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Review

Modification of Enhancer Chromatin: What, How, and Why?

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Emergence of form and function during embryogenesis arises in large part through cell-type- and cell-state-specific variation in gene expression patterns, mediated by specialized *cis*-regulatory elements called enhancers. Recent large-scale epigenomic mapping revealed unexpected complexity and dynamics of enhancer utilization patterns, with 400,000 putative human enhancers annotated by the ENCODE project alone. These large-scale efforts were largely enabled through the understanding that enhancers share certain stereotypical chromatin features. However, an important question still lingers: what is the functional significance of enhancer chromatin modification? Here we give an overview of enhancer-associated modifications of histones and DNA and discuss enzymatic activities involved in their dynamic deposition and removal. We describe potential downstream effectors of these marks and propose models for exploring functions of chromatin modification in regulating enhancer activity during development.

Introduction

Interpretation of genomic information involves integration of cellular history and extracellular environment, which ultimately occurs at the level of chromatin and is mediated by the functionally diversified *cis*-regulatory elements, such as enhancers, promoters, silencers, and insulators. Among those, enhancers play a central role in driving cell-type-specific gene expression and are capable of activating transcription of their target genes at great distances, ranging from several to hundreds, in rare cases even thousands, of kilobases (reviewed in Bulger and Groudine, 2011; Ong and Corces, 2011, 2012). Recent advances in epigenomic profiling technologies, combined with the realization that certain enhancer-associated chromatin features can be effectively used to annotate them, fueled a large number of efforts aimed at genome-wide enhancer annotation in a variety of cell types and organisms, the most extensive of which is represented by the ENCODE project (reviewed in Buecker and Wysocka, 2012; Heintzman et al., 2009; Maston et al., 2012). These studies not only confirmed that enhancers are the most dynamically utilized part of the genome, but also revealed a staggering number of putative enhancer elements, with 400,000 distinct enhancers mapped in a defined set of human cell lines, and current estimate of enhancer number harbored by the human genome at over a million (Dunham et al., 2012). This astounding expansion and diversification of enhancer elements suggests an enormous combinatorial complexity of expression patterns during human development. Currently lagging behind a staggering amount of genomic data is our ability to examine spatiotemporal activity of predicted enhancers in a high-throughput, unbiased, and dynamic way in the context of a developing organism. Nonetheless, resources aimed at relatively broad validation of regulatory regions through transgenic approaches in mouse embryos, such as the Vista Enhancer Browser (http://enhancer.lbl.gov), are being developed. Moreover, although limited in scope of validation, existing studies strongly support predictive power of epigenomic annotation in enhancer discovery (Arnold et al., 2013; Blow et al., 2010; Bonn et al., 2012; May et al., 2012; Visel et al., 2009).

A central feature of enhancers is their ability to function as integrated transcription factor (TF) binding platforms. Enhancer DNA, commonly 200–500 bp in length, contains clustered recognition sites for multiple TFs, representing distinct classes of DNA binders (reviewed in Spitz and Furlong, 2012) (Figure 1A). Generally, enhancer activation requires presence of multiple TFs, including lineage-specific TFs and sequence-dependent effectors of signaling pathways, ensuring integration of intrinsic and extrinsic environmental cues at these elements. The ability of TFs to activate transcription on chromatin templates is dependent on the recruitment of coactivator proteins (Roeder, 2005; Weake and Workman, 2010). Coactivators often lack sequence-specific DNA binding competency, but instead function as histone modifiers (e.g., histone acetyltransferases), ATP-dependent chromatin remodelers, or mediators of long-range crosstalk with basal transcriptional machinery at promoters. In their active state, enhancers are also bound by general transcription factors (GTFs) and RNA polymerase II (Pol II), leading to the production of enhancer-originating RNAs termed eRNAs (Natoli and Andrau, 2012). eRNAs can be either short, bidirectional, and nonpolyadenylated or long, unidirectional, and polyadenylated, but mechanisms that specify directionality and function of eRNAs await further investigation (Natoli and Andrau, 2012).

Enhancers are thought to activate transcription by delivering important accessory factors to the promoter to potentiate either the formation of the preinitiation complex or the transition from initiation to elongation. However, it is not clear whether the enhancer-mediated delivery of these factors is predominantly required to initiate transcription and/or to continuously sustain

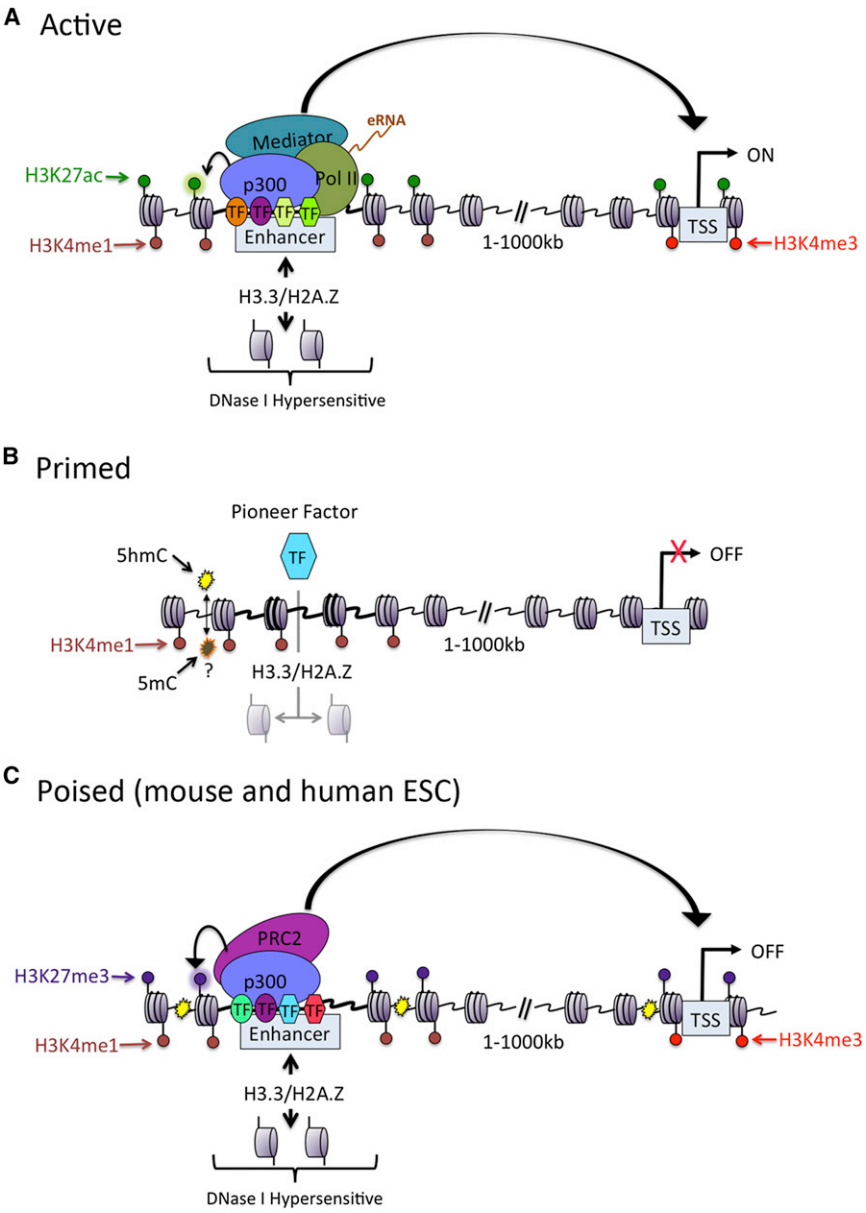

Figure 1. Epigenetic Features of Active, Primed, and Poised Enhancers

(A) Schematic representation of the major chromatin features found at active enhancers. Enhancers are associated with incorporation of hypermobile nucleosomes containing H3.3/H2A.Z histone variants, which compete for DNA binding with TFs. TFs in turn recruit coactivator proteins that can modify and remodel nucleosomes. H3K4me1 and H3K27ac are the predominant histone modifications deposited at nucleosomes flanking enhancer elements.

