Control of the Embryonic Stem Cell State

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Embryonic stem cells and induced pluripotent stem cells hold great promise for regenerative medicine. These cells can be propagated in culture in an undifferentiated state but can be induced to differentiate into specialized cell types. Moreover, these cells provide a powerful model system for studies of cellular identity and early mammalian development. Recent studies have provided insights into the transcriptional control of embryonic stem cell state, including the regulatory circuitry underlying pluripotency. These studies have, as a consequence, uncovered fundamental mechanisms that control mammalian gene expression, connect gene expression to chromosome structure, and contribute to human disease.

Introduction

Embryonic stem cells (ESCs) are pluripotent, self-renewing cells that are derived from the inner cell mass (ICM) of the developing blastocyst. Pluripotency is the capacity of a single cell to generate all cell lineages of the developing and adult organism. Self-renewal is the ability of a cell to proliferate in the same state. The molecular mechanisms that control ESC pluripotency and self-renewal are important to discover because they are key to understanding development. Because defects in development cause many different diseases, improved understanding of control mechanisms in pluripotent cells may lead to new therapies for these diseases.

ESCs have a gene expression program that allows them to self-renew yet remain poised to differentiate into essentially all cell types in response to developmental cues. Recent reviews have discussed ESCs and developmental potency (Rossant, 2008), the nature of the pluripotent ground state of ESCs (Silva and Smith, 2008), ESC transcriptional regulatory circuitry (Chen et al., 2008a; Jaenisch and Young, 2008; Macarthur et al., 2009; Orkin et al., 2008), the influence of extrinsic factors on pluripotency (Pera and Tam, 2010), and cellular reprogramming into ESC-like states (Hanna et al., 2010; Stadtfeld and Hochedlinger, 2010; Yamanaka and Blau, 2010). This Review provides a synthesis of key concepts that explain how pluripotency and self-renewal are controlled transcriptionally. These concepts have emerged from genetic, biochemical, and molecular studies of the transcription factors, cofactors, chromatin regulators, and noncoding RNAs (ncRNAs) that control the ESC gene expression program.

The regulators of gene expression programs can participate in gene activation, establish a poised state for gene activation in response to developmental cues, or contribute to gene silencing (Figure 1). The molecular mechanisms by which these regulators generally participate in control of gene expression are the subject of other reviews (Bartel, 2009; Bonasio et al., 2010; Fuda et al., 2009; Ho and Crabtree, 2010; Li et al., 2007; Roeder, 2005; Surface et al., 2010; Taatjes, 2010). I describe here the regulators that have been implicated in control of ESC state and discuss how they contribute to the gene expression program of pluripotency and self-renewal.

The Stem Cell State

ESCs are perhaps unique in that they've been the subject of virtually every scale of investigation from large-scale genomics and protein-DNA interaction studies to highly focused mechanistic studies of individual regulatory factors. The combined results of these systems-level and molecular approaches offer a definition of embryonic stem cell state in terms of global gene regulation, which serves as both a baseline for understanding the changes that occur as cells differentiate and develop and as a means to understand the basic biology of these cells. For the purposes of this Review, this "state" is the product of all the regulatory inputs that produce the gene expression program of pluripotent, self-renewing cells. The most important regulatory inputs in ESCs appear to come from a small number of "core" transcription factors acting in concert with other transcription factors, some of which are terminal components of developmental signaling pathways.

Transcription Factors

Transcription factors recognize specific DNA sequences and either activate or prevent transcription. Early studies into the transcriptional control of the *E. coli* lac operon created the framework for understanding gene control (Jacob and Monod, 1961). In the absence of lactose, the lac operon is repressed by the Lac repressor, which binds the lac operator and inhibits transcription by RNA polymerase. In the presence of lactose, the Lac repressor is lost and gene expression is activated by a transcription-activating factor that binds a nearby site and recruits RNA polymerase. The fundamental concept that emerged from these studies—that gene control relies on specific repressors and activators and the DNA sequence elements they recognize—continues to provide the foundation for understanding control of gene expression in all organisms.
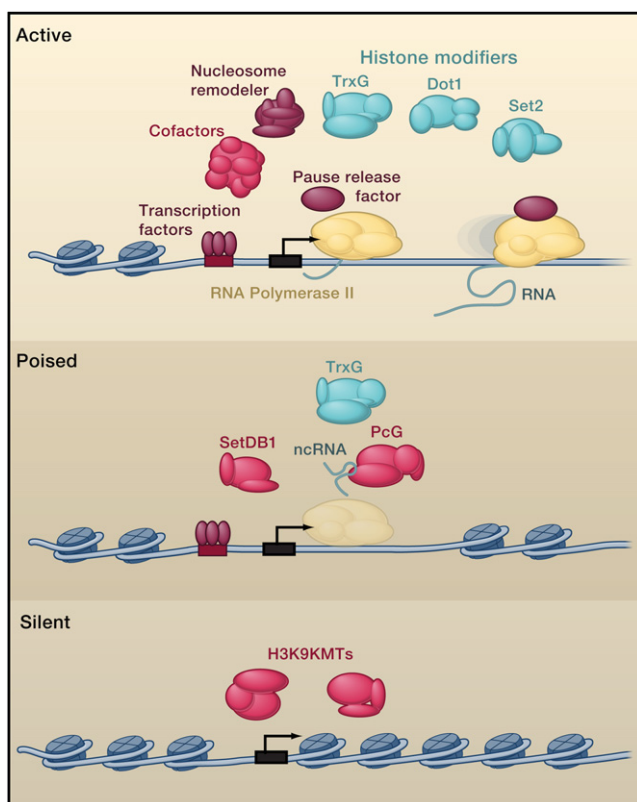
Figure 1. Models for Transcriptionally Active, Poised, and Silent Genes

Transcription factors, cofactors, chromatin regulators, and ncRNA regulators can be found at active, poised, and silent genes. At active genes, enhancers are typically bound by multiple transcription factors, which recruit cofactors that can interact with RNA polymerase II at the core promoter. RNA polymerase II generates a short transcript and pauses until pause-release factors and elongation factors allow further transcription. Chromatin regulators, which include nucleosome-remodeling complexes such as Swi/Snf complexes and histone-modifying enzymes such as TrxG, Dot1, and Set2, are recruited by transcription factors or the transcription apparatus and mobilize or modify local nucleosomes. Poised genes are rapidly activated when ESCs are stimulated to differentiate. At poised genes, transcription initiation and recruitment of TrxG can occur, but pause release, elongation, and recruitment of Dot1 and Set2 do not occur. The PcG and SetDB1 chromatin regulators can contribute to this repression, and these can be recruited by some transcription factors and by ncRNAs. The RNA polymerase II "ghost" in this model of poised genes reflects the low levels of the enzyme that are detected under steady-state conditions. Silent genes show little or no evidence of transcription initiation or elongation and are often occupied by chromatin regulators that methylate histone H3K9 and other residues. Some of these silent genes are probably silenced by mechanisms that depend on transcription of at least a portion of the gene (Buhler and Moazed, 2007; Grewal and Elgin, 2007; Zaratiegui et al., 2007).

In mammals, transcription factors make up the largest single class of proteins encoded in the genome, representing approximately 10% of all protein-coding genes (Levine and Tjian, 2003; Vaquerizas et al., 2009). Transcription factors bind both to promoter-proximal DNA elements and to more distal regions that can be nearby or 100s of kb away. The elements that are involved in positive gene regulation are called enhancers, and these elements are generally bound by multiple transcription

factors. Transcription factors can activate gene expression by recruiting the transcription apparatus and/or by stimulating release of RNA polymerase II from pause sites (Fuda et al., 2009). They can also recruit various chromatin regulators to promoter regions to modify and mobilize nucleosomes in order to increase access to local DNA sequences (Li et al., 2007).

