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Chromatin remodeling and bivalent histone modifications in embryonic stem cells

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Abstract

Pluripotent embryonic stem cells (ESCs) are characterized by distinct epigenetic features including a relative enrichment of histone modifications related to active chromatin. Among these is tri-methylation of lysine 4 on histone H3 (H3K4me3). Several thousands of the H3K4me3-enriched promoters in pluripotent cells also contain a repressive histone mark, namely H3K27me3, a situation referred to as “bivalency”. While bivalent promoters are not unique to pluripotent cells, they are relatively enriched in these cell types, largely marking developmental and lineage-specific genes which are silent but poised for immediate action. The H3K4me3 and H3K27me3 modifications are catalyzed by lysine methyltransferases which are usually found within, although not entirely limited to, the Trithorax group (TrxG) and Polycomb group (PcG) protein complexes, respectively, but these do not provide selective bivalent specificity. Recent studies highlight the family of ATP-dependent chromatin remodeling proteins as regulators of bivalent domains. Here, we discuss bivalency in general, describe the machineries that catalyze bivalent chromatin domains, and portray the emerging connection between bivalency and the action of different families of chromatin remodelers, namely INO80, esBAF, and NuRD, in pluripotent cells. We posit that chromatin remodeling proteins may enable “bivalent specificity”, often selectively acting on, or selectively depleted from, bivalent domains.

Keywords chromatin; chromatin remodeling; embryonic stem cells; epigenetics; histone modifications

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See the Glossary for abbreviations used in this article.

Introduction

The genetic information of a living cell is stored within the DNA. However, additional layers of regulation provide the epigenetic information, which, in concert with transcription factors, enables the same primary DNA sequence to confer different identities to different cell types, developmental stages, disease states, etc.

In eukaryotes, the DNA is wrapped around a histone octamer comprised of a pair of each of the core histones H2A, H2B, H3, and H4, which together form the nucleosome. Nucleosomes are the basic repeating units of chromatin, and they are arranged in a higher order chromatin structure through the binding of linker histones, H1 proteins, between adjacent nucleosomes. Thus, despite having the same genetic makeup, different transcriptional outcomes of different cell types of the same organism are achieved through a variety of epigenetic modifications including DNA methylation, histone post-translational modifications (PTMs), chromatin organization, etc. So far, several histone modifications with physiological importance have been identified, such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, proline isomerization, biotinylation, citrullination, and more [1–3]. Apart from influencing local chromatin structure, these modifications are also recognized by specific adaptor proteins which in turn recruit protein complexes and thereby affect gene regulation. Some of these histone marks such as H3K4me3, H3K9ac, and H3K14ac are associated with actively transcribed genes and some other modifications, for example, H3K27me3 and H3K9me3, are enriched within repressed regions. Activation and repression are believed to occur, at least partly, through charge-mediated chromatin decompaction and chromodomain-containing protein binding, respectively [3,4]. Interestingly, a subset of promoters associated with both activating (H3K4me3) and repressive (H3K27me3) marks, also known as “bivalent” modifications, has been discovered in mouse embryonic stem cells (ESCs) [5,6]. Several recent reviews thoroughly covered the field of bivalency, especially in pluripotent ESCs [7–10]. Here, we focus on the emerging link between bivalent histone modifications and ATP-dependent chromatin remodeling in ESCs. The term “chromatin remodeling” is often used to describe any change or modification to chromatin including histone modification. Here, by “chromatin remodeling”, we specifically refer to the action of the family of ATP-dependent chromatin remodeling factors, described below. When other forms of chromatin structure alterations are referred to, we describe the specific mode of alteration or modification. We will briefly summarize initial and recent experiments establishing the existence and role of bivalent domains in undifferentiated ESCs and during differentiation, and will argue that both H3K4me3 and H3K27me3, and especially the cross talk between them, are intimately linked with chromatin remodeling in pluripotent ESCs.

Glossary

ASH2L	Ash2 (absent, small, or homeotic)-like (<i>Drosophila</i>) protein
BAF250a	AT-rich interactive domain 1A (Swi1 like) protein
Bmi1	B lymphoma Mo-MLV insertion region 1 protein
BRG1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4
Cbx7	chromobox homolog 7 protein
CFP1	CXXC finger 1 (PHD domain) protein
CHARGE	coloboma, heart defect, atresia choanae (also known as choanal atresia), retarded growth and development, genital abnormality, and ear abnormality
CHD	chromodomain-helicase-DNA-binding protein
ChIP	chromatin immunoprecipitation
EED	embryonic ectoderm development protein
esBAF	brahma-associated factor complex associated with embryonic stem cells
ESC	embryonic stem cell
EZH	enhancer of zeste homolog protein
H3K27me3	trimethylated lysine-27 on histone H3
H3K4me3	trimethylated lysine-4 on histone H3
HCNE	highly conserved non-coding elements
INO80	inositol-requiring 80
iPSC	induced pluripotent stem cell
ISWI	imitation switch
LSD	lysine-specific demethylase 1
MLL	mixed lineage leukemia protein
MNase	micrococcal nuclease
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NuRD	nucleosome remodeling deacetylase
OCT4	POU domain, class 5, transcription factor 1 protein
PcG	polycomb group
Phc	polyhomeotic-like 1 (<i>Drosophila</i>) protein
PRC	polycomb repressive complex
PTMs	posttranslational modifications
RBBP5	retinoblastoma-binding protein 5
RING1B	ring finger protein 2
rRNA	ribosomal RNA
SET	SET domain containing protein
SMARCD1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 1 protein
SUZ12	suppressor of zeste 12 homolog (<i>Drosophila</i>) protein
SWI/SNF	SWItch/Sucrose non-fermentable
Tip60	K(lysine) acetyltransferase 5
TrxG	trithorax group
TSS	transcription start site
WDR5	WD repeat domain 5 protein

