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Review

Chromatin regulators: weaving epigenetic nets

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Abstract

In multicellular organisms differentiated cells must maintain their cellular memory, which will be faithfully inherited and maintained by their progeny. In addition, these specialized cells are exposed to specific environmental and cell-intrinsic signals and will have to appropriately respond to them. Some of these stimuli lead to changes in a subset of genes or to a genome-wide reprogramming of the cells that will remain after stimuli removal and, in some instances, will be inherited by the daughter cells. The molecular substrate that integrates cellular memory and plasticity is the chromatin, a complex of DNA and histones unique to eukaryotes. The nucleosome is the fundamental unit of the chromatin and nucleosomal organization defines different chromatin conformations. Chromatin regulators affect chromatin conformation and accessibility by covalently modifying the DNA or the histones, substituting histone variants, remodeling the nucleosome position or modulating chromatin looping and folding. These regulators frequently act in multiprotein complexes and highly specific interplays among chromatin marks and different chromatin regulators allow a remarkable array of possibilities. Therefore, chromatin regulator nets act to propagate the conformation of different chromatin regions through DNA replication and mitosis, and to remodel the chromatin fiber to regulate the accessibility of the DNA to transcription factors and to the transcription and repair machineries. Here, the state-of-the-art of the best-known chromatin regulators is reviewed.

Keywords: chromatin; chromatin regulators; DNA; epigenetic; histone.

Introduction

In multicellular organisms, stem cells can give rise to a diverse array of specialized cell types. These cells will have to appropriately function in their tissue niche and to respond to specific environmental signals, but they must also remember their gene expression pattern once established or, in other words, they must maintain their cellular memory. In addition, their cell fate choices must be faithfully inherited and maintained by their progeny throughout the lifetime of the organism. The canalization of developmental pathways constitute the concept that led Conrad Waddington to introduce the term epigenetics (*epi* -Greek: επι- over, above) in 1942 to define the branch of biology which studies causal interactions between genes and their products which bring the phenotype into being. Waddington compared the path of a cell towards terminal differentiation with a ball travelling downwards along branching valleys; once in its final valley, the ball cannot go to neighboring valleys or return to the beginning. This canalization explains how cellular differentiation pathways become stable and potentially irreversible (1).

However, in certain pathologic situations such as injury, oncogenic stress or inflammation the differentiated phenotype can be compromised and the previously established gene expression settings could be reprogrammed, leading to the reversion, transdifferentiation or desdifferentiation of already lineage-committed cells. In addition, temporally limited overexpression of certain transcription factors *in vitro* allows to derive induced pluripotent stem (iPS) cells from fully differentiated cell types (2, 3) and functional neurons from mouse fibroblasts (4). Therefore, the unidirectional concept of differentiation has been revised and epigenetic processes must maintain the cellular memory in a stable but reversible manner.

The current definition of epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (5) and includes mechanisms such as imprinting, X chromosome inactivation, establishment and maintenance of cell identity, and the propagation of essential architectural features (e.g., telomeres and centromeres). Accordingly, the term 'epigenetic regulator' would be strictly restricted to those chromatin modifying mechanisms with demonstrated self-propagation ability after cell division, such as for DNA methylation, but excludes those mechanisms with an unknown mode of propagation and those mechanisms that act rapid and transiently, such as histone phosphorylation, that allow cells to respond and adapt to environmental stimuli. In addition, a single mechanism proved to be inherited in a specific locus under particular circumstances in a defined cell type will not necessarily ensure the maintenance or the transmission to the progeny of a stable chromatin conformation of other loci in another cellular context. Therefore, we have chosen the term 'chromatin regulators' to describe those molecules able to affect chromatin status. First, we summarize the best characterized changes in the molecular substrate for epigenetic regulation, the chromatin, and we review current literature on those proteins able to affect chromatin modifications or conformation. Next, examples of some intricate connections

among chromatin modifications and regulators are described, to finally provide an overview of current knowledge concerning the recruitment of the chromatin regulators, an essential step for their proper function.

Chromatin and its modifications

The large genome of eukaryotes is packaged into chromatin that fits it into the small volume of a nucleus. In addition to solving a storage problem, chromatin conformation constitutes an additional layer, as well as transcription factors, to regulate gene expression and to guaranty genomic stability maintenance.

The basic component of chromatin is the nucleosome, generated by wrapping approximately 147 bp of DNA around an octamer of core histone proteins. The canonical histone octamer contains two copies each of histones H2A, H2B, H3 and H4 (6). In addition, linker histone H1 associates with the internucleosomal DNA. The highly basic histone proteins and the acidic DNA intimately interact, constituting an extremely stable entity. Successive higher order chromatin structures ultimately compact DNA into thick chromatin fibers, with the highest degree of compaction seen in metaphase chromosomes (7).

Most of the chromatin exists in a closed conformation, in which nucleosomes are compactly packaged, called heterochromatin. In contrast, a minor part of the genome corresponds to euchromatic regions, in which nucleosomes are less compact, form an open chromatin structure, and are transcriptionally permissive, although not necessarily transcriptionally active (8). A remarkable number of complexes act to propagate the conformation of these different chromatin regions through DNA replication and mitosis, and to remodel the chromatin fiber to regulate DNA accessibility to transcription factors and to transcription and repair machineries. These chromatin regulator complexes will affect chromatin conformation and accessibility by covalently modifying the DNA or the histones, substituting histone variants, remodeling nucleosome position or modulating chromatin looping and folding.

Covalent modifications of chromatin

Chromatin conformation can be regulated by two characterized mechanisms. The first mechanism is by directly changing this conformation; for instance, by deposition of acetyl groups that alter histone charges and electrostatic forces or introducing variants or relatively bulky histones that alter histone-DNA interactions and nucleosomal structure. However, most chromatin modifications do not affect nucleosome conformation by themselves, but are marks recognized by chromatin-related proteins, generally acting in multimeric complexes, with the ability to change nucleosome conformation or to recruit other enzymatic activities. The best-known chromatin modifications are DNA methylation and histone covalent modifications. In addition, because post-translational modifications have been found to be dynamic and reversible processes generally mediated by two antagonistic sets of enzymatic complexes, changes in chromatin conformation can result not only from the setting of a new mark but also from the removal of a previously established modification.

