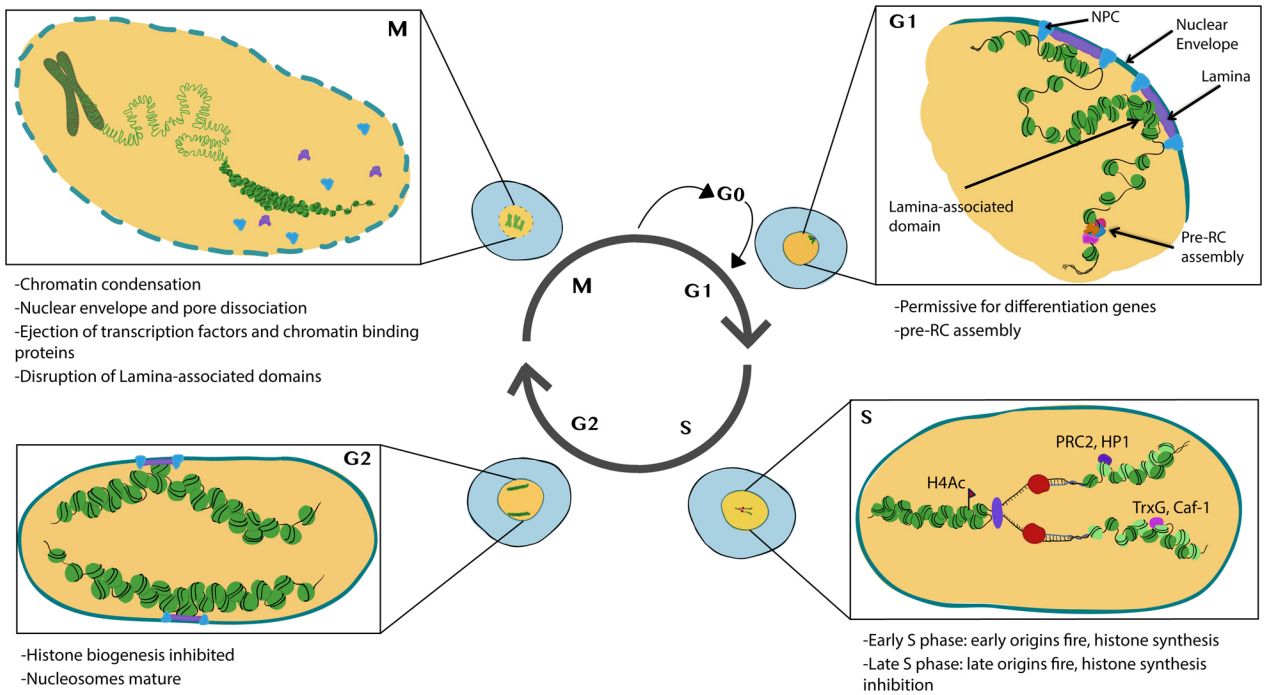

FIGURE 1 | Major features of chromatin and nuclear changes during the cell cycle. Cells in G1 phase exhibit subnuclear domains with some regions associated with nuclear pores and nuclear lamina. Pre-RCs preferentially form at accessible chromatin. During S-phase histones are transcribed and synthesized, DNA is replicated and new (light green) and recycled (dark green) nucleosomes assemble to form nascent chromatin. PTM writers and readers also associate with nascent chromatin. During G2 nucleosomes mature and histone biogenesis is inhibited. During mitosis, chromosomes condense and many transcription factors and chromatin binding proteins are ejected from the chromatin. The nuclear envelope breaks down disrupting nuclear lamina associated domains. Illustration by Nicole Ethen.

We begin our discussion with the regulation of histone biogenesis, key building blocks of chromatin. We then consider how the chromatin state influences the cell cycle through origin firing and chromosome compaction at mitosis. We focus on how the cell cycle impacts chromatin remodelers to coordinate these events and vice-versa. We then take a more global view of the nucleus, to discuss nuclear architecture and how nuclear domains and nuclear pore association impacts gene expression and DNA repair. These topics converge onto issues of how gene expression memory can be transmitted through the cell cycle and we discuss a central question in epigenetics; what are the epigenetic marks inherited through the cell cycle? Finally, we consider how the cell cycle status impacts chromatin to influence cell fate, in instances of cell fate acquisition and in the opposing direction of de-differentiation in nuclear reprogramming.

CELL CYCLE DEPENDENT HISTONE BIOGENESIS

Histones are one of the primary components of chromatin and canonical histones (as opposed to histone variants) are actively synthesized during S-phase, in a manner coordinated with the replication of DNA. The speed of DNA replication is in fact tied to the rate of histone biosynthesis (Groth et al., 2007a; Gunesdogan et al., 2014; Mejlvang et al., 2014), suggesting new histone supply is tightly coupled to immediate demand during S-phase. The canonical histones consist of H1, H2A, H2B, H3, and H4 and they are small and highly positive charged proteins. Two copies of H2A, H2B, H3, and H4 form an octamer, which is wrapped by about 147 bp negative charged DNA (Richmond and Davey, 2003), resulting in the basic structure of the nucleosome. The canonical histone genes form clusters and present as one to several hundreds of copies depending on the species (Hentschel and Birnstiel, 1981; Marzluff et al., 2008). The transcription of histone gene takes place in a subnuclear organelle termed the histone locus body (HLB), containing factors required for the processing of histone pre-mRNAs which have an unusual mRNA structure, with a 3'UTR that forms a stem-loop structure instead of a polyA tail (White et al., 2007; Nizami et al., 2010). It has been suggested that excess free histones may be toxic to cells, explaining the evolutionary pressure for their conserved, yet peculiar regulation (De Koning et al., 2007).

The onset and shut down of histone gene transcription is tightly regulated, in a manner elegantly coordinated with the core cell cycle machinery (De Koning et al., 2007; Groth et al., 2007b). Entry into S-phase is triggered by the activity of the G1-S Cyclin complex, CyclinE/Cdk2. In addition to phosphorylating targets to initiate DNA replication, CyclinE/Cdk2 also phosphorylates nuclear protein ataxia-telangiectasia locus (NPAT), to initiate transcription of the histone genes (Ma et al., 2000; Zhao et al., 2000; Ye et al., 2003). After CyclinE/Cdk2 activity has reached its peak in early S-phase, CyclinE/Cdk2 activity drops due to the degradation of the essential CyclinE component,

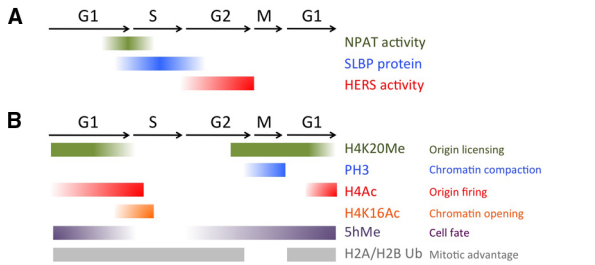

FIGURE 2 | Chromatin modifications and histone biogenesis regulators during the cell cycle. **(A)** Factors controlling histone biogenesis are regulated by the cell cycle to limit histone biogenesis to S-phase. **(B)** Chromatin modifications, including histone PTMs and 5-hydroxy-methylcytosine (5hMe) occur in a cell cycle regulated manner to impact gene expression and nuclear architecture.

thereby preventing further activation of NPAT until CyclinE re-accumulates in the next cell cycle (**Figure 2**).

While this simple mechanism could in theory be sufficient to limit histone biogenesis to S-phase, a direct regulator involved in robustly shutting down histone biogenesis after S-phase was also recently identified in *Drosophila*. The histone gene-specific epigenetic repressor in late S-phase (HERS) protein becomes phosphorylated by the late S-G2 Cyclin complex CyclinA/Cdk1, which localizes it to the histone genes where it acts to silence histone genes after S-phase (Ito et al., 2012). HERS silences histone gene expression by recruiting the repressive chromatin writer Su(var)3-9 for Histone H3 trimethylation at Lysine 9 (H3K9Me3), which subsequently recruits an H3K9Me3 "reader," the transcriptional repressor Heterochromatin protein 1 (HP1). This recruitment of HP1 to the histone genes stably represses histone mRNA expression throughout G2 and early M. Importantly, the activity of the CyclinA/Cdk1 complex is kept low during G1 and early S-phases through the cell cycle-coupled degradation of CyclinA, triggered by the Anaphase Promoting Complex/Cyclosome (APC/C). This window of low CyclinA/Cdk1 during G1 allows cells to "reset" the inhibition of histone gene transcription and prepare for re-activation via the next pulse of CyclinE/Cdk2, to trigger NPAT activation (**Figure 2**).

In addition to the careful regulation of histone mRNA transcription, histone mRNA stability is also tightly regulated to limit transcript accumulation to S-phase. The conserved 3' UTR of metazoan canonical histone transcripts forms a "stem-loop" structure, which binds stem-loop binding protein (SLBP). SLBP is involved in several aspects of histone mRNA metabolism, including histone pre-mRNA maturation, translation and degradation (Marzluff et al., 2008). Perhaps not surprisingly, the SLBP protein itself is cell cycle regulated. SLBP mRNA is synthesized constantly throughout the cell cycle, but SLBP becomes translated just prior to S-phase entry and the protein is degraded at the end of S- phase (Whitfield et al., 2000). SLBP protein stability is controlled by CyclinA/Cdk1, which phosphorylates a phosphodegron to trigger SLBP destruction (Zheng et al., 2003; Koseoglu et al., 2008). Altogether, both activation and repression of histone biosynthesis are very rapid, robust and directly coupled to the Cyclin/Cdk activity oscillations driving the cell cycle (**Figure 2**). This allows histone biogenesis to respond to all the cell fate cues that feed into regulating the speed and dynamics of the cell cycle during development, and under different signaling and environmental conditions.

CHROMATIN ARCHITECTURE IMPACTS THE FORMATION OF ORIGINS FOR DNA REPLICATION

The DNA replication machinery is exquisitely regulated to ensure that the genomic DNA is copied only once within the cell cycle, with the interesting exception of highly specialized cells which re-replicate specific genomic regions to amplify certain genes (Nordman and Orr-Weaver, 2012). Replication is set up in three basic steps; first, the origin recognition complex (ORC complex) somehow identifies and binds to future origins on the chromatin just after mitosis and during early G1 (Mechali, 2010; Alabert and Groth, 2012). Next, during G1 the pre-replication complex (pre-RC) assembles on the ORC-bound locations. Pre-RC formation is marked by Cdt1 and Cdc6 recruitment of the minichromosome maintenance complex (MCM) complex. The successful assembly of a pre-RC then "licenses" origins for the third step, origin firing during S-phase. Firing is triggered in part by Dbf4/Cdc7 kinase (DDK) and CyclinE/Cdk2-dependent phosphorylations of origin complex components, leading to the recruitment of helicases and enzyme complexes for DNA replication (Zegerman and Diffley, 2007; Boos et al., 2013; Ramer et al., 2013).

A fundamental question about DNA replication is where on the genome replication starts. Unlike prokaryotes and yeast, metazoans have no obvious DNA sequence to designate origins of replication. Furthermore, there are estimated to be 30,000–50,000 potential origins of replication in the human genome, only about 10% of which are used within a given adult somatic cell cycle, suggesting most potential origins lie dormant (Alabert and Groth, 2012). This vast excess of origins may be important during rapid embryonic S-phases, and dormant origins can become activated when cells are placed under stress to avoid an S-phase delay (Courbet et al., 2008). It is widely believed that the choice of origins is developmentally controlled (Claycomb and Orr-Weaver, 2005) and consistent with this, different cell types exhibit distinct DNA replication patterns (Hansen et al., 2010).

Genome-wide analysis of DNA replication has expanded the numbers of predicted origins in *Drosophila*, mouse and human cells, and there is a strong correlation between origins and regions of active transcription (Cadoret et al., 2008; Sequeira-Mendes et al., 2009; Karnani et al., 2010; MacAlpine et al., 2010; Mesner et al., 2011). ORC binding, the first step in origin formation, is enriched in nucleosome-depleted regions suggesting DNA accessibility may be a major determinant in origin choice (MacAlpine et al., 2010; Lubelsky et al., 2014). However, not all open chromatin regions can serve as origins, indicating that origin specification involves additional factors yet to be determined. ORCs can also bind heterochromatin, though several additional factors are required to facilitate binding such as (HP1; Pak et al., 1997; Schwaiger et al., 2010; Cayrou et al., 2011), high mobility group protein HMGA1a (Thomae et al., 2008) and leucine-rich repeats and WD40 repeat domain-containing protein 1 (LRWD1) also known as ORCA (Shen et al., 2010). ORCs can also play origin-independent roles in generating repressive chromatin

(Sasaki and Gilbert, 2007), therefore it has been challenging to tease out whether the recruitment and binding of ORC to heterochromatin functions in origin choice or serves other chromatin remodeling roles. In the cases of ORC recruitment by HMGA1a and ORCA, ORC recruitment does promote preRC formation and functional origins, suggesting these proteins facilitate ORC binding for origin formation in heterochromatin (Thomae et al., 2008; Shen et al., 2012).

While ORC binding may be rather widespread in the genome, this is only the first step in origin selection. The assembly of the pre-RC complex, the second step in origin formation, is also influenced by the chromatin state. Regions with high H4 acetylation are enriched for Pre-RC assembly during G1, and histone acetylation can promote origin licensing (Iizuka et al., 2006; Miotto and Struhl, 2008, 2010). The MYST-family histone acetyltransferase (HAT) HBO1 preferentially acetylates H4 on Lysines 5, 8, and 12 and is essential for proper DNA replication in human cells and *Xenopus* egg extracts (Doyon et al., 2006; Iizuka et al., 2006). An acetyltransferase defective HBO1 is unable to load MCMs for pre-RC formation, despite binding properly to origins (Miotto and Struhl, 2010). This suggests chromatin modifiers can specifically influence the step of replication licensing in G1. However, conspicuously, the loss of HBO1 in mice leads to decreased H3K14 acetylation, as opposed to H4 acetylation, and no obvious defects in DNA replication or cell cycle arrest were observed in HBO1 mutant embryos (Kueh et al., 2011). This unexpected finding suggests perhaps other MYST-family acetyltransferases can compensate for the absence of HBO1 *in vivo*, or possibly the role of HBO1 in preRC formation is more cell-type or context-dependent than thought.

Replication licensing also coincides with a specific histone PTM, monomethylation of H4 Lysine 20 (H4K20Me). H4K20Me levels fluctuate during the cell cycle, peaking during M and early G1 and plummeting during S phase (Tardat et al., 2010). The high levels of H4K20Me at mitosis suggest this mark could be involved in the earliest stage of origin choice (**Figure 2**). Indeed, artificially tethering the H4K20 methyltransferase PR-set7 to a non-origin chromatin region is sufficient to promote the ectopic loading of pre-RC components to that site. However, when PR-set7 is inhibited, loading of MCMs for licensing is impaired yet ORC binding to chromatin remains (Tardat et al., 2010). This suggests that H4K20Me may serve to reinforce origin licensing, perhaps acting sequentially in cooperation with HBO-dependent H4 acetylation.

CHROMATIN AND THE TIMING OF ORIGIN FIRING

Not only is ORC binding and origin licensing impacted by the chromatin state, but origins are fired in a sequential way, such that some regions of the genome replicate early while others replicate late in S phase (Mechali, 2010). Such differential timing in origin firing is highly conserved from fission yeast to humans, and whether this has some evolutionary advantage or is simply a consequence of complex nuclear architecture remains unclear. The timing of origin firing is dynamic during development and different between cell types (Hansen et al., 2010; Farrell et al., 2012). Perhaps not surprisingly, the timing of origin firing correlates with the data on sites of Pre-RC assembly at late M- early G1. Early replicating regions are commonly enriched in H4 acetylation and are associated with actively transcribed, accessible chromatin (Kemp et al., 2005; Goren et al., 2008; Schwaiger et al., 2009; Hansen et al., 2010; Lubelsky et al., 2014). In cells treated with histone deacetylase (HDAC) inhibitors, late replicating origins can shift toward earlier replication (Kemp et al., 2005; Goren et al., 2008) suggesting that opening chromatin has functional consequences on origin firing.

A direct relationship between origin firing and H4 acetylation was reported in yeast (Vogelauer et al., 2002) and *Xenopus* (Danis et al., 2004), and was carefully dissected in a study of specialized origins located near the chorion genes in the follicle cells of the *Drosophila* ovary (Aggarwal and Calvi, 2004). The follicle cells are tasked with quickly producing and secreting the eggshell (chorion) for the developing egg in the ovary. In order to accomplish this, the follicle cells amplify the copy numbers in the regions of the genome encoding the chorion genes by repeatedly re-firing origins at a specific stage of development in the ovary (Nordman and Orr-Weaver, 2012). Thus, the level of chorion gene amplification can serve as a read-out for the firing rate of an isolated origin. This unique feature of origin re-firing and re-replication has allowed for detailed *in vivo* genetic analyses of origin firing, unparalleled in any other system.

Acetylation of H4, in particular acetylation at H4K8, directly correlates with the levels of chorion gene amplification and thus origin re-firing (Kim et al., 2011). When the HDAC Rpd3 is tethered to a chorion amplification origin, amplification and origin re-firing becomes hindered (Aggarwal and Calvi, 2004). By contrast, recruitment of the acetyltransferases CREB-binding protein (CBP) and HBO1 to licensed amplification origins promotes re-firing (McConnell et al., 2012). H4 acetylation could promote origin firing through increasing the accessibility of DNA to the helicase complexes needed for replication fork movement, or by facilitating histone octamer eviction for DNA unwinding via the remodeling SWI/SNF and RSC complexes (Ferreira et al., 2007). These models suggest a passive role for the chromatin state in regulating origin firing though, by simply limiting the access or movement of replication enzymes. It would be interesting to examine whether H4 acetylation may also impact or regulate the ability of CyclinE/Cdk2 to phosphorylate its substrates at licensed origins to initiate firing.

In contrast to early replicating origins and origins for gene amplification, late-firing origins are usually associated with a repressive, closed chromatin structure. For example HP1-bound regions near centromeric heterochromatin repeats in *Drosophila* replicate late, and reducing HP1 levels leads to earlier replication of these centromeric repeats (Schwaiger et al., 2010). The later replication of heterochromatin could be due to a reduced density of ORC bound regions, reduced pre-RC formation, or chromatin that is simply less accessible to helicases and replication enzymes. However, it is worth noting that a subset of heterochromatin replicates early in *Drosophila* and fission yeast (Hayashi et al., 2009; Schwaiger et al., 2010; Cayrou et al., 2011). In these cases, paradoxically the HP1/ORC association promotes ORC recruitment and earlier origin firing. Such differential roles for HP1 in heterochromatin replication imply that a compact chromatin structure is not the only factor dictating replication timing, and

beg the question of what other factors can influence the timing of origin firing.

Recent work in early *Drosophila* embryos has investigated the initial formation of late- replicating heterochromatin in detail. The earliest appearance of late-firing origins in *Drosophila* embryos occurs at repetitive satellite DNA during the mid-blastula transition when zygotic transcription is first activated (Shermoen et al., 2010). Farrell et al. (2012) recently discovered that providing a low level pulse of early Cdk1 activity can push the very first late-firing origins in *Drosophila* development to replicate early. This finding is surprising for two reasons. First Cdk1 activity is normally associated with triggering mitosis and preventing re-licensing of replication origins, so a role for Cdk1 in promoting origin firing is unexpected. Second, Farrell et al. (2012) found that Cdk1 can promote the earlier firing of late origins even at a time when these regions of the genome already exhibit a more compacted chromatin structure (Shermoen et al., 2010; Farrell et al., 2012). This suggests that perhaps local Cyclin/Cdk activity may somehow be able to overcome a compacted chromatin structure to influence the timing of origin firing when needed in specific contexts.

Most likely, both local Cyclin/Cdk activity and chromatin structure ultimately impact the timing of origin firing. Importantly, the initial formation of late-firing origins does require activation of the zygotic transcription program (Shermoen et al., 2010) which underscores the close relationship between gene expression, chromatin accessibility and timing of origin firing during development. Methods to examine the 3D structure and organization of chromatin in the nucleus such as Chromatin Conformation Capture, termed "3C" or "Hi-C," have established that different mammalian cell types contain topologically associated chromatin domains or "TADs," thought to be the results of cell-type specific chromatin sub-compartments (Dixon et al., 2012). Recent work from the Gilbert lab has revealed that TADs also share replication timing features, further demonstrating in mammalian cells that cell-type specific nuclear architecture correlates with replication timing (Pope et al., 2014). Their model, derived from analysis of over 30 mouse and human cell types, suggests DNA replication initiates within TADs permissive for transcription but replication forks gradually advance later into TADs that are repressive for transcription. Importantly, whether transcription establishes the nuclear architecture that influences replication timing, or whether replication timing somehow establishes the nuclear subdomains that impact transcription remains unresolved. Since gene expression and nuclear architecture differs between cell types and changes with the acquisition of cell fate (Peric-Hupkes et al., 2010), it is likely that origin usage and the timing of origin firing will be equally as dynamic during development as gene expression.

WHAT ARE THE EPIGENETIC MARKS?

A qualified epigenetic mark should be faithfully transmitted to daughter cells through DNA replication and cell division. Nucleosomes and the associated chromatin architecture must disassemble before replication forks and re-assemble with newly synthesized DNA and histones after forks pass (Margueron and Reinberg, 2010). This poses a challenge for cells to maintain their non-DNA sequence information, such as DNA methylation and histone modifications. The semi-conservative mechanism of DNA synthesis is thought to provide an effective way to ensure the inheritance of DNA methylation through hemi-methylation dependent maintenance methylases such as the cytosine methyltransferase Dnmt1 in mammals (reviewed in Law and Jacobsen, 2010). Dnmt1 is recruited to nascent chromatin by Ubiquitin-like PHD and RING finger domain 1 protein (UHRF1), which recognizes hemimethylated CG dinucleotides (Bostick et al., 2007; Sharif et al., 2007). Dnmt1 can also interact with a component of the moving replication fork, proliferating cell nuclear antigen (PCNA; Chuang et al., 1997), to promote cytosine methylation immediately after new DNA synthesis. However, some common genetic model organisms lack substantial genomic cytosine methylation, such as budding yeast, *C. elegans* and *Drosophila* (Proffitt et al., 1984; Simpson et al., 1986; Takayama et al., 2014), demonstrating that DNA methylation is not a universal epigenetic mark.

The case of inheriting histone modifications seems more challenging. There is no obvious nucleosome template to directly copy and newly synthesized, unmodified histones are incorporated into the nascent DNA (Probst et al., 2009). A model has been suggested for the inheritance of the H3K27Me3 modification through the cell cycle, based on the observation that this modification can directly recruit a complex containing both PTM writing and binding activity, the PRC2 complex (Hansen et al., 2008). PRC2 contains the H3K27Me3 writer, *Enhancer of zeste* (or EZH2 in humans), as well as an H3K27Me3 binding subunit *Extra sexcombs*, (or EED in humans). Importantly, EED binding to the H3K27Me3 modification stimulates the methyltransferase activity of EZH2, thereby providing an intuitive way for the PRC2 complex to propagate the H3K27Me3 modification (Margueron et al., 2009). The model posits that the PRC2 complex is recruited to chromatin by the H3K27Me3 modification in G1, and enough PRC2 is recruited to H3K27Me3 on mature histones that are recycled and re-incorporated into the replicated DNA during S-phase to allow for H3K27 modification on nearby, newly incorporated histones (Hansen et al., 2008; Margueron and Reinberg, 2010). Such a mechanism is not necessarily H3K27 specific, and could be shared with other histone PTMs. For example, H3K9 is di- or tri-methylated by Su(var)3-9, which is read by the chromodomain of HP1. HP1 then further recruits Su(var)3-9, thereby leading to the spreading, or potentially also the maintenance, of H3K9 methylation on new histones (Bannister et al., 2001; Lachner et al., 2001). Similar interactions could also exist between histone acetylation and HATs, which are often located in complexes that contain acetyl-histone readers, such as bromodomain proteins (Dhalluin et al., 1999; Filippakopoulos et al., 2012; Filippakopoulos and Knapp, 2014). Future studies on the association of additional PTM writer/reader complexes with nascent DNA through the cell cycle may support a similar model for propagation of multiple histone PTMs during DNA replication.

Such a model creates a "chicken and egg" type-conundrum though when asking what is the inherited epigenetic mark in dividing cells, as it seems to be both the histone PTM itself and the writer/reader complex. Indeed, recent work in human cell lines seems to support this model. Alabert et al. (2014)

isolated newly replicated chromatin to profile the association dynamics of thousands of chromatin binding proteins and to compare the levels of histone PTMs in nascent chromatin versus mature chromatin. They found that specific histone PTMs such as H3K27Me3 and H3K9Me3 remained similar between nascent and "mature" chromatin, and when the synthesis of new histones is blocked, H3K27Me3 and H3K9Me3 remain abundant on nascent chromatin. This implies that significant amounts of certain PTMs on nascent chromatin can originate from the old recycled histones (Alabert et al., 2014). In further support of the model, they also find the PRC2 complex is present in both nascent and mature chromatin, consistent with rapid recruitment by recycled parental histones carrying H3K27Me3.

However, a very different model for inheritance of the epigenetic mark through S-phase was proposed by a study of early stage *Drosophila* embryos (Petruk et al., 2012). Petruk et al. (2012) found that the H3K27Me3 mark is actually very low during S-phase in cells of the *Drosophila* gastrula and is not detectable on the newly synthesized DNA until later in G2 phase. They reasoned that the true epigenetic modifications should be re-established shortly after DNA replication. To determine which PTMs or chromosomal proteins are in close proximity to the replication machinery, they used a "proximity ligation assay" (PLA) approach. In this assay, proteins or histone PTMs that are within 30–40 nm of replication forks containing PCNA generate a fluorescent signal, with a sensitivity that allows visualization of single molecule interactions *in vivo* (Soderberg et al., 2006). In the *Drosophila* embryo, several histone modification writers and readers including E(z), TrxG, Pc, Caf-1, LID, UTX, and HP1 are tightly associated with the replication forks, and are located on nascent DNA during S phase. However, their corresponding histone PTMs were not associated with replication forks, nor detectable on nascent DNA until ∼1 hr after the passage of replication fork, which is already G2 phase at this stage of development. This suggests that it is the PTM writers that remain associated with nascent chromatin during replication which must act to re-establish PTMs later. Thus, it seems the chromatin binding of the PTM writers rather than the PTMs themselves may serve as a true, inherited epigenetic mark. Although surprising, this work is consistent with a previous study showing that Polycomb remains bound to replicating chromatin *in vitro* (Francis et al., 2009). The methyltransferase SET domain of PTM writers can bind single-stranded DNA *in vitro*, suggesting a manner in which they may be retained on newly synthesized DNA independent of a recruiting PTM (Krajewski et al., 2005). Self-association and oligomerization may be another manner in which PTM writers can be maintained in the absence of a recruiting PTM (Lo et al., 2012) and finally, Polycomb complexes can be recruited to DNA in a sequence-specific manner through Polycomb response elements or PREs, which recruit complexes during early S-phase prior to replication (Lanzuolo et al., 2011). However, it remains unclear in the *Drosophila* embryo whether the PTM writers remain associated with the same specific locations on DNA before and after replication fork passage.

These seemingly conflicting observations of Alabert et al. (2014) and Petruk et al. (2012) are likely due to the developmental stage and cell cycle speed of the model systems under study. For example, in the *Drosophila* embryo it seems relatively few PTMs may have already been established on the mature nucleosomes at the stage of development under study. Indeed the authors show there is little to no H3K27Me3 at the cellular blastoderm stage before gastrulation. Thus perhaps when there are lower levels of established PTMs, they can be preceded by the binding of the histone modifiers in S-phase (Petruk et al., 2012). In contrast, the adult human cells have already heavily established PTMs in the chromatin prior to passage of the replication fork, and thus recycling histones containing PTMs allows them to more readily be used as a template to recruit modifying enzymes and re-establish the necessary chromatin modifications.

A new study using early *C. elegans* embryos throws yet another wrinkle into this epigenetic inheritance problem though (Gaydos et al., 2014). In contrast to the results in *Drosophila*, Gaydos et al. (2014) find that chromatin containing the H3K27Me3 PTM in *C. elegans* retains the mark through several early embryonic cell divisions, even in embryos lacking the H3K27Me3 writer enzyme. A chromosome inherited with the H3K27Me3 mark already established, retains it during early embryonic divisions exhibiting only the expected level of passive dilution due to new histone incorporation. While chromosomes in the exact same embryo-inherited without the H3K27Me3 mark already established, cannot establish it *de novo* until later in development. Thus, it seems clear the H3K27Me3 PTM itself in *C. elegans* embryos serves as an inherited epigenetic mark. Taken together, the studies of Petruk et al. (2012) and Gaydos et al. (2014) suggest there may be different modes of epigenetic inheritance used in different organisms. Perhaps flies use chromatin-bound PTM writers to carry the epigenetic information through early embryonic cell divisions, while worms use the PTM itself? An organism specific answer to the epigenetic inheritance question seems a bit unsatisfying, especially as all the ingredients, the PTMs, the readers, the writers and the S-phase machinery are so well conserved. Hopefully future studies will be able to reveal an underlying unifying concept to explain what is the true inherited epigenetic mark.

CHROMATIN AND CHROMOSOME COMPACTION DURING MITOSIS

To ensure the fidelity of separating identical genetic information into two daughter cells, chromatin undergoes dramatic compaction during the cell cycle into mitotic chromosomes. Mitotic chromosomes are easily recognizable based on their morphology, however, the details of their three-dimensional structure have remained enigmatic. Recent use of advanced Chromosome Conformation Capture methods such as 5C and Hi-C in human cell lines performed at timepoints across the cell cycle, have revealed that mitotic chromosomes exhibit a common structure shared in multiple cell types (Naumova et al., 2013). Mitotic chromosomes appear to be organized as a linear array of chromatin loops of variable size, which are then tightly compressed together longitudinally. The common structure of mitotic chromosomes seems striking, given the cell type-specific subdomains and features of interphase chromatin structure, such as TADs (Pope et al., 2014). This suggests that some cell-type specific chromatin architecture

is lost during mitosis and higher-order chromatin structures form *de novo* after mitosis.

Accompanying this dramatic chromatin compaction is the alteration of chromatin-based activities, such as the cessation of transcription (Martinez-Balbas et al., 1995; Gottesfeld and Forbes, 1997). This is thought to be accomplished in part, by the inhibition of transcription factor binding to the mitotic chromatin. For example, the large C2H2 zinc finger transcription factor family becomes phosphorylated at the conserved linker region during mitosis, which leads to dissociation from mitotic chromatin (Dovat et al., 2002; Rizkallah et al., 2011). Alternatively for specific transcription factors that remain bound to the mitotic chromosome, such as FoxA1 and GATA1, their co-activators can be excluded from mitotic chromatin. This mechanism may allow the transcription factors to act as platforms for timely reactivation of transcription after mitosis, a mechanism termed "mitotic bookmarking" which has been discussed in detail elsewhere (Kadauke et al., 2012; Caravaca et al., 2013; Kadauke and Blobel, 2013; Wang and Higgins, 2013).