In ESCs, the pluripotent state is largely governed by the core transcription factors Oct4, Sox2, and Nanog (Table 1) (Chambers and Smith, 2004; Niwa, 2007; Silva and Smith, 2008). Oct4 and Nanog were identified as key regulators based on their relatively unique expression pattern in ESCs and genetic experiments showing that they are essential for establishing or maintaining a robust pluripotent state (Chambers et al., 2003; Chambers and Smith, 2004; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000). Oct4 functions as a heterodimer with Sox2 in ESCs, thus placing Sox2 among the key regulators (Ambrosetti et al., 2000; Avilion et al., 2003; Masui et al., 2007). Reprogramming of somatic cells into induced pluripotent stem (iPS) cells generally requires forced expression of Oct4 and Sox2, unless endogenous Sox2 is expressed in the somatic cell, consistent with the view that Oct4/Sox2 are key to establishing the ESC state (Hanna et al., 2010; Stadtfeld and Hochedlinger, 2010; Yamanaka and Blau, 2010). Although ESCs can be propagated in the absence of Nanog (Chambers et al., 2007), Nanog promotes a stable undifferentiated ESC state (Chambers et al., 2007), is necessary for pluripotency to develop in ICM cells (Silva et al., 2009), and co-occupies most sites with Oct4 and Sox2 throughout the ESC genome (Marson et al., 2008b), so it is included here as a component of the core regulatory circuitry.

Core Regulatory Circuitry

Two key concepts dominate our understanding of the function of the core transcription factors Oct4, Sox2, and Nanog in control of ESC state (Figure 2): (1) The core transcription factors function together to positively regulate their own promoters, forming an interconnected autoregulatory loop. (2) The core factors co-occupy and activate expression of genes necessary to maintain ESC state, while contributing to repression of genes encoding lineage-specific transcription factors whose absence helps prevent exit from the pluripotent state.

The interconnected autoregulatory loop formed by Oct4, Sox2, and Nanog generates a bistable state for ESCs: residence in a positive-feedback-controlled gene expression program when the factors are expressed at appropriate levels, versus entrance into a differentiation program when any one of the master transcription factors is no longer functionally available (Boyer et al., 2005; Loh et al., 2006). This regulatory circuit likely explains the ability to jump-start the ESC gene expression program during reprogramming by forced expression of reprogramming factors (Jaenisch and Young, 2008). Thus, the ectopically expressed factors activate transcription of the endogenous *Pou5f (Oct4)*, *Sox2*, and *Nanog* genes and thereby initiate the positive-feedback loop that sustains ongoing production of these factors from the endogenous genes in the absence of further input from the ectopically expressed factors. Some factors present in reprogramming cocktails, such as c-Myc, appear to facilitate activation of this interconnected autoregulatory circuitry by stimulating gene expression and proliferation more generally (Rahl et al., 2010).

Table 1. Transcriptional Regulators Implicated in Control of ESC State

Type of Regulator	Function	References
Transcription Factors		
Oct4	Core circuitry	1
Sox2	Core circuitry	2
Nanog	Core circuitry	3
Tcf3	Wnt signaling to core circuitry	4
Stat3	Lif signaling to core circuitry	5
Smad1	BMP signaling to core circuitry	6
Smad2/3	TGF-β/Activin/Nodal signaling	7
c-Myc	Proliferation	8
Esrrb	Steroid hormone receptor	9
Sall4	Embryonic regulator	10
Tbx3	Mediates LIF signaling	11
Zfx	Self-renewal	12
Ronin	Metabolism	13
Klf4	LIF signaling	14
Prdm14	ESC identity	15
Cofactors		
Mediator	Core circuitry	16
Cohesin	Core circuitry	17
Paf1 complex	Couples transcription with histone modification	18
Dax1	Oct4 inhibitor	19
Cnot3	Myc/Zfx cofactor	20
Trim28	Myc/Zfx cofactor	21
Chromatin Regulators		
Polycomb group	Silencing of lineage-specific regulators	22
SetDB1 (ESET)	Silencing of lineage-specific regulators	23
esBAF	Nucleosome mobilization	24
Chd1	Nucleosome mobilization	25
Chd7	Nucleosome mobilization	26
Tip60-p400	Histone acetylation	27
ncRNA Regulators		
miRNAs	Fine-tuning of pluripotency transcripts	28
GC-rich ncRNAs	PcG complex recruitment	29

The vast majority of these regulators were identified in murine ES cells, but most appear to play similar roles in human ES cells. LIF-Stat3 signaling is important for maintenance of murine ESCs and Activin-Smad2/3 signaling has been demonstrated to be important for human ESCs.

References: 1 (Chambers et al., 2003; Chambers and Smith, 2004; Hart et al., 2004; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000; Scholer et al., 1990); 2 (Chambers and Smith, 2004; Masui et al., 2007); 3 (Chambers et al., 2003; Hart et al., 2004; Mitsui et al., 2003); 4 (Cole et al., 2008; Marson et al., 2008b); 5 (Niwa et al., 1998); 6 (Ying et al., 2003); 7 (Beattie et al., 2005; James et al., 2005; Vallier et al., 2005), 8 (Cartwright et al., 2005); 9 (Ivanova et al., 2006; Zhang et al., 2008); 10 (Wu et al., 2006; Zhang et al., 2006); 11 (Han et al.; Ivanova et al., 2006; Niwa et al., 2009), 12 (Galan-Caridad et al., 2007); 13 (Dejosez

Oct4, Sox2, and Nanog collaborate to activate a substantial fraction of the actively transcribed protein-coding and miRNA genes in ESCs (Figure 3A) (Chen et al., 2008b; Marson et al., 2008b). Sites co-occupied by the three core regulators generally have enhancer activity, and transcription of genes adjacent to such sites often depends on at least one of the trio (Chen et al., 2008b; Chew et al., 2005; Matoba et al., 2006). Oct4 and Nanog can bind and recruit multiple coactivators, as described below, accounting for their ability to activate genes.

Oct4, Sox2, and Nanog also occupy repressed genes encoding cell-lineage-specific regulators, and the repression of these genes is essential for ESCs to maintain a stable pluripotent state and to undergo normal differentiation (Bilodeau et al., 2009; Boyer et al., 2005, 2006; Lee et al., 2006; Loh et al., 2006; Marson et al., 2008b; Pasini et al., 2008; Pasini et al., 2004). The loss of these core regulators leads to rapid induction of a wide spectrum of genes encoding lineage-specific regulators, indicating that these genes are poised for activation.

How might Oct4/Sox2 and Nanog act to repress these genes? The SetDB1 and Polycomb group (PcG) chromatin regulators have both been implicated in repression of these lineage-specific regulatory genes. Oct4 can bind sumoylated SetDB1, which catalyzes the repressive histone modification H3K9me3 at many of these genes (Bilodeau et al., 2009; Yeap et al., 2009; Yuan et al., 2009). PcG complexes can associate with nucleosomes with histone H3K9me3 (Margueron et al., 2009) and further contribute to repression through mechanisms described below. It is also possible that Oct4, Sox2, and Nanog activate some level of transcription initiation in the extensive GC-rich promoter regions of these genes. The corresponding GC-rich RNA species produced from these regions might then recruit or stabilize PcG complexes (Guenther and Young, 2010; Zhao et al., 2010). Thus, Oct4 and its partners may recruit SetDB1 through protein-protein interactions and PcG complexes via interactions with both histone H3K9me3 and transcripts produced as a consequence of local transcription activation.