DNA methylation

DNA methylation is a highly stable covalent modification involving the addition of a methyl group onto cytosines in the DNA of many, although not all, higher eukaryotes (9, 10). Cytosine methylation is a non charge-altering modification and, in mammals, occurs almost exclusively at cytosines that precede a guanosine in the DNA sequence (CpG dinucleotides), reflecting the specificity of the enzymes involved.

CpG distribution is not uniform: most of the CpG dinucleotides of the genome are predominantly methylated, and dense DNA methylation is seen in heterochromatin and repetitive sequences (including satellite sequences, centromeric repeats and interspersed repetitive sequences). However, small stretches of CpG-rich DNA regions (CpG islands) are often located near promoter regions, normally unmethylated and largely associated with euchromatic regions (11).

Whereas methylation of the bulk of the genome can contribute to maintain the large amount of non-coding DNA and intragenomic parasitic elements in a transcriptionally inert state, unmethylated CpG islands in promoter regions permit gene expression if appropriate transcription factors are present. DNA methylation has been widely associated with gene transcription silencing, although increased levels of DNA methylation across a region do not, in their self, guarantee that genes within that region will be silenced (12). Therefore, this effect appears to be mediated by an indirect mechanism involving recruitment of methyl-CpG binding proteins that act as recruiters for corepressor complexes, resulting in repressed chromatin and gene silencing (13), although densely methylated DNA regions could also preclude the binding of transcription factors, directly inhibiting gene expression, at particular genes (14). Important exceptions to the unmethylated state of CpG islands are the silenced alleles for imprinted genes and transcriptionally silenced genes in X chromosomes in mammalian females in normal tissues, and CpG island methylation of critical genomic targets (such as tumor suppressor genes) in a variety of pathologic conditions (15).

In mammals, both parental genomes are subject to global demethylation of methylated cytosines in early cleavage embryos (16). The DNA methylation patterns established at this developmental stage are heritable and remain relatively stable in somatic differentiated cells. Therefore, DNA methylation provides highly stable indexing marks that can be inherited from one cell generation to the next.

Histone modifications

Structurally, canonical and variant histones consist of a central globular domain and flexible NH_2- and COOH-terminal

tail domains. Although the globular domain interacts with nucleosomes, the tails protrude from them (6, 17) and are subject to over 100 different post-translational modifications to defined amino acids, including lysine acetylation, serine, threonine and tyrosine phosphorylation, lysine and arginine methylation, ubiquitination, sumoylation and ADP-ribosylation (18) (Table 1). Although histone modifications were first identified in the NH2-terminal tails, amino acids within the globular histone domains are also subjected to modifications similar to those on the tails and are important for nucleosome mobility and positioning, e.g., H4K91 (lysine residue at position 91 of H4) acetylation affects histone octamer formation (19, 20). In addition, linker histone H1 also carries diverse post-translational modifications, including phosphorylation, methylation, ubiquitination and ADP-ribosylation (7).

Some of the above-mentioned histone modifications, such as acetylation and phosphorylation, are relatively labile and can mediate regulation of gene expression over short-term periods. For instance, lysine acetylation reduces the net positive charge of the tails, weakens histone-DNA bounds, opens the chromatin and improves the ability of transcription factors to access DNA sites. Therefore, diverse histone acetylations (K9, K14, K18, K23 on H3 and K5, K8, K12, K16 on H4) have been widely associated with nucleosome remodeling and transcriptional activation, whereas deacetylation of these residues is generally associated with chromatin condensation and transcriptional repression.

Similarly, serine, threonine and tyrosine phosphorylation affects the ionic potential of the nucleosome and has a direct consequence on DNA accessibility and chromatin structure. Histone phosphorylation is also considered a highly dynamic modification and it has been associated with chromatin function in processes such as transcription, DNA repair, mitosis, apoptosis and chromatin architecture maintenance (21–23). Interestingly, the same phosphorylated site within a histone is involved in very different cellular processes. For example, H3S10 phosphorylation can be induced during immediate-early gene activation, in actively transcribed chromatin, or during mitosis, in which most of the chromatin is condensed, suggesting that phosphorylation could modulate different

Table 1 Main classes of chromatin regulators and their function.

Chromatin modification	Writer	Eraser	Reader
Covalent modifications			
DNA			
Methylation	DNMTs: DNMT1, DNMT2 DNMT3A, 3B DNMT3L	DNA glycosylases e.g., ROS1, DME, DML2, DML3 (plants); MBD4, TDG (mammals)	MBDs: MBD1–4; MeCP2 Some zinc finger domains SRA domain
Histone			
Acetylation	HATs: GNAT P300/CPB MYST proteins	HDACs: HDAC subfamily: I, II, IV Sirtuins family	Bromodomains
Phosphorylation	Serine, threonine and tyrosine kinases; e.g., Aurora B (H3); ATM (H2AX)	Phosphatases: e.g., PP1, PP2A	14-3-3 BRCT domain
Methylation	HMTs: KHMTs e.g., SUV39H1 (H3K9); EZH2 (H3K27); MLL (H3K4) RHMTs e.g., CARM1, PRMT4, PRMT5	Histone demethylases: Oxidases: LSD1 domain Hydroxylases: JmJC domain ?	Royal family domains: Chromodomains e.g., HP1 (H3K9); CBX proteins (H3K27) Tudor MBT WD repeat
Ubiquitination	Histone ubiquitin ligases e.g., Ring1B and 2A-HUB/hRUL138 (H2A); RNF8 (H2A, H2B)	Histone deubiquitinases: e.g., 2A-DUB (H2A)	
Other: Sumoylation ADP-ribosylation			
Remodeling	Helicases: SWI/SNF: Bromodomain ATPases ISWI: SANT domain ATPases Mi-2/NURD: Chromodomain8 ATPases INO80 subfamilies		

processes by directly affecting chromatin conformation or indirectly impinging on the recruitment of other factors.