(B) Prior to activation, enhancers can exist in a primed state, characterized by the presence of H3K4me1. Other features that have been associated with enhancer priming are presence of pioneer TFs, hypermobile H3.3/H2A.Z nucleosomes, DNA 5mC hypomethylation, and hydroxylation (5hmC).

(C) Schematic representation of the chromatin landscape surrounding poised enhancers found in human and mouse ESCs. A subset of "primed" enhancers in ESCs is also marked by H3K27me3 and associated with PRC2. These enhancers are bound by TFs and coactivators and communicate with their target promoters.

gene expression. With respect to mechanisms of long-range communication with promoters, two major (and in fact, non-mutually-exclusive) models have been proposed: "looping" and "tracking" (reviewed in Bulger and Groudine, 2011). The former model postulates that enhancer-associated factors are delivered to the promoter by direct interaction between the enhancer and the promoter with looping out of the intervening DNA sequence. The latter model proposes that enhancers activate transcription via tracking of Pol II (or another factor) down the intervening DNA to ultimately connect with the promoter. Among the two, the looping model has gained extensive support with the emergence of technologies such as chromosome conformation capture (3C) and its derivatives 4C and 5C (reviewed in de Wit and de Laat, 2012; Gibcus and Dekker, 2013). On the other hand, the tracking model is only supported by a handful of studies on individual loci and requires more systematic testing in the future.

Several excellent recent reviews covered role of TFs at enhancers, models of long-range communication, and potential impacts of eRNAs and enhancer transcription (Krivega and Dean, 2012; Lagha et al., 2012; Natoli and Andrau, 2012; Ong and Corces, 2012; Spitz and Furlong, 2012). Here, we instead focus on the molecular mechanisms that govern deposition, removal, and function of the enhancer-associated chromatin marks. We also discuss how modification of enhancer chromatin connects to other layers of enhancer regulation.

A Brief Overview of Enhancer Chromatin Features

Occupancy of TFs at enhancers is associated with regions of nucleosomal depletion, exhibiting high sensitivity to DNA nucleases such as the DNase I (Gross and Garrard, 1988) (Figure 1A). However, rather than being nucleosome free, these regions can be associated with nucleosomes containing specialized histone variants H3.3 and H2A.Z, which are deposited at chromatin in a replication-independent manner (Goldberg et al., 2010; Jin et al., 2009). H3.3/H2A.Z-containing nucleosomes are hyperdynamic and are characterized by high salt sensitivity (Henikoff et al., 2009; Jin and Felsenfeld, 2007; Jin et al., 2009). Nucleosomes directly flanking TF binding regions are less mobile and decorated with specific histone modifications, including, but not limited to, H3K4me1 and H3K27ac (Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011; Zentner et al., 2011) (Figure 1A). These two modifications, often in combination with nuclease hypersensitivity data or coactivator

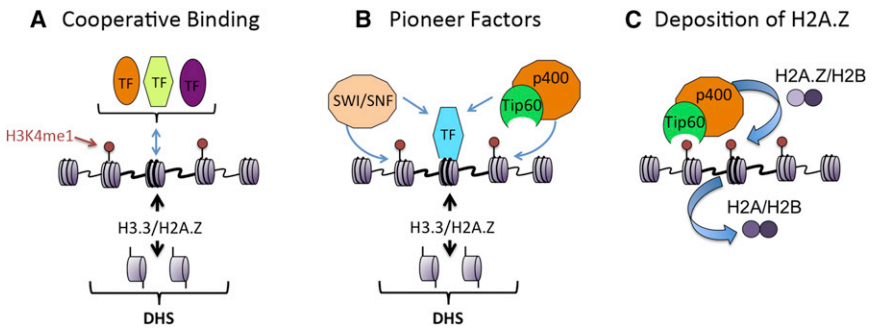

Figure 2. Chromatin Accessibility at Enhancers

(A) Cooperative binding of TFs can overcome the nucleosomal barrier and initiate regulatory events on chromatin.

(B) Enhancers sites can be made available by the action of "pioneer" factors, which can directly associate with nucleosomal DNA, preceding and commonly also enabling occupancy of other TFs via recruitment of the chromatin remodeling activities.

(C) Incorporation of the histone variant H2A.Z, mediated by the TIP60/p400 complex, creates domains of nucleosomal hypermobility that may facilitate initial TF binding events.

occupancy, provide a robust readout of active enhancer positions in the genome and have been utilized for enhancer annotation in a myriad of studies.

Determinants of Chromatin Accessibility at Enhancers

Selection of new enhancer sites during cell-fate transitions is guided by the DNA binding TFs. What triggers the opening of chromatin at enhancers? Simultaneous, cooperative binding of TFs can facilitate the ability to overcome a nucleosomal barrier and initiate regulatory events on chromatin (Figure 2A). Indeed, many TFs cannot bind their target site in the context of nucleosomal DNA in vitro; however, cooperative interactions among multiple factors allow binding (Adams and Workman, 1995). Alternative models of how new enhancer sites are accessed evoke so-called "pioneer" factors, which can directly associate with nucleosomal DNA, preceding and commonly also enabling occupancy of other TFs via repositioning nucleosomes, recruiting chromatin modifiers, or protecting enhancer regions from DNA methylation (Ghisletti et al., 2010; Sérandour et al., 2011; Zaret and Carroll, 2011) (Figure 2B).

An archetypal pioneer factor is FOXA1, which is required for liver specification during development (Lee et al., 2005). FOXA1 has the capacity to access its binding sites in nucleosomal DNA, decompact chromatin, and reposition nucleosomes of the target enhancers in vivo and in vitro (Zaret and Carroll, 2011). Another intriguing example of a pioneer factor is provided by the *Drosophila* protein Zelda, which licenses early zygotic enhancers at the maternal to zygotic transition (MZT) (Harrison et al., 2011; Liang et al., 2008). Unlike other TFs, Zelda can access the majority of its potential binding motifs throughout the genome and create competency for other factors to bind DNA, although the mechanism through which it does so is still unclear (Harrison et al., 2011; Nien et al., 2011). Interestingly, Zelda is not conserved in vertebrates, and it remains to be established whether such global pioneering activities operate in vertebrate early development as well.

