Histone modifications and a choice of variant: a language that helps the genome express itself

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Abstract

Covalent post-translational modifications on histones impact chromatin structure and function. Their misfunction, along with perturbations or mutations in genes that regulate their dynamic status, has been observed in several diseases. Thus, targeting histone modifications represents attractive opportunities for therapeutic intervention and biomarker discovery. The best approach to address this challenge is to paint a comprehensive picture integrating the growing number of modifications on individual residues and their combinatorial association, the corresponding modifying enzymes, and effector proteins that bind modifications. Furthermore, how they are imposed in a distinct manner during the cell cycle and on specific histone variants are important dimensions to consider. Firstly, this report highlights innovative technologies used to characterize histone modifications, and the corresponding enzymes and effector proteins. Secondly, we examine the recent progress made in understanding the dynamics and maintenance of histone modifications on distinct variants. We also discuss their roles as potential carriers of epigenetic information. Finally, we provide examples of initiatives to exploit histone modifications in cancer management, with the potential for new therapeutic opportunities.

Histone marks: an evolving language

The discovery nearly fifty years ago that gene expression correlates with hyperacetylated histones [1] hinted at the importance of factors beyond the DNA sequence and how transcription factors control genome function. In the following years, the identification of histone methylation and phosphorylation [2-4] expanded the repertoire of modifications. Then, work in yeast made the first connection between mutations in histone tails and transcription [5,6], setting the stage for a functional link between histone modifications and gene expression. The connection between acetylation and chromatin function was further supported in Drosophila, using antibodies recognizing acetylated lysines on histone H4 [7]. The characterization of the first histone-modifying enzymes harboring acetyltransferase, deacetylase, methyltransferase, and demethylase activity [8-13]

represented major breakthroughs, by providing a first handle on how to control the modifications. The principle of a dynamic system that responds to cellular stimuli and environmental cues to regulate chromatin structure and function emerged [14]. The presence of these modifications (or "marks") was viewed as acting in two ways: (a) directly, by altering the electrostatic potential and/or the structure of the local chromatin environment to open or restrict access to DNA or (b) indirectly, through the recruitment of effector proteins that carry out a biological event. The latter aspect led to the proposal of the histone code hypothesis that states that histone post-translational modifications (PTMs) act sequentially or in combination to signal downstream events [15,16]. Interestingly, the possibility that these marks could be stably propagated to contribute to the maintenance of cellular identity across many cell

generations placed them as candidate vehicles of epigenetic information [17]. Together, these simple concepts with writers, readers, and erasers initiated a burst of interest spanning a range of scientific disciplines. Yet, we are still debating the existence of a code, defined as one or a combination of marks that signal a particular event. Furthermore, the repertoire of players continues to expand when one considers all histone modifications [18], the enzymes [19], effector proteins [20], and the different histone variants [21,22], and this sophisticated language is evolving with implications in diverse biological activities [23,24]. In this short report, to highlight recent advances in the role of histone marks (modifications and variants), we review novel characterization techniques and provide new insight into their potential inheritance and dynamics in order to discuss emerging approaches in cancer management.

An interdisciplinary effort to deconvolve a complex language

Antibodies that recognize site-specific histone modifications remain crucial tools used to study histone marks [7,25], and are routinely exploited in chromatin immunoprecipitation (ChIP) approaches. Combining ChIP with other techniques, such as with next-generation sequencing (ChIP-seq), enables the mapping of histone marks genome-wide [26-28], whereas combining it with bisulfite sequencing correlates histone marks with DNA methylation [29] and can extend to its other modified forms. One limitation is that these approaches require prior knowledge of the associated protein and/or modification, and the availability of a suitable antibody. Importantly, DNA capture techniques, combined with mass spectrometry, provide opportunities to identify unknown modifications and associated proteins. In one approach, work in yeast introduced a unique DNA sequence that is recognized by a DNA-binding protein that acts as a handle, enabling the purification of a single locus [30]. Alternatively, DNA capture using nucleotide derivatives enables the investigation of histone marks and associated proteins at sites of DNA synthesis [31,32], shedding light onto the maintenance of histone marks following replication. Finally, the recent development of gene editing tools, including transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPRs) [33-35], provides opportunities to analyze the impact of the loss of factors involved in the dynamics of histone marks.