In contrast to acetylation or phosphorylation, histone methylation does not alter the positive charge of the targeted lysine or arginine and functions primarily by influencing the binding of nonhistone proteins such as transcription factors or chromatin remodeling enzymes to nucleosomes, indirectly affecting chromatin structure.

Histone methylation can mark both transcriptionally active and repressive chromatin: whereas H3K4, H3K36 and H3-K79 methylations are often associated with an open chromatin structure surrounding promoter regions of active genes, H3K9 and H3K27 methylations are largely associated with transcriptional silencing, repression and maintenance of stable heterochromatin (24–27). In addition, multiple degrees of methylation can occur and each lysine residue can be mono-, di- or trimethylated and, similarly, arginine residues can be mono- or dimethylated in a symmetric or asymmetric manner (28). The distribution of mono-, di- and trimethylated residues is not uniform, but displays specific and discrete patterns depending on the genomic region. For instance, mono- and dimethylated H3K4 (H3K4me/me2) are broadly distributed, with slight enrichments downstream of the transcription start site (TSS), whereas trimethylated H3K4 (H3-K4me3) is enriched around the TSS and preferentially in sequences downstream of the TSS, a distribution pattern closely related to that of RNA polymerase II (RNAPII) (29, 30).

Although early speculation prompted the notion that methylation might be the ideal epigenetic indexing system in which position and degree of methylated amino acidic residues would unequivocally determine transcriptional activation or repression (31, 32), challenging studies on key developmental genes of embryonic stem cells (ESCs) revealed the coexistence of H3K4me3 (associated with open chromatin and active transcription) and H3K27me3 (associated with a repressive chromatin conformation) on silent promoters in ESCs (33, 34). Later on, these bivalent marks have also been identified in various progenitors and differentiated cell types (35). In these bivalent regions the repressive mark generally overrides the effect of the activation mark, and it primes and maintains in the state of 'hold on' those genes not required in ESCs, progenitor or differentiated cells, but that could be necessary for later differentiation or to respond to diverse stimuli (36).

As can be easily imagined, different covalent marks on histones and DNA are found in combinations and are interconnected to act synergistically to recruit chromatin-associated proteins or transcription factors. For example, whereas H3K9 acetylation is incompatible with methylation of adjacent H3K9 residues, it appears to facilitate H3K4 methylation (37). Another association concerns DNA methylation and histone modifications; this relationship was first noted in plants, in which mutations that interfere with H3K9 methylation abolish DNA methylation (38). In mammals, H3K9 methylation is enriched at promoters with hypermethylated CpG islands when their transcription is impaired, whereas this site is acetylated and unmethylated when these genes are actively transcribed and CpG islands are unmethylated (38). Along the same lines, H3S10 phosphorylation and H4K16 acetylation act cooperatively to promote transcriptional activation (39, 40).

Although not as well documented, other histone modifications in canonical and in variant histones also have important roles in regulating chromatin structure and functions. Among them, histone ubiquitination has been known for more than three decades (41), although its functional significance is now starting to be unveiled.

Ubiquitin is a 76 amino acid protein and protein polyubiquitination (addition of four or more ubiquitin moieties) is a widely used mechanism to target proteins for degradation. However, histone ubiquitination is mostly due to the attachment of a single ubiquitin moiety to a lysine residue, which is not enough to target proteins for turnover (42).

H2A was the first protein found to be ubiquitinated and it represents the most abundant ubiquitination substrate in mammals (5–15% of total H2A). uH2A (monoubiquitinated H2A at lysine 119) is essential for proper DNA repair and gene transcription regulation (43, 44), including transcriptional silencing of *Hox* genes and X chromosome inactivation of female somatic cells (45–47). uH2A mediates a silenced state of RNAPII present at promoters and coding regions of bivalent genes in mouse ESCs and blocks the recruitment of FACT (facilitates chromatin transcription) protein, resulting in RNAPII pausing and inhibition of transcriptional elongation in a subset of regulated chemokine gene promoters (48, 49). In addition, uH2A is thought to cooperate with H3K27me3 to mediate gene silencing and it has been proposed to function downstream of H3K27me3 (45, 46).

H2B ubiquitination affects no more than 2% of the total H2B, but uH2B levels exceed that of uH2A within active chromatin regions. uH2B can regulate H3K4 and H3K79 methylation (50, 51) and it is important for transcription, meiotic recombination and DNA damage checkpoint control (52–54).

H3 and H4 ubiquitination occurs at substantially lower levels (0.05–0.3%) and facilitates cellular response to DNA damage. For instance, H4K91 monoubiquitination, within the core globular domain, is involved in the cellular response to DNA damage, presumably by changes in nucleosome conformation that result in H4K20 exposure to histone methyltransferases (HMTs) (55). In addition, other histones including H1, H2A.Z and macroH2A have also been found to be monoubiquitinated (42).

Since the N-terminal 72 amino acids of the ubiquitin have a tightly folded globular structure, it is very likely that ubiquitination functions to physically modify chromatin conformation, in addition to recruitment of additional factors (26).

Finally, it is worth mentioning that other histone modifications such as sumoylation, ADP-ribosylation, deimination of methylarginine to citrulline, and proline isomerization have also been associated with transcriptional regulation and other cellular functions, but literature on these modifications is scarce.

Chromatin regulators

Chromatin regulators are often being referred to as writers, erasers and readers of the post-translational chromatin modifications, in an attempt to classify the abundant and growing numbers of proteins implicated in chromatin regulation in one way or another. Accordingly, chromatin writers bear a catalytic domain that directly modify the DNA or the histones, such as DNA methyltransferases (DNMTs), HMTs, histone acetyltransferases (HATs), kinases and ubiquitin ligases, among other enzymes. Erasers are those proteins that remove the corresponding mark, whereas readers carry domains able to recognize and bind different marks. For instance, chromodomains or PHD fingers recognize histone methyl-lysine marks, whereas bromodomains recognize specific acetyl-lysine marks (26, 56, 57) (Table 1). Although useful, this classification oversimplifies the nature of many chromatin-modifying proteins, as many writers and erasers also contain reading modules.