DNase sensitivity has been used to probe chromatin accessibility during different stages of the cell cycle. Somewhat surprisingly and in contrast to the Hi-C data mentioned previously, DNase sensitivity is widely preserved from interphase to mitosis (Hsiung et al., 2014). During interphase, DNAse sensitivity generally corresponds to transcription factor binding sites and active gene proximal promoters. While in mitosis, gene expression ceases, higher order chromatin domains are lost and many transcription factors are ejected. So why and how are most DNase sensitive regions maintained during mitosis? First to be precise, there are a few expected alterations to accessibility in mitosis. For example, distal regulatory elements that bind transcription factors are somewhat more likely to lose accessibility during mitosis compared to gene proximal promoters. Second, chromatin modifications and some chromatin modifiers are retained on the mitotic chromosomes and can help to preserve local chromatin structure, even if higher order structures are disrupted, as suggested by the Hi-C data. For example, the trithorax protein MLL maintains its chromatin association during mitosis, and loss of MLL impairs the rapid reactivation of MLL target genes after mitotic exit (Blobel et al., 2009). This process is reminiscent of the mitotic bookmarking described above, and suggests that retention of a few key chromatin modifiers during mitosis may be all that is needed to transmit gene expression information and maintain cell fate through mitosis.

What are the histone PTMs involved in compacting the chromatin at mitosis? The best-documented mitotic chromatin mark is phosphorylation of the H3 N-terminal tails. Four major residues of H3 are phosphorylated during mitosis, T3, S10, T11, S28, in a manner conserved from yeasts to humans (Rossetto et al., 2012). Aurora B is the major kinase responsible for these phosphorylations, which can be counteracted by the Protein Phosphatase 1 (PP1). Insufficient H3 phosphorylation leads to abnormal chromosome condensation and segregation, which is due to impaired recruitment of Condensin I complexes (Adams et al., 2001; Giet and Glover, 2001). The Condensin complex is the major effector of chromosome condensation during mitosis. In the presence of type I topoisomerases, Condensins progressively wind and fold the chromatin fiber into supercoils, which compact to form the mitotic chromosome (Hirano, 2012; Thadani et al., 2012; Aragon et al., 2013). Importantly though, phosphorylation of H3 does more than simply recruit Condensins, it can also modulate the binding of repressive chromatin proteins to mitotic chromosomes. For example, H3K9 the residue adjacent to H3S10 can be methylated and its trimethylation recruits the HP1 reader to form heterochromatin. However, during mitosis the majority of HP1 is released from chromatin, due to phosphorylation on H3S10, which ejects HP1 from binding H3K9Me3 on mitotic chromatin (Fischle et al., 2005). Something similar may also occur with H3K27, which recruits the Polycomb complexes PRC1 when methylated and lies adjacent to the H3S28 phosphosite (Wang and Higgins, 2013).

H4K20 mono-methylation (H4K20Me), the same PTM mentioned earlier to promote pre-RC formation, is also required for proper chromosome condensation (Karachentsev et al., 2005; Sakaguchi and Steward, 2007; Houston et al., 2008; Oda et al., 2009). H4K20me facilitates chromatin condensation in part by antagonizing a second PTM, H4K16 acetylation (H4K16Ac; Nishioka et al., 2002). H4K16Ac inhibits chromatin compaction, and consistent with a role in opening chromatin, its levels normally peak during S phase (Shogren-Knaak et al., 2006) and decrease during mitosis (Rice et al., 2002; **Figure 2**). H4K20Me is also thought to contribute to chromosome compaction in early M phase by binding specific components of the Condensin II complex (Liu et al., 2010). Condensin II binds to interphase chromatin and is thought to mediate early phases of chromatin compaction, well before Condensin I. Altogether this suggests a two-step model for chromatin modifications to promote chromosome compaction at mitosis. First, H4K20Me limits H4 acetylation and recruits Condensin II. This then cooperates with Aurora B triggered H3 phosphorylation to eject H3K9-and possibly H3K27 -bound protein complexes and recruit Condensin I during early metaphase for further compaction (Ono et al., 2003). In this manner, the compaction of the chromatin at mitosis and the ejection of certain chromatin bound factors are directly coupled.

REGULATION OF HISTONE MODIFIERS BY THE CELL CYCLE MACHINERY

While chromatin impacts cell cycle events like origin firing and chromosome segregation at mitosis, the cell cycle machinery also impacts chromatin by regulating the histone modifiers. The activity of certain histone modifiers fluctuates in a cell cycle-dependent manner. Perhaps the best-studied example of this is the regulation of the H4K20 mono-methyltransferase PR-Set7 and its opposing de-methylase, PHF8 (Rice et al., 2002; Liu et al., 2010). Both PR-Set7 mRNA and protein levels peak during G2 and mitosis, only to plummet during G1, consistent with the observed changes of the H4K20Me PTM (Rice et al., 2002). The dynamic regulation of PR-Set7 is in part due to its proteolytic degradation during S-phase. PR-Set7 contains a conserved PCNA-interacting peptide (PIP-box) which mediates its association with the PCNA component of the replication fork. The binding to PCNA during

S-phase is recognized by the E3 ubiquitin ligase CRL4/Cdt2, which leads to degradation of PR-Set7 and PCNA, in order to prevent pre-mature chromatin compaction prior to M-phase (Abbas et al., 2010; Centore et al., 2010; Oda et al., 2010). Conversely, the PHF8 de-methylase becomes phosphorylated by the mitotic Cyclin complex, CycB/Cdk1, resulting in its dissociation from mitotic chromosomes to allow for the accumulation of H4K20Me and subsequent recruitment of Condensin II (Liu et al., 2010).

In addition to H4K20 associated modifiers, cell cycle dependent regulation of other PTM writers has also been reported. EZH2, the mammalian homolog of Enhancer of zeste, E(z) in *Drosophila*, is the major methyltransferase for H3 Lysine 27 and plays a crucial role in differentiation gene silencing through interaction with the Polycomb Repressive Complex 2 (PRC2; Cao et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). EZH2 is a direct target of the core cell cycle transcriptional regulator E2F (Bracken et al., 2003), and is up-regulated in proliferating stem cells or cancer stem cells, where it has been suggested to maintain pluripotency (Varambally et al., 2002; Lee et al., 2006; Sparmann and van Lohuizen, 2006; Simon and Lange, 2008). Several groups also uncovered a direct link between EZH2 and Cyclin/Cdks. The key S-phase and M-phase kinases, CDK1 and CDK2 can phosphorylate EZH2 in a cell cycle dependent manner on Thr350. This phosphorylation reinforces differentiation-associated gene silencing, such as silencing of HOX genes and SOX family members, and is thought to maintain stem cell identity (Chen et al., 2010; Kaneko et al., 2010). However, EZH2 can also be phosphorylated by CDK1 at Thr487, which disrupts the binding of EZH2 to the other PRC2 components, leading to the de-repression of EZH2 target genes, resulting in premature osteogenic differentiation of human mesenchymal stem cells (Wei et al., 2011). Thus, the cell cycle regulation of EZH2 can have both positive and negative outcomes on stem cell identity and differentiation. How these outcomes are balanced in actively proliferating cells remains unclear. Although there is plentiful data suggesting that EZH2 is important for normal cell proliferation and maintaining stem cell identity, whether part or all of these functions occur through PRC2-dependent gene silencing or another role of EZH2 is not known. PRC2-independent roles for EZH2 have been described, including an unexpected function as a transcriptional co-activator (LaJeunesse and Shearn, 1996; Strutt et al., 1997; Lee et al., 2011; Xu et al., 2012). To fully understand how EZH2 coordinates with the cell cycle machinery to promote proliferation and maintain stem cell identity, further investigations will be required.

These specific examples of the cell cycle machinery impacting chromatin modifiers are likely to be only the tip of the iceberg. The Cyclin/Cdk complexes themselves have hundreds of targets, many of which are uncharacterized or remain to be identified (Ubersax et al., 2003; Chi et al., 2008). In addition the myriad of other cell cycle kinases, phosphatases, ubiquitin ligases and their targets are only recently being uncovered on a proteomic scale (Bernal et al., 2014; Kuilman et al., 2014; Li et al., 2014; Lipinszki et al., 2014). Such large-scale approaches are likely to reveal new connections between the cell cycle machinery and chromatin regulators, which lie at the core of coordinating gene expression, with genome duplication and segregation.

GLOBAL NUCLEAR ARCHITECTURE AND THE CELL CYCLE: THE INTERACTION OF CHROMATIN WITH THE NUCLEAR ENVELOPE

Chromatin is not organized randomly within the nucleus during interphase, and microscopic observations of mammalian nuclei revealed that condensed chromatin is localized preferentially in the nuclear periphery, interrupted by stretches of less condensed chromatin at the nuclear pore complexes (NPCs). This distribution of heterochromatin-euchromatin led to the hypothesis that the more open chromatin near nuclear pores represents actively transcribed regions, and that this interaction facilitates the coupling of transcription with mRNA export, a process termed "gene gating" (Blobel, 1985). Consistent with this idea, active genes in yeast have been found to be localized at the Nuclear pore basket, including housekeeping genes and inducible genes that become re-located to the NPCs upon activation (Dieppois and Stutz, 2010; Burns and Wente, 2014). The recruitment of active genes to the NPCs in yeast involves interactions between the Nuclear Basket Nucleoporins or Nups (Mlp1, Nup1) with a HAT complex SAGA, and the TRanscription-EXport complex TREX-2 (Cabal et al., 2006; Luthra et al., 2007). Gene recruitment to these regions is dependent upon specific sequences termed GRS I and II present in the inducible gene promoters (Ahmed et al., 2010).

In higher eukaryotes, the relationship of gene activation and Nuclear Pore binding is complicated due to the recent discovery that several Nups have "off-pore" roles in the nucleoplasm (Capelson et al., 2010; Kalverda et al., 2010; Liang et al., 2013; Buchwalter et al., 2014). In the special, amplified polytene chromosomes of *Drosophila* salivary glands, Nup98 and Nup50 can be observed bound to decondensed chromatin and sites of active transcription microscopically. Nup98 and another Nup, Sec13, are localized to transcribed genes prior to the initiation of transcription, and an RNAi knockdown of Sec13 or Nup98 reduces transcription and RNA polymerase II recruitment, demonstrating functional roles for this binding (Capelson et al., 2010; Kalverda et al., 2010). However, the same Nups can also bind different set of genes when located in the pore vs. nucleoplasm. Recent examination of Nup98 mutant forms that are either solely nucleoplasmic or NPC-tethered showed nucleoplasmic Nup98 binding to genomic regions with high gene expression, marked with Histone PTMs associated with open chromatin (H3K4Me2 and H4K16Ac). In contrast, NPC-tethered Nup98 bound genomic regions with average gene expression, that are low in Histone PTMs associated with transcription (Kalverda and Fornerod, 2010; Kalverda et al., 2010), a finding seemingly opposite to the gene-gating model in yeast. Thus, in metazoans actively transcribed genes bound by Nups are more likely to be found in the nucleoplasm while NPC binding is correlated with lower gene expression levels.

"Transcriptional gene memory" is an interesting case where Nucleoporin binding is associated with future gene re-activation rather than current expression levels. Transcriptional memory is a phenomenon whereby a recently expressed and shut-off

gene is transcriptionally re-activated faster after exposure to the same stimulus for second time, allowing cells to respond quickly to environmental changes. This phenomenon can last through several cell divisions, demonstrating epigenetic inheritance (Brickner, 2009). In yeast, transcriptional memory of the INO1 gene requires a memory recruitment sequence (MRS) sequence in the promoter, incorporation of the H2A variant histone H2Az, and interaction of the promoter with the NPCs (Light et al., 2010). Transcriptional memory is conserved in mammals and also requires Nucleoporin binding. In HeLa cells the HLA-DRA gene induced by Interferon gamma (IFN-γ) exhibits transcriptional memory (Gialitakis et al., 2010), which is inherited through multiple cell divisions and is dependent upon the nucleoporin Nup98 (Light et al., 2013). However, as mentioned previously Nup98 can have both NPC and "off-pore" roles in metazoans, and importantly, the Nup98 interaction with the HLA-DRA promoter in human cells takes place in the nucleoplasm, not at NPCs (Light et al., 2013). In both cases, at yeast and human genes, transcriptional memory is associated with increased dimethylation of H3K4 (H3K4Me2) in the promoters, a mark which is dependent upon the interaction with the Nups (Light et al., 2013). However, H3K4 methylation is apparently not necessary for transcriptional memory, as deletion of the responsible Set1 methylase in yeast does not prevent transcriptional memory at Gal1 and Gal10 loci (Kundu et al., 2007; Laine et al., 2009). Overall, yeast and mammalian cells seem to share a common mechanism regarding transcriptional memory, which requires Nucleoporin binding, but in yeast this interaction occurs at the NPCs, while in mammals it occurs in the nucleoplasm. This distinction may be due to the "closed" nature of mitosis in yeast, where the nuclear envelope does not break down and is therefore is able to carry transcriptional memory through mitosis. In contrast the "open mitosis" of mammals may not be able to maintain transcriptional memory through M-phase and therefore this function has shifted to Nups located in the cytoplasm.

Outside of "gene gating" and transcriptional memory, chromatin binding to NPCs can also be associated with gene repression and silencing. In yeast the nucleoporin Nup170 interacts with the Sir4 subunit of the Silencing InsulatoR (SIR) complex, required for silencing of subtelomeres (Van de Vosse et al., 2013). The mammalian ortholog of Nup170 (Nup155) interacts with the HDAC4, also involved in transcriptional repression, revealing a conserved Nucleoporin function in silencing (Kehat et al., 2011). Because condensed chromatin is often found in the nuclear periphery between NPCs, yet many Nucleoporins are associated with actively transcribed genes, it has been suggested that specific Nups could create "transition zones" between heterochromatin and euchromatin (Van de Vosse et al., 2013), potentially reconciling the seemingly contradictory associations of Nups.

The localization of chromatin to the nuclear periphery, away from pores is suggested to be transcriptionally repressive in yeast and mammals (Andrulis et al., 1998; Malhas et al., 2007). Using this mechanism to silence gene expression involves chromatin movement from the nucleoplasm to the nuclear periphery. Chromosomes maintain certain positions in interphase nuclei (Chubb et al., 2002), and movement of artificial transgenes to the nuclear periphery in mammalian cells has been shown to require cell cycle progression through mitosis (Finlan et al., 2008; Reddy et al., 2008). This may be because the nuclear envelope-chromatin interactions need to be disrupted and re-established, an event driven by the open mitosis in mammalian cells. Importantly, this also suggests post-mitotic cells can use this repressive mechanism to permanently silence genes, and suggests a manner by which forcing cell cycle re-entry of postmitotic cells may promote chromatin re-localization and create a state permissive for cell de-differentiation (Nicolay et al., 2010; Pajcini et al., 2010).

Heterochromatin tethering along the nuclear periphery is mediated by lamins, nuclear cytoskeleton filaments, that connect chromatin to the inner nuclear membrane of the nuclear envelope (Dechat et al., 2008). Lamin-associated aomains (LADs) of the mammalian genome contain a relatively low number of genes and exhibit a repressed chromatin state (Guelen et al., 2008; Peric-Hupkes et al., 2010). LADs have been shown in a number of studies to modulate gene expression, and repositioning genes to a LAD is sufficient to mediate repression (Kosak et al., 2002; Williams et al., 2006; Reddy et al., 2008). One persistent question in the field though, has been how the chromatin associated with LADs can be "remembered" after nuclear envelope breakdown and reformation following mitosis.

A detailed analysis of LAD positioning during the cell cycle was performed using a modified Dam-ID approach, to permanently mark chromatin regions that associate with nuclear lamina, and track their position even after detachment and through the cell cycle (Kind et al., 2013). The study revealed that in a human cell line, LADs are generally found in nuclear periphery during interphase and are enriched for the H3K9Me2 PTM, associated with gene silencing. Interestingly, during mitosis the LADs remain distinct from regions of PTMs associated with transcriptional activity such as H3K27Ac and H3K4me2. However, after mitosis the LADs from the prior interphase do not re-establish a peripheral localization in the nucleus, instead they become distributed stochastically between the nucleoplasm and nuclear periphery. These results suggest that LAD positioning and the PTMs associated with it, are in fact, not mitotically inherited (Kind et al., 2013).

This profound and surprising result raises the question of how such stochastic changes in chromatin dynamics during each cell cycle, and presumably gene expression, can possibly be reconciled with seemingly organized and predictable changes in cell fate during development. One possibility is that LADs may be primarily used to modulate gene expression in postmitotic cells, although studies performed in proliferating fibroblasts suggest this may not be the case (Reddy et al., 2008). Importantly, new single-cell based assays are revealing a surprising amount of stochastic variation in individual cell decisions of quiescence vs. proliferation or differentiation vs. pluripotency, even within clonal cell populations in culture (Kalmar et al., 2009; Dey-Guha et al., 2011; Spencer et al., 2013). Does the inherent unpredictability of chromatin reorganization after mitosis possibly underlie this stochasticity? This will be an interesting question to address in future research.

GLOBAL NUCLEAR ARCHITECTURE AND THE CELL CYCLE: OPEN MITOSIS AND THE NUCLEAR PORE COMPLEX

In metazoan cells where an "open mitosis" takes place, the nuclear envelope breaks down at the onset of mitosis. This involves the disassembly of NPCs, lamin depolymerization, and incorporation of nuclear envelope membranes into the endoplasmic reticulum ER (reviewed in Guttinger et al., 2009). Like other events in mitosis, nuclear envelope breakdown is controlled by the activity of the mitotic Cyclin/Cdk kinases. CyclinB/Cdk1 promotes NPC disassembly by phosphorylation of nucleoporins (Onischenko et al., 2005; Muhlhausser and Kutay, 2007). Peripheral Nups are the first to be dissociated from the disassembling NPCs (Terasaki et al., 2001; Dultz et al., 2008), and Nup98, the Nup involved in transcriptional memory and off-pore regulation of gene expression described earlier, is the first to be displaced (Dultz et al., 2008). Nup98 is phosphorylated at the onset of mitosis by CyclinB/Cdk1, Polo-like kinase1 (Plk1), Nek6, (and possibly other kinases) at 13 residues, most of which are localized to the C-terminal portion of the protein that mediates the interaction of Nup98 with other NPC components (Laurell et al., 2011). When these residues are mutated to sites that cannot be phosphorylated, NPC disassembly is delayed, suggesting that Nup98 phosphorylation is an initial and critical step in NPC disassembly at mitosis.

When mitosis is complete, the nuclear envelope must be re-assembled. NPCs are initially re-assembled through interactions with chromatin, followed by association of membranes to form the closed nuclear envelope. NPC re-assembly starts with the recruitment of the Nup107–160 complex to chromatin during late anaphase, mediated by the AT hook containing transcription factor 1 (AHCTF1) also known as ELYS, a scaffold nucleoporin which has a DNA binding domain for recruiting factors to chromatin (Hetzer and Wente, 2009; Imamoto and Funakoshi, 2012). Subsequently, interaction of Nup107–160 with the transmembrane Nup Pom121 allows the recruitment of membrane vesicles and also mediates interactions with other Nups (Nup93–205). Then, the central pore channel Nups and peripheral Nups are recruited to the NPCs (Guttinger et al., 2009; Capelson et al., 2010; Imamoto and Funakoshi, 2012). How are enough NPCs produced during interphase to be equally divided between daughter cells at the next mitosis? In contrast to post-mitotic NPC re-assembly, where the inactivation of mitotic Cdk1 and dephosphorylation of Nups and other nuclear envelope proteins is required, NPC production during interphase is positively regulated by Cdk activity, in particular Cdk1 and Cdk2 (Maeshima et al., 2010). Interphase NPC assembly initiates with the entrance of the transmembrane Pom121 Nup to the nucleus, and its localization to the inner nuclear membrane (Funakoshi et al., 2011). Interestingly, in this case the ELYS Nucleoporin is not required for assembly (Doucet et al., 2010). The Nup107–160 complex is subsequently recruited, but the detailed sequence for interphase NPC assembly remains unclear (Capelson et al., 2010; Imamoto and Funakoshi, 2012).

Apart from the assembly of NPCs, their distribution in the nuclear membrane during cell cycle progression changes as well. During G1, right after completion of mitosis, NPCs are distributed unequally through nuclear surface, generating "pore-free islands" (Maeshima et al., 2006). These "pore-free islands" are rich in type A Lamins, while regions high in pore density are characterized by the presence of B-lamins and the lamin B receptor (LBR). The distribution of NPCs becomes uniform gradually as the cells progress through S and G2 phases (Maeshima et al., 2006). As NPCs and Lamins both bind chromatin and affect gene expression, the changes in distribution of the nuclear envelope proteins could potentially affect gene expression throughout the cell cycle (**Figure 1**).

DNA DAMAGE AND THE NUCLEAR PORE COMPLEX

How is chromatin tethered to the nuclear pores or nuclear lamina properly replicated during S-phase? The anchoring of chromatin to NPCs turns out to have both positive and negative impacts on genome integrity during replication. For example, replication forks with persistent double strand breaks (DSBs) relocate to NPCs for repair (Nagai et al., 2008). The association of damaged forks to the pores occurs through an interaction with the Slx5/Slx8 complex, a SUMO dependent E3 Ubiquitin ligase, which is bound by Nup84 (Nagai et al., 2008; Perry et al., 2008). While it is not exactly clear why movement to the NPCs facilitates repair, it has been proposed that the nuclear periphery may provide a special permissive environment for additional DSB repair pathways beyond homologous recombination and non-homologous end joining to repair persistent DSBs (Oza et al., 2009).

While recruitment to pores can promote DNA repair, paradoxically, the anchoring of actively transcribed genes to NPCs can also be a source of replication stress. It is thought that as the DNA replication fork proceeds, it will eventually meet the NPC- tethered region actively transcribing genes. The inflexibility of tethered DNA can become a source of tension as the unwinding of DNA occurs during replication fork progression (Branzei and Foiani, 2010), and the tension generated between an actively transcribed region tethered to the NPC and the approaching replication fork is somehow released by the activity of the DNA damage checkpoint kinases and their associated complexes (Bermejo et al., 2011). When the checkpoint response is inhibited, replication forks collapse and firing of dormant replication origins occurs (Bermejo et al., 2011). It remains unclear whether a similar checkpoint mechanism is applied upon replication of transcribed genes that are not tethered to the NPC, for example those bound to other immobile nuclear structures.

The act of DNA replication during S-phase can also be a source of DNA damage (Mazouzi et al., 2014) which if not repaired could in turn lead to acquisition of mutations, cell cycle arrest or even senescence. Apart from chromatin anchoring, Nups facilitate the maintenance of genome integrity also by affecting the nuclear transport of DNA damage repair proteins required during the cell cycle. In human cells the knockdown of Nup153 impairs DNA repair by preventing proper nuclear accumulation of 53BP1 (Moudry et al., 2012). Furthermore, Tpr (Mlp1/Mlp2 in yeast), is a Nup that interacts with Nup153 in the nuclear pore basket as is also essential for proper DNA damage signaling. When Tpr is depleted, the nuclear export of p53 becomes compromised, resulting in nuclear accumulation of p53 and activation of downstream target genes such as p21 leading to premature senescence (David-Watine, 2011). Thus, NPCs influence DNA repair and DNA damage signaling during S and G2 phases in many different

ways, and significantly contribute to the maintenance of genome stability.

CELL CYCLE PHASE AND CELL FATE ACQUISITION

Cellular differentiation and proliferation must be intimately coordinated for proper development and tissue homeostasis. Stem cells pose a special case in this regard, as they must proliferate when needed, yet retain their undifferentiated status (Fuchs, 2009; Lange and Calegari, 2010; Li and Clevers, 2010). The cell cycle of pluripotent embryonic stem (ES) is reminiscent of that in early embryos, characterized by very short gap phases. Upon differentiation G1 phase becomes longer, more similar to adult somatic cells (Singh and Dalton, 2009; Calder et al., 2013; Coronado et al., 2013), and several studies have suggested ES cells initiate differentiation in G1 phase (Mummery et al., 1987; Sela et al., 2012; Chetty et al., 2013; Pauklin and Vallier, 2013). When undifferentiated human ES stem cells are isolated in different phases of the cell cycle, their propensity for spontaneous differentiation in culture varies. G1-phase cells exhibit a high rate of spontaneous differentiation, while S, and G2 -phase cells exhibit reduced spontaneous differentiation (Sela et al., 2012). Interestingly, the propensity of G1 cells to differentiate, is reduced when co-cultured with S and G2 phase cells in direct contact, suggesting cell cycle-dependent cell to cell signaling may be partly responsible for this effect. *In vivo*, the propensity for embryonic neural stem cells to self-renew vs. produce differentiated daughters also varies with changes in the cell cycle (Arai et al., 2011; Hardwick and Philpott, 2014), and manipulation of cell cycle phase length in neural stem cells can alter the balance of self-renewal vs. differentiation in the developing brain in animals ranging from *Drosophila* to mammals (Manansala et al., 2013; Tapias et al., 2014).

What are the molecular mechanisms connecting cell fate acquisition with prolonged G1? Cells in or poised to enter quiescence exhibit reduced Cyclin/Cdk activity and thus reduced phosphorylation of the Retinoblastoma (RB) tumor suppressor, a critical regulator of the restriction point and cell cycle entry (Henley and Dick, 2012; Sadasivam and Decaprio, 2013; Schachter et al., 2013). Human ES cells with hypo- or unphosphorylated RB exhibit the highest propensity to spontaneously differentiate, suggesting even a transient quiescence may consequently promote differentiation (Sela et al., 2012). However, it is important to note that a parallel study in mouse ES cells found no impact on spontaneous differentiation when Cyclin/Cdk activity was directly inhibited and RB was hypo-phosphorylated (Li et al., 2012). Whether these differences may be organism or cell-line specific remains to be determined, but multiple lines of evidence support a relationship between cell cycle changes and cell fate acquisition in human ES cells (Calder et al., 2013; Chetty et al., 2013; Coronado et al., 2013; Pauklin and Vallier, 2013; Singh et al., 2013). While the capacity for ES cells to differentiate may be established during quiescence, there is evidence that in adult cells differentiation is actively inhibited during quiescence through the transcriptional repressor Hes1 (Sang et al., 2008). Inhibition of differentiation during quiescence is critical for adult stem cells, which can spend prolonged periods in an arrested state, yet must retain their stem cell capacity (Fuchs and Chen, 2013). This suggests there will be distinct mechanisms that link the cell cycle with cell fate acquisition in adult vs. ES cells.

A view of the molecular signaling mechanisms that coordinate cell fate decisions with the core cell cycle machinery in ES cells is just beginning to emerge. Work with human ES cells has now revealed a pathway connecting CyclinD/Cdk4 activity to the TGF-β/Smad signaling pathway. TGF-β signaling promotes endoderm fate in human ES cells, but only during a permissive window in early G1. The capacity for endoderm differentiation drops-off upon cell cycle entry, in a manner correlated with increasing G1 CyclinD/Cdk4 activity. Pauklin and Vallier reconciled these observations by showing that CyclinD/Cdk4 regulates the chromatin association of the TGF-β responsive transcription factors Smad 2 and 3. Smad2/3 associate with chromatin in early G1 allowing for expression of TGF-β target genes, but CyclinD/Cdk4–dependent phosphorylation of residues in the Smad2/3 linker regions prevents them from binding chromatin upon cell cycle entry (Pauklin and Vallier, 2013). This simple relationship between CyclinD/Cdk4 activity and Smad2/3 chromatin binding creates a permissive window for endoderm differentiation directly linked to the core cell cycle machinery.

The ability to monitor differentiation and cell cycle dynamics in real-time, at the single-cell level, has been made possible by the use of the Fluorescent Ubiquitylation-based Cell-Cycle Indicator (FUCCI) system (Sakaue-Sawano et al., 2008). This system uses fluorescently labeled cell cycle reporters that are degraded at different cell cycle phase transitions, such that the dynamics of G1, S and G2/M phases can be monitored and quantified. The FUCCI system facilitated the studies of Pauklin and Vallier by allowing them to use flow cytometry to precisely sort stem cells based upon their cell cycle phase. Using a similar approach, also in human ES cells, Singh et al. (2013) examined gene expression changes during the cell cycle. They find that genes expressed specifically during G1 are heavily enriched for roles in development and cell-fate commitment and that these changes in gene expression are dependent upon cell cycle status (Singh et al., 2013). To determine how this cell cycle-dependent gene expression is regulated, they examined global chromatin changes during the cell cycle and unexpectedly found that the cytosine modification 5-hydroxymethylcytosine (5hmC) is increased during late G1, followed by a sharp decline in S-phase, and re-established during G2. Interestingly, the loss of methylation during S phase may be greater than that expected by simple passive loss through the incorporation of new unmodified nucleotides during DNA replication. If this is the case, there may be cell cycle regulated active de-methylation during S-phase in stem cells.

In contrast to the better-known repressive cytosine methylation 5mC, 5hmC is instead associated with active promoters, increased gene expression and genes poised for rapid expression (Jin et al., 2011; Pastor et al., 2011). The cell cycle regulated changes in 5hmC impact developmental gene expression and are associated with the histone PTMs H3K4me3 and H3K27me3, which are the so-called "bivalent" marks, associated with differentiation genes in stem cells. Bivalent domains have been suggested to simultaneously prevent premature expression of differentiation genes in ES cells via the repressive H3K27me3 mark, yet simultaneously keep them poised for rapid expression

upon differentiation via the H3K4me3 mark, although this model is controversial (Vastenhouw and Schier, 2012; Voigt et al., 2013). The work of Singh now adds an extra layer to the puzzle by demonstrating an additional chromatin modification that appears to be under the control of the cell cycle machinery. It remains unknown how and why 5hmC is increased during the G1 phase and re-established at G2, or perhaps more importantly how and why de-methylation occurs during S phase. It will be important to investigate the molecular mechanisms linking genome methylation with the cell cycle machinery in stem cells. While it has been discussed for over two decades that the response of cells to differentiation cues seems to be affected by their cell cycle status, we are just now beginning to decipher the specific mechanisms linking the cell cycle to the chromatin state and the acquisition of cell fate.

THE "MITOTIC ADVANTAGE" AND NUCLEAR REPROGRAMMING

While differentiation and lineage restriction of pluripotent cells seems to be increased during the G1-phase of the cell cycle, multiple lines of evidence suggest the acquisition of pluripotency or potential for nuclear reprogramming is increased during mitosis (Egli et al., 2008). An increase in nuclear reprogramming efficiency at mitosis may seem surprising at first glance, since the use of quiescent G0 nuclei was suggested to be essential to the success of the most famous example of mammalian cloning, Dolly the ewe (Campbell et al., 1996a,b). However, subsequent examples of mammalian cloning demonstrated that actively dividing cells could be efficiently used for donor nuclei (Cibelli et al., 1998). More recent cell reprogramming experiments carried out through cell-fusion of differentiated cells with mouse ES cells to form heterokaryons, suggested that successful reprogramming of chromatin actually requires activation of DNA synthesis within the first 24 h of cell fusion (Tsubouchi et al., 2013). In this case, DNA synthesis was suggested to facilitate nuclear reprogramming by passively diluting existing DNA methylation marks. But there are additional observations suggesting active cell cycling and more specifically mitosis is advantageous for nuclear reprogramming.

