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SURVEY AND SUMMARY

A case study in cross-talk: the histone lysine methyltransferases G9a and GLP

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ABSTRACT

The histone code hypothesis predicts that the post-translational modification of histones can bring about distinct chromatin states, and it therefore serves a key regulatory role in chromatin biology. The impact of one mark on another has been termed cross-talk. Some marks are mutually exclusive, while others act in concert. As multiple marks contributing to one outcome are generally brought about by complexes containing multiple catalytic and binding domains, it appears regulation of chromatin involves a web of writers and readers of histone modifications, chromatin remodeling activities and DNA methylation. Here, we focus on the protein lysine methyltransferases G9a and GLP as examples of this extended cross-talk. G9a and GLP can catalyze the formation of and bind to the same methyl mark via distinct domains. We consider the impact of other histone modifications on G9a/GLP activity and the coordination of activities within G9a/GLP containing complexes. We evaluate the potential impact of product binding on product specificity and on maintenance and propagation of the methyl mark. Lastly, we examine the recruitment of other silencing factors by G9a/GLP. Regulated assembly of specific complexes around key marks may reinforce or alter the biological outcome associated with given histone modifications.

The histone code hypothesis recognizes that the post-translational modification of histones can directly impact chromatin structure or can be read by effector modules. Certain modifications have been correlated with unique transcriptional outcomes and chromatin states. Individually, the modifications of the histone code are bound with weak affinity. Binding modules with the same specificity are found in complexes with differing, and sometimes opposing, activities. For example, G9a and GLP have been described as both co-repressors (1–11) and co-activators (1,12,13). This has led some to doubt the validity of the histone code hypothesis, as they question the ability of a single modification to affect a biological outcome (14–16). Multivalent interactions have been proposed as the answer, where no single modification, but rather patterns of modifications and the network of interacting proteins that can read and write them are responsible for the regulation of chromatin (17,18). We adopt this extended view, and we focus on G9a/GLP as an example of this, because they feature the ability to bind their own product and to serve as an organizing hub for diverse activities.

The impact of one mark of the histone code on another is frequently observed. With the structural and biochemical characterization of more of the players in histone modification, the underlying mechanisms of cross-talk, and the interplay of histone cross-talk and other pathways, such as chromatin remodeling and DNA methylation and repair, are becoming clear (19–25). Mechanistically, cross-talk occurs when one or more binding modules and catalytic domains reside in the same complex or polypeptide, allowing coordination of different activities. Cross-talk can occur prior to catalysis, in which case the recognition of one mark (or its absence) can serve to recruit an enzyme to its substrate in the generation or removal of a second mark. This is essential for targeting and coordination of activities. We detail a second type of cross-talk, product binding, where binding by a reader module follows catalysis. G9a and GLP can bind their products via a domain distinct from the catalytic domain. In other cases, product binding has proved essential to maintain marks after their

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establishment or to spread the modifications. As sequence- and structure-specific DNA-binding modules within the modifier complexes may also play a large role in contributing specificity, we also consider their role in mediating gene-specific silencing in G9a/GLP complexes. Product binding also allows for more complicated signaling on chromatin, where a complex, bound to its product, can act as a platform to integrate diverse signals.

G9a/GLP

The highly similar euchromatic methyltransferases G9a-like protein (GLP, also known as EuHMT1) and G9a (also known as EuHMT2) form a heteromeric complex, and the loss of either substantially reduces mono- and dimethylation of H3K9, which is a marker of silent euchromatin (10,26–28). G9a has also been recognized for its ability to methylate histone H1.4 and other non-histone proteins, including itself (29–32). G9a and GLP bind their H3K9me1 and H3K9me2 products via ankyrin repeat domains (33), which contain a hydrophobic cage present in methyllysine binding modules of diverse folds. This cage binds H3K9me1/2 with approximately equal affinity, but it is too narrow to accommodate H3K9me3.