Bivalent modifications

The first evidence for the existence of bivalent modifications came from studies in pluripotent mouse ESCs [5,6]. Using sequential chromatin immunoprecipitation (ChIP) and tiling arrays, highly conserved non-coding elements (HCNEs) were found to be enriched with bivalent histone modifications H3K4me3 and H3K27me3, marking lowly expressed developmental regulators [6]. Shortly thereafter, early replicating genes were similarly shown to possess bivalent domains [5]. Depletion of the EED subunit of the Polycomb repressive complex 2 (PRC2) led to an almost complete loss of H3K27me3 resulting in an upregulation of the bivalent genes analyzed. Despite the presence of the activating histone marks, the expression of the bivalent genes in both studies varied from very low to no expression, suggesting that these

genes are poised for immediate activation. Supporting this notion, upon differentiation, some of these bivalent modifications were resolved, either losing the H3K27me3 mark permitting their expression, or losing the H3K4me3, rendering them stably silent (Fig 1).

Bivalent histone modifications were also identified in human ESCs [11,12] and induced pluripotent stem cells (iPSCs) [13–15], marking developmentally regulated genes, similar to the situation found in mESCs. Other stem cell types, such as hematopoietic stem cells, were also shown to possess a similar bivalent chromatin architecture, containing thousands of bivalently marked, developmentally regulated promoters [16]. However, although many of these bivalent domains are resolved during differentiation, a subset of promoters retains its bivalent state following even terminal differentiation [17]. Therefore, bivalency may not merely reflect a transient, flexible chromatin state during differentiation, but rather a condition present in most or even all cell types. Supporting this idea, bivalent domains were found in a number of different non-stem-cell lines [14,18], including differentiated human T cells, where weakly expressed genes were found to possess additional acetylation marks on H3K9 and H3K14 along with H3K4me3 and H3K27me3 in their promoters [19,20]. Bivalent domains were reported in several cancer cells as well [21–24], where they were suggested to promote their plasticity and responsiveness, potentially serving as a novel unexplored therapeutic avenue [22]. These studies suggest that bivalent modifications are present in both pluripotent and non-pluripotent cells, where they maintain genes largely in a repressed state, but at the same time, keep them poised for activation until a proper signal is perceived.

Despite the rapidly expanding literature reporting different aspects of bivalent chromatin, whether bivalent domains serve an actual function has recently been questioned [25,26]. In addition, the existence of bivalent domains on the same nucleosome has not been unequivocally demonstrated. Apart from the many ChIP experiments, bivalent chromatin was shown using micrococcal nuclease (MNase) digestion of chromatin followed by liquid chromatography and mass spectrometry [27], suggesting an asymmetric configuration of chromatin on opposite H3 tails. However, all evidence to date relies on population studies, and therefore, the seeming presence of two opposing marks on the same nucleosome may be the outcome of cellular heterogeneity. Single nucleosome resolution, the smoking gun of bivalent chromatin, has yet to be reported.

Establishment and maintenance of bivalent modifications in ESCs

Trithorax group (TrxG) proteins and Polycomb group (PcG) proteins assemble into multimeric protein complexes and are largely, although not exclusively, responsible for the deposition of H3K4me3 and H3K27me3 marks, respectively (Fig 2). The exact mechanism behind the recruitment of these protein complexes to specific sites is not entirely clear; however, initial studies showed that bivalent domains are predominantly associated with CpG islands in ESCs [6]. In addition to DNA methylation, multiple studies have demonstrated that selected histone modifications, several transcription factors (TFs), and some non-coding RNAs (ncRNAs) also play a role in this process [7].