The ability of Oct4, Sox2, and Nanog to positively regulate genes necessary to maintain ESC state while repressing genes that would enable egress from this state explains, in part, the ability of ESCs to self-renew in an undifferentiated state yet remain poised to differentiate into all cell types of the body in response to developmental cues. Additional regulators of gene expression are known to collaborate with Oct4, Sox2, and Nanog to control the ESC gene expression program (Table 1). Many of these regulators have emerged from systems-level
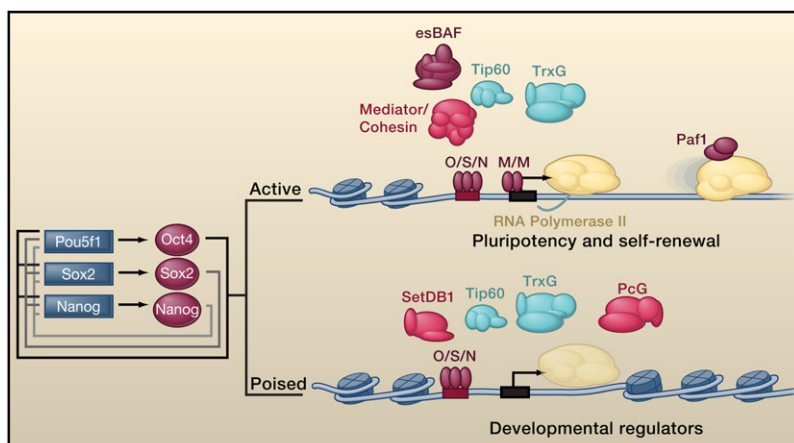
et al., 2008; Dejosez et al., 2010); 14 (Jiang et al., 2008; Niwa et al., 2009); 15 (Chia et al., 2010); 16 (Hu et al., 2009; Kagey et al., 2010); 17 (Fazzio et al., 2008; Hu et al., 2009; Kagey et al., 2010); 18 (Ding et al., 2009); 19 (Kim et al., 2008; Niakan et al., 2006; Sun et al., 2009); 20 (Hu et al., 2009); 21 (Fazzio et al., 2008; Hu et al., 2009); 22 (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Leeb et al., 2010; Li et al., 2010; Pasini et al.; Peng et al., 2009; Shen et al., 2009; Stock et al., 2007; van der Stoop et al., 2008); 23 (Bilodeau et al., 2009; Yeap et al., 2009; Yuan et al., 2009); 24 (Ho and Crabtree, 2010; Schnetz et al., 2010); 25 (Gaspar-Maia et al., 2009); 26 (Schnetz et al., 2010); 27 (Fazzio et al., 2008); 28 (Marson et al., 2008b); 29 (Guenther and Young, 2010; Surface et al., 2010).

Figure 2. Core Regulatory Circuitry
Oct4, Sox2, and Nanog collaborate to regulate their own promoters, forming an interconnected autoregulatory loop. The Pou5f (Oct4), Sox2, and Nanog genes are represented as blue boxes and proteins as red balloons. These core transcription factors (O/S/N) function to activate expression of protein-coding and miRNA genes necessary to maintain ESC state, but they also occupy poised genes encoding lineage-specific protein and miRNA regulators whose repression is essential to maintaining that state. Additional transcription factors, such as the c-Myc/Max heterodimer (M/M), cause pause release at actively transcribed genes. A subset of the cofactors and chromatin regulators implicated in control of ES cell state (Table 1) are shown.

genetic and proteomic screens (Bilodeau et al., 2009; Chia et al., 2010; Fazzio et al., 2008; Hu et al., 2009; Ivanova et al., 2006; Kagey et al., 2010; Liang et al., 2008; Pardo et al., 2010; van den Berg et al., 2010; Wang et al., 2006; Zhao et al., 2010). Although the roles of these regulators are not yet fully understood, they ultimately exert their effects by regulating RNA polymerase II at various steps in transcription.

Control of RNA Polymerase II

Transcription factors control at least two major steps in gene expression (Fuda et al., 2009; Peterlin and Price, 2006; Rahl et al., 2010). Some transcription factors recruit RNA polymerase II to promoters, where the enzyme typically transcribes a short distance (approximately 35 bp) and then pauses or terminates. Other transcription factors recruit a cyclin-dependent kinase (Cdk9/cyclinT) called p-TEFb, which phosphorylates the polymerase and its associated pause control factors, allowing the enzyme to be released from pause sites and fully transcribe the gene. Oct4, Sox2, and Nanog interact with coactivators that bind to RNA polymerase II (Kagey et al., 2010), so the core regulators are involved in RNA polymerase II recruitment. In contrast, c-Myc, which plays important roles in ESC proliferation and self-renewal (Cartwright et al., 2005), does not appear to play an important role in RNA polymerase II recruitment but rather binds to E box sequences at core promoter sites and recruits p-TEFb, thus stimulating pause release (Rahl et al., 2010). A large proportion of the actively transcribed genes in ESCs are bound and regulated by both the core transcription factors and c-Myc (Figure 3A). Thus, Oct4/Sox2/Nanog apparently play dominant roles in selecting the set of ESC genes that will be actively transcribed and recruiting RNA polymerase II to these genes, while c-Myc regulates the efficiency with which these selected genes are fully transcribed. This likely explains why forced expression of c-Myc can enhance reprogramming efficiency and why this transcription factor plays such a potent role in proliferation of many cancer cells (Jaenisch and Young, 2008; Rahl et al., 2010).

Multiple Enhancers and Enhanceosomes

Enhancers are generally bound by multiple transcription factors, forming large nucleoprotein complexes called enhanceosomes, which permit cooperative binding between transcription factors and allow for synergistic and combinatorial effects on gene regulation (Maniatis et al., 1998). The cooperative interactions among transcription factors binding to adjacent DNA sites and to cofactor complexes explains why multiple transcription factors are found together in the genome and why transcription factors bind stably to only a small subset of the millions of DNA sequence motifs present in the vertebrate genome. Many genes have multiple enhancers and thus multiple enhanceosomes (Levine and Tjian, 2003). In *Drosophila*, these multiple, seemingly redundant enhancers have been shown to contribute to phenotypic robustness during embryonic development (Frankel et al., 2010; Hong et al., 2008). That is, normal levels of gene expression are obtained despite environmental and genetic variability so long as genes are equipped with multiple enhancers.

In addition to Oct4, Sox2, Nanog, and c-Myc, the transcription factors Tcf3, Smad1, Stat3, Esrrb, Sall4, Tbx3, Zfx, Ronin, Klf2, Klf4, Klf5, and PRDM14 have been shown to play important roles in control of ESC state (Table 1). The ChIP-Seq data that have been obtained for these transcription factors indicate that they can bind to loci occupied by Oct4/Sox2/Nanog as well as other loci (Figure 3B), forming sites that have been called multiple transcription factor-binding loci (MTL) (Chen et al., 2008b; Kim et al., 2008). Several lines of evidence indicate that most MTL are enhancers. Most MTL are occupied by the p300 cofactor, and the subset of MTL that are occupied by Oct4/Sox2/Nanog are also occupied by the mediator cofactor (Chen et al., 2008b; Kagey et al., 2010). All Oct4/Sox2/Nanog-containing MTL tested to date have been shown to exhibit enhancer activity (Chen et al., 2008b). It is therefore likely that functional enhanceosomes are formed at most MTL.