In G9a or GLP knockout cells, DNA methylation and HP1 binding to euchromatin are lost (10,34). The associated loss of silencing causes embryonic lethality, and differentiation of ES cells in the absence of G9a leads to apoptosis (26). G9a is required for the restriction of cell fate in development, for the silencing of the homeobox gene Oct3/4, which is required for pluripotency (4), for NRSF/REST-mediated silencing of neuronal genes in non-neuronal lineages (35), for PRDI-BF1 (Blimp-1) silencing in B-cell differentiation (5) and for CDP/cut mediated silencing of genes involved in cell differentiation and proliferation (8). The role of G9a/GLP as a gatekeeper of differentiation has been demonstrated by knockdown and with a novel inhibitor, BIX-01294 (36–39). In the absence of the repressive H3K9me1/2 mark, genes normally expressed only in stem cells are induced, and cells are more readily reverted to pluripotent states.

The diverse functions of G9a/GLP appear to require different activities. The isolated catalytic domain targeted to DNA is sufficient for transcriptional silencing (9,40). Conversely, DNA methylation appears to require the ankryin repeat domain, but not histone methyltransferase activity (34,41,42). Lastly, methyltransferase activity has little or no effect on the transcriptional activation of nuclear receptor regulated genes, β-globin or rDNAs by G9a (1,12,13). We consider potential roles for G9a product binding (Figure 1), including competition with methyltransferase activity to restrict procession to trimethylated H3K9, blocking the back modification of the H3K9me1/2 marks by lysine demethylases, spreading of the methyl mark akin to the Su(var)3-9/HP1 system, and recruiting other proteins including histone H1, HP1, DNA methyltransferases and transcription factors.

The influence of other post-translational modifications on G9a/GLP activity

Unlike certain other methyltransferases, such as Set1 or Dot1 (43), G9a/GLP methylation is not dependent on pre-existing marks, but merely the absence of modifications to the target lysine or adjacent residues is sufficient for catalysis. This limited catalytic cross-talk in G9a/GLP relates to their recognition of a fairly short stretch (minimally H3T6-H3T11) of H3 for methylation (30). Acetylated H3K9 and phosphorylated H3S10 or H3T11 block methylation, while H3K4 acetylation or methylation merely reduces activity. Additionally, arginine methylation at H3R8 blocks activity (30,44). Product binding is blocked by phosphorylation of H3S10 (33).

Coordination with other activities

A few studies have probed the spatiotemporal relationships of modifications during transcriptional activation or silencing. One compelling study traced the silencing of the *Dntt* (deoxynucleotidyltransferase, terminal) promoter during differentiation of thymocytes and it clearly established a temporal sequence of events from active to silent chromatin (45). During the maturation of the thymocytes, the promoter was stripped of activating modifications by deacetylation of H3K9 and demethylation of H3K4. The modifications were first lost at the promoter and then spread outwards. Conversely, silencing H3K9 methylation nucleated at the promoter and spread over 10 kb outward from the promoter over time. Transcription was strongly reduced with the loss of the activating modifications and fully quenched when dimethylated H3K9 (H3K9me2) reached ~50% of its maximal value. Lastly, DNA CpG methylation increased as the cells terminally differentiated. Coincident with this process, the gene relocalized to a region of silent heterochromatin. Interestingly, in a cell line with reversible *Dntt* silencing, modifications were nucleated at the promoter, but did not spread. Notably, the study did not identify the H3K9 methyltransferases involved and we include it as a fine example of the coordination of silencing activities, but not of G9a/GLP function. The developmental silencing of the Oct3/4 promoter follows a similar course, with loss of activating modifications and gain of G9a/GLP-mediated H3K9 methylation, followed by association of HP1 and DNA methyltransferases (4).

Similar coordination has been seen in other silencing complexes. Small heterodimer partner (SHP), known for its ability to silence transcription mediated by a number of nuclear-receptors, is able to interact with histone deacetylases (HDACs), a H3K9me2 methyltransferase (G9a) and a silencing SWI–SNF family complex (3). It appears that HDAC activity precedes histone lysine methylation. H3K9me2, in turn, was required for recruitment of the silencing chromatin remodeling complex (3). Similarly, a RE1-silencing transcription factor (REST) complex containing an H3K4me3 demethylase (SMCX), HDACs and H3K9me2 methyltransferases (G9a) is targeted to neuron-restrictive