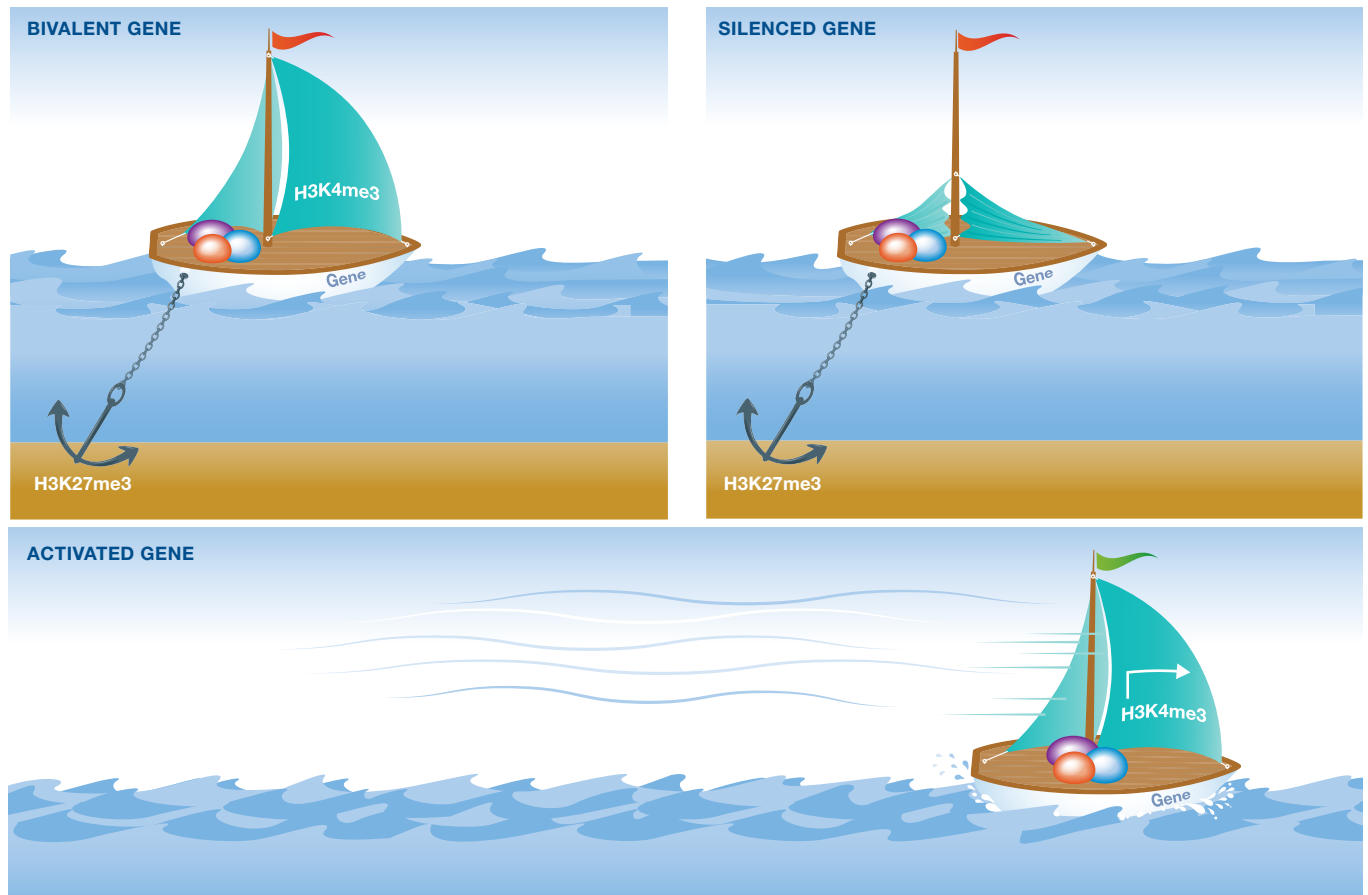


Figure 1. The bivalency concept.

A bivalent gene, depicted as a boat (top left), is ready to go (sail up: H3K4me3) but is held in check (anchor: H3K27me3). Once the sail is down (top right), the gene is stably silenced (only H3K27me3), but if instead the anchor is lifted (bottom), the gene is promptly activated (only H3K4me3). [Correction added on 2 December 2015 after first online publication: "H4K4me3" has been corrected to "H3K4me3".]

H3K4me3

In mammalian systems, SETD1A, SETD1B, and MLL complexes, among others, which share several subunits including WDR5, RBBP5, ASH2L, and DPY-30, catalyze the deposition of the H3K4me3 mark [28] (Fig 2). SETD1A/B complexes seem to be responsible for global H3K4me3 deposition, whereas MLL1–MLL4 complexes likely serve more specific functions. Among those, MLL2, but not MLL1 or SETD1, was shown to act as the main methyltransferase at bivalent promoters [25,26]. MLL1 and MLL2 contain DNA-binding domains termed CXXC or zinc finger CXXC (ZF-CXXC) motifs, which specifically recognize unmethylated CpG islands [29,30]. These motifs act to recruit MLL complexes to chromatin templates by promoting target site recognition. Similarly, SETD1A/B complexes contain a CXXC finger protein 1 (CFP1) subunit, which includes a DNA-binding domain selectively recognizing unmethylated CpGs [31]. Loss of CFP1 most strongly affects H3K4 methylation at promoters of highly expressed genes in ESCs, but not at bivalent gene promoters [32]. Other SETD1A/B and MLL components were also shown to be important in ESCs or early ESC differentiation. Knockdown of WDR5 or ASH2L, for instance, results in aberrant expression programs and defective self-renewal and pluripotency [33–36], and knockdown of RBBP5 or DPY-30 has little

effect on self-renewal but leads to improper ESC neuronal differentiation [37]. Interestingly, knockdown of DPY-30 alters H3K4 methylation specifically at bivalent domains in ESCs [38], suggesting a selective developmentally related function of this subunit. MLL2 depletion also results in skewed differentiation, along all three germ layers [39], and *MLL2*-null mice die before embryonic day E11.5, showing drastically reduced expression of several Hox genes [40]. Taken together, these studies highlight the important role that H3K4 methylation and its maintenance plays in development, pluripotency, ESC biology, and early ESC differentiation.