Although TFs play a major instructive role in guiding genomic position of active enhancers in a given cell type, emerging evidence shows that high mobility of enhancer-associated nucleosomes is not merely a consequence of the competition with TFs, but represents an inherent and important feature of enhancer chromatin. Numerous studies document enrichment of H2A.Z and H3.3 histone variants at both active and poised enhancer and promoter regions of multiple cell types, including embryonic stem cells (ESCs) (Barski et al., 2007; Creyghton et al., 2008; Hu et al., 2012; Jin and Felsenfeld, 2007; John et al., 2008; Ku et al., 2012). Nucleosomes containing H2A.Z and H3.3 appear to be less stable and therefore easier to displace from DNA than canonical nucleosomes (Jin and Felsenfeld, 2007), although question of the biochemical stability of the H2A.Z-containing nucleosomes is complex (caveats and controversies related to this are discussed in Bönisch and Hake, 2012). Nonetheless, it is tempting to speculate that incorporation of H2A.Z into chromatin creates domains of nucleosomal hypermobility that facilitate initial TF binding events. This initial binding can in turn lead to the TF-dependent recruitment of chromatin remodeling complexes such as SWI/SNF and INO80, leading to a subsequent removal of nucleosomes from enhancers (Figure 2C). A recent study provides experimental support for this model. During mouse ESC differentiation to endoderm/hepatic progenitor cells, a subset of H2A.Z occupied regions is targeted for nucleosomal depletion, which is dependent on binding of the pioneer factor FOXA2 and on subsequent action of remodeling complexes SWI/SNF and INO80 (Li et al., 2012). Consequently, knockdown of either FOXA2 or H2A.Z impairs nucleosome positioning, chromatin remodeling, and mouse ESC differentiation to endoderm/hepatic progenitor cells (Li et al., 2012). Another recent study further underscores the role of H2A.Z in promoting binding of TFs and chromatin modifiers at regulatory regions (Hu et al., 2012). Knockdown of H2A.Z in mouse ESCs leads to increased nucleosomal occupancy, concomitant decrease in the Oct4 binding, and diminished association of the MLL and PRC2 methyltransferase complexes with active and poised enhancers and promoters (Hu et al., 2012). Consequently, H2A.Z knockdown in mouse ESCs results in misregulation of both pluripotency and developmental genes, impairing self-renewal and differentiation (Creyghton et al., 2008; Hu et al., 2012). Taken together, these observations suggest that H2A.Z deposition has a broad function in facilitating accessibility of regulatory regions to the DNA binding proteins. Nonetheless, the relationship between TF binding and H2A.Z incorporation may be characterized by a mutual dependency, facilitated by the TF-dependent recruitment of the TIP60/p400 coactivator complex, which acetylates and deposits H2A.Z onto chromatin (Svotelis et al., 2009).

H3K4me1 at Enhancers: A Window of Opportunity for Enhancer Deployment?

H3K4me1 was the first histone modification globally linked to distal regulatory regions through genomic studies (Heintzman

et al., 2007). Analyses of histone modifications over 1% of the human genome conducted in the initial phase of the ENCODE project associated presence of H3K4me1 with distal enhancer regions, in contrast to H3K4me3, which is present at active promoters (Heintzman et al., 2007). This pioneering work provided the first indication that histone modification patterns can be used for genome-wide and cell-type-specific annotation of distal enhancers. It is important to note, however, that presence of H3K4me1 is not unique to enhancers, as it also coincides with large 5′ portions of actively transcribed genes. Moreover, even in the context of noncoding sequences, H3K4me1 is widely distributed and generally covers substantially broader regions than the underlying genetic enhancer elements.

Interestingly, however, presence of H3K4me1 often precedes substantial nucleosomal depletion, H3K27ac, and enhancer activation. In mouse and human ESCs, developmental enhancers are premarked by H3K4me1 (Creyghton et al., 2010; Rada-Iglesias et al., 2011; Zentner et al., 2011), leading to the hypothesis that this modification participates in enhancer priming (Figure 1B). Premarking of enhancers by H3K4me1 in the absence of H3K27ac and prior to their deployment upon differentiation has now been observed in multiple model systems, including hematopoietic system, neural crest development, *Drosophila* mesoderm formation, zebrafish embryogenesis, and cardiac lineage formation (Bogdanovic et al., 2012; Bonn et al., 2012; Mercer et al., 2011; Rada-Iglesias et al., 2012; Wamstad et al., 2012) (Figure 1B). Unlike H3K27ac or Pol II, H3K4me1 appears to persist at enhancers after their disengagement and loss of activation potential, either temporarily (Bogdanovic et al., 2012) or for extended time (Bonn et al., 2012). Based on *Drosophila* studies, it has been suggested that pervasiveness of H3K4me1 at mammalian regulatory regions has in fact been vastly underestimated due to the undersampling of larger mammalian genomes in chromatin preparation for immunoprecipitation sequencing (ChIP-seq) experiments (Bonn et al., 2012). Regardless, instead of being tightly linked to enhancer activity, H3K4me1 perhaps broadly defines (or merely correlates with) a "window of opportunity" within which enhancer activation can occur, for example through facilitating nucleosomal mobility and/or binding of pioneer TFs (Zaret and Carroll, 2011) (Figure 1B). Combinatorial assembly of other appropriate *trans*-activating signals would then much more precisely specify spatiotemporal activity of enhancers within such broadly defined window. Indeed, H3K27ac, a mark associated with active enhancers, is acquired almost exclusively in the context of the pre-existing H3K4me1 (Bonn et al., 2012).

Potential Effectors of Enhancer H3K4 Monomethylation

What could be the molecular function of H3K4me1 in this context? The predominant mechanism through which histone methylation is thought to mediate its downstream effects is via serving as a binding platform for specialized modules, or "readers," which recognize histone tails in a sequence and post-translational modification-dependent manner (Taverna et al., 2007). Reader binding can be either promoted or repelled by the presence of the modification, and intriguing examples of the latter mechanism are provided by the specific recognition of unmodified H3K4 by various repressor proteins, including BHC80 (component of the CoREST complex), Dnmt3L (an essential cofactor for de novo DNA methyltransferases Dnmt3a/b), and Uhrf1 (a factor required for substrate recognition by the maintenance methyltransferase Dnmt1) (Lan et al., 2007; Nady et al., 2011; Ooi et al., 2007) (Figure 4). As all forms of H3K4 methylation interfere with Dnmt3L binding, it is possible that the main function of H3K4me1 is to protect distal regulatory regions from being targeted for de novo DNA methylation.