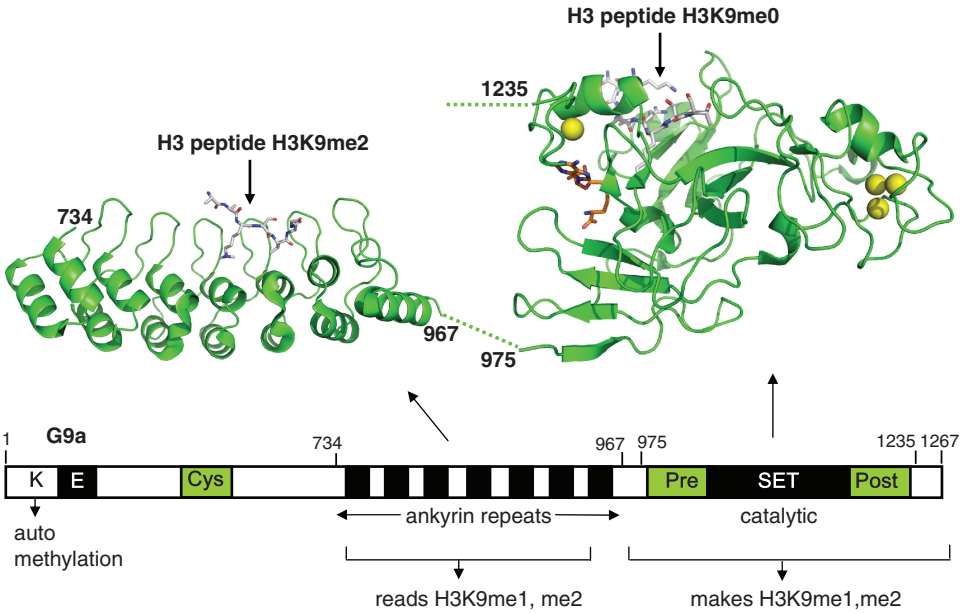

Figure 1. Domain organization of G9a and GLP. G9a and GLP consist of a N-terminal domain with automethylation site(s), the product-binding ankyrin repeats and the catalytic SET domain, which also mediates formation of heteromers (PDB codes 3B95 and 2RFI).

silencing elements (a conserved sequence in the promoters of a subset of neural genes) (46). In this case, H3K4me3 demethylation appeared to coincide with histone deacetylation, which was followed by H3K9 methylation (46). The next challenge is making clear the molecular mechanisms that underlie these patterns.

Interplay of product binding with degree of methylation

Constitutive heterochromatin is distinguished by a high concentration of H3K9me3, while euchromatin/facultative heterochromatin is primarily marked with H3K9me1/2 and limited regions of H3K9me3. *In vitro*, G9a rapidly generates both mono- and dimethyllysine, but it more slowly proceeds to trimethylation (47,48). Although the G9a and GLP knockouts most noticeably reduce global H3K9me1 and H3K9me2, G9a may mediate some trimethylation *in vivo*. Long residence at a target site may allow the formation of trimethyllysine, as G9a tethered to a synthetic mini-locus, as a Gal4 DNA-binding domain fusion generates H3K9me3 *in vivo* (49) and G9a trimethylates itself (29,30,32). *In vivo*, G9a-mediated H3K9me3 has been detected, typically in small regions near promoters. For example, in the absence of G9a, H3K9me3 levels have been found to be reduced at the Oct3/4 promoter, at the promoters of paternally imprinted genes and at a host of other developmentally silenced genes (4,42,50). H3K9me3 reduction at G9a-silenced promoters (but not non-target promoters) is also observed when G9a is chemically inhibited, though the knockout of G9a or GLP had a slightly more potent effect in the same study (38). Since G9a and GLP appear to bring about multiple levels of methylation, potentially based on the duration of their residency on a target, they must strike a balance between proceeding to trimethylation and restriction to lower methylation states. Of particular interest is the distinction between the lower states of H3K9 methylation (me1 and me2), which the G9a and GLP ankyrin repeats can bind (33), and higher states (me2 and me3), which are preferred by HP1 (51). All three degrees of methylation bring about silencing, as even a monomethylating mutant G9a (F1205Y) transgene was able to silence target genes in G9a-null ES cells (47). The ability to reverse a mark and reactivate transcription also relates to the degree of methylation, as some demethylases, such as JHDM2A and LSD1, can demethylate H3K9me1/2 but not H3K9me3 (52,53). Other demethylases, such as those of the JMJD2 family that can act on trimethylysine, would be required for removal of the H3K9me3 mark (54–56).