H3K27me3

The PRC2 complex is responsible for the deposition of H3K27me3 marks at bivalent promoters (Fig 2). The core PRC2 complex is composed of *enhancer of zeste* (EZH2 or EZH1), *embryonic ectoderm development* (EED), *suppressor of zeste 12* (SUZ12), as well as RBBP4 (RbAp48) and RBBP7 (RbAp46) [41]. EZH2 is the catalytic subunit, acting as the methyltransferase of H3K27, which in turn is recognized by chromodomain-containing proteins such as CBX proteins, as well as by the EED subunit itself [42]. In mouse ESCs, CBX7, for instance, the primary CBX protein expressed in ESCs [43,44], was shown to interact with H3K27me3 thereby recruiting

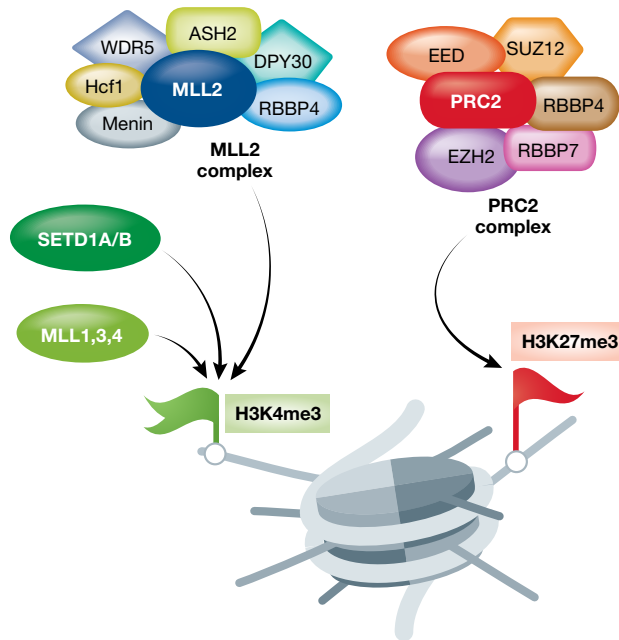


Figure 2. Main protein complexes catalyzing bivalent chromatin marks.

Left: Protein complexes catalyzing H3K4 methylation (green flag). Right: The PRC2 complex catalyzing H3K27 methylation (red flag). Shown are only the main proteins and protein complexes catalyzing H3K4/H3K27 methylation. Less abundant subunits are not depicted. [Correction added on 2 December 2015 after first online publication: “H4K4” has been corrected to “H3K4”.]

the PRC1 complex [43,45]. RING1B and BMI1 subunits of the PRC1 complex in turn deposit the ubiquitination of H2AK119 [46,47]. It was further suggested that the deposition of PRC1 and the H2AK119ub mark (H2A ubiquitylated on lysine 119) are involved in RNA polymerase 2 (RNAPII) pausing in gene bodies, rendering their repression [48,49]. PRC1-related H2AK119ub1 was also shown to recruit PRC2 to chromatin, demonstrating a functional link between the two complexes [50]. Indeed, many developmentally regulated genes are marked with bivalent domains consisting of both PRC1 and PRC2 complexes, but interestingly, a subset of these bivalent domains have been shown to be exclusively bound by PRC2 [51]. Unlike the PRC1/PRC2 double positive bivalent domains, these PRC2-specific bivalent domains usually decorate promoters of genes which are not *bona fide* developmental genes (often encoding for membrane proteins or proteins of unknown functions) and are only weakly conserved. These findings suggest an additional mechanism of silencing. In ESCs, pluripotency factor binding sites often coincide with positioning of core subunits of MLL and PRC2 complexes on bivalent domains [6,34,52]. In addition, key pluripotency components, such as OCT4 and MYC, have been shown to interact with components of the MLL and PRC protein complexes [53,54], suggesting a tight co-regulation between bivalent domains and the pluripotency network. Indeed, depletion of OCT4 in ESCs results in a selective reduction of H3K4me3 levels on selected genes, providing evidence for the tight relationship between the pluripotency network and H3K4me3 levels [34], although whether reduced H3K4me3 levels are a cause or consequence of reduced transcription is still under question [55]. While these observations suggest a functional connection between the maintenance of bivalent

modifications and pluripotency, the exact role that pluripotency factors play at bivalent domains remains largely unclear.

Depletion of individual subunits EED or SUZ12 results in deregulation of lineage-specific genes, although with minimal impact on cell viability and self-renewal [56–59], suggesting that PcG complexes serve little function in ESCs or that in ESCs, alternative compensatory mechanisms exist. In contrast to the situation, when ESCs are kept in an undifferentiated state, ESCs deficient of PRC2 components exhibit aberrant differentiation potential when differentiation is induced [56–59]. These situations parallel the postimplantation lethality phenotypes observed in PRC2 knockout mouse models [60–62]. Concomitantly, depletion of PRC1 components such as RING1B and BMI1 also impairs proper differentiation [63–66]. Simultaneous depletion of RING1B and EED in ESCs provokes an even stronger inclination toward differentiation, although self-renewal can still be preserved under careful culture conditions, and prolonged differentiation results in cell death [59]. Taken together, these knockout models demonstrate that akin to TrxG proteins, PcG proteins—arguably through the control of bivalent target genes encoding developmental regulators—are vital for proper ESC differentiation.