Importantly, the binding of proteins, including antibodies, to histone marks can depend on neighboring modifications [36], potentially altering their specificity, and giving

rise to artifacts. Here, chemical approaches, including advances in peptide synthesis protocols [37-40], have made significant contributions to efforts to better characterize antibody and protein binding using peptide arrays *in vitro* [41,42]. A useful systematic analysis of nearly 200 antibodies using a standardized method employed by the ENCODE consortium is available online [43] (see http://compbio.med.harvard.edu/antibodies/). Peptide arrays have also helped in elucidating the substrate specificity for the modifying enzymes [44-47]. Remarkable advances in chemical biology include the use of label-free assays, which minimize artifacts associated with labels [48,49], and high-throughput platforms, used to identify small molecule modulators of histone modifying enzymes [50,51]. Finally, mass spectrometry (MS) has proven to be a powerful tool, particularly in its quantitative format [52,53], used to identify novel modifications [54,55], crosstalk [56], and to explore their dynamics [57]. This use of mass spectrometry has been extensively reviewed [58-60].

Notably, many assays including ChIP provide only a snapshot of the histone modification landscape, and generally represent an average, over a population of cells. This hinders studies of dynamics, and by averaging over heterogeneous cell populations, key information can be lost or diluted. This has motivated efforts towards single-cell analyses. An early example of single-cell analysis exploited imaging techniques with the use of antibody Fab fragments coupled to fluorophores, a technique termed FabLEM [61], to visualize the dynamics of endogenous modifications in individual living cells. Another study labeled different antibodies with distinct fluorophores to simultaneously visualize multiple marks in single cells isolated using microfluidic devices [62]. Of note, these single-cell approaches tend to assess histone modifications globally across the genome. An alternative approach aims to investigate individual genomic loci in single cells [63] by combining *in situ* hybridization and the proximity ligation assay (PLA) [64]. This assay is compatible with histological sections [63] and could therefore extend to the clinic. Optimization of these techniques and others, such as the development of ChIP-seq at single-cell levels, will open avenues for assays with applications at the bench and in the clinic.

Maintenance of histone marks: should I stay or should I go?

Histone modifications are present not only on nucleosomal histones but also on histones in transit following their synthesis. Exploiting epitope-tagged histone variants in order to purify soluble histone complexes [65] revealed that different pools of non-nucleosomal

histones feature distinct marks [66,67], and some are more prevalent on particular variants [68]. Thus, the issue of the histone variants, whose expression is cell cycle regulated, is a critical component of the picture [21,22].

When present in chromatin, histone modifications are commonly referred to as epigenetic marks, yet often function simply as a signaling module in the short-term and their longevity is still under exploration. To be formally considered an epigenetic mark (as defined by Robin Holliday), the modification should be stably inherited through cell division in the absence of the initiating event [69]. However, dynamics through the cell cycle, in particular during replication (where there is a genome-wide transient disruption of the chromatin organization), challenges heritability. In the wake of the replication fork passage, parental histones are interspersed with newly synthesized histones, diluting parental marks (Figure 1) [70,71]. Notably, evicted parental H3-H4 tetramers are thought to remain intact and to randomly distribute to daughter cells in cultured cells. However, some tetramers could proceed through a dimeric state, and then either rapidly re-associate or remain dimeric and mix with new H3-H4 dimers. Several factors are implicated in these processes to ensure proper packaging of the duplicated DNA [72,73], in particular histone chaperones [74], which represent key players in epigenetic inheritance. Indeed, the histone chaperone anti-silencing function 1 (ASF1) is a candidate to coordinate the recycling of parental and new histones (and associated modifications). Through its connection with the MCM2 helicase [75], ASF1 handles parental H3-H4 dimers and can associate with new H3-H4 dimers, acting as a donor to hand off histones to another H3-H4 chaperone, chromatin assembly factor 1 (CAF-1). CAF-1 acts in a manner coupled to DNA synthesis to deposit the replicative H3.1 variant [65]. Failure in the deposition process can present gaps that are later filled by the histone regulator A (HIRA) chaperone to deposit H3.3 throughout the cell cycle [76]. Importantly, these dynamics may differ in various cellular contexts or cell types. In any event, it is interesting to consider the impact of recycling parental histones and associated marks where nucleosomes feature asymmetric modifications [77] and/or histone variant heterotypic particles [78].