One untested hypothesis is that the G9a and GLP ankyrin repeats could compete with the non-processive catalytic reaction, quenching the reaction at the mono- and dimethyl state and protecting substrates from trimethylation by competition with the catalytic domain. Such competition could come into play whether G9a/GLP themselves are responsible for catalyzing the H3K9me2 to H3K9me3 transition, or another enzyme (such as SETDB1) carries out this process. The ability to limit methylation will be variable, as some G9a substrates will make poor binding partners for the ankyrin repeats.

In particular, for ankyrin repeat binding, H3K9 through H3G13 were observed in the crystal structure. A diglycine motif (H3G12 and H3G13) is required to allow for a sharp turn and mutation to alanine reduced binding (33). These features are largely dispensable for the methylation, where only H3T6-H3T11 are required for G9a/GLP SET domain activity (30). H3R8—which is indispensable for methylation—and preceding residues do not appear in the ankyrin repeat structure. Although the substrate requirements for methylation have been studied exhaustively, the current understanding of binding specificity of the ankyrin repeats comes from a

single target. It would be interesting to determine whether the substrates that are not bound as products are more prone to conversion to trimethylation, either by G9a/GLP or another methyltransferase. Taken together, the histone and non-histone methyltransferase activity of G9a and GLP, potentially moderated by the product binding domains, along with the action of competitive demethylases, may allow a distinction between the 'permanent' silencing associated with imprinting or restriction of cell fate, where HP1 dependent chromatin compaction and DNA methylation are warranted, and more dynamic processes, such as cell-cycle dependent regulation, silencing in cell types with plastic fates, metabolic feedback (e.g. bile-acid metabolism) and cell signaling through nuclear receptors (2,3,6,57).

G9a/GLP product binding and 'gatekeeping'

A second possibility is that G9a and GLP bind their products to protect the modification, competing with demethylases that might back-modify the mark (Figure 2). This 'gatekeeper' function has been suggested by studies showing GLP or G9a, among several other methyltransferases, restrict unliganded nuclear receptors from activating transcription (57). Liganded nuclear receptors require a partner demethylase to promote transcription. This begs the question of whether it is the methyl mark or the physical presence of the methyltransferase and associated complex that serves as the gatekeeper. Treatment with estradiol decreased promoter occupancy of several methyltransferases and the knockdown of the methytransferase itself is sufficient to yield transcriptional activity in the absence of ligand. This suggests active, promoter specific competition between methylases and demethylases. Sequestering methylation through product binding could provide a physical means of blocking removal of the mark. At the same time, the physical presence of the methyltransferase complex could block promoter access and aberrant transcription. Additionally, since G9a/GLP heteromers could bind tails of neighboring nucleosomes, substantial local cross-linking could occur, contributing to compaction of local chromatin (Figure 3).

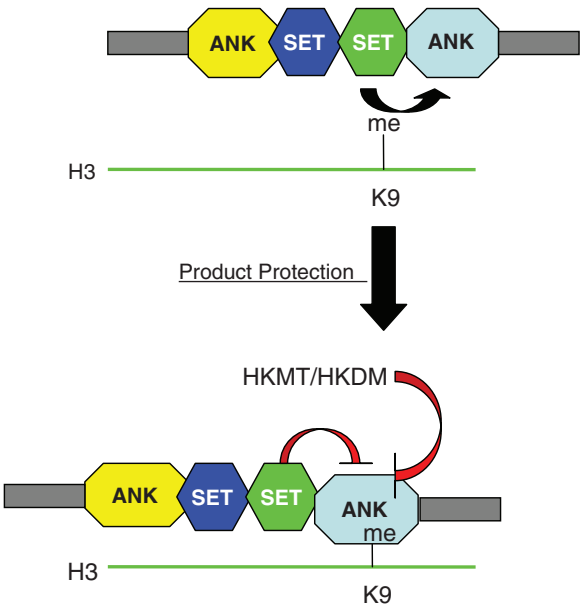

Figure 2. Product protection. Product binding could protect the H3K9me1,2 from Histone Lysine Demethylases (HKDMs), or conversion to H3K9me3 by Histone Lysine Methyltransferases (HKMTs), including that of G9a/GLP.