Chromatin remodeling and bivalency

Accumulating evidence suggests a tight interplay between ATP-dependent chromatin remodeling proteins and bivalent histone modifications. While this connection likely plays a role in most, if not all, cell types, it is particularly pertinent for pluripotent stem cells, because of the relative abundance of chromatin remodelers in ESCs [67], and because of their special connection with bivalency, as described below.

Chromatin remodeling proteins are ATP-dependent complexes that usually contain a catalytic ATPase subunit in addition to regulatory factors mediating protein–protein and chromatin–protein interactions. Chromatin remodelers act to alter chromatin structure by several different mechanisms, including incorporation or ejection of histone octamers, sliding nucleosomes along chromatin templates, and, by histone exchange, altering nucleosome composition [68] (Fig 3). Chromatin remodeling proteins are generally divided into four major families: SWI/SNF (switch/sucrose non-fermentable), CHD (chromodomain-helicase DNA-binding), ISWI (imitation switch), and INO80 (inositol-requiring 80), each involving different protein complexes and often different and even opposing actions. A growing number of chromatin remodeling proteins have been linked to ESC function and pluripotency and were shown to play essential roles in stemness and/or early differentiation and development [69]. Interestingly, at least one member of each of these remodeler families was shown to be essential for early mouse development, before or during implantation [69], demonstrating the importance of chromatin remodelers in pluripotency and early fate decisions.

One of the first clues to the involvement of chromatin remodeling proteins in regulating either H3K4 or H3K27 methylation in developmental genes came from an RNAi screen of chromatin-related proteins in mouse ESCs [70]. Among the dozens of proteins that were identified to have a potential role in maintaining the undifferentiated state in ESCs, the authors specifically identified seven subunits of the Tip60–p400 complex of the INO80 family. Using

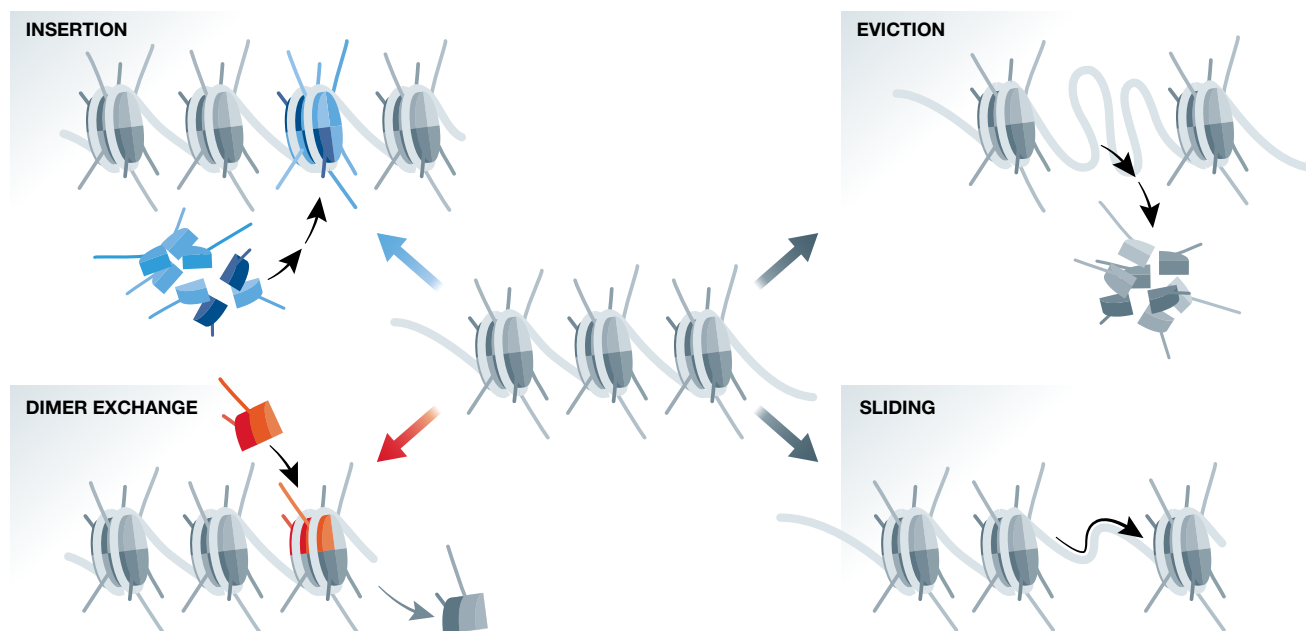


Figure 3. The classical actions of chromatin remodeling proteins.

Shown are different functional outcomes mediated by ATP-dependent chromatin remodeling proteins, including nucleosome insertion (top left), nucleosome eviction (top right), dimer exchange (bottom left), and nucleosome sliding (bottom right). The chromatin remodeling proteins themselves are not depicted.

biochemical and functional assays, the authors found that Tip60–p400 significantly co-localizes with H3K4me3, especially around the transcription start site (TSS), and that it mostly acts to repress gene expression in ESCs. Knockdown of Tip–p400 resulted in 4% deregulated genes, most of which were upregulated. Interestingly, many of the upregulated genes were found to be classical bivalent early differentiation genes, which are normally silent in ESCs, reminiscent of depletion of PcG components in ESCs [70].