DNA modifying enzymes

DNMTs transfer a methyl group from *S*-adenosyl-L-methionine (SAM) to cytosine. In mammals, the DNMT family includes five proteins: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (DNMT3-like). DNMT1 preferentially methylates the cytosine of a CpG if the cytosine on the complementary DNA strand is already methylated and is referred to as maintenance methylase. DNMT1 binds PCNA (proliferating cell nuclear antigen) in the replication fork and is responsible for copying the methylation pattern of parental DNA onto the newly synthesized strand during DNA replication (58). In addition, in early ESCs and cancer cells methylation of previously unmethylated DNA can occur. This so-called *de novo* methylation is preferentially mediated via DNMT3A and DNMT3B.

In mammals, three types of domains confer the ability to read the 5-methylcytosine mark: the MBD (methyl-CpG binding) domain, some zinc finger domains and the SRA (SET and Ring finger-associated) domain (59). Among the MBD-containing proteins (MeCP2 and MBD1–4), MeCP2 was the first to be characterized owing to its selective binding to methylated CpGs in heterochromatic regions. Later on, it was observed that almost all MBD proteins are localized in highly methylated chromatin regions involved in silencing of imprinted genes, endoparasitic sequences and heterochromatin, where they promote genomic stability and transcriptional repression (60, 61). Importantly, genetic defects in MeCP2 are responsible for Rett syndrome, an X-linked postnatal autism spectrum disorder (62).

Methyl-CpG binding proteins often attract other inactivation complexes, including histone deacetylases (HDACs) and lysine histone methyl transferases (KHMTs), which alter the conformation and functional state of the DNA (60).

Removal of DNA methyl marks can occur through two distinct processes, commonly referred to as passive and active demethylation (63). Passive DNA demethylation occurs when maintenance methyltransferases are inactive during the cell cycle following DNA replication, resulting in the absence of methylated cytosines in the newly synthesized strand. Active DNA demethylation involves one or more enzymes and can occur independently of DNA replication. In addition, active demethylation can be direct, erasing DNA methylation through a base excision repair pathway by a DNA demethylase, or indirect, in which methyl-cytosine is first chemically converted to an intermediate product before DNA glycosylase acts (64).

The identity of enzymes that promote active DNA demethylation has been elusive for many years, but recent research in plants led to the characterization of two DNA demethylases, ROS1 and DME, and two related proteins (DML2 and DML3) as 5-methylcytosine DNA glycosylases that initiate a base excision pathway for active and direct DNA demethylation (64). In mammals, although the mechanisms and enzymatic activities involved are still controversial, active DNA demethylation has long been suspected. For instance, passive demethylation, which requires time to occur, cannot account for a very rapid demethylation of the male pronucleus in the zygote of preimplantation embryos (64). Other processes in which a replication-independent demethylase activity must be involved include mammalian gametogenesis (in which a genome-wide demethylation of the parental imprints occurs) and in rapid responses related to long-term memory formation in adult neurons and to cytokine production by the immune system (65). Although biochemical support exists for active and direct DNA demethylation through the DNA glycosylases MBD4 protein and TDG (thymine DNA glycosylase), their enzymatic activity to eliminate 5-methylcytosine is very weak (66, 67) and evidence is accumulating for pathways of active and indirect DNA demethylation based on DNA repair, including mechanisms involved in nucleotide excision, long-patch base excision or mismatch repair (64, 66).

Histone modifying enzymes

One of the most thoroughly studied enzymatic activities is lysine acetylation, which is associated with transcriptional activation (26). HATs do not in themselves show any sequence-specific DNA binding but can associate with sequence-specific DNA binding proteins, targeting them to specific regions. They are grouped into three major families: GNATs (Gcn5-related *N*-acetyltransferases), p300/CBP (CREB-binding protein) and MYST proteins, all of which form multiprotein complexes (56, 68). These families differ in several ways: HAT module size, biological outcome and histone substrate specificity.

Histone acetylation can be reverted by HDACs, which are also found in multiprotein complexes targeted to specific genes and chromosomal regions by association with additional proteins, such as nuclear hormone receptors, MeCP2 and MBD2. HDACs are classified into two families: HDAC subfamily, which requires Zn^{2+} and comprises classes I, II and IV, and the Sirtuin family, which requires NAD^{+} as cofactor. In general, HATs and HDACs do not appear to show much specificity for a particular lysine or acetyl-lysine in the histones, although the limited specificity detected for

some HATs and HDACs in yeast suggests that in some organisms this mark could finely tune gene expression (26).

In contrast to acetylation or deacetylation, the switch between phosphorylation and dephosphorylation of different residues of the histones is controlled by highly specific kinases and phosphatases that dynamically regulate several cellular functions. In mammals, two phosphorylation events have been characterized the most. The first is H3S10 phosphorylation, and prominent among the kinases responsible are those activated by mitogens, cytokines and stress, which include, among others, Aurora kinases, PKB/Akt and Msk1/2 (69). Phosphorylated H3S10 prevents heterochromatin protein (HP1) binding during mitosis and favors transcription initiation by recruiting chromatin remodelers and RNAPII (21, 70). In addition, phosphorylated H3S10 can be recognized by several members of the 14-3-3 phospho-binding protein family, which seem to function as bridges between H3 phosphorylation and histone acetylation during early transcription elongation (40, 71). Once signaling subsides, the action of the kinases is counterbalanced by phosphatases such as protein phosphatase 1 (PP1), which directly interact with H3 and form complexes with HDACs and histone demethylases (72). The second event is H2AX phosphorylation at serine 139 by PI3-K-like kinases, including ATM, ATR and DNA-PKcs, upon DNA damage. Once DNA damage is repaired (see below), several, and probably redundant, protein phosphatases such as PP2A, PP4, PP6 and Wip1 have been shown to dephosphorylate H2AX to silence the checkpoint and restore chromatin structure (73).

In mammals, coordinated balance between histone kinases and phosphatases is critical for physiological processes such as learning and memory formation and its deregulation has been linked to several pathologies such as neuronal dysfunctions and cancer (69, 72, 74).