Recruiting allies to reinforce silencing

H1.4. This role as a chromatin-compacting gatekeeper is reinforced by the ability of G9a to recruit other proteins, including histone H1.4, HP1 and C/EBP (CCAAT/Enhancer-binding protein), to chromatin. Though there are several H1 variants, they are generally silencing and contribute to higher order chromatin structure. H1 positioning serves to block nuclesome repositioning and sliding, and it occludes access of other proteins to DNA [reviewed in (58)]. H1 variants appear to have distinct, non-overlapping roles, and H1.4 participates in dynamic, gene specific silencing (59). G9a methylates H1.4 at lysine 26 (H1.4K26), suggesting a cross-talk where the presence of methylated H3K9 could recruit G9a to methylate H1.4K26, or vice versa. G9a also appears to participate directly in recruiting H1.4 to chromatin (59). It will be interesting to determine if product binding is involved, as the residues flanking K26 appear ideal for ankyrin repeat binding (K_{26}SAGG versus K_9STGG). G9a binding of H1.4K26me could facilitate its placement

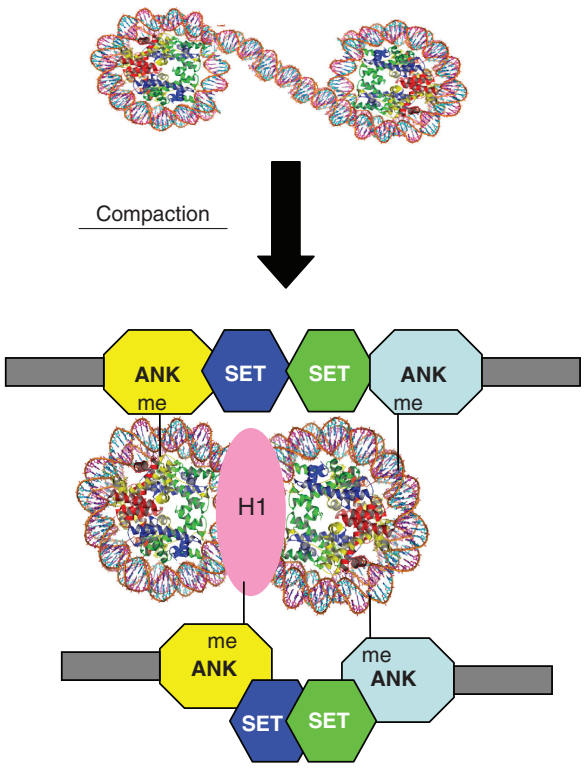

Figure 3. Compaction of chromatin. Polyvalent binding could compact chromatin by crosslinking nucleosomes bearing H3K9 methylation. One member of the dimer, and one H3 tail are shown for simplicity. Alternatively, G9a methylates H1.4, and facilitates its binding to chromatin (though the requirement of product binding has not been formally demonstrated).

onto chromatin and reinforce the alteration of chromatin structure brought about by H1 (Figure 3). The methylation of H1 serves as another mark to reinforce silencing, as methylated H1.4 is a binding platform for HP1 (59,60), for L3MBTL1, which participates in chromatin compaction (59) [reviewed in (61)] and for MSX1, a homeobox transcriptional repressor (62). Completing the cycle, H1.4 lysine demethylases were also identified (59).

HP1. G9a and GLP are required for the binding of HP1 to euchromatin, as the knockout of either greatly diminishes euchromatic HP1 staining, and loss of G9a reduces HP1 occupancy at specific promoters (4,10,63). Paradoxically, although the G9a catalytic domain is sufficient for silencing when targeted to a reporter gene, it is insufficient for HP1 recruitment (9). It appears that G9a targets HP1 to chromatin by automethylating itself (Figure 4a), potentially at multiple sites (29,32). The dominant automethylation site (found in GLP also) mimics the region around H3K9, even containing a phosphorylation site that blocks HP1 binding. Mutation of the target lysine eliminates HP1 binding and causes HP1 to delocalize from G9a containing loci. Interestingly, both relevant studies found G9a automethylation sites to be di- and trimethylated (29,32). Consistent with our proposal that ankyrin repeat binding to mono- and dimethylated products might regulate product specificity, the identified automethylation sites contain substitutions (KTMSK instead of KSTGG in H3) that should render binding to ankyrin repeats impossible. Again, degree of methylation is all-important, as HP1 has a preference for H3K9me2/3 over H3K9me1 (51). The best *in vivo* demonstration of this specificity is provided by the overexpression of a demethylase (GASC1) that removes the H3K9me2/3 marks but cannot act on H3K9me1 (55). The gain in H3K9me1 at the expense of H3K9me2/3 was insufficient to retain HP1 binding (55). The requirement for direct interaction of G9a with HP1 is reminiscent of Suv39H1 mediated targeting of HP1, where not only H3K9 methylation, but also a direct interaction between Suv39H1 and HP1 is required for HP1 residence (9,64).