Once deposited, the landscape of histone modifications and variants may be altered through replication-independent dynamics [76,79] and may have an impact on chromatin function. Indeed, H3.3 accumulates at transcription sites and regulatory elements [76] deposited by either the HIRA or DAXX (death associated protein)

chaperones. Furthermore, a recent example in yeast shows that the Set2-mediated methylation of H3K36 impacts replication-independent dynamics that can alter the acetylation state and give rise to cryptic transcription. Perturbation of this methylation pathway, perhaps by altering the expression or activity of the Set2 methyltransferase or an H3K36 demethylase (KDM4A), promotes nucleosome turnover and results in an accumulation of acetylated histones at the coding regions of genes, allowing transcription factors access to cryptic sites [80]. Histone marks may also be maintained following replication due to histone-modifying enzymes (Figure 2A), some possibly associated with the replication fork [81]. Interestingly, the placement of variants can potentiate the action of enzymes, such as SUV39h1 (suppressor of variegation 3-9 homolog 1), an H3K9 methyltransferase [12]. Indeed, H3K9me1, more prevalent on soluble H3.1 compared to H3.3 [68], is a preferred substrate for SUV39h1 to establish H3K9me3. This leads to the binding of the heterochromatin protein 1 (HP1), a mark of pericentric heterochromatin [82]. Furthermore, existing modifications may recruit enzyme complexes to write modifications on neighboring newly synthesized histones. For example, the polycomb group (PcG) protein complex can recognize and bind to H3K27me3-modified histones. This suggests a model where the PcG complex can bind parental H3K27me3 histones, thereby recruiting enzymatic activity to newly synthesized histones to spread this mark (Figure 2B) [83]. Indeed, in Drosophila, a mutation in the PcG protein EED, which impairs its ability to bind H3K27me3, decreases global H3K27me3 levels [83]. While MS approaches support a model where H3K27me3 remains following replication [31,84,85], a study in Drosophila embryos (using the PLA assay) failed to detect H3K27me3 at replication foci but rather observed the responsible enzyme, enhancer of zeste (E[z]) [86]. It will be interesting to further test this effect with other assays and in different model organisms. Interestingly, recent work in plants shows that histone variants can also impact H3K27 methylation. Here, the enzymes ATXR (arabidopsis trithorax-related protein)5 and ATXR6 specifically monomethylate H3.1K27, as plant H3.3-specific T31 inhibits methylation, an effect not observed with other plant methyltransferases (Figure 