Nonetheless, the presence of H3K4me1 at enhancers in *Drosophila*, an organism lacking Dnmt3 homologs, suggests that other mechanisms may be at play, potentially involving direct recognition of H3K4me1. Although a large number of H3K4 methylation readers has been discovered (Ruthenburg et al., 2007), almost all of these effectors either strongly prefer tri- over monomethylated form (e.g., PHD finger of the TFIID TAF3 subunit [Vermeulen et al., 2007]) or recognize all forms of methylated H3K4 (e.g., chromodomains of the ATP-dependent remodelers CHD1 and CHD7). Interestingly, CHD7 appears to be preferentially associated with enhancer regions (Schnetz et al., 2009). The only reader thus far reported to exhibit preferential binding to H3K4me1 over higher methylation forms is the chromodomain of the acetyltransferase TIP60 (Jeong et al., 2011) (Figure 4). Given that TIP60/p400 complex catalyzes deposition and acetylation of H2A.Z (Altaf et al., 2010), one of the major functions of H3K4me1 in the context of enhancers could be to promote incorporation of the H2A.Z-containing nucleosomes, resulting in more dynamic chromatin structure, facilitating TF accessibility.

What Restricts H3K4me3 at Enhancer Elements?

H3K4me3 is a predominant feature of active promoters, but detectable levels of this modification are also observed at active enhancers bound by RNA Pol II, whereas H3K4me2 is present at both enhancers and promoters (Koch and Andrau, 2011; Pekowska et al., 2011). Nonetheless, compared to promoters, H3K4me3 levels at enhancers are low, and a high ratio of H3K4me1 to H3K4me3 broadly distinguishes enhancers from promoters (Djebali et al., 2012; Heintzman et al., 2007; Koch and Andrau, 2011). In the context of proximal promoters, H3K4me3 is associated with the presence of the initiating form of Pol II, characterized by the Ser5-phosphorylation of the C-terminal domain (Ser5Phos-CTD). Given the widespread binding of Pol II at enhancers, it is intriguing why most enhancers do not acquire high levels of H3K4me3. This is an important issue to address, as the relative enrichment of H3K4me1 to H3K4me3 is currently considered the major epigenomic feature that can differentiate enhancers from promoters. One potential explanation is that Pol II (and Ser5Phos) levels are generally much lower at enhancers than at promoters. However, as discussed below, recently published results suggest that distinct H3K4me1:me3 states reflect both inherent differences in DNA sequence properties of enhancers and promoters, and functional specialization among H3K4 methyltransferase complexes.

Multiple enzymes methylate histone H3 at K4 in mammalian cells, with the major role played by the six methyltransferases of the MLL/Set1 family: Set1a and Set1b (homologous to the

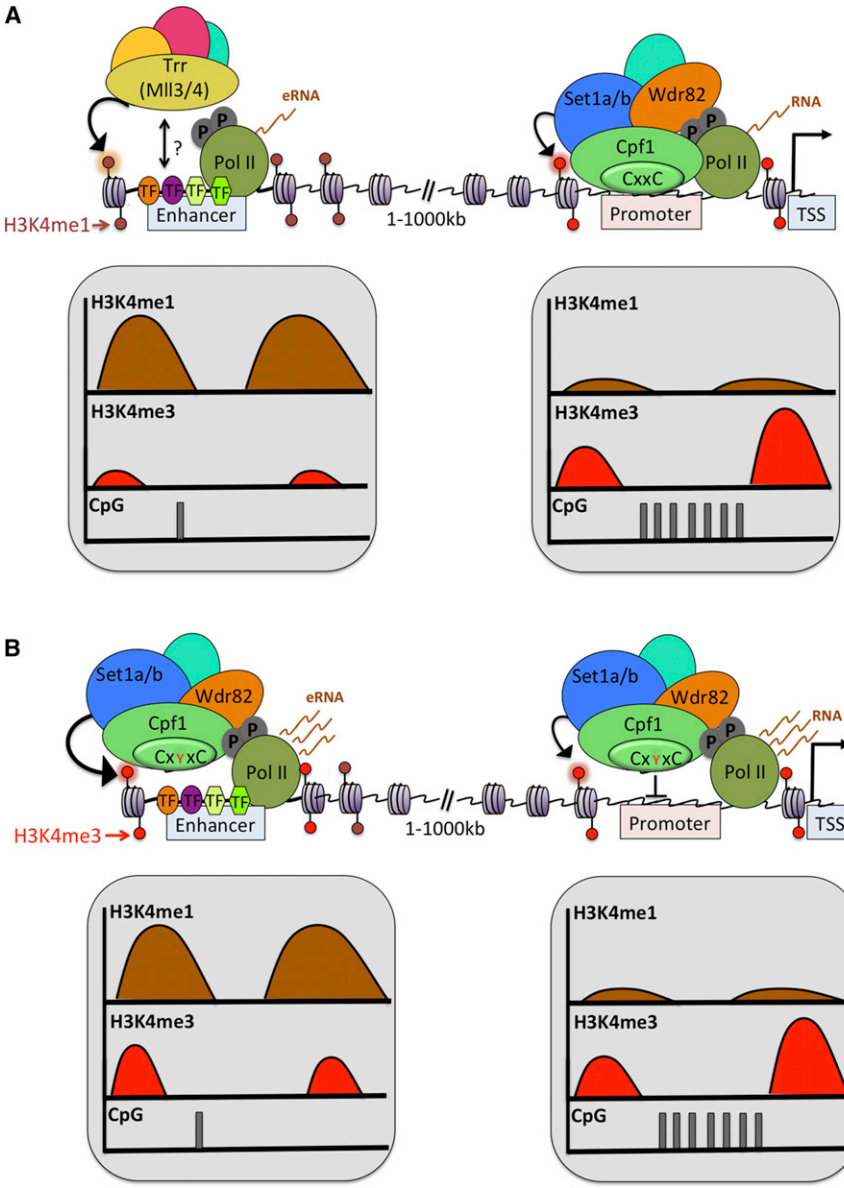

Figure 3. Methylation of H3K4 at Enhancers and Promoters

(A) Enhancers and promoters can be distinguished by the methylation status at the histone H3 lysine 4 (H3K4). Enhancers are enriched for H3K4me1, whereas high levels of H3K4me3 predominantly mark promoters. These differences can be largely explained by the underlying differences in DNA sequence, with high CpG island density observed at most promoters, but not at enhancers. CpG-rich regions are recognized by the CxxC domain of Cfp1, a specific subunit of Set1a/b complex, allowing for its preferential binding and H3K4me3 at promoters. In contrast, other methyltransferases, such as Trr/MLL3/MLL4 complex, are likely responsible for H3K4me1 at enhancers.

(B) Point mutation in the CxxC motif of Cpf1, disrupting CpG island recognition but not the complex assembly, alters binding specificity of the Set1a/b complex, resulting in the ectopic deposition of H3K4me3 at enhancers and leading in turn to aberrantly increased transcriptional activity at the enhancer and, commonly, also at the nearby promoter. See the main text for details.

yeast and *Drosophila* Set1), MLL1 and MLL2 (homologous to the *Drosophila* Trithorax [Trx]), and MLL3 and MLL4 (homologous to the *Drosophila* Trithorax-related [Trr]) (Ruthenburg et al., 2007). Although MLL/Set1 family proteins contain highly similar catalytic SET domains and all are capable of mono-, di-, and trimethylation of H3K4 in vitro, the transition from monomethylation to higher methylation states requires presence of additional accessory subunits, some of which are differentially associated with distinct family members (Shilatifard, 2012). Genetic studies suggest that Set1a and Set1b methyltransferases are responsible for the bulk of the H3K4me3 in metazoan cells (Ardehali et al., 2011; Hallson et al., 2012; Wu et al., 2008). This robust trimethylation activity appears to be mediated in part by the association with Wdr82, an accessory subunit that binds Set1a/b, but not the MLL proteins (Lee and Skalnik, 2008; Wu et al., 2008). Several lines of evidence suggest that metazoan Set1a/b complexes are recruited directly by the Pol II machinery, with Wdr82 tethering Set1a/b to transcription start sites (TSSs) via the Ser5-Phos CTD of Pol II (Ardehali et al., 2011; Clouaire et al., 2012; Lee and Skalnik, 2008). What then prevents accumulation of H3K4me3 at active enhancers, which are also enriched for Ser5-Phos Pol II?