The deposition of a methyl moiety in the histones is carried out by highly substrate-specific enzymes that use SAM as methyl donor. Most HMTs contain SET domains [SET: Su(var)3-9; Ezh2; Trithorax], although not all of these domains have HMT activity. An exception can be found in DOT1, the enzyme that methylates H3K79 but does not carry a SET domain (26).

HMT specificity is not restricted to lysine or arginine position in a defined histone but also discriminates the number of methyl groups to be attached. In addition, arginine HMTs (RHMT) can attach one or two methyl groups to arginine residues allowing variation in the symmetry of these groups. This diversity regarding histone methylation is accomplished by an unusually large family of SET domain-containing proteins (more than 100 members in mammals) (75).

As indicated above, histone methylation can be either activatory or repressive. The first HMT identified was Su(var) 3–9 (Suppressor of variegation 3–9; SUV39H1 in mammals), the major KHMT responsible for H3K9me3, which is associated with gene repression and heterochromatin formation. Other important families of H3K9 KHMT comprise G9a, GLP and SETDB1. These enzymes have different affinities for the un-, mono- or dimethylated states and produce different methylation states. Interestingly, it has recently been shown that the existence of H3K9 methylation multimeric complexes composed, among other proteins, of SUV39H1, G9a, GLP and SETDB1 cooperate to progressively establish H3K9me3 to regulate gene expression and pericentric heterochromatin formation (76). All these H3K9 KHMTs interact with DNMTs, providing a mechanism to maintain H3K9 methylation profiles coupled to DNA replication (76).

Another histone modification largely present in heterochromatin and associated with gene repression is H3-K27me2/3. This mark is established by EZH2 (Enhancer of zeste), of the Polycomb repressive complex 2 (PRC2), which has been found overexpressed in aggressive forms of solid cancers (77). Polycomb group (PcG) genes were initially discovered in *Drosophila*, where they have a specific function in maintaining expression patterns of *Hox* genes (77–79). Although it was initially thought that PcG had a broad regulatory potential restricted to developmental related processes, the list of PcG target genes in various primary and transformed mammalian cell types is continuously expanding (12, 80). In mammals, PcG proteins are classified into two main groups of multimeric protein complexes termed PRC1 and PRC2, although it is increasingly accepted that multiple versions of these two main complexes with alternative subunit composition and distinct functions exist (77, 81). The PRC2 core includes the KHMT EZH2, SUZ12 (Suppressor of zeste 12) and EED (Embryonic ectoderm development). Like EZH2, its close homolog EZH1 forms a PRC2-like complex that also mediates H3K27me2/3, although to a lesser extent than the canonical EZH2/PRC2 complex. Since EED is required for all three methylation states of H3K27, EZH2 and EZH1 may also mediate H3K27 monomethylation (36). Importantly, EZH2 binds to H3K27me3 and would, therefore, be available to copy this methylation on newly incorporated histones during cell division (82).

Deposition of methyl marks associated with active chromatin is carried out by more than ten different H3K4 HMTs in mammals, including SET1a, SET1b and MLL1–4 (Mixed lineage leukemia). MLL proteins are the mammalian homologs of the *Drosophila* Trithorax that regulates maintenance of *Hox* gene expression by counteracting the PcG repressive action (78). SET1 and MLL1–4 are part of multiprotein complexes catalyzing the transfer of mono-, di- and trimethyl groups to H3K4. MLL1 has been shown to interact with the initiating form of RNAPII (83), according to H3K4me3 enrichment around TSS. Importantly, MLL chromosomal translocations lacking the SET domain are extremely frequent in pediatric leukemia (84).

Histone methyl marks are read by several proteins that bear conserved domains of the royal family of protein modules. These include Chromo, Tudor, MBT and WD repeat domains that read distinct methyl signals and subsequently direct different downstream effector proteins in histone signaling (75). For instance, H3K9 methylation is recognized by the chromodomain-containing protein HP1 (75). Likewise, specific chromodomain-mediated interactions occur between methylated H3K4 and CHD1 (chromo-ATPase/helicase-DNA binding domain 1) and between methylated H3-K27 and chromodomain-containing PRC1 proteins (85).

In *Drosophila*, PRC1 core includes Pcgf, Ring1, Phc, and Cbx components. In mammals, duplication of many PcG genes allows the assembly of various, functionally distinct PRC1 complexes depending on cell type and developmental stage (36), and deregulated expression of several PRC1 members has been linked to tumorigenesis (86). In mammals, five CBX proteins have well-conserved chromodomains and display distinct *in vitro* binding specificities towards H3K27me3 and H3K9me3. In addition, CBX proteins interact with RING1A and RING1B (87), with E3 ubiquitin ligase activity, resulting in H2A monoubiquitination (45–47). BMI1 and MEL18, also PRC1 proteins, direct the catalytic activity of RING1A/RING1B towards H2A lysine 119 (88, 89). As indicated above, uH2A blocks RNAPII association with the promoter and inhibits transcriptional elongation (48, 49), although other mechanisms could account for PRC1-mediated gene silencing such as inhibition of the binding of key transcription factors at promoter and/or enhancer regions, chromatin compaction or localization of silenced genes at repressive compartments in the nucleus. Indeed, it has recently been shown that compaction, rather than histone tail ubiquitination, confers *Hox* gene silencing by PRC1 in mouse ESCs (90).

It is also important to note that many genomic sites that accumulate PRC2 are not bound by PRC1, whereas most of the PRC1 accumulating regions also have PRC2 (79). Therefore, methylated H3K27, and probably other methyl marks, are necessary but not sufficient for PRC1 or other readers targeting and suggest that some of these proteins or protein domains can function in combinational recognition of distinct patterns of histone modifications.