In studies using somatic nuclear transfer in *Xenopus*, the use of nuclei that have recently undergone mitosis was shown to increase origin accessibility in the oocyte, which poises the donor nuclei for the rapid S-phase entry and progression required during early *Xenopus* development (Lemaitre et al., 2005). Later work by Ganier et al. (2011) revealed a peculiar ability of *Xenopus* egg extracts, specifically at the metaphase stage, to increase the efficiency of reprogramming mouse fibroblast nuclei to pluripotency. Permeabilized mouse embryonic fibroblasts exposed to mitotic egg extract, but not interphase extract, exhibit decreased histone modifications such as H3K9, H3K4, and H4K20 di- and trimethylation and increased expression of pluripotency-associated genes. When somatic cell nuclear transfer was subsequently performed with the mouse fibroblast nuclei exposed to the mitotic extract, a fourfold increase in reprogramming efficiency was observed (Ganier et al., 2011). This ability of a mitotic egg extract to facilitate mammalian nuclear reprogramming was suggested at least in part, to be due to the extract promoting M-phase entry in the fibroblast nuclei. Indeed, mitotic figures and histone marks associated with mitosis were observed in the fibroblast nuclei exposed to the extract.

How exactly does the mitotic status of a donor nucleus facilitate cell fate reprogramming? Halley-Stott et al. (2014) attempted to address this question recently using a system where permeabilized adult mouse myoblast cells of different cell cycle stages are transferred into enucleated *Xenopus* oocytes, and the activation of mammalian pluripotency genes is used as a readout of reprogramming. They find, consistent with the reprogramming studies of others (Egli et al., 2008; Ganier et al., 2011), that transfer of cells with nuclei in late G2 or M-phase confers a dramatic increase in the responsiveness to reprogramming factors and induction of pluripotency genes, up to 100 times faster than that observed with interphase donor nuclei. They term this phenomenon "mitotic advantage" (Halley-Stott et al., 2014). This mitotic advantage for chromatin reprogramming to pluripotency can be observed in donor nuclei from different cell types and cannot be explained simply by the increased nuclear permeability at mitosis. The authors systematically removed different components from the mitotic chromatin to identify the molecular basis of this advantage. In sum, mitotic advantage appears to require nucleosomes, but cannot be explained by histone acetylation, phosphorylation, or methylation. Rather their data suggest that the loss of ubiquitination on histones H2A and H2B during mitosis (Joo et al., 2007) seems necessary, but is not sufficient to confer a mitotic advantage (**Figure 2**). Future studies will therefore be needed to identify the additional factors involved in mitotic advantage.

The work of Halley-Stott et al. (2014) suggests a permissive window for cell fate reprogramming occurs at mitosis, independent of the dilution of epigenetic marks at S-phase, acting more directly through the rapid expression of pluripotency genes. They suggest the removal of most transcription factors from mitotic chromosomes actually increases their accessibility to reprogramming factors, which allows for rapid induction upon exit from mitosis as soon as transcription resumes (Halley-Stott et al., 2014). Given the stochasticity inherent in the cellular reprogramming progress (Hanna et al., 2009), the rate of pluripotency gene induction after the completion of mitosis is likely key to successful nuclear reprogramming.

CONCLUSIONS AND FUTURE PERSPECTIVES

Extensive connections between the cell cycle machinery and chromatin clearly exist, which impact gene expression and thus, cell fate decisions in important ways. While the use of asynchronous cell culture or mixed lineage tissues has sometimes hampered our ability to see these connections, new tools such as Chromatin Conformation Capture, the FUCCI system, the PLA and modified versions of DamID, are being used in ways that allow detailed views of the cell cycle, chromatin state and cell fate acquisition that were previously impossible. But several key questions remain unresolved. For example, does the gene expression profile of a cell, and thus cell fate, control important facets of the cell cycle such as origin choice and DNA replication timing? Or does the cell cycle status of a cell instead determine its gene expression possibilities and therefore limit choices in cell fate? If the latter is true, how can cell fate be so robustly maintained in some instances

of regeneration or in cases of cell cycle disruption during development? As we learn more about the truly plastic nature of cell fate, we expect to find that the cell cycle influences the probability of acquiring certain cell fate programs, but that multiple cell cycle and cell fate states can be compatible under specific conditions. Future work will continue to uncover new molecular connections between the cell cycle machinery and developmental signaling pathways, to help us finally understand how the cell cycle impacts cell fate.

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Nuclear envelope and genome interactions in cell fate

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The eukaryotic cell nucleus houses an organism's genome and is the location within the cell where all signaling induced and development-driven gene expression programs are ultimately specified. The genome is enclosed and separated from the cytoplasm by the nuclear envelope (NE), a double-lipid membrane bilayer, which contains a large variety of trans-membrane and associated protein complexes. In recent years, research regarding multiple aspects of the cell nucleus points to a highly dynamic and coordinated concert of efforts between chromatin and the NE in regulation of gene expression. Details of how this concert is orchestrated and how it directs cell differentiation and disease are coming to light at a rapid pace. Here we review existing and emerging concepts of how interactions between the genome and the NE may contribute to tissue specific gene expression programs to determine cell fate.

Keywords: nuclear envelope, nuclear pore, nuclear lamina, genome, cell fate, differentiation, gene regulation, nuclear organization

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Introduction

Pluripotent embryonic stem cells (ESCs) from many organisms display strikingly different chromatin structure and overall nuclear architecture when compared with differentiated cells (**Figure 1**). Microscopic visualization of DNA stains in ESC nuclei show diffuse staining indicative of a generally open chromatin state (Efroni et al., 2008; Ahmed et al., 2010). Consistent with this observation, comparisons of pluripotent stem cells with differentiated cells revealed changes in both levels and localization of epigenetic marks within the nuclear space (Bartova et al., 2008; Wen et al., 2009). Such cytological observations of the unique chromatin state of ESCs have been extensively confirmed by genome wide and functional studies of histone modifications and chromatin complexes (Mattout and Meshorer, 2010). Consistent with a decondensed and permissive chromatin state, pluripotent and totipotent cells exhibit higher chromatin mobility (Meshorer et al., 2006; Boskovic et al., 2014).

This large-scale change in overall chromatin structure, condensation and mobility during differentiation is supported by changes in nuclear structure and composition. During cell differentiation, individual genes, as well as larger chromosome regions are repositioned within the nuclear space, and this repositioning correlates with tissue specific gene expression profiles (Schneider and Grosschedl, 2007; Bickmore and van Steensel, 2013). Large-scale chromatin reorganization and gene repositioning during differentiation relies, at least in part, on losing or gaining interactions with major nuclear compartments such as the NE. Components of the NE, including the nuclear lamina, the nuclear membrane (NM) and the nuclear pore complex (NPC), come in close contact with the underlying genome and have

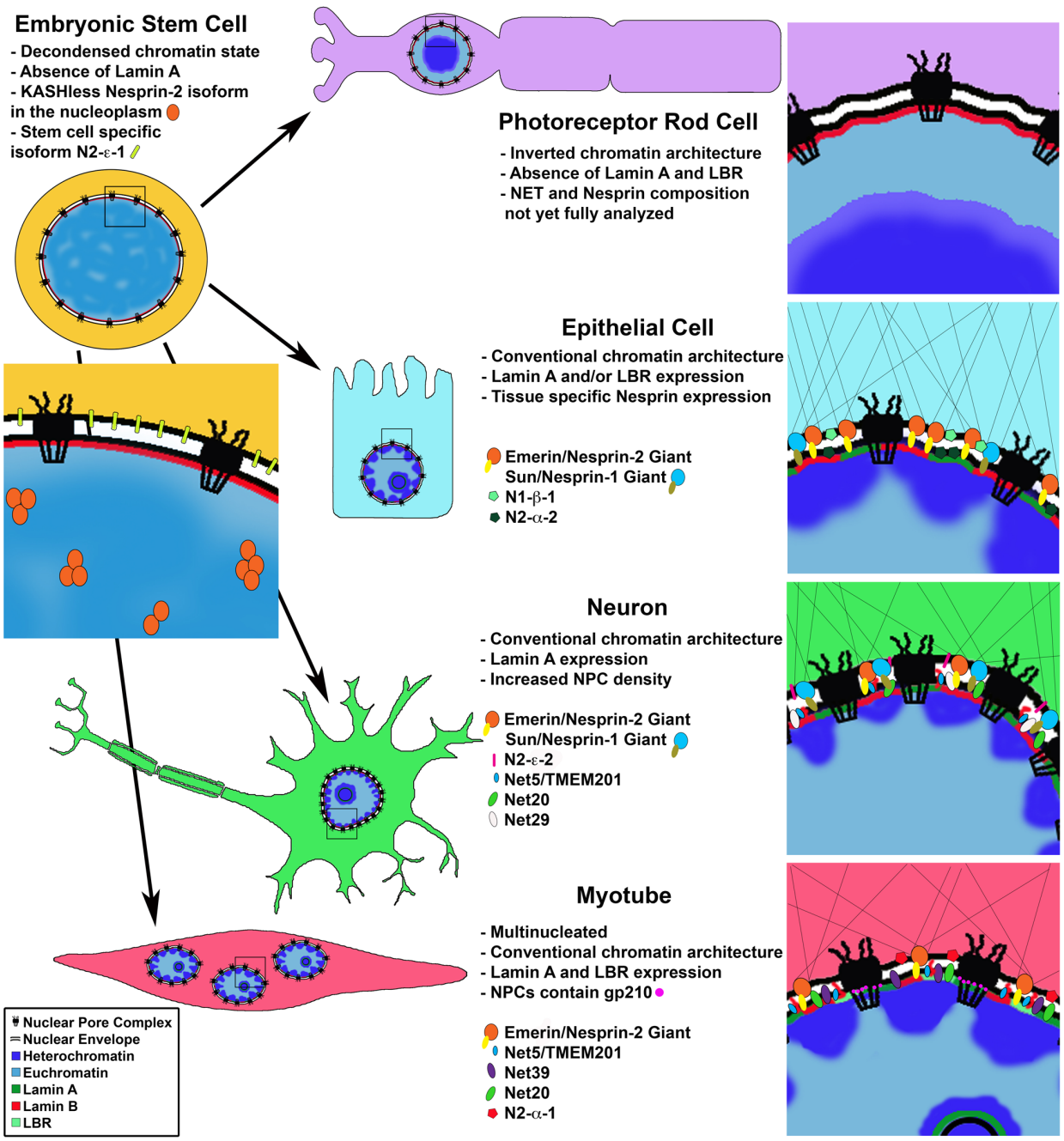

FIGURE 1 | Cell type specific changes in NE composition. Cell differentiation coincides with changes in chromatin organization and protein composition of the nuclear lamina, nuclear envelope and nuclear pore complex. Photoreceptor rod cells lose expression of LBR and Lamin A leading to an inverted chromatin state with heterochromatin in the nuclear interior. Proteomic analysis of differentiated cells shows cell type specific nuclear envelope composition resulting in unique nucleo-cytoplasmic connections influencing cell morphology; and chromatin-NE interactions facilitating intra-nuclear genome reorganization and regulation of gene expression programs. Differentiated muscle cells uniquely express gp210 at the NPC leading to activation of muscle specific genes.

been implicated in a number of chromatin-associated processes (Akhtar and Gasser, 2007; Arib and Akhtar, 2011; Van de Vosse et al., 2011; Amendola and van Steensel, 2014). While several of these processes have been characterized individually, how nuclear components work together to execute tissue specific gene expression programs is still unclear. In this review we aim to outline current understanding of the roles of major NE components in determining tissue specific cell fate and discuss selected examples illustrating their connection to genome organization and function.

The Nuclear Lamina

The nuclear lamina is a meshwork of class V intermediate filament proteins lining the inner nuclear membrane (INM) of the NE (Prokocimer et al., 2009). The lamina is comprised of A and B type Lamins; Lamin A and C are the two major splice variants of a single gene (*LMNA*), while Lamin B1 and B2 are transcribed from distinct genes (*LMNB2* and *LMNB2*; Ho and Lammerding, 2012). Pre-Lamin A undergoes enzymatic cleavage to become mature Lamin A; and all Lamins are subject to a variety of post-translational modifications (Snider and Omary, 2014). Together with Lamin associated proteins, the Lamin filaments are known to provide structural support to the nucleus and to serve as a scaffold for spatial genome organization (Dittmer and Misteli, 2011). Specifically, Lamin proteins are known to function in tethering of heterochromatic and developmentally silenced domains to the nuclear periphery (Guelen et al., 2008; Ikegami et al., 2010; Peric-Hupkes and van Steensel, 2010; Peric-Hupkes et al., 2010), as well as interact with a myriad of proteins affecting chromatin organization and dynamics, such as transcription factors and chromatin remodelers (Ho and Lammerding, 2012). Notably, Lamins, particularly Lamin A, have also been visualized in the nuclear interior, often associated with nucleoli, another nuclear sub-compartment enriched in heterochromatin (Broers et al., 2005; Kind et al., 2013; Kind and van Steensel, 2014; Legartova et al., 2014; Padeken and Heun, 2014). While many mechanistic details remain unknown, it is becoming increasingly clear that Lamins play a pivotal role in the dynamic changes in chromatin and cellular organization required for determination and manifestation of cell fate.

Temporal and Cell Type Specific Expression of Lamins

The B type Lamins (B1 and B2) are expressed in all cell types, while expression of Lamins A and C varies with cell type and developmental stage (Worman et al., 1988a; Rober et al., 1989). Immunofluorescence staining and immunoblotting with isotype specific anti-Lamin antibodies in mouse embryos show low expression of Lamin A/C in ESCs, which increases as cells differentiate (Constantinescu et al., 2006; Eckersley-Maslin et al., 2013). In mice, the increase in Lamin A/C expression is initiated as early as embryonic day 9 and as late as in the adult animal depending on the tissue type (Stewart and Burke, 1987; Rober et al., 1989). In direct support of a role for Lamin A in cell differentiation, experiments in mouse cells testing the effect of Lamin A levels on somatic to iPS cell reprogramming show that depletion of Lamin A accelerates the transition to pluripotency, while cells overexpressing Lamin A take longer to reprogram (Zuo et al., 2012).

Further supporting separate roles for A and B type Lamins, studies of Lamin filaments in amphibian oocytes and HeLa cells indicate that Lamins A, B and C form discrete, but interconnected, lattice structures with differing physical properties (Goldberg et al., 2008; Shimi et al., 2008; Kolb et al., 2011). In agreement with these studies, immunofluorescence staining in mouse embryonic fibroblasts (MEFs) shows non-uniform staining of the nuclear envelope/lamina where Lamin B and Lamin A do not overlap (Legartova et al., 2014). Direct evidence for tissue specific function of Lamin proteins comes from mutations in the human *Lamin A* (*LMNA*) gene, which lead to an array of serious diseases called laminopathies, including cardiomyopathy, muscular dystrophy, lipodystropy, neuropathy and progeria (Dittmer and Misteli, 2011). Together, these data demonstrate that Lamins are expressed in a tissue specific manner and form unique territories in the lamina likely contributing to cell type specific NE composition (**Figure 1**), and support the notion that Lamins play functional roles in cell differentiation, as discussed further below.

Lamins Maintain Heterochromatin at the Nuclear Periphery

Microscopic observations of somatic cell nuclei indicate that in most cell types, heterochromatin is enriched at the nuclear periphery and this enrichment becomes more pronounced with cell differentiation (Wu et al., 2005; Reik, 2007; Ueda et al., 2014). Known epigenetic marks of heterochromatin commonly found at the nuclear periphery include H3K9me1, H3K9me2, H3K9me3, H3K56me3, H4K20me2, H4K20me3, H3K27me2, H3K27me3, and H3K4ac (Eberhart et al., 2013). Reported genome wide Chromatin Immunoprecipitation (ChIP) of the heterochromatin mark H3K9me2 shows that coverage of "large organized chromatin K domains" (LOCKS) grows from 17.5–24% in pluripotent human stem cells to 39.3–44.8% in differentiated cell lines (Wen et al., 2009, 2012). This data combined with DNA adenine methyltransferase identification (DamID) studies of Lamin B1 Associated Chromatin Domains (LADS), exhibits a significant overlap between LOCKS and LADs, which supports a role for Lamin B1 in the peripheral localization of these heterochromatic domains (Guelen et al., 2008; Peric-Hupkes et al., 2010; Amendola and van Steensel, 2014), and agrees with the visually observed changes in chromatin organization during differentiation.

An especially impressive example of the requirement for Lamin expression in heterochromatin organization during cell differentiation comes from studies of retinal rod cells in nocturnal mammals. The authors noticed the conventional nuclear architecture described for most cell types, with heterochromatin lining the nuclear periphery and euchromatin in the nuclear interior, is essentially reversed in retinal photoreceptor rod cells (Solovei et al., 2009). This inverted architecture is thought to have evolved to channel light more efficiently in the eye and has provided a unique and fruitful system, in which to study basic requirements for spatial organization of chromatin.

In a series of elegant experiments the authors demonstrate that during cell differentiation, conventional chromatin architecture requires the sequential expression of first the NE transmembrane protein Lamin B receptor (LBR) and then its replacement by Lamin A/C, with some cell types expressing both proteins (Solovei et al., 2013). The chromatin architecture inversion, with euchromatin at the nuclear periphery and heterochromatin in the nuclear center, in photoreceptor nuclei is a result of loss

of expression of both Lamin A/C and LBR from the nuclear envelope (**Figure 1**). They further show this loss and the subsequent chromatin rearrangements coincide with terminal differentiation of the rod cells. Strikingly, the inversion phenotype was successfully recapitulated experimentally in additional cell types, such as the hair follicle (which does not express Lamin A/C), using LBR null mice, and examination of double null (*Lbr−/− Lmna−/−*) mouse pups indeed showed an inverted phenotype in all post-mitotic cell types studied. Conversely, artificially maintaining expression of LBR, but not Lamin C in these cells was enough to prevent chromatin inversion, suggesting that Lamin C does not bind chromatin directly but perhaps via other nuclear envelope associated proteins such as LEM domain proteins (discussed further below). Although it is not presently clear how the conventional versus the inverted heterochromatin architecture affects cell type specific gene expression, these results support the notion that Lamins B and A/C are needed to position heterochromatin in a cell type specific manner.

Lamins Recruit Differentiation-Specific Genes

Genome wide studies of LADs during neuronal differentiation in mice showed that while ESCs and terminally differentiated cells share a broad LAD structure, smaller sub regions of gene clusters undergo rearrangements corresponding to steps of the differentiation process (Amendola and van Steensel, 2014; Luperchio et al., 2014). For example, genes associated with "stemness," as well as cell cycle related genes, become lamina-associated during differentiation. Conversely, cell type or lineage specific genes were released from the lamina and de-repressed or "unlocked" for expression at a subsequent step in differentiation (Peric-Hupkes et al., 2010; **Figure 2A**).

In support of a role for Lamins in differentiation-specific gene expression programs, B type Lamin knockout mouse models display an array of organogenesis defects, particularly in the brain, yet self-renewal and pluripotency properties of mouse ESCs are not affected (Kim et al., 2011). In *Drosophila*, the gene encoding a critical transcriptional factor *hunchback* was shown to move to the nuclear lamina during differentiation of neuroblast cells to neurons (Kohwi et al., 2013). This gene repositioning correlated with a loss of progenitor cell competence and was found to be dependent on the B type Lamin Dm0. Depletion of Lamin Dm0 extended neuroblast competence, presumably through disruption of targeting the *hunchback* locus to the nuclear lamina. These studies indicate the nuclear lamina is extensively utilized throughout metazoa to stably silence differentiation-specific genes.

How do Lamins bind to heterochromatin or developmentally silenced genes? In addition to reports of a DNA binding domain in Lamin A (Bruston et al., 2010) and *in vitro* interactions of Lamins with DNA and histones (Taniura et al., 1995; Stierle et al., 2003), there are several examples of Lamins interacting with chromatin binding NE proteins, chromatin regulatory machinery and transcriptional regulators. For example, interactions between the lamina and constitutive highly condensed heterochromatin are thought to be mediated via LBR and heterochromatic proteins such as Heterochromatin Protein 1 (HP1), discussed in further detail above and below. Additionally, a recent study identified a new mediator of Lamin-genome interactions, which appears to be utilized by silenced genes in mouse fibroblasts (Zullo et al., 2012). The authors have characterized discrete DNA sequences within LADs spanning the IgH and Cup3a genes able to position these loci to the nuclear lamina and concomitantly silence gene activity. These recurring lamina-associated sequences (LASs) were found to be enriched for a GAGA motif and to bind the transcriptional repressor cKrox in a complex with histone deacetylase 3 (HDAC3) and the lamina-associated NE protein Lap2β. The cKrox/HDAC3/Lap2β complex is necessary for tethering of LAS-containing target genes to the lamina, and represents another key molecular explanation for the coupling of nuclear localization and transcriptional repression. These findings are consistent with a previous study demonstrating the ability of Lap2β to reposition an ectopic binding site to the nuclear periphery and silence expression of genes near the binding site (Finlan et al., 2008). In this example, Lap2β was fused to the bacterial LacI protein, which binds the *lactose operon* (*lacO)* repeats array, introduced into the genome of human culture cells, and the ability of the Lap2β-LacI to silence genes near its target site was similarly found to be dependent on HDAC activity.

On a cautionary note, initial DamID studies of Lamin-chromatin binding sites required a population of cells, and thus the resulting LADs are reflections of both an average of many cells in a population as well as an amalgamation of binding events acquired over the time a DamID fusion protein is expressed. When these studies were repeated using the m6A-tracer technique which is able to label stochastic protein-Dam chromatin interactions in single, living cells, the authors found that at a given time only a subset of the initially described LADs was localized to the periphery while the rest were often located in the nuclear interior and further, this subset often changed following each cell division (Kind et al., 2013; Kind and van Steensel, 2014). Use of the m6A-tracer technique to specifically monitor Lamin A-chromatin binding shows Lamin A binding at the nuclear periphery and also around the nucleoli. These results support previous observations of an intranuclear pool of Lamin A (Moir et al., 2000a,b) and indicate a stochastic nature of Lamin-chromatin binding, which would allow for dynamic binding of LAD sequences to either A or B type Lamins, or Lamin associated proteins, as needed.

The Nuclear Membrane

The nuclear envelope is a double lipid bilayer system made of the INM, directly adjacent and connected to the Lamin filaments, and the outer nuclear membrane (ONM), which is contiguous with the endoplasmic reticulum. The space between these membranes is called the perinuclear space (PNS) and is interrupted by NPCs which fenestrate the NM. Originally viewed as simply a protective barrier for the genome, the nuclear envelope along with its nuclear envelope transmembrane proteins (NETs) and associated soluble proteins are now known to participate in an array

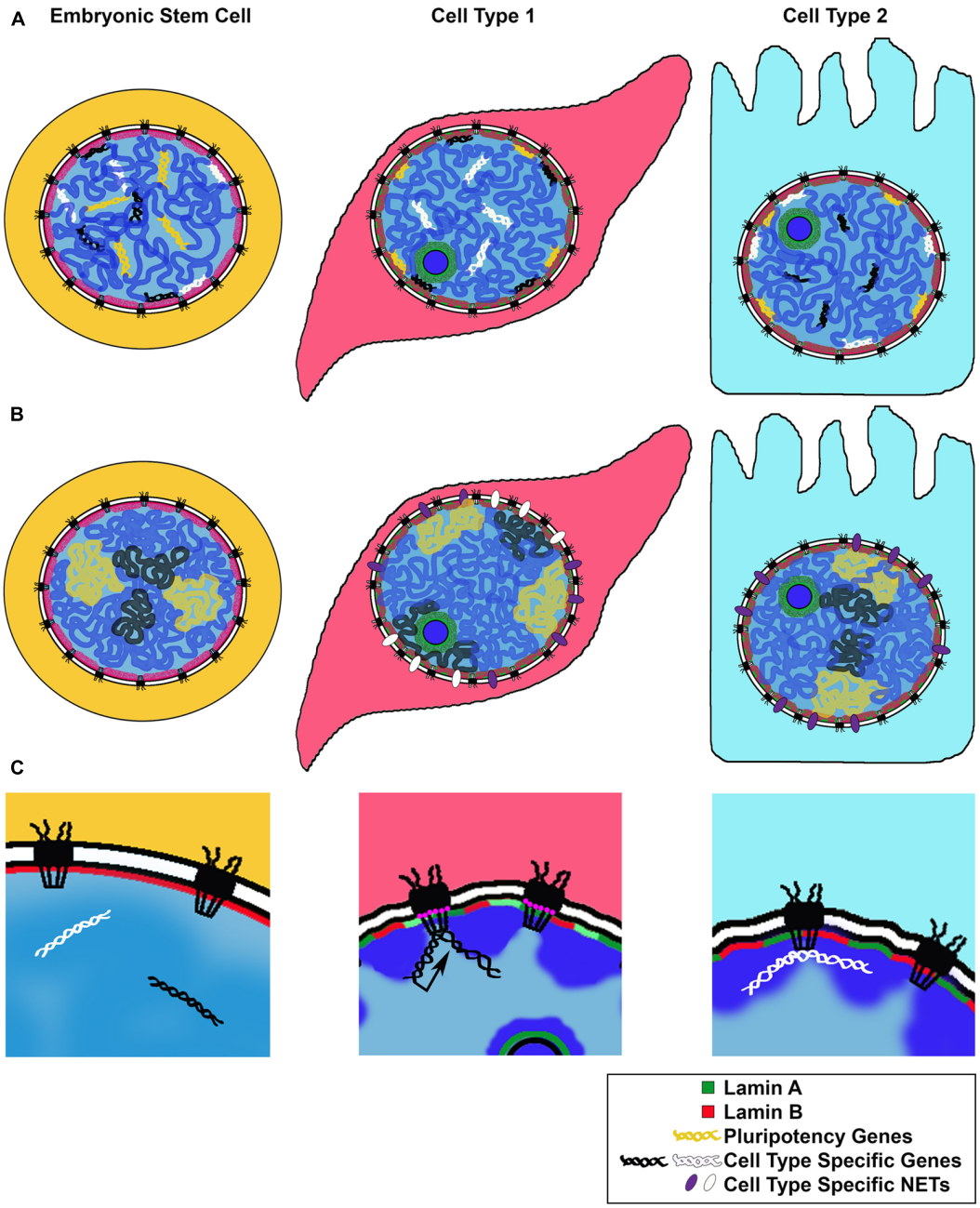

FIGURE 2 | Models for changes in NE-genome interactions during differentiation. We illustrate three proposed mechanisms for genomic rearrangement during cell differentiation. **(A)** Repositioning of cell type specific genes: along the steps to a fully differentiated cell, genes required for pluripotency or an alternate differentiation pathway are repositioned to the transcriptionally repressive nuclear periphery. Genes required for differentiation or cell type maintenance are kept in the nuclear interior. **(B)** Expression of cell type specific NETs during cell differentiation repositions chromosomes or nuclear territories to the nuclear periphery, influencing their transcriptional activity. **(C)** Cell type specific genes can be repositioned to the NPC for transcriptional activation (black arrow) or other regulation (white), such as establishment of chromatin boundaries or non-expressed genes; NPC composition may change depending on the cell type, with some Nups, such as Nup210 (pink circles), expressed only in certain differentiated states.

of cellular functions including genome organization, nuclear migration and positioning, cell cycle regulation, signaling, and cell differentiation (Dauer and Worman, 2009; Chow et al., 2012; Gomez-Cavazos and Hetzer, 2012). While the NM is now accepted as a dynamic interface between the nucleus and cytoplasm, exactly how the NM and its composite proteins are manifesting these processes is still largely unclear. An exciting current area of nuclear study is analysis of the nuclear envelope proteome and characterizing functions of NE proteins in more detail.

The Nuclear Membrane Proteome is Tissue Specific

To date the NE/NM proteome has been analyzed in three tissues – liver (Schirmer et al., 2003; Korfali et al., 2012), muscle (Wilkie et al., 2011) and blood leukocytes (Korfali et al., 2010), as well as mouse neuroblastoma cells in culture (Dreger et al., 2001). The three most recent of these studies were performed under identical experimental conditions and therefore the resulting data sets can be directly compared. These studies identified 1,037 NETs in total, a huge increase compared with only 67 potential NETs known in 2003. The results indicate a surprisingly high degree of tissue specificity in NE protein composition with only 16% of identified transmembrane proteins shared between the three tissues (**Figure 1**). These tissue specific results were directly verified for several novel NETs by immunofluorescence staining and comparison with known tissue specific expression profiles (Korfali et al., 2010, 2012; Wilkie et al., 2011). Further highlighting cell type specific expression of these proteins, in tissues composed of multiple cell types, often, only a subset of cells displayed a clear nuclear rim staining for a given NET (Korfali et al., 2012). Additionally, results of these proteomic analyses correlate with previously annotated protein complexes, reported in the interaction networks by the Johns Hopkins Human Protein Reference Data (HPRD) database. The authors found a preference for NETs proposed to act in a complex according to the interactome data, to have similar tissue type expression profiles (Korfali et al., 2012).

Nuclear Membrane Proteins Reposition Chromosomes

Early work on NETs focused in large part on their role in NE reassembly following cell division. One important outcome of these studies is the finding that many NETs are able to directly bind mitotic chromatin (Ulbert et al., 2006). This finding becomes relevant in the context of cell fate determination as it indicates these NETs have the capacity to bind chromatin also in interphase and thus are able to contribute to three dimensional genome organization and gene expression programs. In addition to the LBR and the INM protein Lap2β examples provided above, other NETs have been found to directly reposition genomic loci to the NE/nuclear lamina. For example, a domain of the NET Emerin, fused to LacI, repositioned a *lacO* array to the INM, and interestingly, this repositioning was found to require passage through mitosis (Reddy and Singh, 2008; Reddy et al., 2008). Similarly, NE targeting by the LacI-Lamin B fusion was found to require cell division (Kumaran and Spector, 2008; Kumaran et al., 2008), suggesting that cells have to break down their nuclear architecture to allow reorganization of NE-genome contacts. Observed redistribution of LAD subsets between the nuclear interior and periphery after mitosis lends further support to this idea (Kind et al., 2013). In terms of cell fate specification, these results suggest that cell cycle exit could effectively "fix"/make static one's nuclear genome organization.

A visual screen for the effects of NETs on chromosome positioning was performed for 22 novel NETs identified from the liver specific proteomic analysis (Korfali et al., 2012) as well as the more familiar NET, Emerin (Zuleger et al., 2013). The ability of transiently expressed NETs to reposition chromosomes was assayed, using chromosome paint and image analysis, in human cell culture. Four of the tested proteins, NET5, NET29, NET39, and NET47 were able to specifically reposition both copies of chromosome 5 to the nuclear periphery. Only NET29 and NET39 had an effect on chromosome 13, and none of the NETs tested effected nuclear positioning of either chromosome 17 or 19. In support of tissue specific chromosome positioning via tissue specific NET expression, the authors correlate peripheral localization of chromosome 5 in liver tissue with preferential expression of NET47 (70% of total expression across tissue types). In kidney cells, which account for only 3% of NET47 total expression, chromosome 5 is found more often in the nuclear interior. Importantly, the authors show by RNAi knockdown that NET positioning of chromosomes at the nuclear periphery is reversible.