2C) [87]. Together, these examples highlight the importance of assay choice and model systems when analyzing histone modifications and suggest that even conserved modifications may be subject to different regulatory mechanisms in various species. Notably, it is important to consider instances where histones do not have the capacity to bear the same modifications either due to mutations [88-91] or the presence of variants, such as the centromeric H3 variant
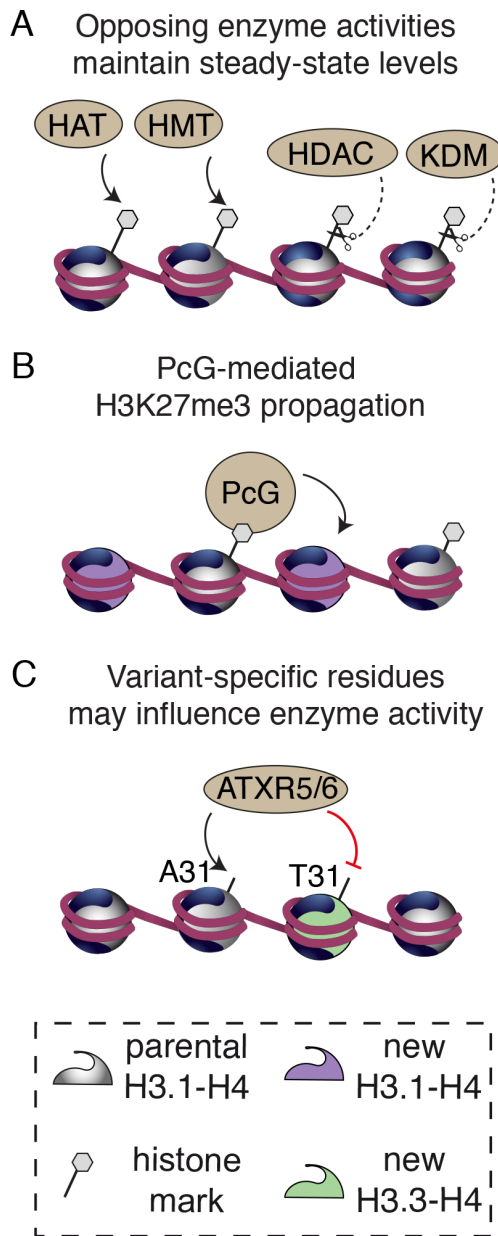
Figure 1. The choreography of histone modifications and variants at the replication fork with their chaperones
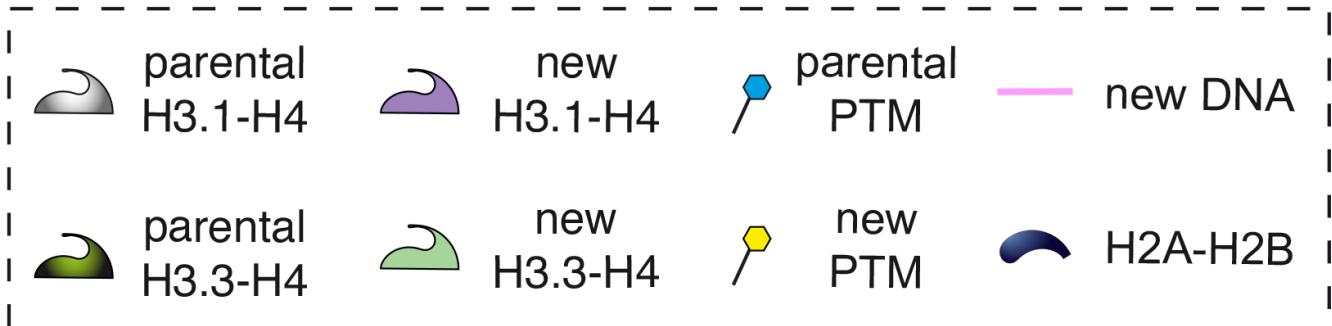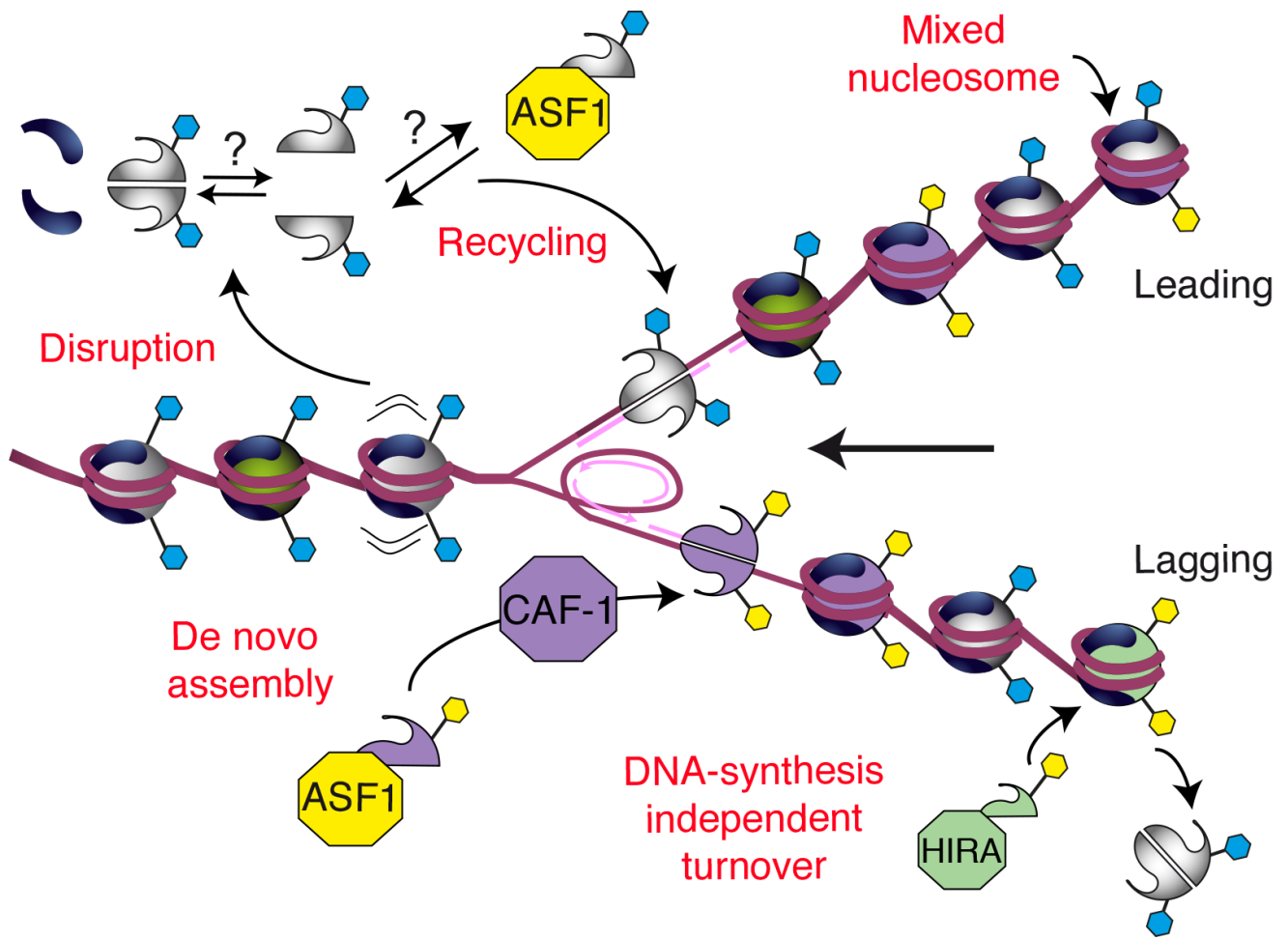
Histone chaperones participate in histone deposition during replication and are key candidates to regulate epigenetic inheritance. Parental H3-H4 histones evicted from chromatin are handled by the chaperone ASF1, which also associates with newly synthesized histones [75]. Parental histones and marks are recycled onto daughter strands along with newly synthesized histones with characteristic marks [68,107], the latter deposited by the chaperone CAF-1. Mixing of parental and new histones is rare [108] but may have functional consequences. Importantly, away from the fork, histones are susceptible to turnover, which can alter the modification landscape.