Histone methylation was thought to be highly stable and that methyl marks could only be removed by relatively slow and limited mechanisms such as passive dilution, histone exchange or controlled histone proteolysis. After the discovery and characterization of the first histone methyl demethylase, LSD1 (lysine specific demethylase 1), in 2004 (91), many other enzymes involved in active histone demethylation have been identified for essentially most of the well-known lysine methyl sites and degrees in histones (92). To date, two different catalytic reactions, oxidation or hydroxylation, carried out by proteins with LSD1 or JmjC (Jumanji C) domains, respectively, can account for lysine demethylation. Whereas LSD1 acts to remove mono- and dimethyl moieties, JmjC-domain-containing hydroxylase-like proteins are able to demethylate mono-, di- or trimethylated lysines.

Lysine demethylases have been identified in a continuously growing number of multiprotein complexes and their specificity will be influenced by the proteins they bind to and by adjacent chromatin marks. For instance, during androgen receptor-activated gene expression, H3T6 phosphorylation prevents LSD1 from demethylating H3K4 (93). Although it is clear that a direct mechanism of action is by counteracting either active or repressive methylation marks, histone demethylases will also affect chromatin conformation and transcription by their associated enzymatic activities, such as deacetylase and nucleosome remodeling activities (94).

In contrast to the abundant data on lysine methylation, the nature of proteins involved in the chemistry of arginine histone methylation is much less well known. Histone arginine methylation (which can also be associated with both transcriptional activation and repression) is controlled by RHMTs, such as CARM1, PRMT4 and 5, which catalyze monomethylation and symmetric and asymmetric dimethylation (26). Often the methylated arginine residues are localized nearby other post-translationally modified histone residues suggesting crosstalk between arginine methylation and other histone modifications. Importantly, a methyl-arginine binding protein has yet to be discovered and it is unclear to what extent protein arginine demethylation occurs, although possible erasers of this mark could involve the deaminase PADI4 or the hydroxylase JMJD6 (95).

As indicated above, the PRC1 protein RING1B is a ligase responsible for H2A monoubiquitination (45–47). Another specific histone H2A ubiquitin ligase, 2A-HUB/hRUL138, is recruited by the N-CoR/HDAC1/3 complex to a specific set of chemokine genes in macrophages (49), and it has been suggested that distinct H2A ubiquitinases, each recruited based on interactions with different corepressor complexes, contribute to distinct transcriptional repression programs (49). In contrast, RNF8 regulates both H2A and H2B ubiquitination at DNA damage sites and is required for mitotic exit (96) and promotes H4K16 acetylation, which is a critical modification for histone replacement by protamines during spermiogenesis (97).

Removal of the ubiquitin moiety is carried out by deubiquitinases. Histone H2A deubiquitinase 2A-DUB (KIAA-1915/MYSM1) is required for full activation of several transcriptional events including androgen receptor-regulated target genes in prostate cancer cells by forming a regulatory protein complex with the p300/CBP-associated factor HAT (98). In *Drosophila*, a PcG protein, BAP1, displays ubiquitin carboxy-terminal hydrolase activity and specifically removes monoubiquitin from H2A (99), suggesting a dynamic balance between H2A ubiquitination by PRC1 and H2A deubiquitination by a PcG deubiquitinase complex.

Several ligases involved in ubiquitination of other histones have been identified, for instance, yeast Rad6/Bre1 and human RNF20/40 for H2B, Cul4-DDB-Roc1 complex for H3/H4, and TAFII250 for linker histone H1, as well as a variety of deubiquitinases (Ubp8, Ubp10, and Usp7) for H2B.

Remodeling proteins

Chromatin structure can be directly modified by remodelers that affect its accessibility. They all contain helicase activity and are broadly classified into the SWI/SNF (bromodomain ATPases), ISWI (SANT domain ATPases), Mi-2/NuRD (chromodomain ATPases) and Ino80 subfamilies (7).

Chromatin remodelers use energy derived from ATP hydrolysis to mobilize nucleosomes, move away some or all of the histones from the nucleosome or to exchange histone variants in a non-covalent manner (100). In this regard, and in contrast to canonical histone deposition into chromatin that occurs exclusively during S-phase, histone variants are

incorporated outside of S-phase. For instance, variant H3.3 is recruited at highly expressed loci, whereas the unique incorporation of the centromere specific histone H3 variant CENP-A specifies the site of centromere identity (101). Therefore, incorporation of different histone variants is linked to specific chromatin conformations required to support specialized cellular functions.

Weaving chromatin networks

DNA organization into chromatin is essential for many eukaryotic processes such as replication, DNA repair or transcription, and elicits specific responses to environmental cues. As can be easily inferred, this enormous functional diversity cannot rely on the presence or absence of a single post-translational modification in the chromatin or the activity of a given chromatin regulator, but will be achieved by complex and highly specific interplays among chromatin marks and regulators that allow a remarkable array of possibilities. This notion is further supported by the continuously growing list of associations among them, and by the fact that most chromatin modifiers are part of multisubunit complexes that display a variety of enzymatic and docking abilities. In addition, recent research reveals that different composition of chromatin regulator complexes is associated with promoters of tissue-specific genes differentially regulated on time, providing additional variability to respond at different paces to diverse stimuli.

Underlying chromatin marks can influence the binding of some chromatin regulators and will determine the pattern of modifications to be generated. For instance, MLL1 shows enhanced methylation activity against highly acetylated histone tail substrates. Accordingly, treatment of cells with HDAC inhibitors not only leads to global hyperacetylation of core histones but to an enhancement in hypermethylated H3K4 (18). Other examples are the preference of PRC2 to methylate H3K27 in CpG-rich sequences, whereas H3K9 methylation and HP1 seem to confer repression to some CpG-poor promoters (12), indicating that H3K9 and H3K27 methylases function at separate targets that differ in sequence composition.