Set1a/b complexes contain another specific subunit, Cfp1, which binds nonmethylated CpG dinucleotides via its CxxC domain (Lee and Skalnik, 2005; Lee et al., 2001) (Figure 3A). Synthetic nonmethylated CpG-dense sequences are sufficient to recruit Cfp1 and establish H3K4me3 domains in vivo (Thomson et al., 2010), suggesting that inherent DNA sequence features influence H3K4me3 targeting to promoters. In contrast to enhancers, most mammalian promoters are characterized by the high density of CpG dinucleotides (so-called CpG islands) (Deaton and Bird, 2011). Interestingly, deletion of Cfp1 in mouse ESCs results in a depletion of H3K4me3 from the CpG island containing promoters of actively transcribed genes, with simultaneous appearance of the ectopic H3K4me3 sites at many distal elements that commonly overlap enhancers (Clouaire et al., 2012). Complementation of the Cfp1 loss with a mutant that is deficient in CpG binding but not other functions rescues H3K4me3 at promoters but does not prevent aberrant H3K4me3 accumulation at enhancers (Figure 3B) (Clouaire et al., 2012). These results suggest that although Set1a/b complex has an intrinsic ability to bind most Pol II sites, including those at enhancers, the main function of

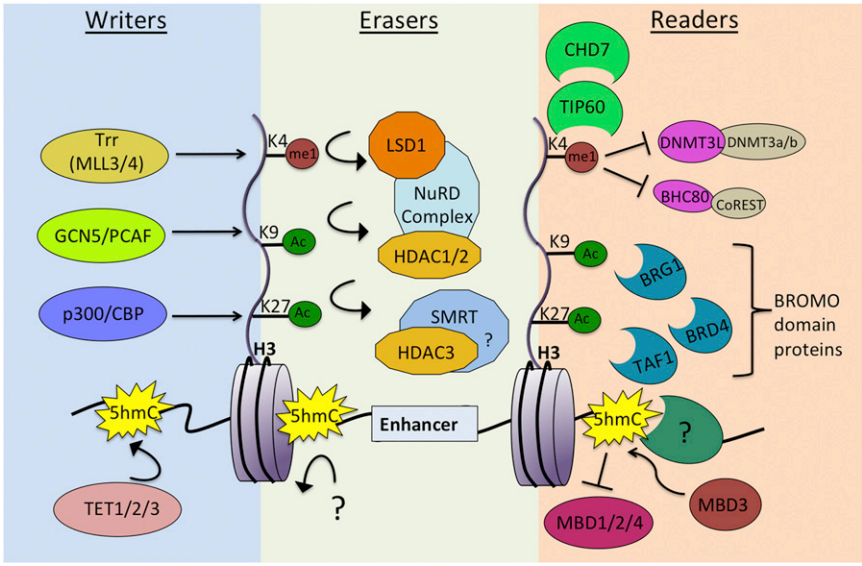

Figure 4. Writers, Readers, and Erasers of Major Enhancers Marks
Proteins capable of adding (writers), removing (erasers), and recognizing (readers) major enhancer-associated chromatin modifications, including H3K4me1, H3K9ac, H3K27ac, and 5hmC, are shown. See the main text for details.

the CpG binding activity of Cfp1 is to restrict its occupancy toward the CpG-island-rich regions, predominantly occurring around TSSs. In agreement, genome-wide coincidence between presence of the CpG islands and H3K4me3 has been observed (Guenther et al., 2007; Mikkelsen et al., 2007). With respect to enhancers, is the restriction of H3K4me3 important for the regulatory function? It appears so, as ectopically H3K4me3 modified distal enhancers are associated with aberrantly elevated level of transcription both from the enhancers and from the nearby promoters (Figure 3B) (Clouaire et al., 2012). Nonetheless, a small subset of enhancers characterized by the high CpG content and Pol II occupancy might in fact display H3K4me3 enrichments comparable to those at promoters. Furthermore, alternative mechanisms must exist to deposit high levels of H3K4me3 at CpG-island-poor active promoters.

Enzymatic Activities Regulating H3K4me1 at Enhancers

The model above explains how Set1a/b H3K4 trimethylase activity is sequestered away from distal elements, but it does not clarify how H3K4me1 mark is written and erased at enhancers. A couple of recent reports indicate that depletion of Trr, a *Drosophila* homolog of MLL3/4, affects global H3K4me1 levels in vivo, whereas knockdown of Set1 or Trx has a minor effect (Ardehali et al., 2011; Herz et al., 2012). Furthermore, protein complexes formed by the Trr or MLL3/4 do not seem to possess a CpG binding activity and are unlikely to be subjected to the above-mentioned promoter sequestration mechanism. Consistent with this notion, genomic binding of Trr in *Drosophila* is observed not only at promoters, but also at intergenic regions and gene bodies, with many of the intergenic sites overlapping enhancers (Herz et al., 2012). Based on these observations, Trr in *Drosophila* and MLL3/4 in mammals represent the major candidate enhancer H3K4me1 methyltransferases (Figure 4). In certain cellular contexts, however, this role may be played by other enzymes capable of H3K4 monomethylation, including MLL1/2 and Set7/9. Individual studies document association of MLL1/2 and Set7/9 proteins with specific enhancers (Jeong et al., 2011; Kawabe et al., 2012; Tao et al., 2011), but it remains to be established whether these enzymes do indeed play a broader role in enhancer monomethylation. Ultimately, histone H3K4me1 is lost or reduced at enhancers after they become disengaged or "decommissioned" after departure of TFs and Pol II; at least during ESC differentiation, this process is mediated through demethylation by LSD1 (Whyte et al., 2012). However, in *Drosophila*, H3K4me1 appears to persist at disengaged enhancers (Bonn et al., 2012).