Additionally, PcG proteins were also shown to be functionally linked to chromatin remodeling proteins in ESCs. This relationship was revealed following knockdown (KD) studies of the core component of the SWI/SNF esBAF complex, BRG1, in mouse ESCs [71]. Expression analysis following BRG1 KD revealed increased transcription of several PcG subunits of both PRC1 and PRC2 complexes including *Bmi1*, *Cbx7*, *Ring1*, *Phc1*, and *Phc2*, promoters of which were directly bound by BRG1, as revealed by ChIP-seq experiments [71,72]. Moreover, a PRC2 component required for ESC differentiation, *Jarid2*, interacts with esBAF [73], counteracting PRC2's methyltransferase activity [74,75]. These results suggest a direct association of chromatin remodeling proteins both with promoters of PcG-related genes and with the PRC2 protein complex itself. However, no co-localization of PRC2 components with BRG1 was found at chromatin on a genome-wide scale [71], hinting that their association is not required for chromatin binding. When BRG1 was knocked out in ESCs, global H3K27me3 levels were not altered, but H3K27me3 displayed a selective elevation in BRG1-activated genes and a decrease in BRG1-repressed genes [76]. This indicates that BRG1 directly regulates the level and distribution of H3K27me3 at its target genes.

The strong connection between SWI/SNF chromatin remodeling proteins and H3K27me3 in ESCs was recently further supported by our own studies [77]. Screening for proteins that are differentially

associated with chromatin between undifferentiated and differentiated ESCs, we identified the chromatin remodeling protein SMARCD1 (BAF60a), an additional component of the esBAF complex. ChIP-seq maps of SMARCD1 in ESCs revealed a distribution not dissimilar from that of H3K27me3 around transcription start sites (TSS) and a significant enrichment in promoters of bivalent genes. Analyzing genome-wide maps of H3K27me3 and H3K4me3 before and after SMARCD1 depletion revealed significant redistribution of these marks. In undifferentiated ESCs, both H3K4me3 and H3K27me3 were slightly elevated around TSSs upon SMARCD1 KD. In contrast, in differentiating ESCs, H3K4me3 was still elevated around TSSs, but H3K27me3 was dramatically reduced, by more than 75% around TSSs. Interestingly, bivalent genes were relatively protected from this wave of H3K27me3 elimination, indicating selective regulation of bivalent genes, by an unknown mechanism. Once again, no apparent changes in global levels were observed, as seen in the BRG1-KO ESCs.

Regulation of bivalent histone modifications by the esBAF complex was further established by an inducible knockout system of the esBAF component BAF250a in mouse ESCs [78]. Mapping of nucleosomes, bivalent histone modifications, and the PcG component SUZ12 before and after BAF250a depletion demonstrated that BAF250a mediates nucleosome occupancy and H3K27me3 levels at the upregulated, but not the downregulated genes in ESCs, and that it exerts its function likely by regulating esBAF and PRC2. BAF250a KO led to an increase in nucleosome positioning and a decrease in H3K27me3 levels, especially in bivalent and developmental gene promoters in ESCs. These alterations, accompanied by aberrant expression of developmental and pluripotency genes, resulted in differentiation defects in the BAF250a KO ESCs [78]. This study supports a synergic role for esBAF and PRC2 in ESCs.

As indicated above, the catalytic subunit of the esBAF complex is BRG1. In human ESCs, BRG1 depletion resulted in elevated levels of the enhancer-associated histone modification H3K27ac at BRG1 target gene enhancers [79]. This suggests that in addition to its function in mediating H3K27 methylation levels at promoters, BRG1 may also act as a selective repressor at enhancer regions. Support for a dual role for BRG1 in regulating H3K27ac enhancer regions on one hand, and in maintaining PcG-mediated promoter repression on the other, came from a recent study of *in vitro* mesodermal differentiation of mouse ESCs [80]. BRG1 co-localization with H3K27ac at distal enhancers is required to maintain their H3K27 acetylation during mesoderm induction, while it is also required to maintain PcG-mediated repression of non-mesodermal developmental regulators during differentiation. Taken together, these studies demonstrate a potential role for esBAF chromatin remodeling proteins in regulating bivalent histone modifications, primarily H3K27 methylation, in ESCs and during ESC differentiation, and provide evidence that the interplay between esBAF and PcG acts both to activate and to silence gene expression programs in ESCs.