In addition, cooperation among different chromatin regulators to establish or maintain a specific chromatin conformation is frequently found. For instance, intricate connections between chromatin regulators are responsible for the maintenance of chromatin conformation in CpG-rich regions. In these regions, pre-existing methylation is recognized by MBD1 that forms a complex with the KHMT SETDB1. In addition, MBD1 can interact with the KHMT SUV39H1 and HP1. Further retention of HP1 in heterochromatin involves both self-association of HP1 proteins and their interaction with SUV39H1 (102). Moreover, DNMT1, that maintains the established DNA methylated pattern, interacts with HDACs and H3K9 KHMTs (103–106). In turn, HDACs can directly induce chromatin condensation by increasing the positive charge of the histones, acting by a *cis* mechanism, but they can also act in *trans*, deacetylating a particular lysine for subsequent methylation that will serve as docking surface for methyl-binding effectors. Therefore, silencing of these CpG regions is tightly maintained on various levels by interconnected mechanisms forming a self-sustaining loop.

Another example is the cooperation between HMTs and histone demethylases. Whereas H3K27 demethylases, that erase a repressive mark, associate with MLL2 to generate the epigenetic profile of active promoters (H3K4me3) (107), H3K4 demethylases, erasers of marks associated with open chromatin, cooperate with PcG to promote selective H3K27-me3 enrichment on repressed promoters (108).

Regulating the function of the chromatin regulators

Chromatin functions cover cellular processes that affect the totality of the genome, such as chromatin condensation or DNA replication, or short regions of the genome, such as transcription or DNA repair (Figure 1). Therefore, the mechanism responsible for the recruitment of different chromatin regulators depends on the particular chromatin environment (euchromatin or heterochromatin) and the required function at a given time and defines the appropriate chromatin regulator function.

A widely used model system for epigenetic maintenance of the chromatin structure is the replication of heterochromatin found at centromeres and other genomic regions, although mechanisms involved in the establishment of these regions during development are less clear. In mammals, these heterochromatin regions are associated with high levels of DNA methylation, low levels of histone acetylation, high levels of methylated H3K9, H3K27 and H4K20, and HP1 enrichment (102) (Figure 1). During replication, DNA polymerases are assisted by PCNA. PCNA, together with NP95, recruits DNMT1 which methylates hemimethylated CpG sites on daughter strands (102). PCNA, and methylated CpG, will further recruit other enzymatic and docking chromatin regulators, allowing the maintenance of chromatin conformation in these regions. Therefore, the DNA replication machinery is responsible not only for the semiconservative inheritance of the DNA sequence but also for DNA methylation and chromatin conformation at the replication fork.

Although it seems paradoxical, transcription can well be a prerequisite for the assembly and maintenance of some forms of silent chromatin. For instance, noncoding RNAs (ncRNAs) transcribed by heterochromatin regions could actively recruit modifying enzymes that help assemble a higher order chromatin structure (i.e., some HP1 proteins have affinity for RNA), contributing to heterochromatin integrity in fission yeast and plants (109). In mammals, ncRNA transcripts from heterochromatin regions have also been detected in early S-phase (110) and, although their role in duplicating the chromatin structure is not yet clear, there is growing evidence that ncRNAs can contribute to epigenetic inheritance. One of the most prominent examples is the ncRNA Xist, which nucleates the repressive chromatin state for almost the entire X chromosome and is involved in its

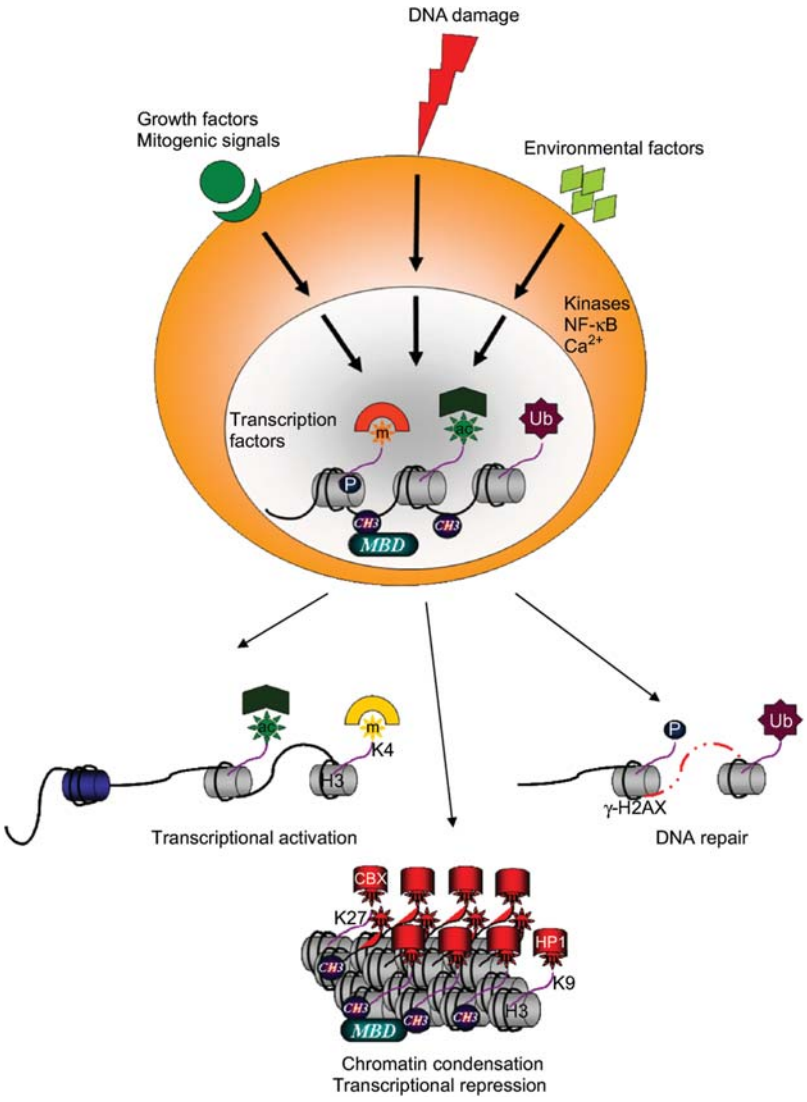

Figure 1 Integrative overview of mechanisms involved in the regulation of chromatin conformation and some of the pleiotropic functions regulated by the chromatin in response to different stimuli.
Eukaryotic cells are challenged to respond to a wide variety of stimuli, such as growth factors, DNA damage agents or environmental conditions. Depending on the nature of these stimuli, different signal transduction pathways are involved, which will result in chromatin modifications (for instance, methylation, histone phosphorylation, acetylation or histone replacement), or to the recruitment and/or activation of transcription factors, replication or DNA damage response machineries, that will be in charge of recruiting or regulating diverse chromatin regulators. Crosstalk among chromatin marks and regulators result in changes in chromatin conformation that modulate different outcomes, such as transcriptional activation or repression, chromatin condensation or DNA repair.

inactivation in mammalian females (111, 112). Another ncRNA is TERRA (Telomeric repeat-containing RNA), which is transcribed towards the chromosomal end and binds chromosome ends, contributing to telomerase regulation (113–115).