The evidence obtained thus far suggests that most Oct4/Sox2/Nanog-regulated genes are co-occupied by one or more of the other transcription factors implicated in control of ESCs (Figures 3A–3C). Examination of the Max gene reveals a typical pattern, where the promoter region contains a site bound by Oct4, Sox2, Nanog, Tcf3, and Essrb and various other sites occupied by c-Myc, Zfx, Ronin, and Klf4 (Figure 3D). Thus the functions of the core regulators (Oct4, Sox2, and Nanog) are augmented by the functions of many the other transcription factors implicated in control of ESC state at actively transcribed target genes.
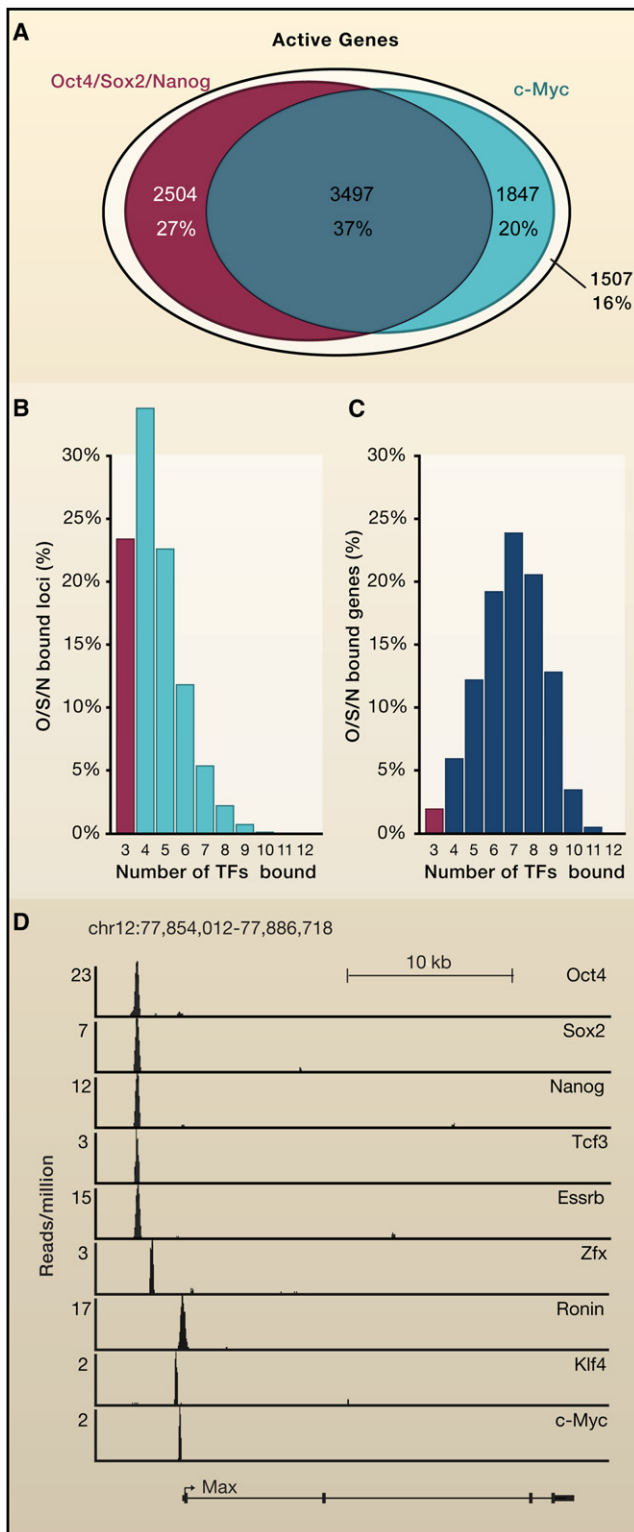
Figure 3. Relationships between Core and Other Transcription Factors in Regulatory Circuitry and Gene Control

(A) Overlap between actively transcribed genes occupied by core transcription factors (TFs) (union of Oct4-, Sox2-, and Nanog-bound genes) and those occupied by c-Myc. Active genes (9355) were defined as the set of genes

Signaling to the Core Regulatory Circuitry

Cells sense and respond to their cellular and biochemical environment through signal transduction pathways, which can deliver information to the genome in the form of activated transcription factors or cofactors. For ESCs, maintenance of the pluripotent state is dependent on the absence or inhibition of signals that stimulate differentiation (Pera and Tam, 2010; Silva and Smith, 2008). ESCs were initially cultured on a layer of irradiated fibroblasts in order to obtain the necessary factors for self-renewal and pluripotency (Smith, 2001; Smith and Hooper, 1983). LIF, Wnt, and ligands of the TGF-β/BMP signaling pathway were among factors supplied by the fibroblasts and found to influence the murine ESC state (Okita and Yamanaka, 2006; Sato et al., 2004; Smith et al., 1998; Williams et al., 1988; Ying et al., 2003).

Remarkably, the transcription factors associated with the LIF, Wnt, and BMP4 signaling pathways (Stat3, Tcf3, and Smad1) tend to co-occupy enhancers bound by Oct4, Sox2, and Nanog, thereby allowing direct control of genes within the core circuitry by these signaling pathways (Figure 4) (Chen et al., 2008a, 2008b; Cole et al., 2008; Tam et al., 2008; Wu et al., 2006; Zhang et al., 2006). Loss of Oct4 leads to a loss of these signaling transcription factors at Oct4-bound enhancers. Thus, signals mediated by these pathways are delivered directly to the enhancers of genes within the core regulatory circuitry and can thereby have profound effects on pluripotency and self-renewal. This likely explains why manipulation of the Wnt signaling pathway can enhance reprogramming (Lluis et al., 2008; Marson et al., 2008a).

Transcriptional Cofactors

Cofactors are protein complexes that contribute to activation (coactivators) and repression (corepressors) but do not have DNA-binding properties of their own. Some cofactors mobilize or modify nucleosomes, and in these cases they are also considered chromatin regulators. Cofactors are generally expressed in most cell types, but ESCs are more sensitive than somatic cells to reduced levels of certain cofactors and chromatin regulators, such as mediator and cohesin (Fazzio and Panning, 2010; Kagey et al., 2010).

occupied by both RNA polymerase II and nucleosomes with histone H3K79me3.

(B) Frequency distribution showing how c-Myc, Tcf3, Smad1, Stat3, Esrrb, Tbx3, Zfx, Ronin, and Klf4 are associated with Oct4/Sox2/Nanog-occupied loci. Oct4, Sox2, and Nanog are the three transcription factors in the first bin, which indicates that 23% of O/S/N-bound loci are not occupied by any of the other transcription factors included in the analysis. Binding was called at a high confidence ($p < 10^{-9}$) threshold within a 50 bp window, so the actual number of factors bound to Oct4/Sox2/Nanog-occupied loci is somewhat higher than indicated in this graph.

(C) Frequency distribution showing how often c-Myc, Tcf3, Smad1, Stat3, Esrrb, Tbx3, Zfx, Ronin, and Klf4 are associated with Oct4/Sox2/Nanog-occupied genes ($p < 10^{-9}$). Oct4, Sox2, and Nanog are the three transcription factors in the first bin, which shows that only 2% of O/S/N-bound genes lack binding to any of the other transcription factors.

(D) Gene tracks showing an example of an actively transcribed gene (Max) occupied by an Oct4/Sox2/Nanog enhancer and other transcription factors implicated in ESC control. At this gene, Tcf3 and Essrb occupy the Oct4/Sox2/Nanog enhancer and Zfx, Ronin, Klf4, and c-Myc bind loci closer to the transcription start site.