Another family of chromatin remodeling proteins which was implicated in regulating H3K4/H3K27 methylation is the *Chromodomain-helicase DNA-binding* (CHD) family of proteins. CHD1 was found to regulate open chromatin and pluripotency in mouse ESCs by its association with H3K4me3 and counteracting heterochromatinization in pluripotent cells [81]. However, it was later concluded that its association with H3K4me3 is specific to active genes and it is in fact exclusively depleted in the dual H3K4/H3K27 regions [82], suggesting a selective role as an activator, leaving the suppressed bivalent domains intact. Since CHD1 interacts with H3K4me3, it raises the possibility that a mechanism to selectively clear CHD1 from bivalent promoters exists. CHD7 on the other hand was shown, using clustering analysis, to be associated with three distinct protein complexes in ESCs: an enhancer signature cluster, a c-MYC/n-MYC-enriched cluster, and a PcG cluster, containing SUZ12, RING1B, and EZH2 [83], suggesting, among other things, a function for CHD7 in PcG-mediated gene regulation. Supporting this notion, depletion of the CHD7 homolog Kismet in *Drosophila* resulted in global elevation of H3K27 methylation levels, demonstrating a role for CHD7/Kismet in counteracting PcG activity [84]. CHD7 was also shown to associate with the PBAF chromatin remodeling complex during embryogenesis and human ESC differentiation to promote neural crest migration and neural crest gene expression programs [85]. CHD7 mutations or other causes of failure to activate neural crest migration have been implicated in the development of CHARGE syndrome, a complex genetic disease affecting the nervous system, heart, vision, ears, and more [86]. These studies highlight a

link between CHD proteins and H3K27 methylation, thereby affecting bivalency albeit indirectly.

The CHD family of proteins also includes two prominent members of the NuRD chromatin remodeling complex, namely CHD3 and CHD4. The NuRD complex was shown to be essential for proper ESC differentiation [87] and was shown to deacetylate H3K27 in ESCs, enabling the subsequent recruitment of PcG proteins and trimethylation of H3K27. It therefore controls the balance between H3K27 acetylation and methylation, thereby enabling cell differentiation [88], although it likely does not act directly at bivalent promoters since it is repelled by H3K4me3 [89]. Further supporting the tight connection between NuRD and PcG during development is the association between CHD4 and the H3K27 methyltransferase EZH2 required during astroglial differentiation. CHD4 was found to be essential for EZH2 association with key astroglial gene promoters, suppressing their expression in non-glial cells, and depletion of CHD4 promotes gliogenesis *in vivo* [90]. A similar role for NuRD, mediated by CTBP2, was also seen in differentiating ESCs during the exit from pluripotency: NuRD facilitates H3K27 deacetylation followed by recruitment of PcG and H3K27 methylation [91]. Finally, NuRD was also shown to play a role in regulating H3K4me3- and H3K27me3-marked bivalent rRNA genes [92]. Although the latter study was not performed in ESCs, given the importance of the NuRD complex and rRNA transcriptional regulation in pluripotency, it is fair to speculate that it likely plays a similarly important role there too. Consistent with NuRD's role in regulating bivalency, the complex was shown to interact not only with PcG proteins, as discussed above, but also with the H3K4 methyltransferase MLL1 [93]. But perhaps even more importantly, it was also shown to interact with the H3K4 demethylase LSD1, which occupies the large majority of active genes, as well as approximately two-thirds of bivalent genes (2003 out of 3,094), in ESCs. Thus, through its association with the NuRD chromatin remodeling complex, LSD1 acts to silence pluripotency genes during early differentiation [94].

Taken together, these studies demonstrate that chromatin remodeling and bivalency, and most significantly PcG-mediated H3K27 methylation, are oftentimes functionally linked in ESCs and during differentiation, acting to resolve bivalent domains into stably activated or stably repressed states (Table 1; Fig 4). Because this connection between chromatin remodeling and bivalency is only beginning to emerge, the examples provided above are sometimes sketchy or indirect, with only H3K4 or H3K27 being affected. However, as more convincing data are gradually accumulating, it is becoming increasingly clear that remodelers, among other chromatin factors, act to shape and regulate bivalent chromatin

Table 1. Chromatin remodelers involved in regulating bivalency.

Remodeler	Complex	H3K4me3	H3K27me3	Bivalent	References
Tip60-p400	INO80	✓			[70]
BRG1	esBAF		✓	✓	[71]
SMARCD1	esBAF	✓	✓	✓	[77]
BAF250a	esBAF		✓	✓	[78]
CHD7	–		✓		[83]
CHD3/4	NuRD	✓	✓	✓	[88]

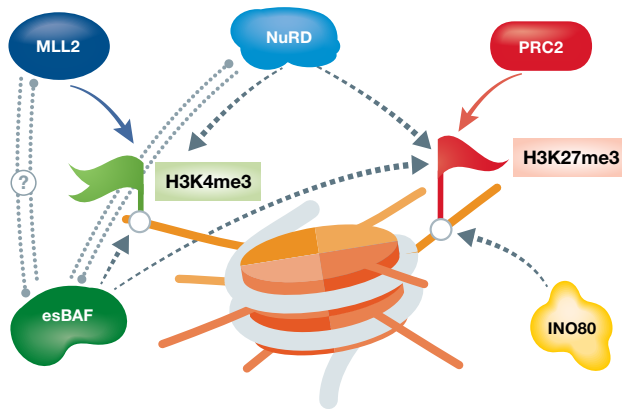


Figure 4. Chromatin remodeling complexes regulating bivalent nucleosomes.