Regarding transcriptional regulation, different mechanisms can account for the specific recruitment of chromatin regulators to target genes. One possibility is that the general transcription machinery could serve as a docking platform to recruit chromatin regulators, possibly providing a positive feedback. In fact, the initiating form of the RNAPII has been shown to interact with MLL1 (83), and it has been proposed that the CxxC motifs (which bind preferentially to unmethylated CpGs) present in MLL1 and MLL2 could be involved in target selection (12).

In addition, the study of the epigenetic regulation of pluripotency networks has revealed the interplay between transcription factors involved in the maintenance of the pluripotency and chromatin regulators, and several chromatin modifiers that confer gene silencing are bound by OCT4, NANOG, SOX2 and/or SAL4 (1). Importantly, temporally limited overexpression of different combinations of transcription factors is sufficient to reactivate endogenous pluripotency genes and to regain a developmental potency akin to that of ESCs (2, 3), reflecting the ability of these tran-

scription factors to extensively recruit those chromatin regulators required to reprogram differentiated cells for iPS derivation.

In addition to genome-wide connections between transcription factors and chromatin regulators, concrete and specific associations between both players to precisely control the expression of a number of genes upon different stimuli have also been reported. For instance, in yeast the p38 functional homolog, Hog1 kinase, regulates the response to osmotic stress by favoring the recruitment of SWI/SNF, RNAPII and other components of the general transcription machinery to target promoters (116) (Figure 1). Likewise, phosphorylation of MEF2D by p38 kinases mediates the recruitment of the HMT MLL complex to the chromatin of muscle genes, thereby promoting H3K4me3 (117).

Less clear are the mechanisms involved in PcG recruitment to chromatin to mediate transcriptional repression, despite several genome-wide mapping studies in a variety of tissues and cell types to identify PcG targets. Because PcG complexes do not bind their target DNA in a sequence-specific manner, current models propose that a combination of several DNA binding factors, and maybe components such as ncRNAs, could lead to PcG tethering (79). In addition, PcG binding sites strongly correlate with the presence of repressor motifs and absence of motifs capable of conferring transcriptional activity within CpG islands in mammalian pluripotent cells (118), suggesting that DNA-binding factors and CpG islands are likely to be working combinatorially (79).

Another chromatin function involving relatively short genomic regions is DNA repair. In response to genotoxic stress, the decision either to repair the DNA or to undergo apoptosis relies on H2AX dephosphorylation at tyrosine 142, which is phosphorylated in unstressed cells (119). Whereas phosphorylated Y142 prevents binding of repair factors, dephosphorylation of H2AX at Y142 by EYA protein tyrosine phosphatases facilitates H2AX phosphorylation at serine 139 (119). This phospho-motif is recognized by the MDC1 complex (Mediator of DNA damage checkpoint I) that recruits early proteins to foci, such as the ubiquitin ligase complexes UBC13 and RNF8. As a consequence, a cascade of ubiquitination reactions is triggered, affecting H2AX, H2A and other core histones, possibly functioning in DNA damage signal amplification (120). Later on, the DNA repair machinery (and possibly some of the proteins involved in DNA replication) will fix and restore DNA sequence and chromatin marks and conformation by recruiting several chromatin regulators (Figure 1).

Finally, it is important to stress that the function of the chromatin regulators can also be affected by post-translational modifications, although less is known about them. For instance, DNMT1 can be methylated, and this modification reduces its stability, whereas phosphorylation of HP1β by CK2 (casein kinase II) causes its release from methylated histone H3K9 (1). Also, an interesting function for *O*-linked β-*N*-acetylglucosamine glycosylation (*O*-GlcNAcylation) in the regulation of PcG and Trithorax members have been identified, and *O*-GlcNAcylation of MLL5 is required for the H3K4 methyltransferase activity of MLL5 (79).

Expert opinion

During the past few years, great progress has been made in identifying chromatin marks and in the characterization of several proteins involved in chromatin regulation, although new marks and chromatin regulators await to be discovered. In addition, genome-wide studies have shed light on the preferences of a given chromatin modification or modifier for specific genomic regions and revealed cross interactions among different chromatin regulator complexes. Importantly, the epigenetic nature of some chromatin marks at specific chromatin regions during mitosis has been or is about to be established. Much of this understanding comes from the study of model organisms (mostly yeasts and stem cell-based differentiation models in mice), which will continue to provide important information as they are pure population systems that can be easily genetically modified. However, although useful for creating a conceptual chromatin regulation framework, this information should not be extrapolated to other cellular contexts and to other genomic regions in different lineage cells from other organisms. In addition, identification and characterization of the full complement of proteins within the variety of chromatin regulator complexes is a challenging task.

Outlook

The enormous efforts of the scientific community have laid the foundations of chromatin regulation, but there are many aspects that remain unveiled. First, we must remain open to the identification of new candidates for chromatin regulation, such as ncRNA or proteins involved in asymmetric division or in meiosis. Equally important will be the regulation of different regions of the genome in specific cell lineages under physiological and pathological conditions and the regulation of chromatin regulators by environmental stimuli in humans. In addition, in the coming years we must expect to have new clues on how different chromatin regulators contribute to chromatin organization in high-order structures, an old concept recently rescued for PcG-mediated repression (90). A more detailed knowledge of these mechanisms will allow the identification of targets for the pharmacological treatment of diseases such as cancer or immunologic and neurologic disorders.

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