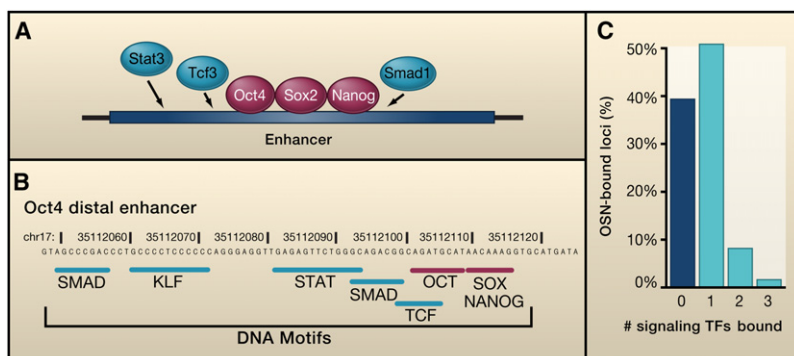ChIP-Seq data were obtained from GSE11431, GSE11724, GSE12680, and GSE22557.

Figure 4. Signaling to Core Regulatory Circuitry
(A) Model of an enhancer where transcription factors associated with Wnt, LIF, and BMP4 signaling (Stat3, Tcf3, and Smad1) occupy sites near the core regulators.
(B) Oct4 distal enhancer provides an example of a DNA element that is bound by the core regulators and signaling transcription factors and contains sequence motifs for each of these factors.
(C) Frequency distribution showing how often signaling transcription factors (Stat3, Tcf3, and Smad1) are associated with Oct4/Sox2/Nanog-bound loci throughout genome. Binding was called at a high confidence ($p < 10^{-9}$) threshold within a 50 bp window, so the actual percent of Oct4/Sox2/Nanog-bound loci that are occupied by signaling transcription factors is somewhat higher. ChIP-Seq data were obtained from GSE11431, GSE11724, GSE12680, and GSE22557.

Transcription factors that occupy active enhancers bind coactivators such as p300 and mediator, which in turn bind and control the activity of the transcription initiation apparatus (Conaway et al., 2005; Malik and Roeder, 2005; Roeder, 1998; Taatjes, 2010). The p300 and mediator coactivators are very large multisubunit complexes that can accommodate simultaneous interactions with many transcription factors. The p300 cofactor occupies most active promoters in ESCs (Chen et al., 2008b). Reduced levels of p300 do not appear to adversely affect ESCs but rather have a profound effect on ESC differentiation (Chen et al., 2008b; Zhong and Jin, 2009).

Recent studies have shown that mediator physically links Oct4/Sox2/Nanog-bound enhancers to the promoters of active genes in the core regulatory circuitry of ESCs (Figure 5) (Kagey et al., 2010). The mediator recruited to the Oct4/Sox2/Nanog-regulated promoters associates with the cohesion-loading factor Nipbl, which provides a mechanism for cohesin loading at these sites. The mediator/cohesin complex forms a looped chromosome architecture between enhancers and core promoters that is necessary for normal gene activity. Mediator and cohesin co-occupy different promoters in different cell types, thus generating cell-type-specific DNA loops associated with the gene expression program of each cell.

Mediator plays an important role in the transcriptional response to signaling. The CDK8 kinase subunit of the mediator complex can influence the activity of signaling transcription factors (Alarcon et al., 2009; Fryer et al., 2004; Gao et al., 2009; Taatjes, 2010). For example, CDK8-mediated phosphorylation of the linker region within Smad1/5 or Smad2/3 complexes can activate these transcription factor complexes, but it also targets them for proteasomal degradation. A dynamic cycle of transcription factor activation and destruction ensures that continuous pathway activation is necessary for continuous gene activation and may facilitate rapid changes in cell state when signaling is altered.

Cohesin and condensin complexes mediate essential changes in chromosome morphology associated with expression and maintenance of the genome (Nasmyth and Haering, 2009; Wood et al., 2010), and ESCs are highly sensitive to reduced levels of these key structural components of chromatin (Fazzio and Panning, 2010; Kagey et al., 2010). The association of cohesin with mediator and its contribution to both gene activity and DNA looping in ESCs makes it both an essential transcriptional cofactor and a key chromatin regulator (Kagey et al., 2010). The presence of similar cohesin/condensin complexes in prokaryotes suggests that these proteins existed before histones and may thus have more ancient roles in structuring DNA than nucleosomes.

ESCs are also sensitive to changes in the levels of the Paf1 complex, which is associated with RNA polymerase II at active genes (Ding et al., 2009). Based on studies in yeast, the Paf1 complex couples transcription initiation and elongation with histone H3K4 and H3K36 methylation (Krogan et al., 2003). In ESCs, the Paf1 complex may also play this role, as knockdowns lead to reduced levels of histone H3K4me3 at actively transcribed genes (Ding et al., 2009).

Corepressors that have been implicated in control of ES cell state include Dax1, Cnot3, and Trim28 (Fazzio et al., 2008; Hu et al., 2009; Sun et al., 2009). Overexpression of Dax1 causes ESC differentiation, likely due to an inhibitory interaction with Oct4 (Sun et al., 2009). Cnot3 and Trim28 co-occupy many promoters with c-Myc and Zfx and probably contribute to control of proliferation and self-renewal. They differ somewhat in the additional promoters they occupy, which might explain why loss of Cnot3 causes ESCs to differentiate into trophectoderm, whereas loss of Trim28 causes cells to differentiate into the primitive ectoderm lineage (Hu et al., 2009). The mechanisms involved in ESC gene regulation by Cnot3 and Trim28 are not yet well understood, but Trim28 can interact with HP1 and SetDB1 to facilitate formation of repressive chromatin (Cammas et al., 2004; Schultz et al., 2002).

In summary, ESCs are especially sensitive to reduced levels of certain cofactors, such as the mediator and PAF1 complexes, possibly because a large portion of the ESC genome is transcriptionally active and these cofactors are limiting. ESCs are also sensitive to the loss of specific corepressors, which apparently exert their control by acting on Oct4 directly or through repressive chromatin-modifying activities.

Chromatin Regulators in ESC Gene Activity and Silencing

Eukaryotic genomes are packaged into nucleosomes (Kornberg and Thomas, 1974; Olins and Olins, 1974), which provide a means to compact the genome and to influence gene expression. Early studies showed that nucleosomes can affect transcription in vitro (Knezetic and Luse, 1986; Lorch et al., 1987)
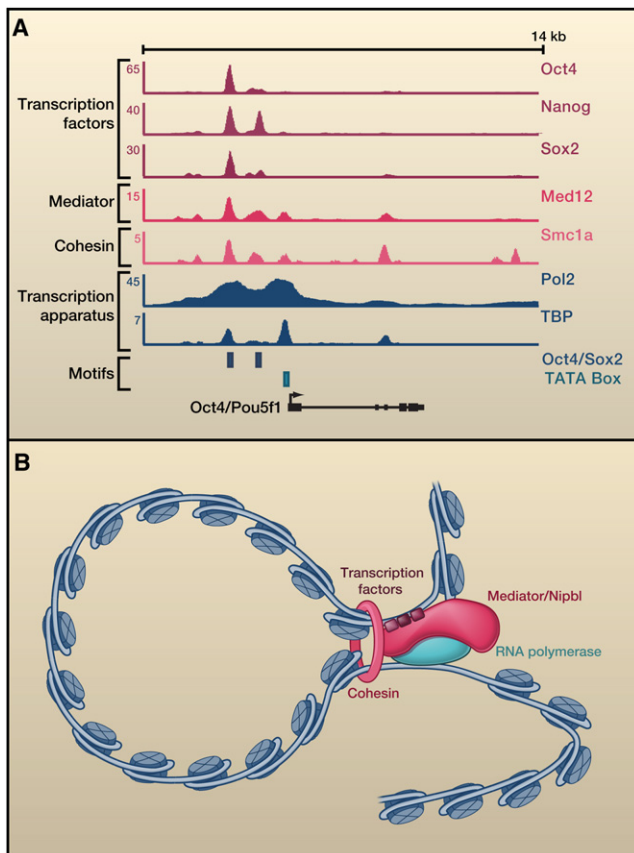
Figure 5. Mediator and Cohesin Contribute to Gene Control in Core Circuitry

(A) ChIP-Seq data at the Pou5f gene for transcription factors, mediator and cohesin, and the transcription apparatus (Pol2 and TBP). Note evidence for crosslinking of most components to both enhancer elements and core promoter. The numbers on the y axis are reads/million. ChIP-Seq data were obtained from GSE11431, GSE11724, GSE12680, and GSE22557.