A big open question relates to the mechanism of methyltransferase recruitment to distal regulatory regions. MLL proteins have been reported to interact with cell-type-specific and signaling-dependent TFs, including Pax proteins, nuclear receptors, and beta-catenin, suggesting a TF-mediated recruitment mechanism (Kawabe et al., 2012; Lee et al., 2006; Mo et al., 2006; Patel et al., 2007; Sierra et al., 2006). Given that H3K4me1 commonly precedes enhancer activation and occupancy of the relevant enhancer transactivating TFs, it would be most parsimonious if H3K4 monomethyltransferase recruitment was linked to the pioneer factor activity. In support, in several cellular models, appearance of H3K4me1/2 was shown to coincide with binding of the pioneer TFs such as FOXA1 and PU.1 (Heinz et al., 2010; Sérandour et al., 2011). However, other reports indicate that FOXA1 is in fact guided by the preexisting landscape of H3K4me1/2 (Lupien et al., 2008). Furthermore, many observations document presence of H3K4me1 over fairly broad regions surrounding enhancers, suggesting spreading of the activity beyond the immediate vicinity of the TF binding site. Potential mechanism of such longer-range stabilization involves histone-mediated interactions or association with noncoding RNAs transcribed in *cis* (Wang et al., 2011; Wysocka et al., 2005). Still, deposition of the methyl mark seems to precede Pol II binding and enhancer-initiated transcription, suggesting a more complex relationship between enhancer activity and in situ transcripts. Finally, although H3K4me1 primes enhancer regions, not all potential enhancers are premarked in pluripotent cells and H3K4me1 patterns show a significant degree of cell type specificity, at least in mammalian cells. These observations beg the question of how deposition of H3K4me1 is regulated in anticipation of developmental decisions.

Histone Acetylation at Enhancers

The ability of TFs to activate transcription is dependent on the recruitment of coactivator proteins, many of which have histone acetyltransferase (HAT) activity. Indeed, the first demonstration that histone modifications play a regulatory role in gene expression came from the realization that the *Tetrahymena* homolog of a well-known yeast transcriptional coactivator, Gcn5, is a HAT (Brownell et al., 1996). Subsequent investigations identified many distinct HATs with coactivator activity and showed that TF-dependent transcriptional activation from chromatinized templates is strongly stimulated by histone acetylation (Brown et al., 2000; Lee and Workman, 2007). While most of the early studies focused on acetylation of proximal promoters, it should come as no surprise that distal enhancers, which in metazoan organisms are the major sites of combinatorial TF assembly, are also occupied by HATs and acetylated. In recent years, binding of p300 or CBP, two highly homologous HATs, has been used for genome-wide enhancer mapping in different cell types and tissues (Ghisletti et al., 2010; Heintzman et al., 2009; Kim et al., 2010; Rada-Iglesias et al., 2011; Visel et al., 2009). p300/CBP are ubiquitously expressed and recruited, often cooperatively, by a broad range of sequence-dependent activators representing distinct TF classes (Goodman and Smolik, 2000). This general function makes p300/CBP particularly suited for tissue-specific enhancer annotation. Nonetheless, as discussed below, a subset of regulatory regions dependent on HATs other than p300/CBP will inevitably be missed in such analysis.

One of the main substrates acetylated by p300/CBP in vivo is H3K27 (Jin et al., 2011; Pasini et al., 2010; Tie et al., 2009) (Figure 4). Presence of H3K27ac distinguishes active enhancer states from those poised for activation or disengaged (Bonn et al., 2012; Creyghton et al., 2010; Heintzman et al., 2009; Rada-Iglesias et al., 2011; Zentner et al., 2011). As a consequence, H3K27ac enrichment at distal elements shows a high degree of cell-type specificity, even more so than p300 binding itself. For example, in human ESCs, poised developmental enhancers (discussed in more detail later) are bound by p300, but lack H3K27ac (Rada-Iglesias et al., 2011). These observations suggest that enhancer acetylation is regulated at multiple steps: (1) p300 recruitment and (2) direct or indirect modulation of its enzymatic activity. To prevent premature acetylation by the preloaded p300, counteracting mechanisms must exist, such as turnover by deacetylases, direct inhibition of p300 enzymatic activity or mutually exclusive relationship with H3K27me3.

Although significant attention has focused on H3K27ac, it is not the only, or perhaps not even the most prominent acetylation mark at enhancers. For one, p300/CBP have other major substrates, such as H3K18ac (Jin et al., 2011). Moreover, other HAT complexes are known to function as coactivators for sequence-dependent TFs, and thus should associate with enhancers. In support of this notion, the Gcn5/PCAF HAT complex ATAC, as well as H3K9ac, an acetylation mark preferentially catalyzed by Gcn5/PCAF, have been detected at enhancers (Ernst et al., 2011; Karmodiya et al., 2012; Krebs et al., 2011; Zentner et al., 2011) (Figure 4). In addition, in human T cells, a subset of TIP60 and MOF genomic binding sites overlaps intergenic DNase hypersensitivity regions, suggesting association of these MYST family acetyltransferases (and their respective acetylation residues) with a subset of enhancers (Wang et al., 2009). In fact, the majority of active enhancers is probably occupied by multiple HATs (as are promoters), whereas some elements may preferentially recruit one activity over others. A specific repertoire of HATs and acetylation marks at a given enhancer is likely a reflection of types of bound TFs, their cooperativity in HAT recruitment, and the presence of modulatory inputs on enzymatic activities.

Consequences of Enhancer Acetylation

Acetylated lysine residues are recognized by bromodomains, present in diverse nuclear proteins, including HATs themselves (e.g., p300, CBP, PCAF, and Gcn5), ATP-dependent remodelers (e.g., BPTF, WSTF, BRG1, and BRM), TFIID components (e.g., TAF1 and TAF1L), and factors regulating transcriptional pause release (e.g., BRD4) (Filippakopoulos and Knapp, 2012). In the context of promoters, many examples illustrate that histone acetyl-lysine recognition is important for stabilization of bromodomain-containing complexes on chromatin (Sanchez and Zhou, 2009) (for a comprehensive view of histone-binding activity of human bromodomain proteins, see Filippakopoulos et al., 2012). It is important to bear in mind, however, that bromodomain proteins can also recognize nonhistone acetylation, and thus in some cases they might be tethered to enhancer regions via recognition of acetylated TFs rather than histones, as has been demonstrated for acetylated GATA1 and bromodomain protein BRD3 (Lamonica et al., 2011). Regardless, bromodomain proteins associated with distinct aspects of transcriptional regulation, including preinitiation complex assembly (e.g., TAF1), initiation (e.g., various HATs and BRG1), and transition to elongation (e.g., BRD4) broadly associate with putative distal enhancers in ChIP-seq studies (De et al., 2011; Rada-Iglesias et al., 2011; Wang et al., 2009; Zhang et al., 2012) (Figure 4). This complexity probably reflects the fact that in addition to being major sites of TF-dependent coactivator assembly, enhancers are also active sites of transcription. While many of the aforementioned proteins are probably initially recruited to enhancers via bromodomain-independent mechanisms, acetyl-lysine recognition may stabilize and amplify activating signals (for example, through effective tethering of various HATs) and contribute to the cooperative mechanisms.