This study yields several important conceptual findings: firstly, it provides examples of tissue specific NET expression, giving rise to unique NE compositions correlating with cell type (**Figure 1**). Secondly, these results suggest that NETs bind specific chromosomes in a reversible manner, linking chromosome positioning with differentiation (**Figure 2B**). In this manner, tissue specific NETs may function to reposition entire chromosomes or large chromosomal regions to the nuclear periphery, which may further assist or stabilize the silencing of specific developmental genes by association with the nuclear lamina (**Figure 2A**). Thirdly, multiple NETs can act to position the same chromosome, perhaps via cellular regulation of relative abundance of different NETs. Lastly, in addition to tissue specific expression levels, several of the NETs in this study appear to have tissue specific splice variants. Together these provide another layer of regulation to how a cell might fine-tune its gene expression profile during differentiation by utilizing NETs to position chromosomes at the NE in a tissue specific manner.

Nuclear Membrane Proteins Regulate Chromatin State

Lamin B receptor, discussed above, is an INM protein shown to interact directly with Lamin B and the chromodomain heterochromatic protein HP1 (Worman et al., 1988b; Schuler et al., 1994; Ye and Worman, 1996; Ye et al., 1997). Initial characterization of LBR indicates it forms oligomeric structures which, in contrast to the smooth nuclear rim staining observed for Lamin proteins, localize into discrete microdomains in the NE (Makatsori et al., 2004). More recent experiments using a Celluspots peptide array of 384 histone tail peptides showed the nucleoplasmic domain of LBR binds a specific set of heterochromatin marks, namely H4K20me2, H4K20ac, H4R19me2s, H4R19me2a and H4R23me2s ("a" and "s" refer to arginine methylation patterns: asymmetric or symmetric, respectively; Hirano et al., 2012). To verify these binding partners *in vivo* the authors showed the ChIP fraction obtained using an anti-LBR antibody was significantly enriched for H4K20me2 and that this

heterochromatin mark indeed localized to the nuclear periphery. As H4K20me2 is widespread throughout the genome, binding of LBR to the additional, less common, methylated histone residues provides a possibility for further specificity in tethering unique heterochromatin or developmentally silenced sites to the nuclear periphery. Fluorescence recovery after photobleaching (FRAP) experiments analyzing mobility of LBR truncation mutants revealed domains involved in interactions with histone H4, but not with Lamin B1 or B2 are required for formation of stable LBR microdomains in the NE. To investigate a role for LBR in heterochromatin formation, *in vitro* experiments using atomic force microscopy to measure chromatin compaction showed incubation of recombinant LBR with reconstituted chromatin resulted in highly aggregated chromatin fibers compared with controls. This study further demonstrated that LBR itself has the ability to repress transcription of a reporter plasmid.

Together with the previously discussed role for LBR in maintaining a conventional chromatin architecture (Solovei et al., 2013) and reports of *in vivo* effects of LBR depletion or mutation (Worman, 2005), the study described above suggests a differentiation specific function for LBR in formation and maintenance of heterochromatin at the nuclear periphery via roles in chromatin compaction and transcriptional repression. LBR provides a clear example of a NET physically and functionally bridging Lamins to heterochromatin at the nuclear face of the INM.

Nuclear Membrane Proteins are Linked to the Cytoskeleton

Thus far we have discussed changes within the nucleus that lead to or occur with changes required for cell fate determination. However, often during cell differentiation there are significant physical changes in cell shape and size as well as in nuclear positioning, and sometimes the formation of multinucleate cells. Almost a decade ago a complex physically linking the nucleoskeleton and cytoskeleton (the LINC complex) was first described (Padmakumar et al., 2005; Crisp et al., 2006). The finding that Lamin binding proteins of the INM interact with cytoskeletal binding proteins of the ONM via the PNS was the first evidence of NPC independent communication between the nucleus and cytoplasm. The SUN proteins are INM specific with their SUN domain extending into the PNS. The SUN domain interacts with the C-terminal KASH (Klarsicht-ANC-Syne-homology) domain of Nesprins, extending in most cases, from the ONM into the PNS. Nesprins are further structurally characterized by a spectrin repeat rod domain and a variable N-terminal domain which interacts with cytoskeletal elements including actin and plectin (Wilhelmsen et al., 2005). To date the LINC complexes have been implicated in a variety of cell processes including nuclear size, shape and positioning, cell migration and polarity as well as mechano-sensory signal transduction (reviewed in Lombardi and Lammerding, 2011; Razafsky et al., 2011; Neumann and Noegel, 2014) In addition to roles in NE embedded LINC complexes at the ONM, Nesprin-2a, lacking a transmembrane domain has been shown to exist within the nuclear interior and Nesprin-2 has been shown to directly interact with Lamin A (Haque et al., 2010; Yang et al., 2013).

The Nesprin protein family continues to grow with the four Nesprin coding genes currently described in mammals giving rise to an ever-increasing number of isoforms (Apel et al., 2000; Wilhelmsen et al., 2005; Zhang et al., 2005; Roux et al., 2009). Evolutionary conservation analysis of these gene sequences indicates they are the result of two whole-gene duplication events followed by individual rearrangements (Simpson and Roberts, 2008). Both Nesprin-1 and Nesprin-2 genes have internal promoters which give rise to shorter isoforms. At present while Nesprin-3 has two known isoforms, Nesprin-1 has 21 identified isoforms and Nesprin-2 has 14. While only one Nesprin-4 variant has been reported, its expression appears specific to secretory epithelial cells.

Of the known LINC complex components, expression of both SUN and Nesprin proteins appear to exhibit temporal and tissue specific expression patterns (**Figure 1**; Randles et al., 2010; Razafsky et al., 2013). A recent study of the expression patterns of Nesprin isoforms in a panel of 20 human tissues and 7 human cell lines (including ESCs) reveals complex expression profiles of Nesprin isoforms (Duong et al., 2014). Quantitative PCR was used to examine the distribution of expression of nine Nesprin isoforms from Nesprin-1 and Nesprin-2. The results indicate unique Nesprin profiles for each tissue or cell line. Perhaps expectedly, ESCs display a unique "Nesprinome" void of Nesprin-1 isoforms. ESCs predominantly express Nesprin-2 giant as well as the two smaller isoforms N2-e-1 and N2-a-2. Of further interest, when localization of Nesprin-2 giant was examined in ESCs, rather than the nuclear rim localization observed in differentiated cell types, the protein was visualized within the nucleoplasm. The authors found that this Nesprin-2 species lacks the KASH domain revealing a novel nucleoplasmic role for this protein. Nesprin isoform distribution in differentiated tissues was highly variable. For example, liver tissue was reported to have 95% relative abundance of Nesprin-2 giant, while heart tissue has 36% and brain only 8%. While more work is needed, these results suggest an important role for Nesprins in determining cell identity and the transition from Nesprin-1 to Nesprin-2 isoforms as a signature of cell differentiation.

A separate study of Lamin, SUN and Nesprin expression profiles in the developing mouse central nervous system confirmed many of the conclusions made above (Razafsky et al., 2013). The authors found unique expression profiles for all three of these protein families corresponding to differentiation stage and cell type. Notably they found that as differentiation progressed, lower molecular weight isoforms of the Nesprin giants became predominant. They additionally confirmed the presence of KASH-less isoforms of Nesprin1 in CNS tissues.

One can imagine a model where Nesprin isoforms are expressed in response to developmental signals and then themselves confer cytoskeletal changes as well as alter 3D organization of the genome to promote further tissue specific gene expression. They can do so via relaying these signals to INM proteins or through their own, yet undetermined, nucleoplasmic roles. Additionally, changes in nuclear size, shape and relative position within the cell can potentially influence

the kinetics of nuclear processes and thus gene expression. Perhaps the most exciting implication of the LINC complex lies in its connection of the chromatin-associated INM proteins to cytoskeletal proteins via Nesprins, suggesting that cytoplasmic forces can directly move or alter nuclear chromatin positioning. This idea is supported by studies in both *Caenorhabditis elegans* and mice functionally linking cytoskeletal components to proper chromosome pairing and movement, as well as telomere clustering during meiosis via Sun/KASH protein bridges (Sato et al., 2009; Morimoto et al., 2012; Horn et al., 2013; Woglar and Jantsch, 2014). The large number of NE proteins and their splice variants, expressed in a tissue specific manner, connecting chromatin to the cytoskeleton, provide a window into the complex and interconnected mechanisms utilized by the cell nucleus to manifest its ultimate destiny (**Figure 1**).

The Nuclear Pore Complex

The NPCs are multi-component protein complexes that form selectively permeable channels through the NE. The primary function of the NPCs is to mediate nucleo-cytoplasmic transport of molecules and thus allow communication between the nucleus and the cytoplasm (Wente and Rout, 2010; Raices and D'Angelo, 2012). They are estimated to be the largest protein complexes in the cell, ∼90–120 MDa in human cells. The NPC is composed of multiple copies of ∼30 individual components, termed Nucleoporins (Nups). The overall structure of the NPC is highly conserved and displays an eightfold rotational symmetry. Its core consists of a ring of membrane-embedded scaffold subcomplexes built around a central transport channel. The NPC core is further connected to its auxiliary structures, such as the meshwork of phenylalanine glycine repeat containing Nups (FG Nups), which fill the central channel and form the permeability transport barrier, the cytoplasmic filaments and the nuclear basket, which extends into the nuclear space (D'Angelo and Hetzer, 2008). Interestingly, individual Nups display highly variable rates of association with the nuclear pore (Rabut et al., 2004). While the core scaffold Nups have been shown to be remarkably stable once assembled into the NPC, with residence times exceeding one cell cycle, many of the non-scaffold Nups, such as the FG Nups and Nups of the nuclear basket were found to be highly dynamic, able to move on and off the pore with kinetics of seconds to a few hours.

Via its transport functions, the NPC plays an obvious role in gene regulation by controlling export of generated RNA and import of transcription and signaling factors. Yet, in addition to its canonical transport role, the NPC and individual Nups have been shown to play a role in genome organization and gene expression via direct binding to specific genomic locations (Casolari et al., 2004; Taddei et al., 2006; Brown et al., 2008; Capelson et al., 2010; Kalverda et al., 2010; Vaquerizas et al., 2010; Ikegami and Lieb, 2013; Liang et al., 2013; Ptak et al., 2014; Sood and Brickner, 2014). Multiple studies in a variety of genomes have identified the presence of specific Nups at active and silent genes, and have revealed a functional requirement for NPC components in execution or maintenance of select transcriptional programs and chromatin states, as detailed below. Additionally, a number of Nups have been demonstrated to be critical for certain paths of tissue specific differentiation. An intriguing possibility that arises from these studies is the potential ability of the NPC to integrate its transport and genome-binding roles, bridging for instance, the nuclear import of developmental transcription factors to their activating function at target promoters. In this manner, the NPC has emerged as a new scaffold for genome organization, and may play a role as a nexus of developmental signaling, able to coordinate transport, spatial genome organization and gene expression.

Nuclear Pore Proteins Drive Tissue Specific Differentiation

Tissue specific expression of Nups has not been systematically analyzed in mutli-cellular organisms, but many individual examples that point to tissue specific roles of Nups have been reported. For instance, Nup50, a dynamic Nup, is highly expressed in the mammalian neural tube and the testis, particularly in the male germ cells (Trichet et al., 1999; Smitherman et al., 2000), while Nup45 exhibits variable expression in select mouse and rat cell lines (Hu and Gerace, 1998). Several Nups have been reported to change expression during cardiomyocyte differentiation (Perez-Terzic et al., 2003), as well as in response to cardiac hypertrophy (Chahine et al., 2015). Publically available genome wide expression studies in various cell types and organs also readily show differential expression of Nups. For instance, RNA Sequencing (RNA-Seq) and *in situ* RNA hybridization studies of the early *Drosophila* embryo revealed that Nups vary in their expression patterns relative to embryonic segments and developmental time points (Combs and Eisen, 2013), suggesting that different Nups are linked to different developmental pathways.

Strikingly, a number of tissue specific pathologies in humans and tissue specific phenotypes in model organisms have been described for mutations in a variety of both stable and dynamic Nups (Capelson and Hetzer, 2009; Xu and Powers, 2009; Raices and D'Angelo, 2012). For example, inherited cases of a cardiac disorder atrial fibrillation have been mapped to a missense mutation in the human Nup155, a stable Nup, which is highly expressed in the heart, liver and skeletal muscle (Zhang et al., 2008). Additionally, a mutation in the FG Nup Nup62 has been shown to underlie the familial form of infantile bilateral striatal necrosis (Basel-Vanagaite et al., 2006). Nup133, another stable Nup of the NPC scaffold, was found to be required for neuronal differentiation in the mouse embryo, and ESCs carrying a functionally null mutation in Nup133 are not able to undergo terminal differentiation into neurons (Lupu et al., 2008). Interestingly, a component of the same NPC scaffold sub-complex, ELYS, affects neuronal, retinal and intestinal development and proliferation in zebrafish (Davuluri et al., 2008; de Jong-Curtain et al., 2009). A large number of plant Nups, including Nup96, Nup160, ELYS and Tpr, have been reported to affect a diverse array of tissue specific processes, such as flowering, hormone signaling and immune function (Meier and Brkljacic, 2009). In *Drosophila*, several Nups, including Nup98/Nup96, Seh1 and Nup154 were uncovered to play a

role in gametogenesis (Gigliotti et al., 1998; Parrott et al., 2011; Senger et al., 2011), where mutations in these Nups disrupt germ cell differentiation and cause sterility in males and females. In *C. elegans*, Nups such as the homolog of Nup98 were demonstrated to be critical for the formation of germline-specific P granules (Voronina and Seydoux, 2010), and multiple Nups have been shown to be required for normal embryonic development (Galy et al., 2003). Knowledge of the molecular mechanisms behind most of these developmental defects remains incomplete, and the connection of the NPC to chromatin organization may provide a new perspective to understanding these phenotypes.

Perhaps the most remarkable and well characterized example of a nuclear pore component playing a role in differentiation is that of Nup210. The transmembrane nucleoporin Nup210 is absent in mouse progenitor myoblasts and ESCs, but its expression is sharply upregulated during differentiation of these lineages into myotubes and neuroprogenitors, respectively (D'Angelo et al., 2012). Nup210 was further shown to be functionally necessary for these differentiation events, suggesting the NPC undergoes a compositional change required for the developmental programs of these cell types. Interestingly, the general transport properties of the NPC appear to remain unchanged by the addition of Nup210. Yet the expression of a subset of developmental genes was found to be dependent on Nup210 during myogenesis, indicating again a possible role of an NPC component in direct gene regulation to specify cell fate (**Figure 1**).

Wnt signaling, a central developmental signaling pathway of multi-cellular organisms, has also been repeatedly linked to the nuclear pore (Sharma et al., 2014). Wnt signaling relies on β-catenin as the primary transducer of activating signals from the plasma membrane to the nucleus, resulting in regulated shuttling of β-catenin between the nucleus and the cytoplasm. Nuclear import of β-catenin has been shown to be independent of the normal nuclear localization signal (NLS)/importins-regulated transport, and instead to involve direct interactions with a number of FG Nups, such as Nup62 and Nup358 (Sharma et al., 2012). Once in the nucleus, activated β-catenin associates with transcription factors of the LEF-1/TCF family and together, they induce transcription of Wnt target genes. One such member of the LEF-1/TCF family, TCF-4 has been shown to be sumoylated by Nup358, which carries a SUMO E3 ligase activity, and this sumoylation increases the transcription activity of TCF-4 and its binding to β-catenin (Shitashige et al., 2008). An additional key component of the Wnt pathway, APC, which is required for stabilizing and thus activating β-catenin, has been similarly reported to interact with specific FG Nups, such as Nup153 and Nup358 (Collin et al., 2008; Murawala et al., 2009).

These findings illustrate that Wnt pathway components are regulated by FG Nups both in terms of transport and function. Given the indispensable nature of Wnt signaling in stem cell maintenance, embryonic development and cell migration, these connections heavily implicate Nups in both normal development and oncogenic transformation. Intriguingly, the pluripotency state itself has been postulated to be regulated by the NPC via controlling levels of the pluripotency factors Oct4, Sox2 and Nanog in the nucleus (Yang et al., 2014). Together, these studies underscore the functional roles of the NPC in regulating developmental states and transitions. The mechanisms of these roles will be a fruitful subject for future investigations in the field's efforts to fully understand cell fate determination.

Nuclear Pore Proteins Facilitate Transcription

The phenotypes of Nups in tissue specific differentiation, described above, can result from either the transport or the genome regulatory roles of Nups, or possibly, from the integration of both. Multiple examples of cell type specific transport have been reported, and proposed transport mechanisms of Nups in development have been reviewed recently (Hogarth et al., 2005; Xylourgidis and Fornerod, 2009; Raices and D'Angelo, 2012). Here, we concentrate on recent work on the emerging roles of Nups in transcription and chromatin function, which may provide an alternative mechanism for the tissue specific roles of the NPC.

A functional relationship between nuclear pores and nuclear organization of chromatin was originally proposed based on EM close ups of mammalian nuclei that show frequent association of what appears to be decondensed chromatin with nuclear pores (Capelson and Hetzer, 2009). Such lighter stained, decondensed chromatin is thought to correspond to active regions of the genome that are more permissive to transcription. The observed correlation between NPCs and open/active chromatin was the basis for the 'gene gating hypothesis' (Blobel, 1985), which proposed that NPCs preferentially interact with and possibly regulate active genes to promote coregulation of transcription and mRNA export. Such images also suggested that the NPCs somehow participate in the establishment or maintenance of decondensed active chromatin.

A large amount of work in the yeast system has provided evidence for the role of the NPC in transcriptional activation. Genome wide studies in *Saccharomyces cerevisiae* demonstrated that some Nups, such as Mlp1, Nup2 and Nup60 often occupy regions of highly transcribed genes (Casolari et al., 2004, 2005), and revealed an interaction between the NPC component Nup2 and promoters of select active genes, termed the "Nup-PI" phenomenon (Schmid et al., 2006). Inducible yeast genes such as INO1, GAL and HXK1 are targeted to the NPC upon activation, and this association has been shown to be functionally important (Taddei et al., 2006; Light et al., 2010). Mechanistically, NPC-genome contacts in yeast have been shown to involve components of the histone acetyltransferase (HAT) SAGA complex (Rodriguez-Navarro et al., 2004; Cabal et al., 2006; Luthra et al., 2007), and mRNA export complexes TREX2 and THO-TREX (Rougemaille et al., 2008), as well as a transcription factor Put3 (Brickner et al., 2012).

A recently proposed function of the NPC-gene interactions that is especially relevant to cell fate control is a potential role in epigenetic memory of transcriptional events. The inducible yeast genes INO1, GAL and HXK1 have been shown to remain associated with the NPC for multiple generations, following their initial induction and during subsequent repression (Tan-Wong et al., 2009; Light et al., 2010). Interestingly, this association with the NPC was found to be important for the enhanced transcriptional

response during reinduction, suggesting that binding of the NPC to recently transcribed genes primes them for later reactivation and in this manner, serves as a memory mark of transcriptional events. For GAL1 or HXK1, the maintenance of this transcriptional memory was found dependent on the nuclear basket Nup Mlp1, a homolog of the mammalian Nup Tpr (Tan-Wong et al., 2009). For INO1, it requires binding of Nup100 (mammalian Nup98), as well as changes in chromatin structure of the gene promoter, such as incorporation of the histone variant H2A.Z (Light et al., 2010).

In metazoa, the roles of the NPC in transcriptional activation, chromatin structure and epigenetic memory should be particularly important for tissue specific development. In support of this idea, several studies have analyzed genome wide chromatin binding of Nups in *Drosophila* and reported binding of a subset of fly Nups to developmental genes (Capelson et al., 2010; Kalverda and Fornerod, 2010; Vaquerizas et al., 2010). In the fly genome, Nups such as Nup98, Sec13, Nup50 and FG Nups such as Nup62 are recruited to loci actively transcribed by RNA Polymerase II (RNAP II) or to genes undergoing developmental induction, where they were found to be functionally necessary for full activation (Capelson et al., 2010; Kalverda and Fornerod, 2010). Additionally, Nups of the nuclear basket such as Nup153 and Mtor were shown to bind the genome in long stretches, termed Nup Associated Regions (NARs), which were similarly enriched for active genes (Vaquerizas et al., 2010). Such NARs further suggest that NPC components contribute to global chromatin organization, similarly to Lamins. Interestingly, *C. elegans* NPC components were recently found to associate specifically with targets of RNA Polymerase III (RNAP III), such as *tRNA* and *snoRNA* genes, where they appear to be functionally required for correct RNA processing (Ikegami and Lieb, 2013). Since expression of RNAP III targets such as *tRNA* genes has also been shown to be highly tissue specific (Dittmar et al., 2006), these findings suggest that Nups may contribute to cell fate via regulation of both RNAP II and RNAP III targets.

Intriguingly and in line with their dynamic behavior, *Drosophila* Nups have been shown to be recruited to their target genes in the nucleoplasm, away from the NE embedded NPCs, suggesting that the ability of Nups to regulate or support active chromatin can be carried out at any location in the nucleus (Capelson et al., 2010; Kalverda et al., 2010; Vaquerizas et al., 2010). Both the off pore mode of Nup-gene interactions and the binding of Nups to developmentally induced genes were recently also observed in human cells. Genome wide binding studies of human Nup98 in ESCs, neural progenitor cells and differentiated IMR90 fibroblasts revealed large tissue specific differences in Nup98 target genes and demonstrated that a subset of genes activated during ESC differentiation are recruited to the NPC (Liang et al., 2013). Together, these studies in metazoan systems support the notion that the NPC or individual Nups bind and promote activation of genes induced in a lineage specific manner, thus constituting another important NE linked complex with a role in gene expression and cell fate (**Figure 2C**). In this manner, the NPC may represent a distinct nuclear environment that promotes a permissive chromatin state at the nuclear periphery, functionally opposed to the roles of the Lamins and NETs (**Figures 2A,B**), but perhaps providing an accessible scaffold for switching between silenced and activated states during cell differentiation.

In support of the link of the nuclear pore to chromatin structure, suggested by the early EM images, several histone modifying enzyme complexes have been linked to the NPC. In addition to the reported interaction of the yeast NPC with the SAGA HAT complex (Rodriguez-Navarro et al., 2004; Cabal et al., 2006; Pascual-Garcia et al., 2008), *Drosophila* Nup98 was found to associate with histone modifying complexes such as the histone methyl transferase Trithorax (Trx), the fly homolog of Mixed Lineage Leukemia (MLL), and the Non-Specific Lethal (NSL) Complex, which carries a conserved HAT males absent on the first (MOF; Pascual-Garcia et al., 2014). MOF, as part of the fly dosage compensation complex that maintains transcriptional hyperactivity of the male X chromosome, has also been shown to associate with Nups Nup153 and Mtor (Mendjan et al., 2006). Since both Trx/MLL and NSl/MOF are critical epigenetic regulators, these interactions further implicate Nups in the epigenetic memory of transcription, suggested by yeast studies. Interestingly, the memory function of yeast Nups appears to be conserved in human cells. HeLa cells treated with interferon gamma (IFN-γ) show faster reactivation of IFN-γ inducible genes than cells never exposed to IFN-γ (Light et al., 2013), demonstrating that these genes are marked as recently transcribed. As its yeast homolog Nup100, Nup98 was found to be required for propagating this memory through cell divisions, since Nup98-depleted cells lose the enhanced transcriptional response to IFN-γ repeated exposure. In this case, chromatin structure again appears to be involved, as the deposition of histone H3 lysine K4 di-methylation at target gene promoters is gained during the memory acquisition and lost as a result of Nup98 knock down. Together, these findings highlight transcriptional and epigenetic regulation of genes by Nup binding as a likely mechanism for some of the tissue specific phenotypes of Nups and a new regulatory aspect of cell fate determination.

Nuclear Pore Proteins Contribute to Chromatin Organization

In addition to transcribing genes, the NPC has been implicated in binding silenced genomic regions and chromatin boundary elements (**Figure 2C**). The earliest genome wide binding analysis of various Nups in yeast demonstrated that the stable yeast Nup84 (mammalian Nup107) binds to loci that are not enriched for transcriptional activity, and thus termed "neutral" chromatin (Casolari et al., 2004). Subsequently, ChIP analysis of another stable NPC component, Nup93, in human cells similarly demonstrated that the Nup93 binding targets in HeLa cells included nontranscribing regions, enriched for silent histone modifications (Brown et al., 2008). Recently, a study carried out in budding yeast revealed a direct functional involvement of the stable Nup170 (mammalian Nup155) in maintenance of silent heterochromatin (Van de Vosse et al., 2013). Nup170 was identified at repressed genomic regions such as ribosomal protein and subtelomeric genes, and was demonstrated to be required for their silencing via interactions with the chromatin remodeling remodels the structure of chromatin (RSC) complex and the silent

information regulatory (SIR) complex component Sir4. It appears that the NPC can bind both active and silent genes, likely through using different Nup components, each of which has the ability to interact with different types of chromatin regulatory complexes.

Boundary elements or insulators are critical for the establishment and maintenance of the correct genome architecture in a cell type specific manner (Van Bortle and Corces, 2012). Their main property involves the ability to separate chromatin domains of varying activity states from each other, as for example, insulating euchromatin from heterochromatin. Recently, a role in delineating euchromatin and heterochromatin domains has been described for the nuclear basket Nup Tpr in mammalian culture cells (Krull et al., 2010). Depletion of Tpr resulted in the loss of the decondensed chromatin regions associated with the NPCs and allowed the spread of heterochromatin into the nuclear regions underlying the nuclear pores, as assessed by EM. Interestingly, the binding sites of the NE embedded NPCs (not the dynamic components) in fly S2 culture cells were found to be enriched for the binding sites of a well characterized insulator protein Suppressor of Hairy Wing [Su(Hw); Kalverda and Fornerod, 2010]. The ability of the NPC to function as an insulator between active and silenced regions has also been demonstrated in yeast. A genetic screen for proteins with boundary activity, using a reporter gene positioned next to a heterochromatic domain, identified several exportins and Nup2 as being able to insulate the reporter gene from silencing (Ishii et al., 2002). Additionally, several Nups such as Nup2 and Nup60 were found at the tRNA insulator of the yeast silenced HMR mating locus, although their depletion did not compromise insulating activity (Ruben et al., 2011). Binding of stable Nups was similarly reported at *tRNA* genes in *C. elegans* embryos (Ikegami and Lieb, 2013), further supporting the notion that the NPC may serve a conserved boundary function in eukaryotic genomes.

These studies lend the view of the NPC as another important scaffold for spatial genome organization (**Figure 2C**), which bears direct relevance to the establishment of cell type specific gene expression programs. Whether the metazoan NPC primarily functions as a scaffold for expression of RNAP II and RNAP III genes, for establishment of chromatin boundaries or for additional regulation of silenced genes remains to be fully deciphered. It is possible that this large protein complex can accommodate interactions with all three types of loci via different Nups. Furthermore, the stable proteins of the NPC have been shown to be remarkably long-lived. Once assembled, the NPC core essentially does not turn over during the entire life span of post-mitotic cells, such as neurons (D'Angelo et al., 2009; Toyama et al., 2013). This extreme stability makes the NPC a well suited nuclear scaffold for establishing long term genome organization and thus transcription programs.

Interplay Between NE Components

Rather than thinking of these compartments individually, accumulating evidence portrays the NE as a machine with many components working together to affect gene expression programs and differentiation. Many of the known NE components of the nuclear lamina, the NPC and the NM are known to associate with each other, and this high level of interplay makes it difficult to separate the functions of these compartments. Current work indicates that Lamin A isoforms interact with integral and associated NM proteins, which are expressed in a tissue specific manner, thus further contributing to tissue specific genome conformations and gene expression profiles. As discussed above, Lamins have been shown to interact with several NM proteins including LBR, Emerin, Man1, Lap2a as well as barrier to autointegration factor (BAF; Ho and Lammerding, 2012). These interactions are required for many of the reported NE-genome contacts and for supporting repressive effects that the nuclear lamina can exert on gene expression. Additionally, Lamins are known to associate with the Sun and Nesprin proteins, which form the LINC complex connecting chromatin and the Lamina to the cytoskeleton. Nuclear envelope retention of some of these proteins, such as Emerin, as well as a subset of less characterized NETs has been shown to be Lamin A dependent (Sullivan et al., 1999; Malik et al., 2010).

Nuclear pore complex components such as Nup153 and Nup88 have also been shown to interact with the Lamins (Ho and Lammerding, 2012). But although they appear to contact each other closely in nuclear space, the precise molecular relationship between nuclear lamina and nuclear pores is still unclear. A recent study provided an example of this relationship in the *Drosophila* testes stem cell niche, where Lamin Dm0 was found to regulate ERK and epidermal growth factor (EGF) receptor signaling to maintain cyst stem cells and support differentiation of germ stem cells (Chen et al., 2013). This function of Lamin is carried out via Nups, such as Nup153, which results in nuclear retention of phosphorylated ERK in the cyst stem cells. Here, the nuclear lamina appears to contribute to setting up the correct composition of the NPC, which in turn regulates developmental EGF signaling to control the stem cell niche. Future studies of the interplay between nuclear lamina, the NPC, the INM proteins and the LINC complex components are sure to yield exciting new aspects of developmental regulation.

Conclusion

Accumulating evidence has demonstrated tissue specific presence and functions of various NE components. Much of that knowledge supports the model that many of these functions are carried out via cell type specific interactions between the NE and the genome, which contribute to the correct establishment of tissue specific gene expression (**Figure 2**). Tissue specific expression of Lamin isotypes appears to be important for tethering heterochromatin to the nuclear periphery and for repositioning critical developmental genes to a silencing nuclear compartment (**Figure 2A**). This role of nuclear lamina is closely linked to and likely executed through the functions of INM proteins, which have the ability to interact with chromatin bound regulators and histone modifying complexes. Expression of NETs has been shown to be highly cell type specific and likely drives the reorganization

of chromosomes and large genomic regions needed for certain paths of differentiation (**Figure 2B**). Finally, components of the NPC are functionally implicated in regulation of developmentally induced active genes and in setting up boundaries between chromatin domains (**Figure 2C**). The composition of the NPC may vary depending on the cell type, with some Nups such as Nup210 being added to drive critical differentiation steps. Developmentally regulated genes and boundaries may thus be recruited to the NPC in a tissue specific manner, or recruit Nups to their intranuclear locations. An exciting new direction stemming from these models is how developmental signaling factors that enter the nucleus during differentiation of particular lineages may cofunction with NE proteins and influence their genomic binding.

Together the presented data also illustrate the interconnected roles of nuclear compartments essential for cell fate determination, from the earliest steps of chromatin structure rearrangement to the last stages of morphological and other changes. Perhaps a more accurate view of the NE-genome interplay involves a myriad of overlapping mechanisms with increasing specificity during differentiation. The NE composition may be another "cellular code" for specifying tissue specific gene expression programs through its contacts with the underlying chromatin. Similarly to other highly complex regulatory networks, future applications of the systems biology view of this "code" may be particularly beneficial for fully understanding the role of the NE in genome function and cell fate.