(B) Model for DNA looping by mediator and cohesin. Oct4, Sox2, and Nanog bind mediator, which binds RNA polymerase II at the core promoter, thus forming a loop between the enhancer and the core promoter. The transcription activator-bound form of mediator binds the cohesion-loading factor Nipbl, which provides a means to load cohesin. Both mediator and cohesin are necessary for normal gene activity. This model contains a single DNA loop, but multiple enhancers may be bound simultaneously, generating multiple loops.

and in vivo (Han and Grunstein, 1988; Kayne et al., 1988). Subsequent studies revealed that gene expression can be influenced by proteins that modify histones (Brownell et al., 1996) or mobilize nucleosomes (Cote et al., 1994; Imbalzano et al., 1994; Kwon et al., 1994) and these have come to be known as chromatin regulators. Chromatin regulators are generally recruited to genes by DNA-binding transcription factors, the transcription apparatus, or specific RNA species (Guenther and Young, 2010; Li et al., 2007; Roeder, 2005; Surface et al., 2010).

Some chromatin regulators are essential for ESC viability, including SetDB1 and the cohesin/condensin protein complexes (Dodge et al., 2004; Fazzio and Panning, 2010; Kagey et al., 2010), whereas others contribute to the stability of ESCs or establish a state that is essential for differentiation (Leeb et al., 2010; Meissner, 2010; Niwa, 2007). The chromatin regulators

that contribute to these states fall into four classes: cohesin/condensin protein complexes (discussed above), histone-modifying enzymes, ATP-dependent chromatin-remodeling complexes, and DNA methyltransferases.

Histone-Modifying Enzymes

The chromatin regulators known to have the most profound impact on ESC state are histone-modifying enzymes that repress genes encoding lineage-specific developmental regulators. These include the PcG protein complexes, SetDB1, and Tip60-p400. PcG and Trithorax group (TrxG) genes were discovered in *Drosophila melanogaster* as repressors and activators of Hox genes (Schuettengruber et al., 2007). TrxG proteins catalyze trimethylation of histone H3 lysine 4 (H3K4me3) at the promoters of active genes and facilitate maintenance of active gene states during development, in part by antagonizing the functions of PcG proteins. PcG protein complexes catalyze ubiquitylation of histone H2A lysine 119 (H2AK119u) and trimethylation of histone H3 lysine 27 (H3K27me3) and function in ESCs to help silence genes encoding key regulators of development yet allow them to remain in a state that is "poised" for activation during differentiation (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Bracken et al., 2006; Endoh et al., 2008; Landeira et al., 2010; Lee et al., 2006; Li et al., 2010; Pan et al., 2007; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009; van der Stoop et al., 2008). PcG proteins are thought to inhibit transcription, at least in part, by restraining poised RNA polymerase molecules (Stock et al., 2007; Zhou et al., 2008b). ESCs lacking PcG protein complexes can be established but are unstable and tend to differentiate; when they do differentiate, they fail to execute differentiation programs appropriately (Leeb et al., 2010).

Multiple histone H3 lysine 9 methyltransferases have been implicated in control of ESC state (Bilodeau et al., 2009; Yeap et al., 2009; Yuan et al., 2009). A subset of the silent genes that encode lineage-specific developmental regulators, including those involved in generating the extraembryonic trophoblast lineage, are occupied and repressed by SetDB1, which catalyzes methylation of histone H3 lysine 9. Thus, multiple repressive mechanisms, involving methylation of H3K27 and H3K9 and ubiquitylation of histone H2A, are used to silence genes encoding lineage-specific developmental regulators.

The Tip60-p400 complex has multiple activities, among which is histone acetylation, and loss of this complex affects ESC morphology and state (Fazzio et al., 2008). It is found associated with active promoters in ESCs and appears to be recruited in two ways, directly by the H3K4me3 mark and indirectly by Nanog. Interestingly, the complex is also associated with nucleosomes with H3K4me3 at PcG-occupied genes encoding lineage-specific regulators, where it apparently facilitates repression of these poised genes. Because Tip60-p400 is generally found associated with active genes, its repressive function may derive from its potential role in facilitating transcription of ncRNAs that recruit or stabilize PcG complexes, as described below.

ATP-Dependent Nucleosome Remodeling

ATP-dependent nucleosome-remodeling complexes can be recruited by transcription factors and modified histones to the promoters of genes, where they enhance or reduce the access of transcriptional components to DNA sequences with resulting positive or negative effects on gene activity (Clapier and Cairns,

2009; Ho and Crabtree, 2010). Components of multiple ATP-dependent nucleosome-remodeling complexes have been implicated in control of ESC state (Table 1) (Bilodeau et al., 2009; Gaspar-Maia et al., 2009; Ho and Crabtree, 2010; Klochendler-Yeivin et al., 2000; Schnetz et al., 2010). A complex purified from ESCs called esBAF has been shown to be associated with the promoters of genes under the control of Oct4, Sox2, and Nanog, and core subunits of this complex are essential for ESC maintenance (Ho and Crabtree, 2010). Chd1, a member of the chromodomain helicase DNA-binding (CHD) family of ATP-dependent chromatin remodelers, is associated with the promoters of active genes, and Chd1-deficient ESCs are incapable of giving rise to primitive endoderm (Gaspar-Maia et al., 2009). Another member of the CHD family, Chd7, is associated with active Oct4/Sox2/Nanog-bound enhancers in ES cells, where it is thought to fine-tune the expression levels of ESC-specific genes (Schnetz et al., 2010). Unlike mutations in esBAF and Chd1, which affect ESC state, the effects of changing Chd7 dosage are subtle and do not appear to affect pluripotency or self-renewal. Thus, multiple ATP-dependent nucleosome-remodeling complexes are present at many key ESC genes.

DNA Methylation

DNA methylation is essential for mammalian development and is required in most somatic tissues. Although five DNA methyltransferases (Dnmt1, 2, 3a, 3b, and 3l) are expressed in ES cells and 60%–80% of all CpG dinucleotides are methylated (Meissner, 2010), ESCs can be established and maintained in the absence of Dnmts and DNA methylation. However, Dnmt-deficient ESCs are markedly deficient in differentiation (Jackson et al., 2004), which is likely due, at least in part, to their inability to completely silence genes encoding Oct4 and Nanog during differentiation (Feldman et al., 2006).