Abbreviations: ASF1, anti-silencing function 1; CAF-1, chromatin assembly factor 1; HIRA, histone regulator A; PTM, post-translational modification.

Figure 2. Histone modification dynamics and maintenance mechanisms

A   Opposing enzyme activities maintain steady-state levels

B   PcG-mediated H3K27me3 propagation

C   Variant-specific residues may influence enzyme activity

parental H3.1-H4   new H3.1-H4

histone mark   new H3.3-H4

(A) Enzymes having opposing activities maintain steady-state levels of histone marks. (B) A model where the polycomb group protein complex propagates H3K27me3 by binding to the parental histone mark, which recruits the responsible enzyme to methylate neighboring nucleosomes [83]. (C) Histone variant-specific residues can influence how enzymes entertain their substrates, as shown with the ATXR5/6 methyltransferases in plants, where T31 (S31 in mammals) inhibits monomethylation of H3K27 [87].
Abbreviations: ATXR, arabidopsis trithorax-related protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; (note H is sometimes replaced with K to represent lysine methyltransferase – see [109]); KDM, lysine demethylase; PcG, polycomb group protein.

CenH3 (also known as CENP-A) [92], which lack the target residue and how this can impact chromatin integrity.

Code red! Histone marks in cancer
Genomic and epigenomic factors are now recognized as contributing to cancer [93-96]. Interestingly, recent evidence showcases links between the genome and epigenome, leading to new questions about how they cooperate in order to impact cancer progression and whether this can be exploited to better manage patient care. A pioneering study identified mutations in histone H3, including H3K27M, in pediatric glioblastoma [88], a finding recently supported in several new reports [89-91,97]. Biochemical evidence shows that even a fraction of histones presenting this mutation is sufficient to impact global H3K27 methylation levels [98]. Additionally, new data have shed light on how histone marks impact genome stability. The overexpression of KDM4A, which demethylates H3K9 and H3K36, leads to site-specific copy gain of regions often amplified in human tumors [99]. Interestingly, SUV39h1 or HP1γ (which binds H3K9me3) antagonizes this effect, highlighting the importance of balancing opposing enzyme activities [99]. Furthermore, a selective inhibitor for an H3K79 methyltransferase kills acute leukemia lines bearing mixed lineage leukaemia (MLL) translocations [100], bringing hope of a therapy for this disease that has limited treatment options. It will be important to better understand the links between genome and epigenome as this may identify novel candidates for targeted therapeutic intervention, particularly in cancers with distinct genome anomalies.

The histone-modifying enzymes are common targets for identifying small molecule modulators. However, highly conserved active sites among similar enzymes have challenged the development of selective inhibitors. Interestingly, the sensitivity to histone deacetylase (HDAC) inhibitors varies depending on the protein complex [101], suggesting that altering the concentration of the inhibitor may target different HDAC complexes and thereby different groups of genes. An emerging strategy is to target the effector proteins, such as the bromo- and chromodomains, which bind acetylated and methylated lysines, respectively. Indeed, the demonstration that small molecule bromodomain inhibitors, such as JQ1 [102] and i-BET [103], can alter gene expression has motivated additional screens for other inhibitors. The Structural Genomics Consortium (SGC) has identified several potential epidrugs and has made them and corresponding structural information available to the research community (for more information see www.thesgc.org), advancing our understanding of how epidrugs impact cell function. Another approach that has gained momentum is combining "epidrugs", either with other "epidrugs" or with classical therapeutic

regimes. Indeed, combining certain demethylase inhibitors with pan-HDAC inhibitors has a synergistic effect on cell death in glioblastoma cells, providing a proof-of-concept to test other combinations of epidrugs for enhanced sensitivity [104]. Interestingly, changing the chromatin structure through epidrugs also sensitizes cells to radiation [105]. This could enable lower doses and higher selectivity to cancer cells and minimize harmful side effects. Epidrugs have also shown promise in slowing the onset of resistance or reversing the resistant phenotype [106]. The future success of epidrugs in the clinic will depend on a better understanding of how these compounds function in cells, thus necessitating studies integrating several aspects of chromatin dynamics. Furthermore, identifying markers that could be exploited as companion diagnostics will help identify the patients that will most likely clinically benefit, improving overall patient care.

Conclusions and perspectives

The past few years have seen significant progress in our understanding of histone marks and their implications in diverse biological processes. However, several important questions remain. How can mutations in histones found in certain cancers alter the histone modification landscape and thus chromatin function? How do enzymes of opposing activities cooperate to establish a modification landscape with proper dynamics? Are histone modifications propagated through cell division and if yes is this inheritance due to the presence of recycled parental marks or a cellular memory that acts to re-establish the marks? Are variations in the dynamics of the chromatin landscape a cause or consequence of tumorigenesis? What is the impact of epidrugs on the global histone modification landscape, genome integrity, and on the proteome, featuring thousands of modification substrates? The answers to these questions should help place histone modifications within the network of other chromatin regulators and strengthen the link with both development and disease. In this respect, the histone variants deserve consideration in the histone code debate as the variants themselves impact the modification landscape as well as the dynamics of the genome itself, exemplified during DNA damage [79]. Code or not, the continuous development of new assays, including single-cell approaches, should help provide further insight into several aspects of histone modifications, such as cause or consequence of particular marks, and help identify novel biomarkers and targeted candidates for therapeutic intervention.

Abbreviations

ASF1, anti-silencing function 1; CAF-1, chromatin assembly factor 1; ChIP, chromatin immunoprecipitation; ChIP-seq, chromatin immunoprecipitation with next generation sequencing; HDAC, histone deacetylase; HIRA, histone regulator A; HP1, heterochromatin protein 1; MS, mass spectrometry; PcG, polycomb group protein; PLA, proximity ligation assay; PTM, post-translational modification; SGC, Structural Genomics Consortium; SUV39h1, suppressor of variegation 3-9 homolog 1.

Disclosures

The authors declare that they have no disclosures.

Acknowledgments

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