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REVIEW
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Transcriptional control of stem cell fate by E2Fs and pocket proteins

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E2F transcription factors and their regulatory partners, the pocket proteins (PPs), have emerged as essential regulators of stem cell fate control in a number of lineages. In mammals, this role extends from both pluripotent stem cells to those encompassing all embryonic germ layers, as well as extra-embryonic lineages. E2F/PP-mediated regulation of stem cell decisions is highly evolutionarily conserved, and is likely a pivotal biological mechanism underlying stem cell homeostasis. This has immense implications for organismal development, tissue maintenance, and regeneration. In this article, we discuss the roles of E2F factors and PPs in stem cell populations, focusing on mammalian systems. We discuss emerging findings that position the E2F and PP families as widespread and dynamic epigenetic regulators of cell fate decisions. Additionally, we focus on the ever expanding landscape of E2F/PP target genes, and explore the possibility that E2Fs are not simply regulators of general 'multi-purpose' cell fate genes but can execute tissue- and cell type-specific gene regulatory programs.

Keywords: stem cell fate, neural precursor cell (NPC), pocket proteins, transcription, epigenetics, stem cells, cell cycle, E2F transcription factors

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Introduction

Since the discovery of the retinoblastoma protein (pRb) as a potent tumor suppressor two and a half decades ago, the pocket protein (PP) family (including pRb, p107 and p130) and their best characterized interacting partners, the E2F transcription factor family, have been under intensive scientific investigation. It is now clear that the PP and E2F proteins are not only important regulators of cellular proliferation but of multiple cellular processes, many of which impact cell fate decisions. While cell cycle-independent roles for E2Fs and PPs have been known for some time (Lee et al., 1994), what remains to be fully clarified, however, are the mechanisms by which the PP and E2F families control such diverse functions.

The advent of genomics and other systems biology approaches to study the role and mode of action of transcription factors has contributed greatly to our mechanistic understanding of E2F/PP function. Additionally, work by many groups, predominantly over the past decade, focused on linking causative target genes to non-canonical E2F/PP biological functions has greatly enriched our view of E2Fs and PPs as regulators of not only cell cycle control, but also key cell fate decisions. Collectively, these studies suggest that E2fs and PPs are dynamic transcriptional regulators that can control diverse cellular functions by regulating genes directly involved in those processes, potentially in a highly tissue-specific manner.

In this review we discuss the current understanding of how the classical cell cycle regulatory pathway impacts cell fate decisions at the level of E2F/PP-dependent transcriptional regulation.

Specifically, we highlight findings that position E2F and PP factors as fundamental regulators of cell fate control in stem and progenitor populations. Furthermore, we discuss emerging mechanisms by which E2Fs and PPs may execute cell type-specific gene regulatory programs in order to regulate cell fate control in a specialized manner.

Cell Cycle Regulation by E2Fs and PPs

The eukaryotic cell cycle is controlled in large part by the cyclical expression of important effector molecules. For example, the expression of enzymes that participate in DNA replication or chromosome segregation typically occurs when these proteins are needed, in S or M phase, respectively. While a great deal of regulation of this process occurs at the level of controlled synthesis-degradation of certain regulatory proteins (most notably the cyclins), transcriptional control by sequence-specific E2F transcription factors and their regulation by PPs is also heavily implicated as a central mechanism driving cell cycle regulation [reviewed in Dick and Rubin (2013)].

To date, eight *E2F* genes, giving rise to 10 distinct E2F proteins, have been identified in mammals [reviewed in Chen et al. (2009b)]. While E2F factors exhibit varying degrees of sequence and structural differences, the DNA binding domain is strikingly well-conserved among family members. This befits findings that E2F family members typically exhibit significant overlap in their target genes in a given tissue (Xu et al., 2007). The classical view of E2F/PP activity in cell cycle control (Cam and Dynlacht, 2003) is that unphosphorylated PPs form transcriptional repressive complexes with repressor E2Fs (E2F3b, E2F4, and E2F5) in quiescent and early G1 phase cells, to silence the expression of cell cycle regulatory and effector genes. In the presence of mitogenic stimuli, cyclin D-CDK4/6 initiates the phosphorylation of PPs, which leads to the disruption of the E2F/PP repressive complexes and nuclear export of the E2F factors. Concomitantly, activator E2F proteins (E2F1, E2F2, and E2F3) become expressed and stimulate the transcription of cell cycle genes that allow cells to pass the G1/S transition.

An Expanded Role for E2Fs and PPs in Controlling Stem and Progenitor Cell Fate Decisions

As a central regulator of proliferation and cell cycle exit, the E2F/PP pathway is functional in essentially all cell types, and during all stages of development. Investigations into the biological roles of cell cycle regulatory proteins beyond fibroblasts and tumor-derived cell lines, specifically within tissue-specific primary stem and progenitor cell populations, have revealed that this pathway controls a number of cellular processes, many of which impact key stem cell fate decisions. This is exemplified collectively by findings that loss of pRb and/or the other PPs results in stem cell expansion in many tissues, often accompanied by decreased cell survival, inhibition of differentiation, or altered lineage choices upon differentiation [reviewed in Sage (2012), Cai et al. (2013), De Sousa et al. (2014)]. Deregulation of E2F activity is strongly implicated in driving many of these phenotypes, and the existing literature now suggests a fundamental widespread role for these transcriptional regulators in cell fate determination.

Similar to the strong evolutionary conservation of a role in cell cycle regulation (Dimova et al., 2003; Stevaux et al., 2005; Kirienko and Fay, 2007; Hirano et al., 2008; Acharya et al., 2012; Korenjak et al., 2012; Kudron et al., 2013), E2F/PP-mediated control of stem cell fate decisions also appears to be deeply conserved. The PP and repressive E2F orthologs in the highly regenerative freshwater planarian (*Smed-Rb* and *Smed-E2F4-1*, respectively) are required for the self-renewal, maintenance and survival of pluripotent adult stem cells in this system (Zhu and Pearson, 2013). Additionally, a clear role for E2Fs and PPs in regulating stem cell fate decisions was in fact first demonstrated in the plant species *Arabidopsis thaliana*. In this system, functional suppression of the single PP RBR or over-expression of the transcriptional activator E2Fa leads to a specific increase in the number of stem cells in the root meristem; conversely, *RBR* overexpression causes these cells to rapidly differentiate (Wildwater et al., 2005). *RBR* loss also results in an expanded stem cell pool and aberrant fate determination in the male germline (Chen et al., 2009c).

A Multi-Tissue Cell Fate Regulatory Role for E2F and Pocket Proteins

The earliest indications that the functional importance of the cycle machinery extends beyond the regulation of cell cycle progression in mammalian systems came from analysis of *Rb1* knockout mice. *Rb1*-deficient embryos die between embryonic day 13.5–15.5 and they are marked by ectopic mitoses and extensive apoptosis throughout the developing nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Morgenbesser et al., 1994). This demonstrated a potential novel role for pRb in cell survival. These and subsequent studies additionally revealed an essential role for pRb in cell cycle exit and cellular differentiation, predominantly within the myoblast, neural, erythroid, and trophoblast stem cell lineages (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994; Slack et al., 1998; de Bruin et al., 2003; MacPherson et al., 2003; Wu et al., 2003). The aberrant trophoblast stem cell differentiation induced by pRb loss was later attributed to deregulated E2f3 activity (Wenzel et al., 2007) and antagonism between the E2f3 and E2f7&8 factors (Ouseph et al., 2012). Further, conditional loss of pRb in muscle precursors (Rb-flox:Myf5-Cre) led to a reduced differentiation capacity and increased rates of apoptosis (Huh et al., 2004), demonstrating the cell autonomous nature of these effects. Together, these phenotypic studies suggested an essential role for pRb in embryonic development and post-natal survival, characterized by widespread roles in cellular proliferation, survival, and differentiation.

Deficiency in PPs other than pRb revealed additional roles for this family in differentiation and survival. Compound deficiency for both pRb and either p107 or p130 results in phenotypes similar to *Rb1* knockouts, but these mice die earlier and display an exacerbation of proliferative and apoptotic phenotypes in a number of tissues, including the central nervous system (CNS; Lee et al., 1996; Lipinski and Jacks, 1999; Sage et al., 2000; Berman et al., 2009). Mice lacking both p107 and p130 also exhibit

perinatal lethality and have defects in chondrocyte and epidermal differentiation (Cobrinik et al., 1996; Ruiz et al., 2004). Finally, loss of all three PPs demonstrated an essential role in early development and pluripotency, as these mice die by E9.5-11.5 with evidence of widespread elevated proliferation and cell death (Wirt et al., 2010). Furthermore, triple PP-deficient human embryonic stem cells (ESCs) exhibit cell cycle arrest and death, by activation of p53 and p21 signaling (Conklin et al., 2012). Thus, loss of PPs leads to marked defects in development and differentiation of many cell and tissue types.

In the tumor prone retina, pRb is required in a cell autonomous manner for progenitor cell exit and differentiation of rod photoreceptor cells (Zhang et al., 2004), while the PP family is together required to maintain horizontal interneurons in a post-mitotic state (Ajioka et al., 2007). In the absence of PPs, horizontal cells maintain their differentiated state but begin to clonally expand, giving rise to metastatic retinoblastomas. pRB loss in human retinal cone cells has also been demonstrated to drive cell cycle exit and to promote retinoblastoma-like tumor development (Xu et al., 2014). E2fs themselves are also heavily involved in the proliferation, survival, and differentiation of distinct neuronal cell types in the retina (Chen et al., 2007, 2013). Additionally, E2F1 and hyper-phosphorylated pRB play important roles in post-mitotic neurons in the adult brain, specifically in effecting the calpain-induced neuronal cell death observed in a number of CNS neurocognitive disorders, including HIV-induced encephalitis, Alzheimer disease, Parkinson's disease, and amyotrophic lateral sclerosis (Giovanni et al., 2000; Jordan-Sciutto et al., 2001; Ranganathan and Bowser, 2003; Höglinger et al., 2007; Akay et al., 2011; Zyskind et al., 2015). Thus, determination and maintenance of cell fate by E2F and PPs is a key feature underlying both tissue homeostasis and disease phenotypes.

Mice deficient in only p107 or p130 suffer much less severe phenotypes than pRb knockouts, and are viable and fertile (Cobrinik et al., 1996; Lee et al., 1996). However, a deeper analysis of these models, particularly for p107, revealed key functions for the E2F/PP pathway in not only differentiation, but also direct regulation of stem and progenitor cell maintenance. For example, p107 is required in the developing and adult forebrain to both promote neuronal differentiation and limit neural precursor cell (NPC) expansion (Vanderluit et al., 2004, 2007), and for proper lineage commitment in adipose stem cells (Scimè et al., 2010; De Sousa et al., 2014).

Many PP-mediated phenotypes that impact fundamental stem and progenitor cell fate decisions have been shown to be fully or at least partially E2F-dependent, typically due to a clear transcriptional-based mechanism (Chen et al., 2007, 2009a, 2013; McClellan et al., 2007, 2009; Wenzel et al., 2007; Chong et al., 2009a; Shamma et al., 2009; Hu et al., 2012; Rotgers et al., 2014). Additionally, E2F-deficiency alone, even loss of single E2F family members, disrupts cell fate regulation in a number of cell types (McClellan and Slack, 2007; Ruzhynsky et al., 2007; Asp et al., 2009; Chen et al., 2009a, 2013; Chong et al., 2009b; Julian et al., 2013; Suzuki et al., 2014). Thus, it is clear that transcriptional regulation mediated by E2Fs and PPs is an important functional mechanism underlying stem cell fate determination (**Figure 1**).

Intersection of Cell Cycle Regulation with Cell Fate Control

Cell cycle dynamics are in fact tightly connected with stem cell fate. Cellular differentiation occurs when a primitive, progenitor cell type acquires more specialized functions, and many cell differentiation events are accompanied by changes in proliferation status. For instance, skeletal muscle precursors irreversibly exit the cell cycle once they terminally differentiate into myocytes (Bischoff and Holtzer, 1969; Nadal-Ginard, 1978; Olson, 1992), and slow-dividing stem cells of the intestinal crypt give rise to transit-amplifying precursors that proliferate quickly before undergoing terminal differentiation into one of the intestinal cell types [reviewed in Potten and Loeffler (1990)]. The self-renewal or differentiation potential of pluripotent stem cells is tightly linked to cell cycle phase, where G1 phase cells are poised for differentiation (Sela et al., 2012; Chetty et al., 2013; Singh et al., 2013). Similarly, NPCs lengthen their G1 phase and shorten S phase upon commitment to differentiation (Takahashi et al., 1995; Calegari et al., 2005; Arai et al., 2011), and disrupted cell cycle dynamics severely affect the balance between NPC populations and newly born neurons in the brain (Lange et al., 2009; Lim and Kaldis, 2012). Gain- or loss-of-function studies have revealed key roles for cell cycle proteins in controlling cellular processes and cell fate decisions that influence cortical development, neurogenic output and the number and behavior of neural stem and progenitor cells (these studies will be discussed further below). As cell cycle regulation and cell fate decisions are so closely interconnected, one might therefore argue that the non-canonical activities of E2Fs/PPs that have now been identified in stem and progenitor cells are a secondary consequence of cell cycle control.

One potential mechanism by which E2F- and PP-dependent regulation of cell cycle genes may indirectly influence cell fate decisions is through alteration of cell cycle kinetics. The "cell cycle length hypothesis" postulates that the time spent by tissue progenitors in the G1 phase might increase the ability of these cells to respond to differentiation cues, for example the response to certain morphogens (Lange and Calegari, 2010). In this scenario, PPs would control cell fate in cooperation with E2F factors by silencing their canonical cell cycle target genes, blocking S-phase entry and lengthening the G1 phase. This mechanism has been proposed for adipose and neural precursors (Calegari and Huttner, 2003; De Sousa et al., 2014). However, a number of recent studies have highlighted direct, cell cycle-independent roles for E2Fs in controlling many of these diverse processes. Phenotypic studies in a number of tissues have offered clear evidence that E2Fs and PPs can regulate cell fate decisions that impact stem cell maintenance and differentiation without simultaneously affecting cell cycle dynamics (Ceol and Horvitz, 2001; McClellan et al., 2007; Vanderluit et al., 2007; Chen et al., 2009a; Chong et al., 2009b; Wenzel et al., 2011; Julian et al., 2013; Kareta et al., 2015). A primary, fundamental role for E2Fs outside of cell cycle regulation is further supported by findings in the nematode *Caenorhabditis elegans*, where the E2F, DP and pRb orthologs *elf-1*, *dpl-1* and *lin-35*, are essential for fertility by controlling differentiation of precursor cells during vulval

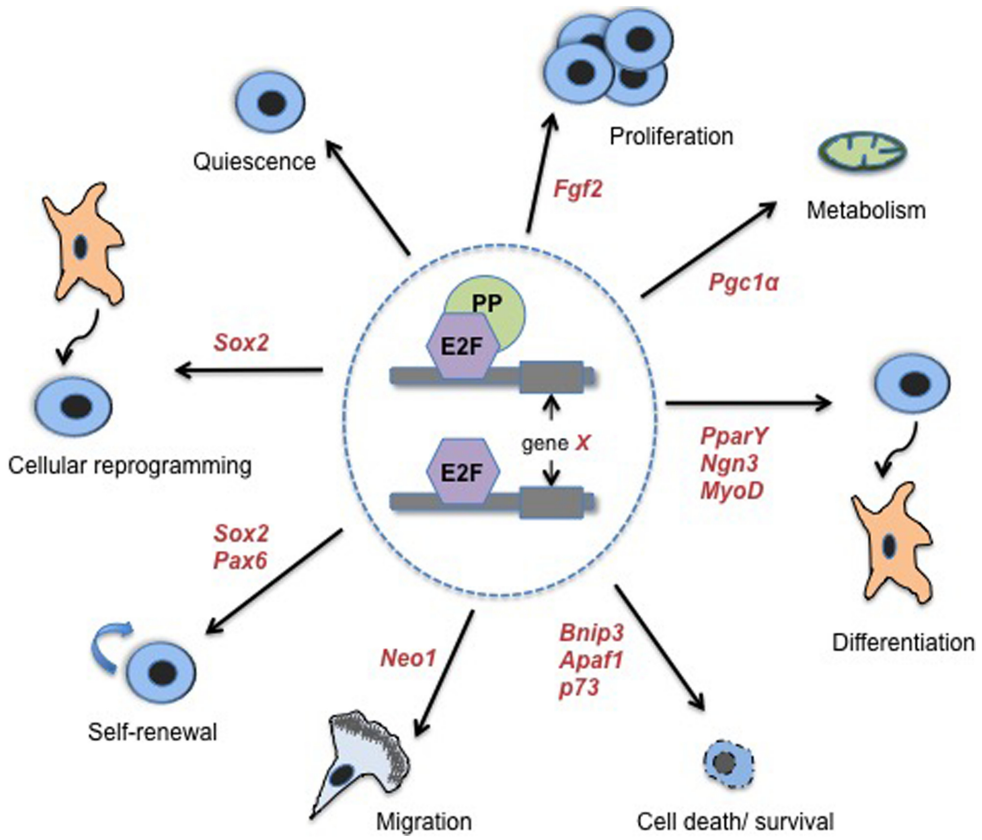

FIGURE 1 | E2Fs/PPs control diverse classes of cell fate regulatory processes and associated genes. Transcriptional regulation of non-classical, cell cycle-independent, genes by E2Fs and PPs has been associated with a number of biological processes that impact stem cell fate. These processes include: quiescence, proliferation, metabolism, differentiation and lineage choice, cell death and survival, migration of newly committed cells, stem cell self-renewal, and cellular reprogramming (specifically, reprogramming of fibroblasts to pluripotent SCs). Where examples are known, select cell fate-associated genes that have been confirmed as functional targets of E2Fs/PPs are indicated in red italicized font. References for cell death/survival genes are as follows: (Irwin et al., 2000; Moroni et al., 2001; Hershko and Ginsberg, 2004; Tracy et al., 2007). See the main text or **Table 1** for additional references.

development by antagonizing Ras-MAPK signaling, as opposed to regulating proliferation (Ceol and Horvitz, 2001; Myers and Greenwald, 2005).

A pivotal question remains, however, in determining whether E2Fs and PPs can truly regulate cell fate processes in a direct, cell cycle-independent manner. As the basic functional role of E2Fs is to transcriptionally regulate gene expression, it is important to establish whether they can directly regulate expression of genes that control cell fate processes.

Unbiased Identification of E2f Target Genes Suggest a Widespread Transcriptional Role in Cell Fate Determination

Despite the extensive and ever increasing evidence of a fundamental biological role for E2F and PPs in stem cell fate control, the underlying cellular mechanisms are only beginning to be clarified. As concerted E2F/PP activity ultimately affects transcriptional regulation of E2F target genes, it is highly likely that the genes that are bound and regulated by E2Fs are pivotal elements of how this pathway controls cell fate decisions. A range of potential scenarios exist, however, whereby E2Fs may have the capacity to regulate genes directly involved in cell fate regulation, or these effects may instead be indirect, caused by secondary effects of cell cycle gene expression and changes in cell cycle dynamics. Understanding these mechanisms at the gene regulatory level is therefore paramount to determining the true nature and extent of E2F/PP function in stem cell biology.

Early Identification of E2F and PP Target Genes

Among the first genomic studies to take a global look at E2F targets were those employing DNA microarrays to perform gene expression profiling after gain- or loss-of-function of E2F and PP family members. One serious limitation of this approach stems from its inability to distinguish direct and indirect gene regulatory relationships (Ishida et al., 2001; Kalma et al., 2001; Markey et al., 2002; Polager et al., 2002; Stanelle et al., 2002;

Vernell et al., 2003; Blais and Dynlacht, 2007). For this reason, experiments of chromatin immunoprecipitation (ChIP) coupled to promoter DNA microarray hybridization were undertaken. The earliest studies used microarrays with long, PCR-generated probes limited to a subset of known genes, typically focused on promoter regions of cell cycle-related genes. These investigations were instrumental in reaffirming previous findings that PPs and E2Fs directly regulate a large cohort of genes associated with proliferative control (Cam and Dynlacht, 2003; Blais and Dynlacht, 2004, 2007; Bracken et al., 2004; Dimova and Dyson, 2005). These canonical target genes include those encoding: key cell cycle regulators (e.g., Cyclin proteins, E2Fs themselves), nucleotide synthesis and DNA replication enzymes (e.g., TK, DHFR, DNA polymerase alpha), DNA repair proteins (e.g., RAD51, the Fanconi anemia proteins), and proteins involved in chromosome organization and segregation (e.g., histones, HMG1, SMC proteins).

This canonical view of E2F-dependent regulation of cell cycle associated genes has come from studies carried out not only in human or rodent cells, but also in flies, nematodes, and plants (Dimova et al., 2003; Stevaux et al., 2005; Kirienko and Fay, 2007; Hirano et al., 2008; Acharya et al., 2012; Korenjak et al., 2012; Kudron et al., 2013). Thus, the role of the E2F/PP signaling node as a key cell cycle regulator, as well as the basic mechanisms of gene regulation by E2Fs and PPs, has deep evolutionary roots.

E2Fs and PPs as Widespread Regulators of Genes Associated with Stem Cell Fate

Two key advances have contributed to changing the way we now look at the degree of functional diversity of the E2F/PP pathway. First, rapid technological advances in systems biology have increasingly allowed us to perform larger, genome-wide scale screens that are less biased and more likely to provide a comprehensive view of transcription factor targets than the earliest studies of E2F/PP target genes. Second, screens have been performed in a larger diversity of cell types, and in various cell differentiation paradigms, going beyond fibroblasts and cancer cell lines. The data gathered from these studies have revealed that E2Fs and E2F/PP complexes target the promoters of numerous genes with a much broader range of functional associations than was originally perceived, not only the canonical set of cell cycle genes, many of which directly instruct key cell fate decisions. The unbiased identification of E2F/PP target genes, together with the analysis of genetic knock-out animal models, has revealed an incredible diversity of function for the E2f and PP families that cannot be fully appreciated solely with the classical cell cycle regulatory model.

In mammalian cells, large-scale ChIP-chip and ChIP-Seq analyses of E2F target genes that have been performed to date are predominantly focused on identifying E2F1 and E2F4 binding sites, and have been reported in a relatively limited panel of cultured and immortalized cell types (Conboy et al., 2007; Xu et al., 2007; Lee et al., 2011). Unbiased identification of genes bound by E2F3 (including both the E2F3a and E2F3b isoforms) have to our knowledge been reported to date only in C2C12 myoblasts and myotubes (Asp et al., 2009), in mouse embryonic fibroblasts (von Eyss et al., 2012) and in primary murine NPCs (Julian et al., 2015). E2F3a&b, however, exhibit pivotal roles in a number of tissue systems and cellular processes, including gross mammalian embryonic development (Tsai et al., 2008), neurogenesis (Chen et al., 2007; McClellan et al., 2007, 2009; Julian et al., 2013), myogenesis (Asp et al., 2009), Sertoli cell maturation and survival (Rotgers et al., 2014), and maintenance of trophoblast stem cells (Wenzel et al., 2007). Given its broad functional roles, unbiased identification of E2F3 target genes in a greater diversity of cell types will therefore be greatly informative of the conserved and possible tissue-specific mechanisms by which E2F/PPs regulate cell fate decisions.

Despite the relatively limited data currently available, genome-wide DNA binding studies have been instrumental in establishing novel cellular functions for the E2F/PP pathway. Furthermore, they have significantly expanded both the cell cycle-independent roles in which this pathway is implicated, as well as the extent to which it is thought to be integrated transcriptionally in each of these functions. These studies have revealed that E2Fs bind to the regulatory regions of not 100s, as was our previous understanding, but 1000s of genes, in a relatively consistent manner across cell types. Whereas this pathway has been broadly implicated in the regulation of genes involved in not only cell cycle control, but also apoptosis, development, and differentiation for some time (Müller et al., 2001), genome-wide analyses are now demonstrating that E2F factors are in fact poised to control a large network of often 100s of genes involved in each of these biological functions. Additionally, recent studies that have expanded analyses to identify genes bound or regulated at the expression level by E2F/PPs outside of cancerous and immortalized cell lines, specifically in pluripotent, epidermal, muscle and neural stem cells (Asp et al., 2009; Lorz et al., 2010; Yeo et al., 2011; von Eyss et al., 2012; Kareta et al., 2015) have revealed large groups of target genes involved in many specialized functions that influence cell fate. These functions broadly include the regulation of cellular metabolism, quiescence, stem cell self-renewal, and tissue-specific differentiation programs. These findings, and the large number of potential target genes uncovered for each process, suggest a widespread transcriptional role for E2Fs and PPs in stem cell fate regulation.

Functional Evidence that E2Fs and PPs Control Transcription of Cell Fate-Associated Genes

Functional assays and analyses of genetic mouse models have provided important biological confirmation that E2Fs and PPs can indeed affect cell fate outcomes in stem and progenitor cells by transcriptionally regulating genes that directly control cell fate processes. E2F and PP family members have been implicated as important biological regulators of a number of processes that impact stem and progenitor cell fate, including: death and survival, quiescence, self-renewal, proliferation, differentiation, and migration (**Figure 1**). While classical cell cycle control is implicated in some processes, such as quiescence (Sage et al., 2003; Lorz et al., 2010; Andrusiak et al., 2013), many genes that have

direct, seemingly cell cycle-independent roles in cell fate regulation have been validated as true functional E2F/PP targets (**Figure 1** and **Table 1**). Although the number of such validated genes is currently limited, considering the large number of potential target genes uncovered by genome-wide analyses, the findings that have been made provide important proof of concept that this cell fate regulatory mechanism is important across multiple cell lineages.

Regulation of Genes that Promote or Inhibit Differentiation

A number of genes that directly control progenitor cell commitment to differentiation or lineage choice have been validated as biologically relevant E2F/PP target genes. For instance, E2f1 stimulates expression of the Peroxisome proliferator-activated receptor PPARγ in adipogenic progenitors to promote their expansion, while E2f4 conversely represses PPARγ expression to limit expansion and promote adipocyte differentiation (Fajas et al., 2002b). Functional analysis of myogenesis, along with direct identification of the genes both bound and regulated at the mRNA level by E2fs in proliferating and differentiating myoblasts revealed that E2f3b is required to repress expression of key myogenic factors, such as MyoD, during differentiation (Asp et al., 2009). Furthermore, E2f1 stimulates pancreatic differentiation by activating expression of the Ngn3 promoter in embryonic endocrine precursors (Kim and Rane, 2011). In the CNS, regulation of the neurogenesis and migration related genes *Dlx1/Dlx2* and *Neo1* (Neogenin) are linked to pRb and E2f-mediated control of interneuron specification and neuronal migration during development (Andrusiak et al., 2011; Ghanem et al., 2012). E2F/PP-mediated transcriptional regulation of factors that potentiate differentiation has also been linked to tumorigenesis, where E2f1-mediated transcription of PPARγ and Fatty acid synthase (Fasn) drives proliferation and survival of medulloblastoma tumors (Bhatia et al., 2012; Bhasin et al., 2013), and activation of multiple Notch pathway genes by E2fs serves to limit tumor expansion in hepatocellular carcinoma (Viatour et al., 2011).

Regulation of Stem Cell Maintenance Genes by E2Fs and PPs

Recent studies have substantiated a direct transcriptional role for E2Fs and PPs in not only the regulation of differentiated cell fates, but also in the control of stem cell self-renewal and proliferation. Studies of E2F/PP biological function and associated target genes in the CNS in particular have significantly increased our understanding of how E2F/PP activity can impact stem cell function. In the adult CNS, loss of E2f1 leads to a reduction of neural stem and progenitor cell divisions in the proliferative zones, resulting in reduced hippocampal neurogenesis (Cooper-Kuhn et al., 2002). In the retina, pRb and E2f3a together control differentiation, specifically of starburst amacrine cells (Chen et al., 2007). In the developing telencephalon, however, p107 and E2f3a/b regulate the balance between NPC maintenance, self-renewal and differentiation, and these activities are strongly associated with transcriptional regulation of the core stem cell self-renewal/maintenance genes *Sox2* and the Notch/Hes pathway (Vanderluit et al., 2004, 2007; Julian et al., 2013). E2f4 also promotes neural stem cell self-renewal, and this has been linked to regulation of the Sonic Hedgehog (Shh) pathway (Ruzhynsky

TABLE 1 | Listed here are the stem and progenitor cell populations for which E2Fs and PPs have demonstrated cell fate regulatory roles.

Stem cell population	E2F and PP factors implicated	Cell fate process affected	Target gene(s)	Reference (for target genes)
Pluripotent SC	pRb, p107, p130, E2f2, E2f4	Self-renewal, Reprogramming to pluripotency, Survival	*Sox2***	Yeo et al. (2011), Li et al. (2012), Kareta et al. (2015)
Neural and retinal precursors	pRb, p107, E2f1, E2f2, E2f3, E2f4	Self-renewal, Proliferation, Differentiation Migration, Survival	*Sox2*, *Pax6*, Notch and Shh pathways, *Fgf2, Dlx1, Dlx2, Neo1, Nrp1***	Vanderluit et al. (2004, 2007), Jiang et al. (2007), McClellan et al. (2007, 2009), Ruzhynsky et al. (2007), Andrusiak et al. (2011), Ghanem et al. (2012), Julian et al. (2013, 2015)
Myoblast	pRb, p107, E2f1, E2f3, E2f4	Proliferation, Differentiation, Survival	*MyoD***	Asp et al. (2009)
Hematopoietic SC	pRb, p107, p130, E2f8	Quiescence, Expansion, Differentiation		
Adipogenic progenitor	p107, E2f1, E2f4	Proliferation, Differentiation	*PPARγ*	Fajas et al. (2002b)
Osteoblasts	pRb, E2f1	Differentiation	*Alpl*, *Bglap*	Flowers et al. (2013)
Liver oval SC	pRb, E2f1	Quiescence, Expansion	Notch pathway	Viatour et al. (2011)
Pancreatic/endocrine SCs	E2f1	Proliferation, Differentiation	*Ngn3*	Kim and Rane (2011)
Trophoblast SC	pRb, E2f3, E2f7, E2f8	Proliferation, Differentiation, Survival		
Spermatogonial SC	pRb	Self-renewal		

*Also indicated are the specific E2F and PP factors that have been implicated functionally, as well as the cell fate regulatory process known to be controlled by E2F/PPs, in each stem/progenitor cell type. Where examples are known and have been functionally validated, we give key examples of direct cell fate regulatory genes that are transcriptional targets of E2Fs/PPs. The references listed are specific to the target genes given. **Indicates that genome-wide analyses (represented among the references) have uncovered additional putative target genes in that cell type. The cell fate process 'expansion' encompasses the potential for both proliferation and/or self-renewal.*

et al., 2007), another core regulator of neural stem cell maintenance. Additionally, p107 and E2f3 control NPC proliferation in the developing brain through regulation of the fibroblast growth factor *Fgf2* (McClellan et al., 2009).