Noncoding RNAs in ESC Regulatory Circuitry

The idea that ncRNA might regulate genes was proposed at the dawn of studies on regulation of gene expression (Britten and Davidson, 1969; Jacob and Monod, 1961). It is now clear that ncRNA is involved in regulation of many important biological processes, including X inactivation, dosage compensation, imprinting, polycomb repression, and silencing of repeated elements, as described in several recent reviews (Lee, 2009; Surface et al., 2010; Wilusz et al., 2009; Zaratiegui et al., 2007). Indeed, a variety of ncRNA species have been implicated in control of ESC state (Table 1). These include miRNAs, which can regulate the stability and translatability of mRNAs and, acting in this fashion, play essential roles in normal ESC self-renewal and cellular differentiation. They also include longer ncRNAs of various types, which have been implicated in recruitment of chromatin regulators such as the PcG complexes (Bracken and Helin, 2009; Guenther and Young, 2010; Surface et al., 2010; Wilusz et al., 2009; Zhao et al., 2010).

miRNAs and Control of ESC Identify

Multiple lines of evidence indicate that miRNAs contribute to the control of early development. ESCs deficient in miRNA-processing enzymes such as dicer and DCGR8 show defects in differentiation and proliferation (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). Two key themes have emerged from studying the regulation of miRNA genes in ESCs (Marson

et al., 2008b). First, the core regulators Oct4/Sox2/Nanog activate genes for miRNAs that are preferentially expressed in ESCs, and these miRNAs contribute to cell state maintenance and cell state transitions by fine-tuning the expression of key ESC genes and by promoting the rapid clearance of ESC transcripts during differentiation. Second, the core regulators co-occupy repressed lineage-specific miRNA genes with SteDB1 and PcG complexes, thus poising them for expression during differentiation.

The core circuitry controls the expression of miRNAs that fine-tune the expression of key transcripts and promote the rapid clearance of ESC-specific transcripts during differentiation (Figure 6). Several miRNA polycistrons that specify the most abundant miRNAs in ESCs and that are silenced during early differentiation are positively regulated by Oct4/Sox2/Nanog (Marson et al., 2008b). These include the mir-290-295 cluster, and miRNAs with seed sequences in this family have been implicated in cell proliferation (He et al., 2005; O'Donnell et al., 2005; Wang et al., 2008) and have been shown to rescue the proliferation defects observed in miRNA-deficient ES cells (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007, 2008). Furthermore, the zebrafish homolog of this miRNA family, miR-430, contributes to the rapid degradation of maternal transcripts in early zygotic development (Giraldez et al., 2006), and this miRNA family also promotes the clearance of transcripts in early mammalian development (Farh et al., 2005).

The core transcription factors and PcG complexes co-occupy genes for miRNAs that are repressed in ESCs but become selectively expressed in cells of the immune system (mir-155), pancreatic islets (mir-375), neural cells (mir-124 and mir-9), and differentiating ESCs (mir-296) (Figure 6A) (Marson et al., 2008b). This set of miRNA genes is thus poised to contribute to cell-fate decisions during development in the same fashion as genes encoding lineage-specific transcription factors that are co-occupied by the core regulators and PcG complexes. Some of these miRNA genes are rapidly induced upon ESC differentiation and facilitate loss of ESC state; for example mir-296 targets Nanog mRNA (Tay et al., 2008) (Figure 6B). Other poised miRNA genes, such as those specifying mir-155, mir-375, mir-124, and mir-9, are induced in a tissue-specific manner during development.

ncRNAs and Polycomb-Mediated Silencing

Recent studies indicate that a broad spectrum of ncRNA molecules recruit or stabilize PcG complexes at specific sites in the ESC genome. Specific ncRNA molecules have been shown to recruit PcG complexes to the X-inactivation center X(ic), the *kcnq1* domain, the *INK4b/ARF/INK4a* locus, the *HOXD* locus, and many other genomic loci in ESCs and other cell types (Gupta et al., 2010; Pandey et al., 2008; Rinn et al., 2007; Tsai et al., 2010; Yap et al., 2010; Zhao et al., 2010; Zhao et al., 2008). Noncoding RNAs of various lengths are transcribed bidirectionally by RNA polymerase II from a majority of promoters (Core et al., 2008; Guenther et al., 2007; He et al., 2008; Seila et al., 2008); some of these are able to bind PcG complexes, which tend to occupy genes near promoter sites (Guenther and Young, 2010; Kanhere et al., 2010; Surface et al., 2010; Zhao et al., 2010). Polycomb Repressive Complex 2 (PRC2), one of the PcG complexes, has been shown to bind RNA species of 200–1200 nucleotides that
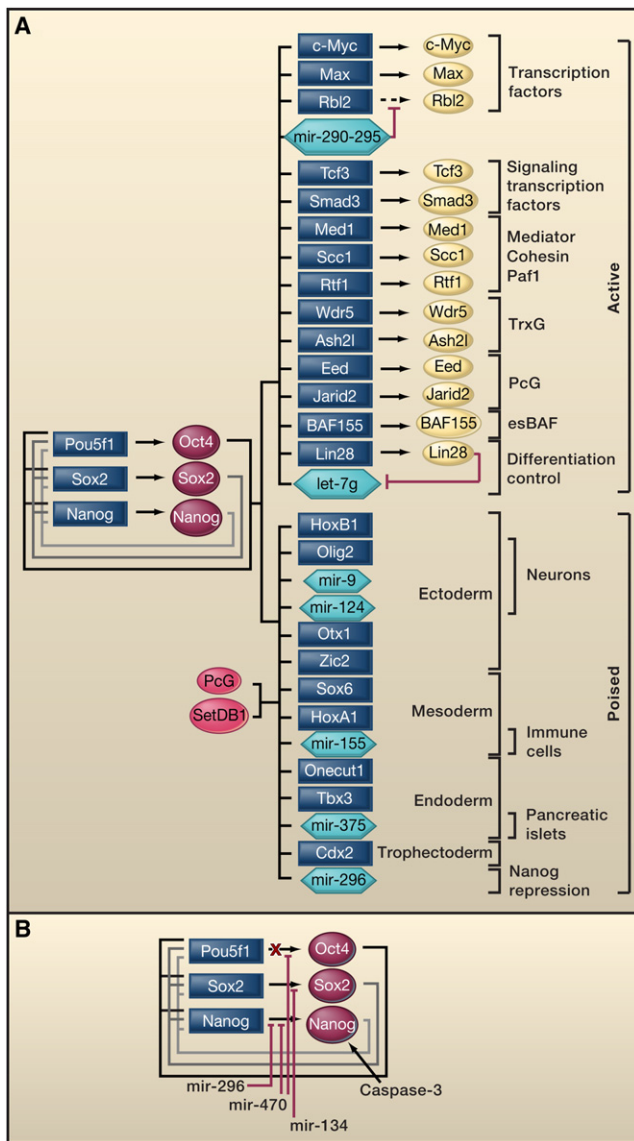
Figure 6. Selected Components of ESC Core Regulatory Circuitry and Its Disruption during Differentiation

(A) This model of core regulatory circuitry incorporates selected protein-coding and miRNA target genes. Oct4, Sox2, and Nanog directly activate transcription of genes whose products include the spectrum of transcription factors, cofactors, chromatin regulators, and miRNAs that are known to contribute to ESC state. Oct4, Sox2, and Nanog are also associated with SetDB1- and PcG-repressed protein-coding and miRNA genes that are poised for differentiation.

(B) The loss of ESC state during differentiation involves the silencing of the Pou5f1 gene, the proteolytic destruction of Nanog by caspase-3, and miRNA-mediated reduction in Oct4, Nanog, and Sox2 mRNA levels.

originate from approximately 20% of the sites in the ESC genome that are occupied by PRC2 (Zhao et al., 2010). PRC2 can also bind to RNA species of 50–200 nucleotides (Kanhere et al., 2010). Polycomb Repressive Complex 1 (PRC1), another of the PcG complexes, can also bind specific RNA species (Yap et al., 2010). Thus, PcG complexes may generally be recruited, stabilized, and thus regulated by binding to ncRNAs that are tran-

scribed from a large number of promoter regions in ESCs. This may also help to explain why transcripts are occasionally observed from genes occupied by PcG complexes.