An intriguing possibility is that in addition to bromodomain protein-mediated roles, histone acetylation may directly affect enhancer function through attenuating nucleosomal stability, promoting chromatin decompaction and/or regulating enhancer-promoter communication. Due to the charged nature of both the histone octamer and the DNA, electrostatic interactions play a key role in the nucleosome's overall stability. These interactions can be affected by charge-nullifying modifications such as lysine acetylation, particularly occurring in the context of histone globular domains (Fenley et al., 2010). Furthermore, histone acetylation can have a direct effect on chromatin structure. For example, H4K16ac was shown to disrupt 30 nm fiber formation and impede ability of chromatin to form cross-fiber interactions (Shogren-Knaak et al., 2006). Specifically in relation to potential enhancer mechanisms, communication between distant DNA regions on nucleosomal arrays is dependent on

the presence of histone tails (Kulaeva et al., 2012). Computational models predict that this dependence is a consequence of transient electrostatic internucleosomal interactions mediated by the N-terminal tails of the core histones (Kulaeva et al., 2012). Therefore, acetylation of histone tails, which affects their electrostatic charge, could regulate long-distance enhancer-promoter communication on the chromatin fiber, but this hypothesis awaits experimental validation in future studies.

Interplay between Enhancers and H3K27me3

One of the surprising outcomes of recent epigenomic studies was the discovery of the widespread priming of differentiation-associated enhancers by H3K4me1. Interestingly, in pluripotent cells, such as human and mouse ESCs, a large subclass of enhancers enriched for H3K4me1, but lacking H3K27ac, is also marked by H3K27me3 and bound by the Polycomb complex PRC2 (Rada-Iglesias et al., 2011; Zentner et al., 2011) (Figure 1C). These elements, termed "poised enhancers," are located near key early developmental genes and share most of the properties of active enhancers, including similar levels of nucleosomal depletion, p300, and BRG1 binding. Yet, poised enhancers are unable to drive gene expression in pluripotent cells, although they acquire such ability during differentiation, coincidentally with the loss of H3K27me3 and gain of H3K27ac (Rada-Iglesias et al., 2011). Surprisingly, poised enhancers in human ESCs are already looped to their target promoters, whereas in other cell types, efficient promoter communication is largely associated with active enhancers (J. Dekker, personal communication) (Sanyal et al., 2012). Consequently, differentiated cell types, but not ESCs, show a high correlation of enhancer-promoter looping events with gene expression (J. Dekker, personal communication) (Sanyal et al., 2012). Observed long-range interactions of poised enhancers in ESCs might be mediated by the Polycomb proteins, as has been reported in other contexts (Tiwari et al., 2008; Tolhuis et al., 2011). Consistently, genes located in proximity of H3K27-methylated enhancers commonly have so-called bivalently marked promoters, characterized by the simultaneous presence of the H3K4me3 and H3K27me3 and association with PRC2.

Regardless, H3K27me3 enrichment has not been broadly observed at enhancers in other cell types, suggesting that poised enhancers in ESCs may reflect unique Polycomb regulation in pluripotent cells. Nonetheless, the interplay between distal enhancer activity and H3K27me3 status at promoters probably occurs in many different tissues and contexts. For example, in erythroid cells, enhancer function is required for Polycomb eviction from α-globin promoter, and this mechanism is dependent on the enhancer-mediated recruitment of the H3K27me3 demethylase, Jmjd3 (Vernimmen et al., 2011). Interestingly, a second known mammalian H3K27me3 demethylase, UTX, is a specific subunit of the MLL3/MLL4/Trr complex (reviewed in Shilatifard, 2012; Swigut and Wysocka, 2007), which, as we discussed, is a major candidate for the enhancer H3K4 monomethyltransferase.

Complexity of Enhancer Histone Modification Patterns

Although a fairly limited set of modifications, H3K4me1/3, H3K27ac, and H3K27me3, is ubiquitously utilized to map and distinguish active and poised enhancers and promoters, these marks represent only a fragment of the full repertoire of histone modifications decorating distal regulatory regions. For example, various acetylation marks, including H3K9ac and H3K18ac, were detected at putative enhancers (Ernst et al., 2011; Zentner et al., 2011), as was histone crotonylation (Tan et al., 2011), H3K79me2/3 (Bonn et al., 2012; Djebali et al., 2012), and, in the context of poised enhancers, H3K9me3 (Zentner et al., 2011). An interesting study demonstrated that in *Drosophila*, enhancers are phosphorylated at H3S10 and H3S28 and that recruitment of JIL1, kinase responsible for the H3 phosphorylation, is required for the CBP-dependent H3K27ac (Kellner et al., 2012). Thus, kinase signaling cascades may regulate enhancer activity not only through modulation of TF binding, but also by directly affecting chromatin states.

What other histone modifications can we expect to find at enhancers? Probably those catalyzed by TF-dependent coactivators with enzymatic activities. Various HATs certainly make a good case, and others might include arginine methyltransferases CARM1 and PRMT1, which function as nuclear receptor coactivators, and catalyze H3R17me and H4R3me, respectively. The next few years will almost certainly bring discoveries of many new enhancer-associated marks, their readers, writers, and erasers, and the regulatory interplay among them. Adding to this complexity, recent analyses of epigenomic data point toward another layer of diversity associated with chromatin states at enhancer elements: pervasive asymmetry of histone modification signals and nucleosome positioning in relation to the TF and Pol II occupancy (Kundaje et al., 2012). Such asymmetry suggests that enhancers have inherent orientation determined by (or reflected in) their chromatin environment.

Functionality of Histone Modifications at Enhancers: Can We Test It?

Although chromatin signatures are highly predictive of enhancer position and activity, direct evidence supporting functionality of enhancer chromatin modifications in vivo is still lacking. This is in large part a consequence of technical difficulties in performing functional experiments that would unambiguously distinguish cause from consequence. In the absence of better methodologies, many studies utilized one (or both) of two approaches: (1) knockout/knockdown of relevant modifying and demodifying enzymes, and (2) specific mutations of the reader domains, affecting their ability to recognize modified target residue. Although the former approach is useful and revealed chromatin modifying enzymes to be major regulators of development, differentiation, and disease, it is plagued by two major caveats, which are that histone-modifying proteins often have major nonenzymatic functions and that their enzymatic activity is not limited to histones. The second aforementioned approach is more direct and informed by the expansive structural studies, which underscore specificity of most reader modules both for the type of the modification and the surrounding amino acid sequence (Taverna et al., 2007). One relevant example of utilizing reader domain mutations to assess functional consequences of enhancer-associated modification is provided by the study on the role of TIP60 during the estrogen receptor (ER) response (Jeong et al., 2011). Point mutations of the TIP60

chromodomain, which affect H3K4me1 recognition, also impair TIP60 recruitment to the ER-responsive enhancers and perturb hormone activated gene expression (Jeong et al., 2011).

A more direct approach to access functionality of specific modifications in vivo would involve replacement of wild-type histones with versions mutated at specific residue(s) of interest. However, with the exception of certain rare variants, such replacement experiments are generally not feasible in vertebrates, which have many copies of each canonical histone gene, in multiple locations throughout the genome. Nonetheless, histone H3 replacement experiments in a simple multicellular organism, *Neurospora crassa*, demonstrated that point mutations in either of the K4, K9, or K27 residues are incompatible with organismal viability (Adhvaryu et al., 2011), suggesting a key functional role of these histone tail residues (and presumably their modifications) in complex organisms. However, even in cases where histone replacements could be introduced, interpretation is still complicated by the fact that certain amino acids can be modified by a multitude of marks (for example, lysines can be acetylated, butyrylated, crotonylated, methylated, ubiquitylated, and sumoylated), and specific histone residues are indeed known to be subject of distinct posttranslational modifications. Moreover, since enhancer-associated marks are also commonly present at promoters, replacement experiments would not distinguish enhancer-specific versus promoter-specific function. Clearly, new technologies, perhaps utilizing site-specific incorporation of unnatural amino acids into proteins in vivo (Davis and Chin, 2012), will be required to precisely address questions of histone mark functionality in the future.