These studies reveal a highly dynamic role for PPs and E2F factors in CNS development and homeostasis. Furthermore, they heavily implicate transcriptional regulation of non-canonical, cell fate-associated genes as a driving mechanism behind E2F/PP-dependent function in stem and progenitor populations. In line with the situation in the CNS, E2F/PP activity has also recently been implicated in controlling the self-renewal potential of pluripotent ESCs in mammals. Transcriptomic and transcription factor motif analyses in human ESCs suggested a fundamental role for E2F factors in the self-renewal of pluripotent stem cells (Yeo et al., 2011), which has been confirmed functionally (Conklin et al., 2012; Suzuki et al., 2014). This study suggested that E2Fs were highly integrated in the self-renewal network, and our recent bioinformatics analysis of E2f3 and E2f4 direct binding sites in murine NPCs confirmed that E2fs do indeed bind to an extensive network of genes fundamental for self-renewal and stem cell function (Julian et al., 2015).

This identification of E2Fs as important regulators of stem cell self-renewal and associated core regulatory genes has important implications not only for tissue homeostasis and development, but also the tumorigenic or tumor suppressive role of E2F and PP factors. An interesting possibility is that cancer may arise due to a loss of the ability to control expression of stem cell self-renewal genes, in addition to *bona fide* cell cycle genes. Supporting this assertion, two recent studies demonstrated a pivotal role for E2F/PP complexes in inhibiting cellular reprogramming to pluripotency. Specifically, two forms of transcriptional repressive complexes, pRb/E2f as well as p130/E2f4 in complex with the Cyclin-dependent kinase inhibitor p27, were shown to function as inhibitory blocks to reprogramming (Li et al., 2012; Kareta et al., 2015). Intriguingly, the underlying mechanisms were independent of cell cycle control, but due to transcriptional repression of Sox2 expression during the reprogramming process. Furthermore, the ability of pRb/E2f to repress Sox2 expression appeared to be a critical tumor-suppressive mechanism (Kareta et al., 2015). These studies, together with the findings of E2f-dependent *Sox2* regulation in NPCs (Julian et al., 2013), establish the E2F/PP regulatory node as an essential regulator of one of the most fundamental stem cell identity genes, importantly in two primary cell types that rely heavily on Sox2 for maintenance of their stem cell pool.

Regulation of Genes that Control Cell Death and Survival

A role for E2Fs and PPs in mediating cell death and/or survival has been functionally described in many lineages. Although this biological role has been known for some time, the mechanisms affecting cell death in stem and progenitor cells due to deregulation of E2Fs or PPs is not fully clarified. Nevertheless, p53-dependent mechanisms have been highly implicated, and while recent evidence suggested a non-transcriptional role for pRb in apoptotic induction (Hilgendorf et al., 2013), a number of genes that are involved in both the mitochondrial apoptotic signaling cascade as well as autophagy regulation have been demonstrated as downstream or direct targets of E2Fs and/or PPs (Hiebert et al., 1995; Sherr, 1998; Irwin et al., 2000; Moroni et al., 2001; Nahle et al., 2002; Vorburger et al., 2002; Hershko and Ginsberg, 2004; Hershko et al., 2005; Tracy et al., 2007; Ianari et al., 2009; Conklin et al., 2012; Bertin-Ciftci et al., 2013; Sung et al., 2013; Benson et al., 2014). Furthermore, a specific biochemical interaction between pRB and E2F1 is required for regulation of both E2F1-induced apoptosis and expression of E2F-dependent apoptotic genes (Dick and Dyson, 2003; Julian et al., 2008; Carnevale et al., 2012), strongly suggesting that transcriptional regulation by E2F/PP is a primary mechanism by which this pathway controls cell death.

Transcriptional Regulation of Metabolism by E2Fs/PPs

In addition to cell cycle dynamics and execution of stem cell-specific gene regulatory networks, a plethora of recent work has revealed an essential role for metabolic adaptations in driving the stem cell state. Specifically, it has become clear that stem cells inhibit oxidative metabolism and depend on metabolic pathways that rely heavily on glycolysis for energy production (Folmes et al., 2013; Ochocki and Simon, 2013). Given this knowledge, recent findings demonstrating a requirement for E2F and PP factors to both inhibit oxidative phosphorylation/promote glycolytic pathways in muscle and adipose tissue and to repress expression of genes associated with oxidative metabolism, such as PGC1α, are particularly intriguing (Scimè et al., 2010; Blanchet et al., 2011). While the functional relevance of E2F/PP-dependent regulation of core metabolism genes has not been investigated in stem cell populations, genome-wide studies have identified numerous metabolism-related genes as putative E2F targets (Asp et al., 2009; Yeo et al., 2011; Julian et al., 2015). Importantly, these discoveries in addition to others discussed here, place E2Fs and PPs as pivotal transcriptional regulators of multiple essential biological processes and regulatory programs that control stem cell fate decisions (**Figure 1**).

Mechanisms of Cell Fate Gene Regulation by E2F/PPs

As discussed above, the cell fate-associated processes with which E2Fs and PPs have been functionally implicated are diverse. In mammals, the PP and E2F families are now known to impact cell fate determination in many lineages. This includes neuronal, mesenchymal, hematopoietic, muscle, intestinal, mammary gland, liver, trophoblast, spermatogonial, and pluripotent stem and progenitor cells [for a thorough review on many of these lineages, see (Daria et al., 2008; Viatour et al., 2008; Ouseph et al., 2012; Sage, 2012; Hu et al., 2013; Yang et al., 2013; Suzuki et al., 2014; Rotgers et al., 2014; Kareta et al., 2015]. Thus the current evidence suggests that these proteins play instructive roles in stem and progenitor cell types that encompass all embryonic germ layers, as well as extra-embryonic, germ cell, and pluripotent lineages (**Figure 2** and **Table 1**). While our understanding of

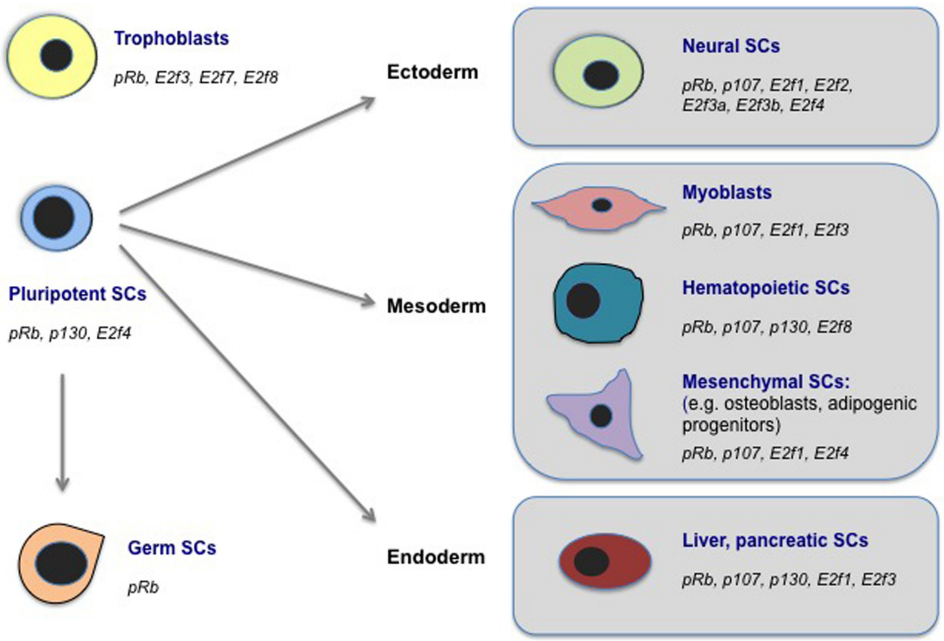

FIGURE 2 | E2Fs and PPs regulate cell fate in diverse stem cell populations. Here, we highlight examples of mammalian stem cell (SC) populations in which biological roles for E2Fs and/or PPs in cell fate regulation, outside of classical cell cycle control, have been documented. These cell types include SC populations representing all three embryonic germ layers (ectoderm, mesoderm, endoderm), as well as pluripotent SCs (in ESCs as well as during cellular reprogramming to iPSCs), germ (spermatogonial) SCs, and extra-embryonic trophoblasts. We indicate the E2F and PP factors that have been implicated in cell fate regulation in each SC type. See the main text or **Table 1** for references.

the full extent of stem and progenitor lineages that are affected by E2F/PP fate control is incomplete, these findings suggest that E2F/PP-dependent mechanisms are pervasive and perhaps fundamental for stem cell fate control. Additionally, they suggest that E2F and PP factors may have the capacity to regulate unique classes of cell fate regulatory genes in different tissue types. While the extent of tissue-specific gene regulation by E2F/PPs is poorly understood, emerging data suggests that it is extensive and likely to involve multiple mechanisms to influence target gene selection.

Regulation of Tissue-Specific Genetic Networks

A long-standing theory proposed to explain the ability of cell cycle regulators to potentiate tissue-specific differentiation programs, on a biological level, is that PPs interact with transcriptional co-factors other than E2Fs that are unique to specific tissues and that regulate tissue-specific target genes. Indeed, pRb has been shown to complex with transcription factors other than E2Fs in a manner that affects progenitor cell differentiation, one prominent example being its interaction with Runx2 in the osteoblast lineage to regulate the expression of osteoblast-specific genes (Thomas et al., 2001). An interaction between pRb and MyoD in muscle cells has also been reported (Gu et al., 1993), but it is likely that the interplay between the PP and the master regulator of myogenesis occurs indirectly, through competition for binding to the transcriptional co-repressor HDAC1 (Mal et al., 2001; Puri et al., 2001). pRb has also been shown to repress adipocyte differentiation by interacting with PPARγ and recruiting HDAC1 to its target promoters (Fajas et al., 2002a), and to stimulate adipogenesis by interacting with CEBP transcription factors (Chen et al., 1996). Additionally, stabilization of the homeobox protein Pdx1 through a direct interaction with pRb is necessary for embryonic pancreas development and adult β-cell function (Kim et al., 2011). It is likely that more tissue-specific interactions of this kind will be discovered as this line of investigation progresses.

Independently from these possibilities, however, the evidence that is now emerging from a deeper analysis of E2F target genes in individual cell types suggests that a prominent mechanism by which cell cycle regulators control tissue-specific cell fate decisions is through E2F-dependent regulation of networks of cell fate regulatory genes that are specific to that lineage. Due to the accumulating evidence that E2Fs and PPs can control cell fate processes without affecting cell cycle dynamics, and the expanding number of direct E2F target genes that control tissue-specific stem cell fate decisions (Fajas et al., 2002b; Ruzhynsky et al., 2007; Asp et al., 2009; McClellan et al., 2009; Andrusiak et al., 2011; Ghanem et al., 2012; Li et al., 2012; Julian et al., 2013; Kareta et al., 2015), we anticipate that further investigations into the genome-wide binding sites of E2Fs in different cell types will solidify the hypothesis that E2Fs target large networks of tissue-specific target genes. Speaking to this, a recent study identified extensive tissue-specificity in the binding sites of E2F and PP orthologs in germline and somatic cell populations in *C. elegans* (Kudron et al., 2013). In mammalian cells, comparative analysis of E2f3-bound

gene promoters in murine NPCs and myoblasts showed that while cell cycle-related target genes are common to both cell types, there is a large degree of tissue-specificity among E2f3 target sites, specifically at those genes involved in differentiation and development-related processes (Julian et al., 2015; **Figure 3**). To understand how widespread this phenomenon is, it is imperative that systematic analysis of genome-wide E2F binding sites, and corresponding gene expression analyses, be performed in a much more expansive group of mammalian tissues and primary cell types.

Diverse Transcriptional Roles of E2Fs and E2F/PP Complexes

Multiple lines of evidence suggest that E2F/PP-mediated regulation of cell fate-associated genes does not closely follow the canonical view that E2Fs1-3a are predominantly transcriptional activators, and the remaining E2Fs are predominantly repressors that function in cooperation with a PP. First, the fact that genomic binding studies have identified both 'activator' and 'repressor' E2Fs at seemingly active promoters, in multiple stem and progenitor cell types, does not support this canonical view (Asp et al., 2009; Yeo et al., 2011). A number of observations from single gene-focused analyses have further revealed that E2F transcriptional function is more complex than the canonical model suggests. Unexpectedly, the E2f3-mediated regulation of Sox2 in NPCs was paradoxically found to be dependent on a transcriptional activation role for E2f3b and a repressive role for the classical 'activator' E2f3a (Julian et al., 2013). Additionally, this repressive role for E2f3a appears to function in concert with p107, an atypical binding partner for E2f3a as it is was thought to only form PP-containing complexes with pRb. Interestingly, E2f3a has also been shown to mediate repression in starburst amacrine cells in the retina, this time through collaboration with pRb (Chen et al., 2007). Additionally, a role for pRb/E2f1 complexes in gene activation has been demonstrated at select genes involved in osteogenic, adipogenic, and myogenic differentiation (Flowers et al., 2013), while alternatively, pRb-independent gene repression by E2f3b, across a large panel of genes, has also been observed (Asp et al., 2009).

Intriguingly, the simultaneous identification of biologically functional E2f3b activator and E2f3a/p107 repressor complexes at the same target gene, Sox2, in proliferating NPCs, suggests that a homeostatic level of E2F and PP family members is required to ensure proper regulation of at least some target genes. By this mechanism, proper biological function would be dependent on finely tuned transcriptional regulation by E2F and PPs, as opposed to strict 'on/ off' activation or repression by specific transcriptional complexes. The requirement for opposing regulation of Sox2 by E2fs/PPs to regulate the balance between NPC maintenance and differentiation (Julian et al., 2013), as well as findings that both pRB over-expression and deficiency in human ESCs induces cell cycle arrest and death (Conklin et al., 2012), and that both loss or gain of E2f expression drives survival defects in retinal progenitors (Chen et al., 2009a; Chong et al., 2009b), are testaments to this possibility.

Together, these findings paint a more malleable picture of transcriptional regulation by E2Fs and PPs than what the canonical model suggests, where the potential combinations of E2F and PP factors at DNA sites and their resulting transcriptional effects are in fact diverse and not clearly predictable. The complexes that are formed and their transcriptional effects at cell fate regulatory target genes are likely to be influenced by a number of factors, including cell type- and state-specific expression profiles of E2F and PP family members and additional co-factors, as well as the chromatin environment that surrounds particular E2F-bound sites. Thus, it is likely that transcriptional regulation by E2Fs and E2F/PP complexes can vary significantly at different genomic sites and in different cellular states.

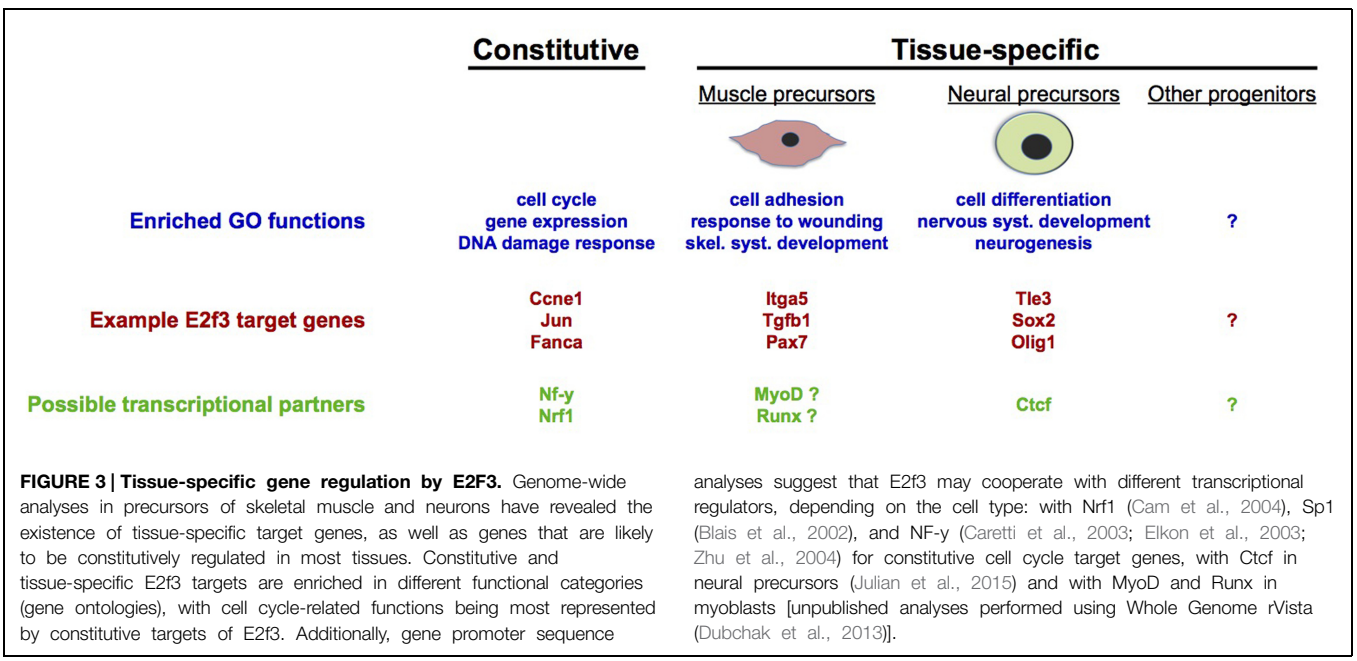

FIGURE 3 | Tissue-specific gene regulation by E2F3. Genome-wide analyses in precursors of skeletal muscle and neurons have revealed the existence of tissue-specific target genes, as well as genes that are likely to be constitutively regulated in most tissues. Constitutive and tissue-specific E2f3 targets are enriched in different functional categories (gene ontologies), with cell cycle-related functions being most represented by constitutive targets of E2f3. Additionally, gene promoter sequence analyses suggest that E2f3 may cooperate with different transcriptional regulators, depending on the cell type: with Nrf1 (Cam et al., 2004), Sp1 (Blais et al., 2002), and NF-y (Caretti et al., 2003; Elkon et al., 2003; Zhu et al., 2004) for constitutive cell cycle target genes, with Ctcf in neural precursors (Julian et al., 2015) and with MyoD and Runx in myoblasts [unpublished analyses performed using Whole Genome rVista (Dubchak et al., 2013)].

Transcriptional Co-Factors and E2F Target Gene Selection

Given the association of E2F and PP factors with cell fate regulation in what is now known to be a considerably broad range of cell types, especially with the potential tissue-specificity of this phenomenon, understanding how these proteins are able to physically discern between their canonical cell cycle regulatory genes and their non-canonical targets becomes an important question. The fact that many E2F/PP-dependent cell fate regulatory functions can be functionally separated from cell cycle control suggests that these proteins are recruited to 'cell cycle' and 'cell fate' genes with the help of different transcriptional partners.

While a prospective analysis of potential E2F co-factors that may specifically regulate cell fate genes has not been reported, two co-factors for E2F3, to date the most highly implicated E2F family member in cell fate control, have been identified. Specifically, the E-box transcription factor TFE3 has been shown to interact uniquely with E2F3 through its marked box domain and to regulate proliferation and the expression of select genes in cooperation with E2F3 (Giangrande et al., 2003; Nijman et al., 2006). Additionally, a recent study demonstrated that the SNF2-like helicase protein HELLS interacts with E2F3 in the context of tumorigenesis to induce cell cycle entry and proliferation, and that the two appear to synergistically activate select target genes (von Eyss et al., 2012). These studies implicated TFE3 and HELLS as E2F3 co-factors largely in the context of proliferative control. It is possible that these interactions have the same functional consequence in all cell types; however, it may also be the case that TFE3 and HELLS are important factors in the recruitment and/or activity of E2F3 to cell fate regulatory genes in stem cell populations. As the functional implications and conservation of these interactions have not been extensively characterized, this possibility warrants further investigation.

In addition to potential co-factors that may recruit E2Fs to target sites, recruitment of E2Fs to target genes can also be regulated by mechanisms that compete for their ability to bind DNA. For instance, the Cyclin-dependent kinase CDK5 is a potent cell cycle suppressor in post-mitotic neurons (Zhang et al., 2008), and the underlying mechanism is due to the ability of a CDK5-p35 complex to directly bind E2F1, consequently disrupting the ability of E2F1 to interact with DP1 on DNA at various cell cycle-related genes (Zhang et al., 2010). As enzymatically active CDK5 is restricted to post-mitotic neurons, studies have largely focused on determining its function in this cell type. However, the mechanism described here is not dependent on enzymatic activity, and since CDK5 is broadly expressed (Tsai et al., 1993) this unique E2F regulatory mechanism may be important in other cell types. It is unclear at the moment if such a mechanism may similarly contribute to the regulation of cell fate regulatory genes by E2Fs in neural cells or other lineages, but it is a promising possibility that this or a similar mechanism contributes to E2F target gene specificity.

Another intriguing possibility is that interaction between E2Fs and PPs with enhancer regions may underlie the ability of these proteins to bind to potentially unique sets of cell fate associated genes in different cell types, as enhancers are key mediators of cell type-specific gene regulation. The recent finding that a significant proportion of E2F4 binding sites are directly associated with enhancers lends credence to this idea (Lee et al., 2011). Furthermore, gene promoter sequence analysis of E2f3-bound promoter sites has identified a few select factors that may discern common and tissue-specific E2f target sites in NPCs and myoblasts (Julian et al., 2015; unpublished data; **Figure 2**). Intriguingly, further bioinformatic analyses revealed CTCF as a potential novel co-factor for E2f3 at cell fate genes specifically in NPCs. CTCF is a well-known insulator protein associated with enhancer regions and, as recently demonstrated, with a sub-population of promoter sites (Shen et al., 2012; Phillips-Cremins and Corces, 2013). Given the particular importance of CTCF in neuronal development (Hirayama et al., 2012), enhancer–promoter connections mediated between CTCF and E2F represents a particularly promising mechanism for NPC-specific cell fate gene regulation by E2F/PPs.

As the identity of protein complexes found at enhancer regions and their interactions with promoters is a major mechanism dictating cell type-specific gene expression, this is an exciting finding that suggests E2Fs may influence tissue-specific cell fate control by coordinating enhancer–promoter interactions at key cell fate associated genes. Application of ChIP-Seq to identify truly unbiased genome-wide binding sites of additional E2F factors in a greater diversity of cell types will importantly reveal how widespread this phenomenon is among the E2F family. Coupling this approach with genomic structural analyses, such as Hi-C technology, which allows for identification of chromatin loop domains and associated chromatin marks and binding proteins (Rao et al., 2014), will provide an important perspective on the potential functional implications of these interactions.

Conclusion and Perspectives

The relatively recent technical progress in systems biology approaches to understanding gene regulation on a genome-wide level has revealed an extensive diversity of function for the classical cell cycle regulatory E2F/PP pathway. We now know that transcriptional regulation of extensive sets of cell fate regulatory genes by E2Fs and PPs is an important regulatory mechanism underlying key cell fate decisions in a number of cell types. Emerging evidence also suggests that the E2F/PP signaling node is able to mediate cell type-specific gene expression programs. While advances over the past few years have greatly expanded our view of the functional importance of transcriptional regulation by E2Fs and PPs, the mechanistic understanding of their role in stem cell fate regulation is in its infancy. We need a better understanding of which stem cell populations rely on E2F/PP activity when making key cell fate decisions, as well as which epigenetic co-factors contribute to gene class and cell type-specific gene expression.

Moving forward it will be important to continue to exploit advances in systems biology approaches that allow for truly

genome-wide analyses of transcription factor binding sites in order to understand the full extent of E2F/PP function in stem cell fate control. Correlation of putatively identified target genes with gene expression signatures, co-factor binding, and both two- and three-dimensional chromatin structure will shed important mechanistic insight on the epigenetic role of E2Fs/PPs in cell fate decision making.

An important question for future investigations, which is currently largely unaddressed, is how E2Fs and PPs may regulate cell fate genes in post-mitotic cells. There is extensive evidence that PPs and E2Fs can repress cell cycle entry in post-mitotic cells and that they can participate in the formation of multi-protein repressive complexes in these cell types to repress classical E2F cell cycle target genes [reviewed in Blais and Dynlacht (2007), Dick and Rubin (2013), Herrup (2013)]. It is therefore likely that E2Fs and PPs are important regulators of cell fate-associated genes in post-mitotic cells, in both normal and disease settings, perhaps to repress the stem cell state, or to maintain differentiation and survival. Gaining a clearer understanding of the mechanisms underlying epigenetic cell fate regulation by E2Fs/PPs by addressing these key questions will have important implications in the contexts of tumorigenesis and disease, development, tissue homeostasis, and regeneration.

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Molecular targets of chromatin repressive mark H3K9me3 in primate progenitor cells within adult neurogenic niches

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Histone 3 Lysine 9 (H3K9) methylation is known to be associated with pericentric heterochromatin and important in genomic stability. In this study, we show that trimethylation at H3K9 (H3K9me3) is enriched in an adult neural stem cell niche- the subventricular zone (SVZ) on the walls of the lateral ventricle in both rodent and non-human primate baboon brain. Previous studies have shown that there is significant correlation between baboon and human regarding genomic similarity and brain structure, suggesting that findings in baboon are relevant to human. To understand the function of H3K9me3 in this adult neurogenic niche, we performed genome-wide analyses using ChIP-Seq (chromatin immunoprecipitation and deep-sequencing) and RNA-Seq for *in vivo* SVZ cells purified from baboon brain. Through integrated analyses of ChIP-Seq and RNA-Seq, we found that H3K9me3-enriched genes associated with cellular maintenance, post-transcriptional and translational modifications, signaling pathways, and DNA replication are expressed, while genes involved in axon/neuron, hepatic stellate cell, or immune-response activation are not expressed. As neurogenesis progresses in the adult SVZ, cell fate restriction is essential to direct proper lineage commitment. Our findings highlight that H3K9me3 repression in undifferentiated SVZ cells is engaged in the maintenance of cell type integrity, implicating a role for H3K9me3 as an epigenetic mechanism to control cell fate transition within this adult germinal niche.

Keywords: epigenetics, histone methylation, pericentric chromatin, Papio anubis, SVZ, neurogenesis

INTRODUCTION

Chromatin is functionally classified as euchromatin and heterochromatin, which are crucial for epigenetic controls of gene expression. Underlying the specialized chromatin structure around centromere and telomere, H3K9me3 was identified to be heterochromatin-enriched histone code to silence gene expression and prevent chromosomal instability (Czvitkovich et al., 2001; Lachner et al., 2001; Peters et al., 2001, 2002; Black et al., 2012). For instance, a previous study has shown that loss of H3K9 methylation in Drosophila causes DNA damage in heterochromatin and mitotic defect (Peng and Karpen, 2009). In mice, loss of H3K9me2/me3 causes the disruption of heterochromatin and increases telomere length (Peters et al., 2001; Garcia-Cao et al., 2004; Benetti et al., 2007). H3K9 methylation is also involved in pluripotency of embryonic stem cells (ESCs) and multipotency of neural precursor cells (NPCs), in which the pluripotent genes (e.g., Nanog, Oct4) and non-neural genes (e.g., GATA4, NODAL) gain H3K9me3 that lead to long-term repression during differentiation of human ESCs into NPCs (Golebiewska et al., 2009; Hirabayashi and Gotoh, 2010). Thus, H3K9me3 plays a repressive role in numerous neuronal and non-neuronal genes (Roopra et al., 2004; Schaefer et al., 2009) in addition to its known function in genome stability. Among all lysine methyltransferases, the KMT1 family composed of G9a/GLP and Suv39h1/h2 are characterized to be essential for H3K9me1/2 and H3K9me3 modifications, respectively (Czvitkovich et al., 2001; Lachner et al., 2001; Peters et al., 2001; Black et al., 2012). Studies from KMT1 knock-out mice demonstrated that loss of H3K9 methylation contributes to behavioral abnormalities and cognitive impairment (Schaefer et al., 2009) in addition to its protective role in genome stability. In this work, we found that H3K9me3 is enriched in the subventricular zone (SVZ), where adult neurogenesis occurs.

The SVZ is the largest neural stem cell niche, which harbors stem/progenitor cells for adult neurogenesis. The SVZ contains slowly dividing neural stem cells (NSCs) with astrocyte-like morphology. In the rodent model, NSCs give rise to transit-amplifying cells, which subsequently give rise to immature neuroblasts. These neuroblasts migrate through the rostral migratory stream (RMS) and generate interneurons in the olfactory bulb (Alvarez-Buylla and Lim, 2004; Ihrie and Alvarez-Buylla, 2011). Numerous studies have demonstrated that extracellular signals such as growth factors or morphogens have significant effects on either self-renewal of NSCs or lineage commitment

(Doetsch et al., 2002; Alvarez-Buylla and Lim, 2004; Zheng et al., 2004; Jackson et al., 2006; Ihrie et al., 2011). Additionally, the intracellular effectors of adult neurogenesis include cell-cycle inhibitors (p16/INK4A and p21) (Molofsky et al., 2006), transcription factors (Doetsch et al., 2002; Shi et al., 2004; Kohwi et al., 2005; Roybon et al., 2009; Qu et al., 2010; Ihrie and Alvarez-Buylla, 2011), and epigenetic mechanisms (Ming and Song, 2011). One such epigenetic mechanism includes histone modifications (Ming and Song, 2011). In this study, we demonstrate that H3K9me3 has a distinct distribution pattern within cell populations in the rodent and non-human primate SVZ. Yet, the molecular targets of H3K9me3 in this adult germinal niche remain unknown. Therefore, we developed a technique to purify subpopulations of SVZ cells from baboon brain (*Papio anubis*) (Sandstrom et al., 2014) for genome-wide analysis by using ChIP-Seq. In addition to genes involved in cell cycle and proliferation, we found that H3K9me3 enriched for genes functioning in axon and neuron projection, cellular maintenance/organization, cell signaling, and post-translational acetylation as well. A further integrated ChIP-Seq and RNA-Seq analysis revealed that 35% of H3K9me3-enriched genes are silenced, many of which are known to function in neuronal-, hepatic-, and immunological-cell type activation. In light of previous studies showing that H3K9me3 is a chromatin repressive mark, we anticipate that H3K9me3 is critical for the maintenance of cell identity within this adult neurogenic niche through its repressive function to protect against improper lineage differentiation within the SVZ.

RESULTS

H3K9me3 IS EXPRESSED IN GERMINAL ZONES WITHIN THE ADULT BRAIN

Using co-immunostaining with antibodies specific for H3K9me3 (a pericentric chromatin staining pattern, **Figure 1B**) and cell type specific markers in the SVZ (**Figure 1I**) for the gross anatomy of 8-week (P56) old adult mouse brain (**Figure 1A**), we found that H3K9me3-positive cells are co-localized with GFAP which labels quiescent and active NSCs (**Figure 1C**), Vimentin positive active NSC (**Figure 1D**), and PSA-NCAM positive neuroblast populations as well (**Figure 1E**). As Mash1 is commonly used in the mouse to denote cells as "transient amplifying cells," we applied co-immunostaining of Mash1 and H3K9me3 in the mouse SVZ, and found colocalization of H3K9me3 and Mash1 (**Figure 1F**). We then performed a 2 h EdU administration in mice to label quickly dividing transit-amplifying cells and neuroblasts, and found colocalization of EdU and H3K9me3 (**Figures 1G,H**). These results show that H3K9me3 is present in undifferentiated SVZ cells within rodent brain.