A single schematic bivalent nucleosome is shown (orange) marked with both H3K4me3 (green flag, left) and H3K27me3 (red flag, right). Chromatin remodeling complexes which were shown to regulate either or both marks are shown in green (esBAF), blue (NuRD), and mustard (INO80). Dotted arrows represent suggested regulation; dotted double lines represent potential interaction.

domains. For example, non-canonical, replication-independent histone variants including H2A.Z and H3.3 have been recently reported to be associated with bivalent chromatin [95–98]. In ESCs, H2A.Z is found in active and bivalent promoters, both of which are enriched with H3K4me3, but absent from repressed H3K4me3-negative promoters [96]. H2A.Z depletion caused derepression of poised genes with concomitant loss of PcG components from bivalent promoters [95], suggesting a potential co-regulation of H2A.Z and PcG. However, a direct interaction between PcG and H2A.Z has not been reported so far. Along the same lines, H3.3 was shown to be required for the deposition of H3K27 methylation at bivalent promoters in a PRC2-dependent manner [98]. When H3.3 or its chaperone, HIRA, is depleted, H3K27me3 mark is reduced at bivalent promoters, along with reduced PRC2 occupancy and reduced nucleosome turnover [98], albeit with minimal transcriptional changes. This implies that other compensatory mechanisms regulating developmental genes in ESCs are in place, but together portrays a picture which suggests that histone variants and their remodelers might also be a part of the bivalency apparatus.

Concluding remarks

Almost a decade has passed since the original discovery of bivalent nucleosomes in ESCs [5,6]. While it was tempting to speculate that bivalent promoters are restricted to developmentally regulated genes, enabling a quick transition to an active or a stable silent state, it is clear today that bivalency is more complicated, extending to different gene families in multiple different cell types. Furthermore, it is increasingly recognized that regulation of the bivalent state is highly complex involving a variety of different proteins and regulators. Here, we highlighted the family of ATP-dependent chromatin remodeling proteins, which is emerging as an important player in regulating bivalent domains, especially in the context of pluripotent stem cells. While TrxG and PcG proteins provide the mechanisms of action for H3K4/H3K27 methylation, we speculate

Sidebar A: In need of answers

- (i) Is bivalency a single-cell phenomenon, or a result of population heterogeneity?
- (ii) If bivalency is within a single cell, are bivalent histone marks present within the same allele?
- (iii) If so, do bivalent histone marks appear within the same nucleosome?
- (iv) If they reside within same nucleosome, are the bivalent marks distributed symmetrically or asymmetrically within the histone pair of the nucleosome?

To answer these questions unequivocally, bivalent histone marks must be monitored at a single-nucleosome resolution. Single nucleosomes can be reconstituted *in vitro* or digested using micrococcal nuclease (MNase) and immobilized for visualization.

- (i) Does bivalency play a functional role in ESCs or in any other cell type?
- (ii) What is the role of bivalent promoters in terminally differentiated cells?

Complete selective depletion of bivalent domains may be difficult to achieve, although a recent study [26] demonstrated little effect on early differentiation following depletion of MLL2 which conferred selective depletion of H3K4me3 on bivalent promoters. Additional studies of selective depletion of bivalent marks through the use of genetics or CRISPR/Cas9 approaches will determine the role, if any, that bivalent domains play in pluripotency and embryonic development.

- (i) Which of the histone variants are associated with bivalent domains?

Pull-down of variant modified nucleosomes followed by MS approaches, or single nucleosome assays once single-nucleosome resolution is achieved, will determine the exact composition/modifications of histone variant-containing nucleosomes.

- (i) How do chromatin remodeling proteins regulate bivalent histone marks?
- (ii) Do pluripotency factors, in concert with chromatin remodeling complexes, regulate bivalent domains?

Interaction analyses, mutational studies, rescue experiments, and functional assays will help achieve mechanistic insights into the regulation of bivalent domains by chromatin remodeling proteins and/or pluripotency factors. Understanding the mechanism by which chromatin remodelers regulate the level and distribution of bivalent chromatin domains will be key to establish a direct functional connection between chromatin remodeling proteins and bivalency.

that chromatin remodeling proteins may provide the required bivalent specificity. It is important to note that bivalency is not restricted to the H3K4me3/H3K27me3 pair; several, albeit more haphazard, examples were documented. For example, trophoblast and extraembryonic endodermal stem cells were shown to contain a large fraction of H3K4me3/H3K9me3 bivalent modifications but little H3K4me3/H3K27me3 bivalency [99], and a unique H3K4me1/H3K27ac/H3K9me3 trivalent signature was observed during the transition from fibroblasts to induced neurons [100]. In both these cases, the “non-canonical” bivalent/trivalent signature enables a quick transition from a closed to an open chromatin state akin to the situation proposed for the “canonical” K4/K27 marks. The next challenge would be to identify meaningful patterns and combinations of histone modifications, decipher their potential roles, and understand whether such chromatin signatures are the cause or the consequence of their suggested function. In addition, programmable

nucleases (ZFN, TALEN, and especially CRISPR/Cas9, and mutants thereof) are already emerging as powerful tools that enable the catalysis or removal of specific modifications at bivalent loci, making it possible to study downstream effects on the corresponding genes [101–104]. More specialized systems such as photoactivatable CRISPR switches could take this idea further even to the single cell level [105,106]. The combination of epigenetic reprogramming assays, single cell technologies, and multilevel epigenomic landscape analyses will help decipher the specific roles that multivalent domains and their connection with chromatin remodeling play in pluripotency, ESCs, and development.

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Conflict of interest

The authors declare that they have no conflict of interest.

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