ESCs and iPSCs

Induced pluripotent stem cells (iPSCs) have been generated from a broad range of murine and human somatic cells by using forced expression of Oct4, Sox2, and other transcription factors (Hanna et al., 2010; Stadtfeld and Hochedlinger, 2010; Yamanaka and Blau, 2010). Fully reprogrammed murine iPSCs are apparently equivalent to ESCs in developmental potency and gene expression, although some iPSCs can retain a memory of their somatic program. A few murine iPSCs have been shown to be capable of generating "all-iPSC" mice and thus have a developmental potency equivalent to ESCs (Boland et al., 2009; Kang et al., 2009; Stadtfeld et al., 2010; Zhao et al., 2009). In one study with genetically matched murine ESCs and iPSCs, no consistent gene expression differences were observed, except for transcripts within the imprinted Dlk1–Dio3 gene cluster (Stadtfeld et al., 2010). Similarly, few differences were observed in a comparison of gene expression and histone modifications in human ESCs and iPSCs (Guenther et al., 2010). However, some iPSCs do retain an epigenetic memory of their donor cells (Kim et al., 2010b; Polo et al., 2010; Lister et al., 2011). These results indicate that ESC state and thus ESC regulatory circuitry is re-established in fully reprogrammed iPSCs, but that a limited memory of the gene expression program of the cell of origin can be observed in some iPSCs.

Transitioning from ES to Specialized States

The ESC regulatory circuitry is reconfigured when cells are stimulated to differentiate (Figure 6B). ESC differentiation involves the loss of Oct4 and Nanog through transcriptional and posttranscriptional mechanisms, activation of lineage-specific transcription factors and miRNAs, and changes to the subunit composition of cofactors. Silencing of the Pou5f1 gene is mediated by trans-acting repressors such as ARP-1, COUP-TF1, and GCNF, nucleosome modification by the G9a H3K9 methyltransferase, and promoter DNA methylation by Dnmt3a/3b (Ben-Shushan et al., 1995; Feldman et al., 2006; Fuhrmann et al., 2001). Nanog undergoes proteolytic destruction by caspase-3 (Fujita et al., 2008). Specific miRNAs (mir-134, mir-296, and mir-470, for example) contribute to reduce the levels of Oct4, Nanog, and Sox2 mRNAs (Tay et al., 2008). Loss of the key ESC transcription factors leads to downregulation of the miRNA regulator Lin28, with consequent maturation of the Let-7 miRNAs, which are highly expressed in somatic tissues where they inhibit self-renewal genes (Melton et al., 2010; Viswanathan et al., 2008). Differentiation is also accompanied by modifications in the subunit composition of mediator, BAF, and TFIID complexes (Deato et al., 2008; Deato and Tjian, 2007; Ho and Crabtree, 2010; Taatjes, 2010). In summary, the signals that stimulate ESCs to differentiate cause changes in all classes of regulators discussed here: transcription factors, cofactors, chromatin regulators, and ncRNAs.

Insights into Disease Mechanisms

The study of ESC control has provided new insights into mechanisms that are involved in several human diseases. For

example, improved understanding of the functions of transcription factors such as c-Myc, cofactor complexes such as mediator and cohesin, and chromatin regulators such as TrxG and PcG has provided new insights into the molecular pathways affected by mutations in these regulators.

Key aspects of the ESC gene expression program are recapitulated in cancer cells (Ben-Porath et al., 2008), and it has been argued that this is largely a consequence of c-Myc (Kim et al., 2010a). c-Myc amplification is the most frequent somatic copy-number amplification in tumor cells (Beroukhim et al., 2010). Tumor cells that overexpress c-Myc have enhanced expression of proliferation genes, and this is likely due to the role of c-Myc in recruiting P-TEFb to effect RNA polymerase II pause release at these genes (Rahl et al., 2010). This insight suggests that therapeutic agents that target control of transcription elongation may be valuable for treating tumors that overexpress c-Myc.

Mutations in the genes encoding mediator, cohesion, and the cohesion-loading factor Nipbl can cause an array of human developmental syndromes and diseases. Mediator mutations have been associated with Opitz-Kaveggia (FG) syndrome, Lujan syndrome, schizophrenia, Transposition of the Great Arteries (TGA) syndrome, and colon cancer progression (Ding et al., 2008; Firestein et al., 2008; Muncke et al., 2003; Philibert and Madan, 2007; Risheg et al., 2007; Schwartz et al., 2007). Mutations in Nipbl and cohesin are responsible for most cases of Cornelia de Lange syndrome, which is characterized by developmental defects and mental retardation and appears to be the result of misregulation of gene expression rather than chromosome cohesion or mitotic abnormalities (Krantz et al., 2004; Strachan, 2005; Tonkin et al., 2004). Knowledge that mediator, Nipbl, and cohesin are linked at active promoters suggests therapies that might compensate for partial loss of transcriptional activity. The CDK8 kinase resides within a subcomplex of mediator that has repressive activities (Knuesel et al., 2009; Taatjes, 2010), so it is conceivable that small-molecule antagonists of CDK8 would lead to an increase in transcriptionally active mediator/cohesin assemblies.

Mutations that affect the functions or levels of TrxG and PcG chromatin regulators have been implicated in a variety of cancers (Bracken and Helin, 2009; Krivtsov and Armstrong, 2007). The study of these regulators in ESCs and in cancer cells has revealed how repression of lineage-specific transcription factors and cell-cycle regulators may contribute to cancer phenotypes. Chromatin regulators with enzymatic activities are a new class of targets for small-molecule drug discovery, and we can expect new developments in this field in the near future.

Summary and Outlook

How do regulators of the ESC gene expression program produce a self-renewing cell capable of differentiating into all the cells of the adult? Part of the answer is that the core transcription factors positively autoregulate their own expression, activate transcription of a large fraction of the active genes, and contribute to the poised state of lineage-specific genes. The core transcription factors frequently share enhancers with signaling transcription factors, so signal transduction pathways can deliver signals directly to the genes regulated by the core factors. At actively transcribed genes, additional transcription factors implicated in proliferation and other aspects of self-renewal bind to sites that can be separate from the core enhancers and modulate RNA expression levels though mechanisms that include release of paused polymerases. The core factors help create a poised state by recruiting repressive chromatin regulators to genes encoding lineage-specific factors.

Many of the regulatory features of ESCs probably operate to control cell identity in other cell types. Reprogramming and transdifferentiation experiments support the idea that a small number of master transcription factors can control cell state in various cell types (Graf and Enver, 2009; Vierbuchen et al., 2010; Zhou et al., 2008a). If this model holds true for most cell types, then identification of the master transcription factors of all cell types would significantly improve our understanding of cell identity. The concept that some transcription factors control transcription initiation and others transcription elongation is almost certainly operative in all cell types, suggesting that improved models of global transcriptional control will depend on ascertaining which of these functions applies to each transcription factor. The ability of signaling pathways to transmit information about the cellular environment to enhancers bound by master regulators seems likely to be general, and if this is the case, better understanding of the cell-type-specific effects of certain signaling pathways will be at hand. The emerging evidence that repression of ESC genes by PcG complexes can involve RNA species transcribed in the vicinity of the repressed genes makes it important to determine whether PcG complexes are generally recruited or stabilized by local transcription in other cell types, and if so, to learn what controls such transcription.

ESCs will continue to provide a powerful system for discovering the molecules and mechanisms that regulate mammalian cell state and a resource for understanding the changes that occur as cells differentiate. There are, however, many interesting challenges that must be met in order to more fully understand the basic regulation of these cells, the process of mammalian development, and how regulation goes awry in disease. These include, but are not limited to, determining the dynamic changes that occur as cells migrate through the cell cycle to self-renew or to differentiate, ascertaining the influence of natural cellular environments, and understanding the impact of genetic variation.

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