The comparisons of brain volume and structure across primate species and human have shown significant correlation between baboon and human (Kochunov et al., 2010; Rogers et al., 2010), we therefore examined the H3K9me3 distribution pattern in the baboon SVZ (**Figure 2A**). It is worth noticing that GFAP-positive astrocytic ribbon lies in this niche and also extends toward the lateral ventricle (**Figures 2B,C**), resembling the previous finding in adult human brain (Sanai et al., 2004). Thus, the overall architecture of baboon SVZ geographically represents the SVZ in adult human brain (Sanai et al., 2004, 2011), corroborating the extent to which findings in baboon SVZ are relevant to human SVZ. We found that H3K9me3 is associated with GFAP- and Vimentin-positive NSCs and also PSA-NCAM-positive neuroblasts in the baboon SVZ (**Figures 2D–G**), suggesting that H3K9me3 has function in these cell populations.

To further quantify the percentages of co-localization between H3K9me3 and SVZ subpopulations, we carried out flow cytometry analysis for dissociated SVZ cells after micro-dissection of SVZ from baboon brain. Flow cytometry analysis reveals approximately 45 and 20% of GFAP- and Doublecortin (DCX)-positive populations contain H3K9me3, respectively (**Figure 3**). Consistent with immunostaining results, the great majority of Vimentin- and PSA-NCAM-positive cells (∼95%) are colocalized with H3K9me3 (**Figure 3**). This quantification confirms that H3K9me3 is enriched in undifferentiated SVZ cells, while the abundance of H3K9me3 varies across different cell populations within the SVZ.

H3K9me3 EPIGENETIC LANDSCAPE IN THE SVZ CELLS

While results from our study highlight the distinct localization pattern of H3K9me3 in the SVZ of rodent and non-human primate, the molecular targets of H3K9me3 in this germinal niche remain to be determined. Currently, most of the genome-wide associated studies in neural stem/progenitor cells are conducted in culture to obtain sufficient materials for analyses. However, the *in vitro* cell culture system cannot fully recapitulate *in vivo* epigenetic landscape since the metabolites yielded from cultured condition including acetyl and methyl donors can alter the status of histone acetylation and methylation (Black et al., 2012). To ascertain characteristics of SVZ cells as they exist *in vivo* for identification of the genomic loci that carry the H3K9me3 modification, we developed a technique to purify SVZ cells directly from the baboon brain within a short post-mortem interval (<20 min). We utilized conjugated Dynabeads with antibodies against SVZ cell type-specific markers to purify dissociated cells following dissection of the SVZ (**Figure 4A**) (Sandstrom et al., 2014). This technical innovation preserves the nature of distinct SVZ cell types and is ideal for genome-wide analysis to uncover H3K9me3 enriched loci in the SVZ through ChIP-Seq with antibodies specific for H3K9me3. Additionally, this approach ensures that H3K9me3 positive cells from the adjacent striatum are excluded from the genome-wide analysis of the SVZ cells. DNA obtained from each ChIP pull-down was sequenced to high depth (200 million tags; 36 bases) by using Illumina HiSeq2000 sequencer (**Figure 4A**). We did additional runs of ChIP-Seq as replicates with different vendor's antibody H3K9me3 and independent sample preparation. This approach yielded a confident list of H3K9me3 enriched loci across independent sets of deep-Seq. Because the baboon gene annotation is not currently available, we alternatively compiled, processed, and aligned the sequence reads to the Jan. 2006 rhesus macaque (*Macaca mulatta*) draft assembly, Mmul_051212 (Gibbs et al., 2007) (http://www.ncbi.nlm.nih.gov/genome/assembly/237568/) and the UCSC genome browser version: rheMac2 (http://hgdownload.cse.ucsc.edu/downloads.html#rhesus). Of note, there is only 2% difference at the genomic level between baboon

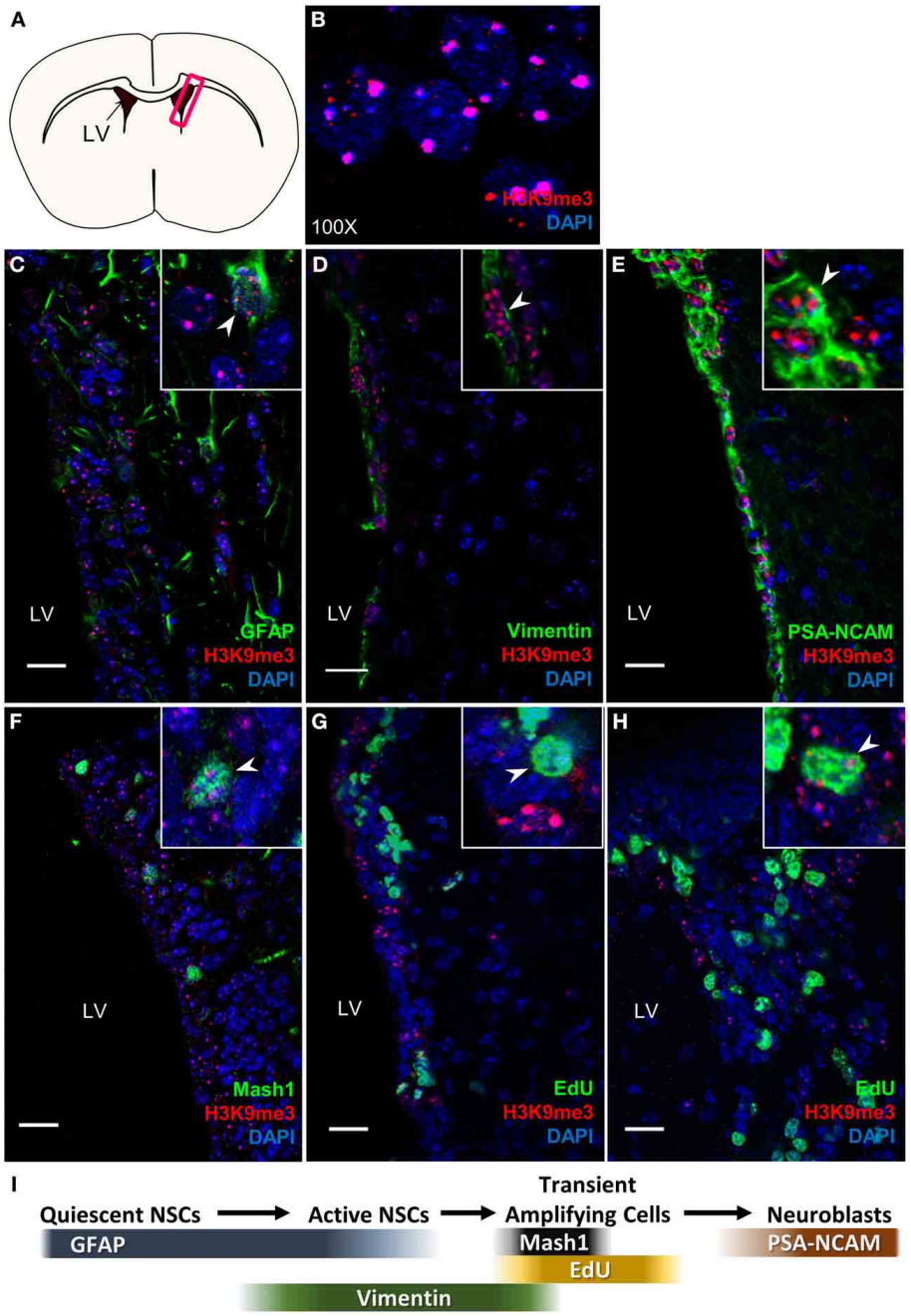

FIGURE 1 | H3K9me3 distribution in mouse SVZ. (A) Coronal cross-section view of mouse forebrain, box indicates location of SVZ. **(B)** H3K9me3 staining pattern in SVZ cells (100X). **(C–E)** Double labeling of H3K9me3 (red) and SVZ cell-type specific markers: **(C)** NSC marker—GFAP; **(D)** Active NSC cell marker—Vimentin; **(E)** Neuroblast marker—PSA-NCAM. **(F)** Small population of H3K9me3-positive cells is colocalized with Mash1. **(G,H)** Small population of EdU$^+$ cells is colocalized with H3K9me3. **(I)** Scheme displays cell type specific markers at different neural progenitor stages. LV, lateral ventricle; Images represent 12 μm coronal sections at 40X magnification; Scale bar = 20 μm.

and rhesus macaque, thus, we were able to identify 863 unique H3K9me3-enriched genes from independent sets of ChIP-Seq (FDR = 0.05) by using the MACS2 and closest-features program from the BEDOPS tool set (Neph et al., 2012) (**Figure 4B**; **Supplemental Table 1i**). Gene ontology with functional annotation analysis reveals that molecular functions associated with these genes include acetylation, axon/neuron projection, and protein targeting/import (**Figure 4C**; **Supplemental Table 1ii**) with connection to neurological disorders. While imprinted genes are known to be involved in broad aspects of biology including brain function, imprinting dysregulation has been associated with several neurodevelopmental and neurological disorders. To

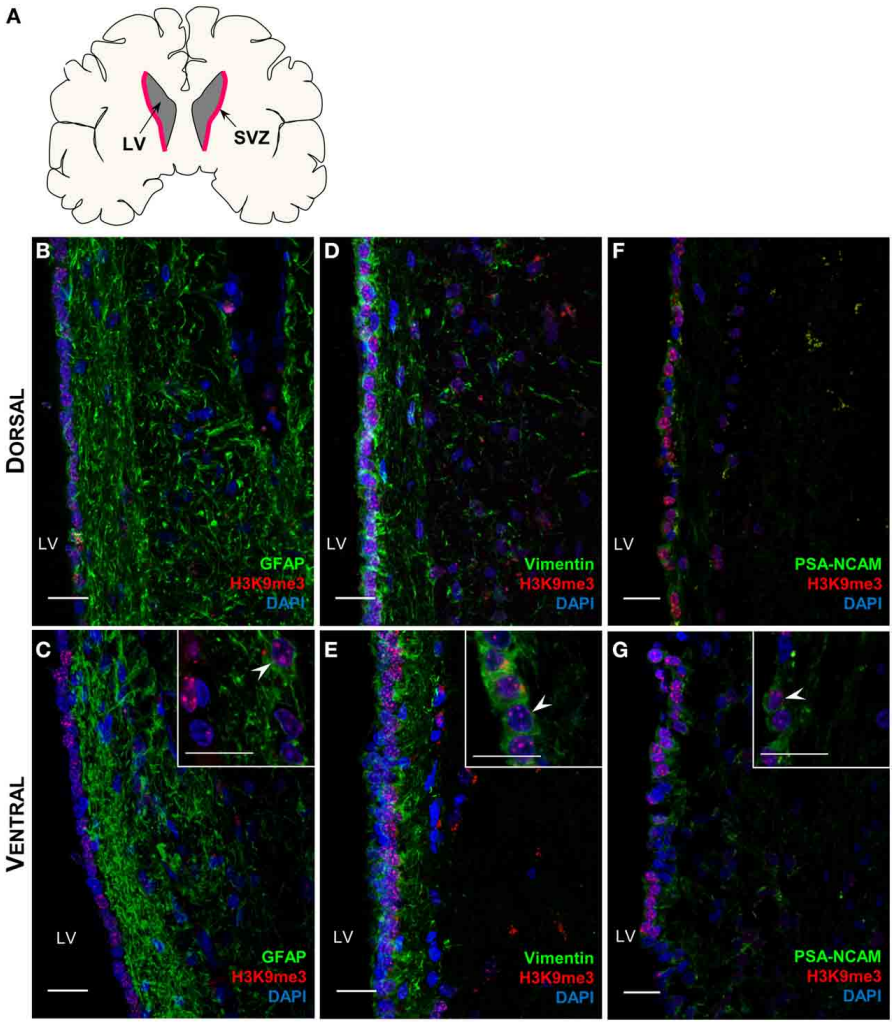

FIGURE 2 | Distribution of H3K9me3 in the neural progenitor populations of the baboon SVZ. (A) Coronal cross-section schematic of baboon forebrain, red highlighted area annotates SVZ. **(B–G)** Double labeling of H3K9me3 (red) and neural progenitor cell specific markers (green): **(B,C)** Only a small population of H3K9me3 positive cells co-localize with GFAP (NSC marker) in dorsal and ventral SVZ. **(D,E)** An extensive population of Vimentin positive cells is co-localized with H3K9me3 along the entire SVZ. **(F,G)** Enrichment of H3K9me3 persists in PSA-NCAM (neuroblast marker) positive population throughout dorsal and ventral of SVZ. LV, lateral ventricle; Images represent 60 μm sections at 40X magnification; Inserts are 100X magnification; Scale bars = 20 μm.

elucidate whether there are imprinted genes among H3K9me3 targets in the SVZ cells, we undertook an overlap comparison between the lists of identified imprinted genes (Luedi et al., 2005) and H3K9me3 targets identified from our ChIP-Seq analysis. We found a total of 11 genes that are imprinted and enriched with H3K9me3. These genes have known functions in CNS fate commitment, glial differentiation, neural projection, and neurite outgrowth, suggesting a role of H3K9me3 in collaboration with imprinting mechanism to maintain the populations of undifferentiated SVZ cells. Among the H3K9me3-enriched genes ($n = 863$), the top biological networks predicted by Ingenuity Pathways Analysis (IPA) are involved in (1) cell morphology and cellular assembly; (2) post-transcriptional and post-translational modifications; (3) protein synthesis; (4) cell cycle; and (5) cellular growth and proliferation (**Figure 5**; **Supplemental Table 1iv**). Additionally, the top biological pathways by IPA prediction include protein ubiquitination as well as signaling pathways involving AKT, BAX, c-JUN, MDM2, p300, P53, PP2A, and PTEN (**Figure 6**; **Supplemental Figures 1–3**).

To further characterize the functional consequence of H3K9me3 enrichment on target genes, we performed RNA-Seq for purified baboon SVZ cells and applied integrated analysis between ChIP-Seq and RNA-Seq. We identified 562 genes enriched with H3K9me3 are detectable by RNA-Seq, which are associated with cellular assembly/maintenance/organization, post-transcriptional and translational modifications, signaling pathways, and DNA replication. We also found that 301 of H3K9me3-enriched genes involved in axon/neuron or hepatic stellate cell activation and immunological response are not detectable by RNA-Seq (p-value = 2.2e-16) (**Figure 4D**;

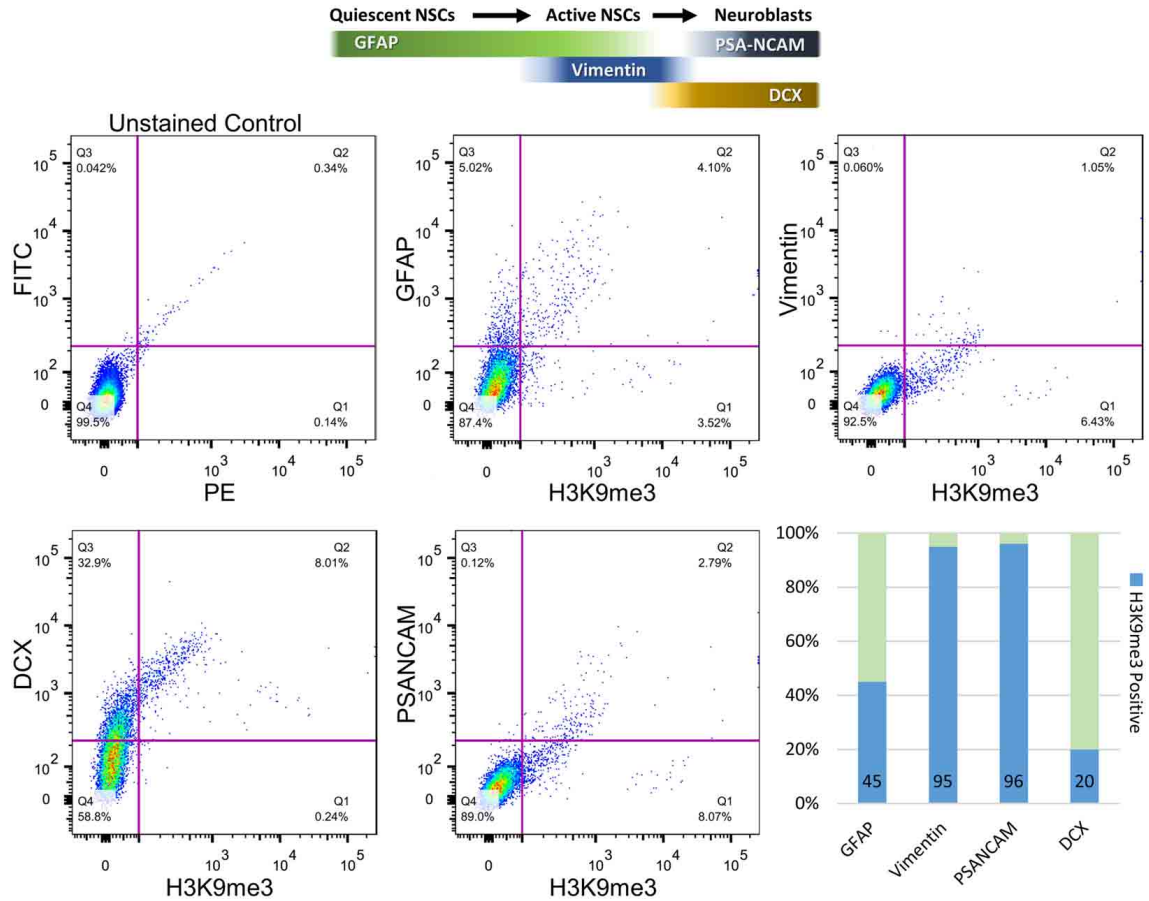

FIGURE 3 | Quantification of co-localization percentages between H3K9me3 and subpopulations of SVZ cells by flow cytometry. After SVZ dissection, dissociated cells were analyzed by flow cytometry with antibodies against H3K9me3 and cell-type markers including GFAP (quiescent and active NSC marker), Vimentin (relatively active NSCs), PSA-NCAM (migrating neuroblast), and DCX (early and migrating neuroblast marker). Bar graph represents undifferentiated SVZ cells for each population positive for GFAP, Vimentin, PSA-NCAM, and DCX (light green). Blue annotates H3K9me3-positive percentage within each SVZ subpopulation.

Supplemental Table 1iii). Given that H3K9me3 can act as short-term and long-term repression, we anticipate 35% (301/863) of H3K9me3-enriched genes to be under long-term repression. Lastly, H3K9me3-enriched genes in the acetylation category from our GO analysis (**Figure 4C**) were previously characterized with roles in histone acetylation that regulate gene expression or in protein acetylation that regulates protein stability, localization, and interactions with other molecules. Numerous H3K9me3-enriched genes associated with cellular assembly, maintenance, organization, and signaling are known to be regulated by histone acetylation (Cho and Cavalli, 2014). In addition to histone acetylation, a set of H3K9me3-enriched genes with connection to neurological disorders linked to basal ganglia malfunction are known to have post-translational acetylation on the protein as well (Lopez-Atalaya et al., 2013; Valor et al., 2013a,b). To further explore the relationship between H3K9me3 enrichment and the set of 162 H3K9me3-enriched genes in the acetylation category, we carried out an integrated analysis and found that 138 of H3K9me3-enriched genes in acetylation category are detectable by RNA-Seq, whereas 24 genes are not detectable by RNA-Seq (**Supplemental Figure 4**). IPA analysis reveals that the 24 undetectable genes are involved in protein degradation and endocrine system development, while the 138 detectable genes are associated with signaling, nervous system development and function, as well as cellular assembly, maintenance, morphology, and organization. Since histone acetylation is highly associated with activation of gene expression and protein acetylation is critical for stability and localization of protein, we anticipate that the interplay between H3K9me3 and acetylation on common sets of genes may modulate the balance of long- and short-term repression.

DISCUSSION

Negative regulation of transcription is important in establishing and maintaining cell-type specific gene expression patterns. One such negative regulation can be achieved by H3K9me3 epigenetic repression. During development, many neuronal genes are subject to repression outside the nervous system to maintain neuronal specificity. For instance, neuronal genes in

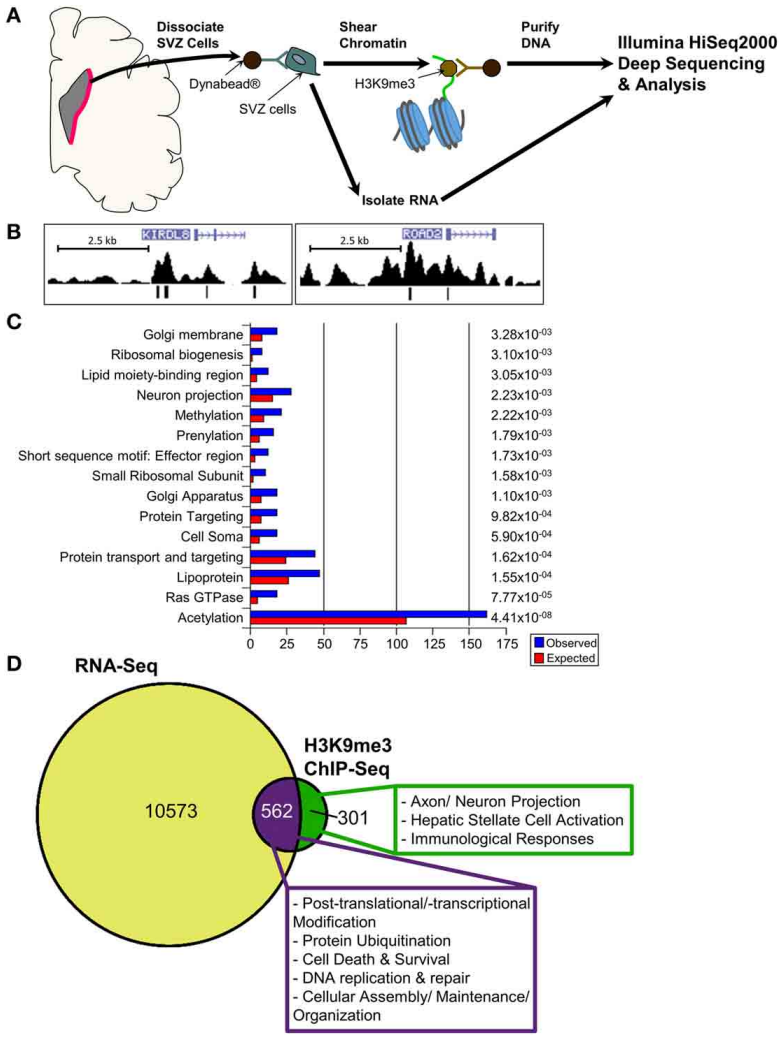

FIGURE 4 | ChIP-Seq and RNA-Seq analyses for *in vivo* SVZ cells from baboon brain. (A) Scheme displays purification of *in vivo* baboon SVZ cells and processing for ChIP-Seq with H3K9me3 antibody and for RNA-Seq. **(B)** Peak views of representative H3K9me3 enriched genes KIRDL8 and ROAD2. [⋙] within the genes indicate the transcript direction and strand sense. H3K9me3 signal is "mapped read density" after normalization to unmodified H3—vertical bars in peak track annotate significant reads within the density plot. The H3K9me3 peaks were called at FDR0.05, which is derived from a comparison of mapped read enrichment relative to a local background model based on the binomial distribution. **(C)** Top significant GO list for H3K9me3-enriched genes, *p*-value shown at right side. **(D)** RNA-Seq analysis uncovered 11099 genes in the undifferentiated SVZ cells are detectable at transcriptional level. While 562 detectable genes are enriched with H3K9me3 (purple portion), 301 of H3K9me3-enriched genes are not detectable (green portion). For characterization of these detectable or undetectable H3K9me3-enriched genes in baboon SVZ cells, IPA prediction reveals the top canonical networks and pathways under H3K9me3 regulation in the undifferentiated SVZ cells as shown in the box.

terminally differentiated fibroblasts are silenced. In this regard, the chromatin repressive mark H3K9me3 was identified to participate in either short- or long-term repression. Although epigenetic mechanisms controlling cell fate specification have been intensively studied in the developing embryonic central nervous system (Lim et al., 2009; Hirabayashi and Gotoh, 2010), whether the specification or maintenance of multipotency of neural progenitors in the adult brain relies on similar epigenetic regulations as in developing brains remains unknown. In this study, we demonstrate that H3K9me3 is associated with NSCs and is persistently enriched in the neuroblast population in both the adult rodent and baboon SVZ. Given the structural and genomic correlations between baboon and human, findings in baboon are more relevant to human with regards to the molecular targets of H3K9me3 in this germinal niche.

To uncover the molecular targets of H3K9me3 in adult baboon SVZ, we developed a technique to overcome the spatial complexity of the SVZ and to purify undifferentiated SVZ cells for genome-wide analysis. Our approach will be of considerable interest to those applying the genome-wide cutting edge techniques for cell lineage study. We identified that H3K9me3 is enriched for genes involved in the network of cell cycle and the signaling pathways of PTEN, MDM2, and AKT. These findings implicate that epigenetic regulation through

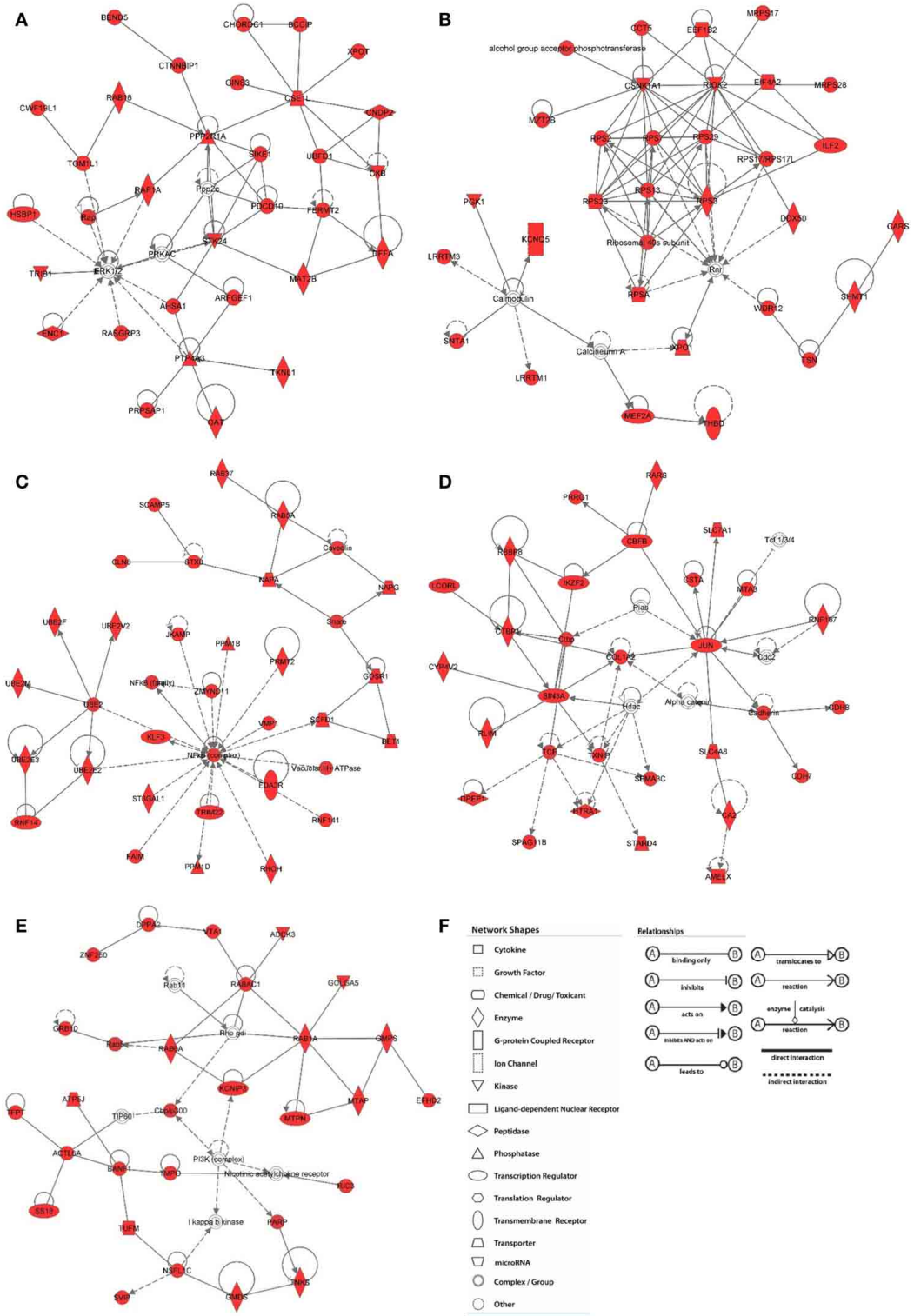

FIGURE 5 | Network analysis of H3K9me3 enriched genes in baboon SVZ cells. Top networks were predicted by Ingenuity Pathway Analysis software. The shaded focus genes (red highlight) were enriched with H3K9me3 identified by ChIP-Seq analysis. Node shape reflects the role of each element in the network and the direction and arrowhead shapes of each edge represent different types of interactions (key at panel **F**). **(A)** Cell morphology and organization; **(B)** RNA post-transcriptional modification; **(C)** Post-translational modifications; **(D)** Cell cycle; **(E)** Cellular growth and proliferation.

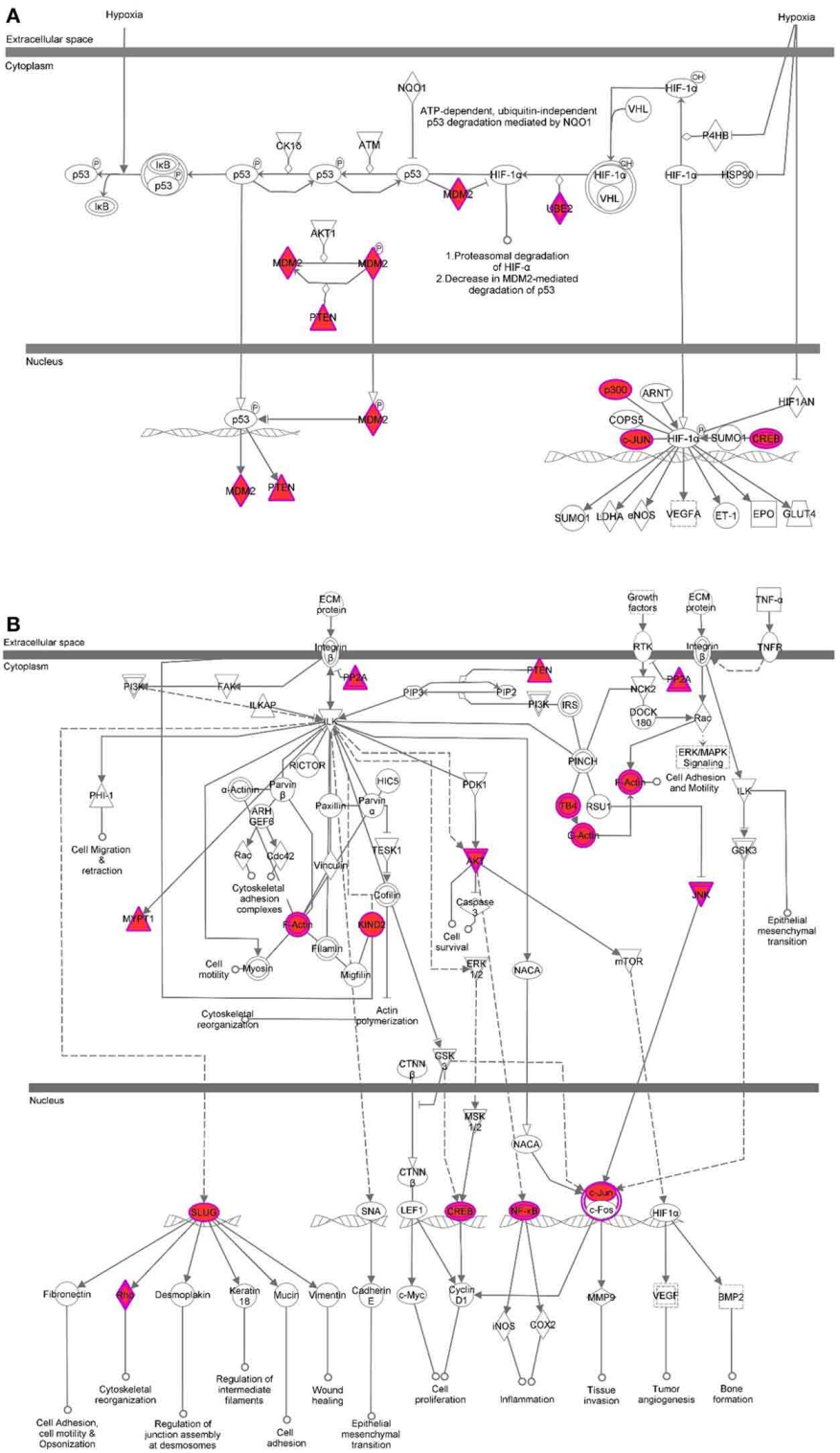

FIGURE 6 | Pathway analysis for H3K9me3 enriched genes in baboon SVZ cells. Top canonical pathways were predicted by Ingenuity Pathway Analysis software. The shaded focus genes (red highlight) were enriched with H3K9me3 identified by ChIP-Seq analysis. The node shape reflects the role of each element in the pathway and the direction and arrowhead shapes of each edge represent different types of interactions (see the key at **Figure 5F**). Representative signaling pathways involve AKT, c-JUN, CREB, MDM2, p300, PTEN, PP2A induced by either hypoxia or growth factors are illustrated **(A,B)**.