DNA Methylation at Enhancers: Driver or Passenger?

DNA methylation at 5-methylcytosine (5mC), preferentially established at CpG dinucleotides by DNA methyltransferases (DNMTs), has been predominantly implicated in genome silencing in various biological processes (reviewed in Jones, 2012). Recent genome-wide analyses document strong anticorrelation between enrichment of active enhancer histone marks and DNA methylation density (Koch and Andrau, 2011; Schmidl et al., 2009; Stadler et al., 2011; Thurman et al., 2012) Similarly, presence of TFs and coactivator binding inversely correlates with DNA methylation (Neph et al., 2012; Stadler et al., 2011; Thurman et al., 2012). Moreover, since active enhancer signatures show high degree of cell-type specificity, so do sites of DNA hypomethylation (Bock et al., 2012; Lister et al., 2009; Stadler et al., 2011). These observations are in agreement with the predominant function of 5mC in gene silencing, and with the long-held view that DNA methylation interferes with binding of many TFs. They also illustrate that DNA methylation at regulatory elements is much more dynamic than previously appreciated.

Nonetheless, correlation alone does not distinguish between two scenarios: (1) DNA methylation plays an active role in shaping enhancer landscapes via eviction of TFs from their cognate sites and (2) TFs are drivers of hypomethylated states, whereas DNA methylation passively fills in sites vacated by TFs departed from decommissioned enhancers. A couple of recent reports argue in favor of the second scenario (Stadler et al., 2011; Thurman et al., 2012). Stadler et al. demonstrated that binding of TFs (in this case, CTCF and REST) is sufficient to induce the formation of hypomethylated regions, even in the context of pre-existing DNA methylation at these sites. On the other hand, TF loss allows for subsequent remethylation (Stadler et al., 2011). In agreement with the latter notion, comparisons among a large number of ENCODE data sets revealed significant negative correlation between expression of a given TF and methylation of its cognate binding sites within the DNase hypersensitive regions; this anticorrelation held true for over 70% of all interrogated TFs (Thurman et al., 2012). While these observations suggest a passive role for DNA methylation in filling in decommissioned enhancers, future work is needed to uncover the means through which TFs can access previously methylated DNA or induce changes in DNA methylation patterns. We already discussed one potential mechanism, which may prevent redeposition of DNA methylation at sites associated with H3K4me1 via interference with Dnmt3L binding. Intriguingly, the first glimpse of a putative active demethylation mechanism is provided by the robust association of developmental enhancers with 5 hydroxymethyl-cytosine (5hmC).

A New Kid on the Block: 5-Hydroxymethylcytosine

Although 5mC has long been regarded as a stable, highly heritable mark, recent discoveries revealed that 5mC undergoes enzymatic oxidation by the Tet family proteins to produce 5 hydroxymethyl-cytosine (5hmC), an intermediate on a pathway to active DNA demethylation via a couple of alterative biochemical pathways (reviewed in Dahl et al., 2011; Kriukienė et al., 2012; Tan and Shi, 2012). 5hmC has been detected in genomes of several cell types, including ESCs, where it positively correlates with gene activity and is found at promoters, gene bodies, and enhancers (Ficz et al., 2011; Pastor et al., 2011; Stroud et al., 2011; Szulwach et al., 2011; Valinluck et al., 2004; Wu and Zhang, 2010). With respect to the latter elements and in contrast to 5mC, 5hmC coincides with H3K4me1 and H3K27ac and follows active enhancer marks during differentiation (Sérandour et al., 2011; Stroud et al., 2011). A recent report provided the first single-base-resolution 5hmC map in mouse and human ESCs, revealing that 5hmC is most abundant at both poised and active enhancers (Yu et al., 2012), rather than at CpG-rich promoters, as previously suggested (Pastor et al., 2011; Wu and Zhang, 2011). 5hmC enrichment at enhancers is characterized by a bimodal distribution flanking TF binding sites, by the inverse correlation with 5mC, and by the high concordance with H3K4me1 (Yu et al., 2012). These results are consistent with an attractive hypothesis that, at least in cell types in which Tet enzymes are expressed, enhancer priming/poising involves active DNA demethylation through the 5hmC intermediate.

Curiously, however, 5hmC is distributed around, but not within, TF consensus motifs (Yu et al., 2012), whereas 5mC depletion is strongest directly at the TF motifs (Neph et al., 2012). Therefore, a model whereby 5hmC only functions as a step in removal of 5mC to allow TF binding is perhaps too simplistic. In fact, 5hmC may be a part of enhancer activation process in its own right by counteracting transcriptionally restrictive chromatin states or recruiting heretofore-unidentified effector proteins. Several methyl-CpG binding proteins present in repressor complexes, such as MBD1, MBD2, and MBD4, preferentially bind 5mC over 5hmC, whereas MBD3 was shown

to exhibit a reverse preference (Yildirim et al., 2011) (Figure 4). Finally, the fate of 5hmC itself needs to be further explored in the context of enhancers: is it destined for further oxidation to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)? Does it always lead to demethylation or perhaps more direct mechanisms exist for turning it back into 5mC after enhancer decomissioning?

Concluding Remarks

Understanding that enhancers share common chromatin features has revolutionized our ability to discover them in a genome-wide, cell-type-specific, and conservation-independent manner. Such genome-wide analyses revealed unexpected abundance and dynamics of enhancer elements in the mammalian genomes. Despite technical difficulties in directly testing the functional impact of enhancer modifications in a cellular or organismal context, evidence begins to emerge that, beyond merely serving as a convenient indexing system, chromatin modifications provide an important layer of enhancer regulation. As we discussed here, they might contribute to the combinatorial assembly of transcriptional complexes, promote enhancer accessibility for TFs, prime regulatory elements for future use, and perhaps even modulate long-range communication with promoters. Nonetheless, insights into the precise mechanisms regarding functional consequences of enhancer chromatin modifications are still lacking, and new technologies and approaches will be required to obtain them. Moreover, given that enhancers and promoters share many features, such as common chromatin modifications, as well as the association with transcriptional machinery and transcription itself, traditional lines functionally separating enhancers from promoters is beginning to blur. It will be critical to delineate in the future which of these similarities might result from limitations of our current assays (such as indirect crosslinking in ChIP experiments) and which reflect true functional differences between the two classes of elements, specified by the inherent diversity of their sequence features. Finally, a big challenge for the future will be to understand how chromatin states relate to other regulatory inputs that are being integrated at enhancers, such as information encoded by the genome, cellular history, and extracellular signaling.

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