H3K9me3 is associated with cellular growth and proliferation. Intriguingly, significant sets of H3K9me3-enriched genes with identified functions in axon/neuron projections and hepatic or immunological activity are not expressed. As mature axon/neuron and non-neuronal genes should be silenced in this germinal niche, we reason that the repression through H3K9me3 is a mechanism to secure the identity of undifferentiated SVZ cells. In summary, as neurogenesis proceeds, numerous genes must go through active, poised, and repressed states to coordinate lineage commitment. The extent of neurogenesis can be inferred from integrated regulations through signaling pathways and different genetic/epigenetic mechanisms. Our findings suggest that H3K9me3 regulates adult neurogenesis, at least in part, by suppressing a subset of genes to maintain SVZ niche properties and to tightly regulate lineage specification in order to coordinate proper timing for adult neurogenesis.

MATERIALS AND METHODS

All animal experiments were approved by the guidelines of the Institutional Animal Care and Use Committee of the University of Texas at San Antonio (UTSA) and Texas Biomedical Research Institute/Southwest National Primate Research Center (SNPRC) at San Antonio.

IMMUNOSTAINING AND CONFOCAL IMAGING

Mouse

Mice were trans-cardially perfused and fixed using 1X $PBS^{(-)}$ and 4% paraformaldehyde (PFA), respectively. Brains were cryoprotected in 30% sucrose prior to OCT embedding. For co-localization staining, 12 μm frozen sections were processed for immunostaining with antibodies against H3K9me3 (Upstate #07-422; 1:500), Glial Fibrillary Acidic Protein (GFAP)—clone GA5 (Millipore #MAB3402; 1:500), Vimentin (Sigma V2258; 1:500), Polysialic Acid-NCAM (PSANCAM)—clone 2-2b (Millipore #MAB5324; 1:500), Mash1(Abcam #ab38556; 1:500), and EdU 5-ethynyl-2′-deoxyuridine (Life Technology, #C10337 Click-iT® EdU).

Baboon

Coronal slices of fresh baboon forebrain were taken to obtain the SVZ and adjacent brain regions, which were subsequently, fixed in 4% PFA overnight and then cryoprotected in 30% sucrose before OCT embedding. For co-localization staining, 60 μm floating sections were processed for immunostaining with the antibodies listed above.

Vectashield with DAPI (Vector Laboratories Ltd # H-1200) was used for mounting medium and nuclear counter stain. Secondary antibodies AlexaFIuor 488 (Molecular Probes, 1:1000) and AlexaFluor 594 (Molecular Probes 1:1000) were utilized for fluorescent labeling. Mouse SVZ images were acquired under a Zeiss510 confocal microscope (40X and 100X oil immersion objectives). Baboon SVZ images were taken under a Zeiss710 two-photon confocal microscope (40X and 100X oil immersion objectives). Z-stacks were projected to single plane using Zen 2012 (Carl Zeiss Microscopy; black edition). All images were analyzed using ImageJ (NIH; version 1.47).

CELL TYPES PURIFICATION FOR ChIP-Seq ANALYSIS

Antibody against SVZ cell type markers, such as GFAP, Vimentin, PSA-NCAM, or Doublecortin was manually conjugated to Dynabeads (Dynabeads®-Protein A, Life Technology). We then used the Dynabeads-conjugated antibody to purify cells immediately dissociated from SVZ microdissection. Briefly, cells from fresh dissected baboon SVZ were immediately dissociated with Accutase, subsequently equilibrated in binding buffer containing phosphate-buffered saline (PBS), saponin, 1X protease inhibitor cocktail (Roche), and subjected to Dynabeads-conjugated antibody purification. The purified cells were crosslinked in 1.1% formaldehyde before chromatin shearing by Diagenode Bioruptor. The resulting sheared chromatin fragments in a size range between 200–500 base pairs were then incubated with H3K9me3 antibody-conjugated Protein A Dynabeads overnight. Additional runs of ChIP-Seq were performed as replicates. Each replicate included independent sample preparation and a different vendor H3K9me3 antibody to ensure no bias was introduced through a specific antibody (Millipore/Upstate #07-442; Active Motif #39162; Life Technology Dynabeads protein A).

For normalization, the aliquot of sheared chromatin fragments were incubated with antibody against total histone 3- conjugated Protein A Dynabeads (unmodified H3 antibody, Millipore #05-499; 1:1000). Subsequently, enriched chromatin fragments were eluted, de-crosslinked purified for library preparation (Illumina Library Kit), and sequenced with 200 million tags through Illumina HiSeq2000 sequencer. 56 ng of H3K9me3 ChIP-DNA were applied for library preparation and 5pM of libraries were loaded into Illumina sequencer. The resulting 121,743,940 pass filter reads were aligned for peak calls.

SEQUENCE ALIGNMENT AND PEAK CALLING

The Rhesus macaque (rheMac2) gene annotation derived from the NCBI RefSeq project (http://nar.oxfordjournals.org/content/33/suppl_1/D501.full) was constructed and is maintained at the UCSC genome browser. Alignments were generated by using the Bowtie alignment program version 0.12.7., with a maximum of 2 mis-matches allowed in the mapping reads. Aligned read enrichments were detected using the MACS2 peak finding program. Peak calls were generated by normalizing to unmodified H3 DNA, and using an FDR of 0.05. ChIP-Sequencing raw data and processed peak calls have been deposited to GEO under the accession ID GSE59074.

GO, NETWORK, AND PATHWAY ANALYSIS

Duplicate gene references were removed prior to GO, network, and pathway analyses. DAVID Functional Annotation Tool (DAVID Bioinformatics Resources 6.7, NIAID/NIH) was utilized to perform GO analysis and significance cutoff was set at a p-value of <0.05. Network and canonical pathway analyses were performed using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, Redwood City, CA, USA). The top 5 networks and pathways presented in this paper were determined by IPA (p-value < 0.003 and Fischer's Test Score > 34, respectively).

RNA-Seq ANALYSIS

Total RNA was extracted from purified baboon SVZ cells using TRIzol reagent and sequencing libraries were generated with Illumina RNA-Seq library kit. Paired-end RNA-deepSeq (76 base pair; >300 million tag reads; 269,081,636 mapped reads) were aligned to hg19. DESeq was used to normalize raw read counts; and Cufflink reports read counts and estimated FPKM (fragments per kilobase of exon per million fragments mapped; http://cufflinks.cbcb.umd.edu/faq.html#fpkm). Genes with expression values >1 FPKM were considered for subsequent analyses. RNA-Sequencing data have been deposited to GEO under the accession ID GSE58527.

FLOW CYTOMETRY

Disassociated SVZ cells were incubated on ice for 10 min with anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen) to block FcRs. Cells were then incubated on ice for 1 h with primary antibodies (anti-H3K9me3, GFAP, Vimentin, PSA-NCAM, and Doublecortin), and subsequently labeled with Alexa Fluor 488- and PE-conjugated secondary antibodies (BD Biosciences). Primary antibody resources: H3K9me3 (Millipore/Upstate #07-442; 1:500); Doublecortin (Millipore clone2G5 #MABN707; 1:250); Polysialic Acid-NCAM, clone 2-2b (Millipore #MAB5324, Lot# 1966892; 1:250); Glial Fibrillary Acidic Protein, clone GA5 (Millipore #MAB3402, Lot#1993774; 1:250); Vimentin (Sigma V2258; 1:250); For analysis, controls including the positive controls stained with each antibody separately, isotype controls, and the unstained cells were used for gate compensation. Flow data was acquired on a LSR-II flow cytometer (BD Biosciences) configured with an argon 488 laser with a 505 LP dichroic and 525/50 filter to detect Alexa fluor 488 and a green 510 laser with a 735 LP dichroic and a 575/26 filter to detect PE. Compensation and data analysis was performed using FlowJo software (Tree Star, Inc, Ashland, OR).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00252/abstract

Supplemental Figure 1 | Protein Ubiquitination Pathway. One of top four canonical pathways predicted by Ingenuity Pathway Analysis for H3K9me3 enriched genes in baboon SVZ cells. The shaded focus genes (red highlight) were enriched with H3K9me3 identified by ChIP-Seq analysis. The node shape reflects the role of each element in the pathway and the direction and arrowhead shapes of each edge represent different types of interactions (see key at bottom-left panel).

Supplemental Figure 2 | P53 signaling pathway. One of top four canonical pathways predicted by Ingenuity Pathway Analysis for H3K9me3 enriched genes in baboon SVZ cells. The shaded focus genes (red highlight) were enriched with H3K9me3 identified by ChIP-Seq analysis. The node shape reflects the role of each element in the pathway and the direction and arrowhead shapes of each edge represent different types of interactions (see key in **Supplemental Figure 1**).

Supplemental Figure 3 | Hepatic stellate cell activation pathway. One of top four canonical pathways predicted by Ingenuity Pathway Analysis for H3K9me3 enriched genes in baboon SVZ cells. The shaded focus genes (red highlight) were enriched with H3K9me3 identified by ChIP-Seq analysis. The node shape reflects the role of each element in the pathway and the direction and arrowhead shapes of each edge represent different types of interactions (see key in **Supplemental Figure 1**).

Supplemental Figure 4 | The integrated analysis between RNA-Seq and the set of H3K9me3-enriched genes in the acetylation category from our GO analysis. 138 of H3K9me3-enriched genes in acetylation category are detectable by RNA-Seq, whereas 24 genes are not detectable by RNA-Seq. For characterization of these detectable or undetectable H3K9me3-enriched genes in baboon SVZ cells, IPA prediction reveals the top canonical networks and pathways as shown in the box.

Supplemental Table 1 | (i) Summary of Enriched Loci of H3K9me3; **(ii)** Gene Ontology by DAVID for H3K9me3-enriched genes in Baboon SVZ Cells; **(iii)** Integrated analyses for ChIP-Seq and RNA-Seq; **(iv)** A subset of H3K9me3-enriched genes was characterized as imprinting genes.

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Dynamic reprogramming of chromatin: paradigmatic palimpsests and HES factors

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Temporal and spatial control of transcription in development is dictated to a great extent by transcriptional repressors. Some repressor complexes, such as Polycomp-group proteins, induce relatively long-term non-permissive states, whereas others such as hairy/enhancer of split (HES) family repressors are linked to dynamically modulated chromatin states associated with cycling expression of target genes. The mode of action and specificity of repressors involved in mediating this latter form of epigenetic control are unknown. Oscillating expression of HES repressors controlled by signaling pathways such as Notch suggests that the entire ensemble of HES–associated co-repressors and histone modifying complexes readily cycle on and off genes. Dynamic interactions between these factors and chromatin seem to be crucial in maintaining multipotency of progenitor cells, but the significance of such interactions in more differentiated cells is less well understood. We discuss here how genome-wide analyses and real-time gene expression measurements of HES regulated genes can help decipher the detailed mechanisms and biological importance of highly dynamic transcriptional switching mediated by epigenetic changes.

Keywords: HES, hairy, oscillatory gene expression, repression, chromatin

INTRODUCTION

Dynamic cellular processes in biological systems require modulated and adaptable responses at the level of gene expression. Variations in the internal and external environment provoke short-and long-term changes in gene expression, which help maintain cellular physiology; these controls are also a fundamental point of evolutionary changes (López-Maury et al., 2008). Some variability in output of gene regulatory networks (GRN) is an inescapable consequence of molecular noise, including stochastic switching of promoter activity or "bursts." Such random fluctuations can be easily propagated to downstream genes or buffered out, and may play significant roles in physiological regulation, differentiation, adaptation and evolution (Eldar and Elowitz, 2010). In addition to the impact of stochastic molecular processes on gene expression, organisms from bacteria to animals have evolved a wide variety of specialized oscillatory gene expression mechanisms to respond to predictable and unpredictable environmental fluctuations and effect developmental programs (Young and Kay, 2001; Paszek et al., 2010). The levels of mechanistic complexity vary among oscillatory systems, but they share common regulatory principles, including negative feedback loops (**Figure 1A**). These core features were successfully used to design simple synthetic oscillatory networks that accurately predict the dynamic behavior of biological systems, which are generally more complex and feature robustness to genetic and environmental influences (Elowitz and Leibler, 2000; Cookson et al., 2009; Tigges et al., 2009).

DESIGN AND FUNCTION OF OSCILLATING GENE NETWORKS

A classic example of oscillatory transcriptional regulation is the ability of the circadian clock to adjust output of many genes in preparation for predictable daily changes in light, food, and temperature (Bell-Pedersen et al., 2005). Although regulation is highly complex, the core of the vertebrate molecular clock is based on transcriptional activation of genes under control of the CLOCK and BMAL1 activators. These factors drive expression of many genes during the day, including the PER and CRY repressors, which feedback inhibit and block CLOCK/BMAL1 action during the nighttime (**Figure 1B**; Ko and Takahashi, 2006; Baggs and Hogenesch, 2010). Repression is relieved by phosphorylation, ubiquitination, and degradation of PER and CRY, leading to a feedback loop with a period of ∼24 h (Busino et al., 2007).

Genome-wide studies have revealed associated rhythmic changes of histone marks corresponding to oscillatory expression of thousands of genes coordinating biological cycles through a complex regulatory network (Feng et al., 2011; Koike et al., 2012). A recent study from the Takahashi laboratory provided a comprehensive overview of chromatin-associated dynamics of circadian cycling in the murine liver. Using time-dependent ChIP-seq analysis of transcription factors (BMAL1, CLOCK, NPAS2, PER1, PER2, CRY1, CRY2, p300, and CBP), RNA Pol II, and histone marks (H3K9Ac, H3K27Ac, H3K4me1, H3K4me3, H3K36me3, and H3K79me2), the authors identified three phases in the circadian clock corresponding to genes in a transcriptionally poised, activated, and repressed states (Koike et al., 2012).

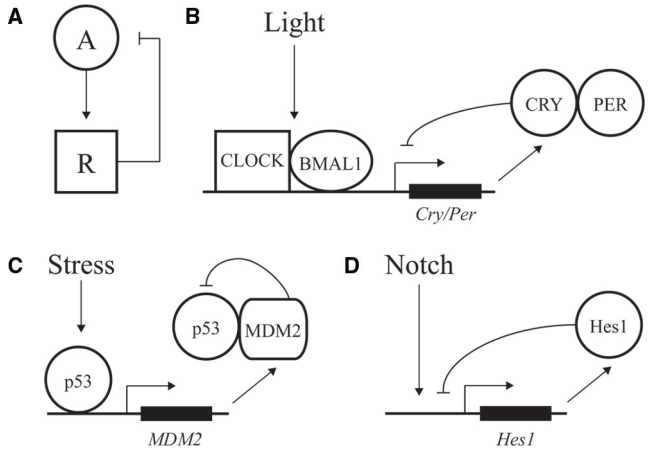

FIGURE 1 | Negative feedback loops at the core of transcriptional oscillators. (A) Diagram of a simple negative feedback loop for oscillatory behavior. An activator "A" increases activity of a repressor "R," which in turn decreases activity of the activator. **(B)** Major factors driving daily oscillations of the circadian clock, whereby CLOCK/BMAL1 drive expression of the inhibitory factors CRY/PER. **(C)** Stress and DNA damage activation of the p53 pathway, whereby 5–9 h. ultradian oscillations in p53 activity drive expression of p53 inhibitor MDM2. **(D)** Hes1 expression is driven by Notch signaling and feedback inhibited by Hes1, with an oscillation of ∼2–3 h.

In addition to predictable daily cycles, cells need to respond to rapid changes and variations during development and growth. Ultradian oscillations often feature a time period of minutes to hours and are triggered by intrinsic and environmental signals. One of the best described such instances is represented by the p53 pathway; this transcription factor can display dynamic behavior in response to DNA damage and other cellular stress to protect cells against malignant transformation (Batchelor et al., 2011). p53 expression is regulated by a negative feedback loop. The MDM2 regulator normally keeps p53 activity at low levels by binding to the factor's DNA binding domain, inducing a change in subcellular localization from the nucleus to the cytoplasm, and inducing ubiquitylation for eventual degradation of p53 (Wu et al., 1993; Haupt et al., 1997). After DNA damage, the p53 protein is phosphorylated, preventing the interaction of MDM2 with p53 and resulting in activation of p53 (Kruse and Gu, 2009). p53 transcriptionally activates expression of many genes including *MDM2*, resulting in a time-delay feedback inhibition that can exhibit oscillations of both p53 and Mdm2 (**Figure 1C**; Lev Bar-Or et al., 2000; Lahav et al., 2004; Bose and Ghosh, 2007). Depending on the dynamic control of p53, different cellular responses can be elicited. Cells can undergo a transient cell cycle arrest and recover from the DNA damage (Purvis et al., 2012). In addition to transient responses, the p53 pathway also triggers terminal fates such as apoptosis and senescence. In contrast to oscillatory output, sustained p53 expression affects the expression of a different set of genes, leading to senescence (Purvis et al., 2012). Therefore, depending on the dynamics of the input, distinct chromatin and regulatory changes can be imparted on a gene network to transmit information and alter cellular fate.

Oscillations are also seen in differentiation and embryonic development. One of the best-studied examples involves the transcriptional repressor Hes1 that controls the differentiation of neurons and formation of somite segments in the vertebrate hindbrain (**Figure 1D**; Kageyama et al., 2007; Koike et al., 2012). Hes1 belongs to the conserved family of hairy/enhancer of split (HES) transcriptional repressors that recruit common co-repressors of the Groucho/TLE family (Davis and Turner, 2001; Aloia et al., 2013). The eponymous Drosophila Hairy repressor functions as a so-called long-range repressor that remodels large blocks of chromatin upon transcriptional repression. Hairy mediates widespread and coupled loss of active histone marks H4Ac, H3K27Ac, H3K4me1, and H3K4me3 on many embryonic genes (Li and Arnosti, 2011; Kok et al., in review). Furthermore, Hairy represses its own transcription by removing these active marks, consistent with the previously observed autoregulatory mechanism of related mammalian HES proteins (Kageyama et al., 2007).

A conserved feature of regulatory pathways involving HES proteins is the role of Notch signaling. Upon ligand binding, Notch is cleaved and released from the plasma membrane to translocate to the nucleus, where it associates with and activates the *Hes1* promoter. Hes1 protein negatively regulates its own promoter, establishing a feedback loop (Fischer and Gessler, 2007). This feedback loop can induce oscillations in Hes1 protein levels (Kageyama et al., 2007). Periodic temporal expression of Hes1 plays a crucial role in formation of somites, which give rise to the vertebrae, ribs, skeletal muscles and dermis (Aulehla and Herrmann, 2004). These segments are formed from the anterior region of the presomitic mesoderm (PSM) by periodic Notch signals. Notch coordinates Hes1 oscillations, which progress from the posterior to anterior region of the PSM. One wave of expression of this so-called segmentation clock lasts 2 h, marking the boundary for a new somite that forms at the end of the embryo (Pourquié, 2003). In this setting, temporal oscillations are converted into a spatial pattern of somite boundaries. A large number of genes involved in cell signaling are periodically expressed during this segmentation process in mouse (Dequéant et al., 2006). Comparison of the mouse, chicken and zebrafish PSM oscillatory transcriptomes revealed networks of 40–100 conserved cycling genes that are activated downstream of the Notch, Fibroblast Growth Factor and Wnt pathways (Krol et al., 2011). Thus, the segmentation clock is controlled by conserved multiple signaling pathways. The common oscillatory genes in all vertebrates include at least one member of the Hes/Her family. However, the identity of cyclic genes varies from species to species as well, indicating evolutionary plasticity of the segmentation networks (Krol et al., 2011).

In contrast to the fate-determining effects of Hes1 oscillations in the PSM, cyclic behavior of Hes1 in neuronal progenitor cells (NPC) is associated with stabilization of the undifferentiated phenotype. In these cells, Hes1 mRNA, protein, and activity oscillate with a 2 h period (Hirata et al., 2002). Hes1 represses transcription of proneural transcription factors such as *Ascl1*, inducing oscillations in levels of that factor. Interestingly, self-renewal of NPCs and their eventual proper differentiation is achieved only when Hes1 and downstream genes are periodically expressed (Imayoshi and Kageyama, 2014). Sustained expression of Hes1 constitutively

in NPCs represses proneural genes, blocking proliferation and inducing quiescence (Baek et al., 2006). This observation indicates that active division of NPCs is dependent on the oscillatory expression of fate determination factors. Neuronal fate choice is determined by sustained expression of Ascl1 after cell division. During differentiation, Hes1 oscillations cease as Notch inputs diminish, leading to upregulation of Ascl1 (Imayoshi et al., 2013). Using a light-activatable system, the impact of oscillating and sustained expression of Ascl1 on proliferation and differentiation of NPCs was tested. A 3 h periodic expression of Ascl1 supported proliferation of NPCs, whereas sustained expression resulted in differentiation (Imayoshi et al., 2013). Similar roles for Hes1 oscillation has been observed in embryonic stem cells (Kobayashi et al., 2009).

The types of chromatin dynamics occurring on genes entrained under the circadian clock system have not been well documented for oscillations involving ultradian factors such as HES proteins and other bHLH transcription factors. However, a recent study suggested that the Ascl1 bHLH factor, which shows oscillatory expression complementary to that of Hes1 in neuronal progenitors, is critical for formation of open chromatin during reprogramming through its activities as a pioneer factor on enhancers (Wapinski et al., 2013). Less is known about the chromatin modifying properties of Hes1 itself, however, the homologous Drosophila protein Hairy has a direct role in chromatin modification, and this protein impacts the chromatin state of hundreds of loci on a genome-wide scale (Li and Arnosti, 2011; Kok et al., in review). As HES transcription factors share common structural features, including DNA binding and effector domains, as well as conserved developmental roles, the biochemical properties are likely to be similar.

How general are the dynamic chromatin responses associated with activation and repression of genes such as those targeted by HES factors? The time-delays associated with activating or repressing promoters are a function of dynamics of protein complexes. Even in steady-state situations, transcription factors are observed to continuously associate and dissociate with target loci, a feature not revealed by ChIP experiments but that is demonstrated by direct imaging as well as *in vitro* approaches (Voss and Hager, 2014). However, as observed for the prolactin promoter, stochastic chromatin processes can render promoters refractory to stimulation. Such refractory periods would block transmission of dynamic signals (Harper et al., 2011). Indeed, high-resolution temporal measurement of mRNA of many mammalian genes from single cells reveals that distinct regulatory regions confer gene-specific switching rates with different refractory periods (Suter et al., 2011). Such differences may cause differential oscillation of genes in response to stimuli. Fine time-scale analysis of global gene expression triggered by the inflammatory cytokine TNF showed oscillations in $>$ 5000 genes that are involved in multiple pathways, with different genes oscillating either very rapidly or after a lag phase (Sun et al., 2008). Cyclic interaction of transcription factors with promoters can extend from seconds for bursting promoters to minutes for developmental oscillators to hours for circadian clocks. A single promoter may experience both fast (2 min) and slow (40 min) periodic binding of a single transcription factor, as with Ace1 occupancy of the yeast *CUP1* promoter (Karpova et al., 2008). The authors suggest that fast cycling is responsible for the initial period of gene expression, while slow cycling represents the fine-tuning of expression levels associated with slow-period oscillating nucleosome occupancy. A short-period ultradian cycling has also been described for the estrogen receptor, involving periodic binding and assembly of chromatin complexes in mammalian cells, however, recent high-resolution studies of RNA polymerase activity have not supported this picture (Hah et al., 2011; Voss and Hager, 2014).

In development, oscillatory circuits affect not only specific networks of genes relating to patterning, as described for Hes1, but also can include many synchronized genes not linked to circadian control. Large-scale transcriptome analysis in *C. elegans* larvae revealed robust ∼8 h cycling of thousands of genes, which may be related to developmental processes such as molting (Hendriks et al., 2014). In contrast to the simple synthetic biology circuits tested in bacteria, such large-scale oscillatory behavior likely involves more components than a single negative feedback loop (Sun et al., 2008). The coordinated expression of many genes in these systems indicates that persistent chromatin changes are not likely to prevent genome-wide oscillatory coordination, thus the dynamic chromatin changes found for HES factors are likely to be representative of many regulatory mechanisms.

OSCILLATORY BEHAVIOR AND CHROMATIN DYNAMICS

The biochemical mechanisms by which transcriptional oscillations can be induced are in many cases better understood than the physiological significance of such dynamics. In the case of circadian regulated genes, adaptation to predictable environmental changes, such as food availability, temperature or light, is a clear driver of such dynamics. In development, the dynamic readout of HES activity represents a morphological pattern generator. In other cases, it is not clear whether the cycling is a necessary feature of the system, or tolerated as an also-acceptable form of control that may or may not have superior regulatory properties. Arguing against a view that cycling occurs by chance is the likelihood that randomly propagated oscillations though a multi-level network should eventually cancel out, thus it is likely that there is selection for coordinated responses at some level. Depending on the nature of downstream targets, cycles of transcriptional output may be "integrated" to a steady-state approximation of the average level of signaling, or it may be "propagated," if dynamics of the downstream gene expression is as fast as the cycling signal (Hoffmann, 2002; **Figure 2A**).

Oscillatory behavior may be eventually damped by several layers of a gene regulatory cascade. For example, in the case of cyclical expression of Hes1, expression of several downstream targets also alternates, but the overall undifferentiated state of the cell—represented by the global activity or inactivity of many genes—stays constant, indicating that at least at a larger scale, such oscillatory behavior is subsumed into a stable phenotype. Alternatively, the oscillatory action at one level of a GRN may better ensure that a particular level of expression within a critical range is maintained, rather like a singer who uses vibrato to hold a particularly difficult note (Imayoshi et al., 2013). At the same time, the interlocking feedback loops that permit oscillation also provide the control points that can be shifted to move a

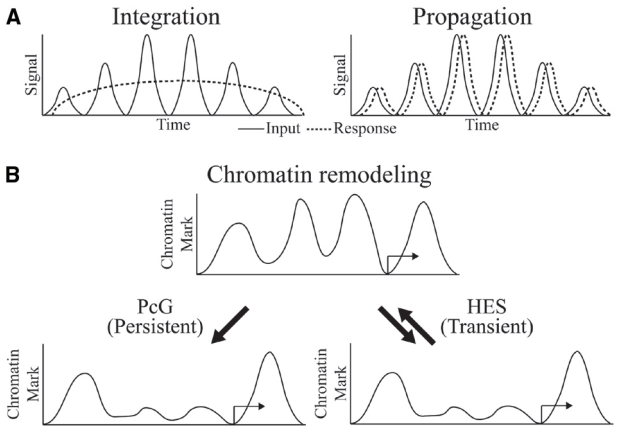

FIGURE 2 | Oscillator inputs and possible outcomes. (A) The oscillating input signal can be integrated or propagated to generate a sustained or dynamic response, respectively. Time on the x-axis might be minutes to hours. **(B)** Outcome at the chromatin level might be persistent and long-term in integrated response by PcG regulation or transient and reversible in propagated response by HES regulation.

cell into a different gene regulatory, and eventually differentiated state. These arguments are attractive in pointing out possible adaptive features of oscillatory regulation, however, testing the null hypothesis is difficult. It may be that just as transcriptional "bursting" is an inevitable consequence of micro-scale chromatin movements, longer period, regular transcriptional oscillations may be system properties that arise as a secondary consequence of core properties of the system, such as robustness. Alternatively, or in addition, many oscillations that are observed are consequences of a few key dynamic drivers that must show periodic changes; the ancillary downstream changes may not important for natural selection acting on gene expression (Paszek et al., 2010; Cheong and Levchenko, 2010).

What is known about the required chromatin dynamics that are associated with oscillatory gene regulation? Circadian regulated genes exhibit cyclical chromatin responses that reset every day (Koike et al., 2012). In the developmental settings for Hairy and HES protein activity, the targets of these proteins are often active only transiently, implying very dynamic chromatin responses. For instance, the activators of *ftz*, a gene that is repressed by Hairy, are present on the genome for only minutes during early embryogenesis, and repressive countermeasures would be required only for a similarly brief time. Indeed, we find that in cases of artificial induction of Hairy, dramatic chromatin deacetylations are quickly reversed as soon as Hairy levels drop, indicating that the repressor is working against a background of cellular chromatin modifying activities that quickly restore a landscape to the status quo ante (K. Kok, unpublished observations). Hes1 action, although not studied at the chromatin level, must similarly be transient in terms of perdurance, as downstream transcriptional targets quickly follow changes in the levels of Hes1 over a period of hours. Thus, in general, HES protein directed alterations to genome-wide chromatin states may be very transient (**Figure 2B**). In some regulatory circuits, we do know that chromatin states are locked in, preserving a particular epigenetic mark through multiple mitoses—these markers involve Polycomb complexes in Drosophila and higher metazoans, as well as DNA methylation signals in vertebrates. Significantly, both of these systems can be deployed in alternate modes, so that in some instances DNA methylation and Polycomb-regulated effects are transient (Aloia et al., 2013). Are global chromatin modifications just reflections of gene regulatory effects rather than drivers of the system? To what extent are these chromatin changes important for setting the boundary conditions for oscillatory gene responses? Systems and synthetic biology approaches will converge with developmental gene regulation to deliver answers to these intriguing questions.

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