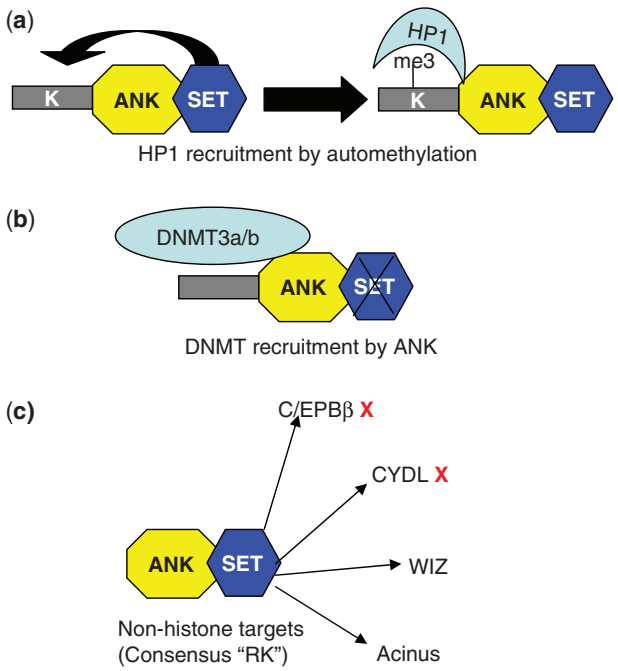

Figure 4. Recuritment. (**a**) G9a automethylation recruits HP1. (**b**) DNA methyltransferases interact with G9a in a manner not dependent on catalytic activity. (**c**) G9a/GLP have been shown to bind or to methylate a number of targets. G9a methylation of C/EPBβ blocks transcriptional activation, and G9a methylation of the CYDL chromodomain blocks its ability to bind histone (red X's). Many other targets have been identified, some of which may also be bound as products.

As HP1 itself has been implicated in the recruitment of other protein and DNA methyltransferases in the formation of not only silent heterochromatin but also euchromatin, this could prove an important gateway into other mutually reinforcing mechanisms of silencing (65). Some studies have proposed a direct interaction between G9a and the DNA methyltransferases (see below). However, HP1 binding to automethylated G9a or H3K9me2/3 could serve to recruit DNMT3a and DNMT3b. Indeed, HP1 knockdown has little effect on G9a occupancy at the TNFα promoter, but DNMT3a, DNMT3b and CpG methylation were lost in the absence of HP1 or G9a. This suggests that G9a is the key organizer of silencing at that promoter and that recruitment of DNMTs requires HP1 as a bridge (63).

DNA methyltransferases. There is a surprising lack of cross-talk involved in G9a-mediated DNA methylation. G9a and GLP are both required for normal levels of DNA methylation in facultative heterochromatin and interact with DNA methyltransferases. An attractive model emerges, where G9a and GLP might establish H3K9 methylation, bind to it, and then attract DNA methyltransferases directly, or through HP1. However, several studies have shown the catalytic activities of G9a and GLP to be dispensable for DNA methylation, and that G9a-mediated DNA and histone methylation are independent pathways, either of which can accomplish silencing (34,41,42). This implies that G9a and GLP play a more structural role in the context of DNA methylation, where their presence, but not activity, recruits DNA methyltransferases to target loci. Perhaps they bridge the DNA-binding partners of G9a (see above) and DNA methyltransferases. Indeed, G9a appears to interact directly with the *de novo* DNA methyltransferase DNMT3a and DNMT3b through its ankyrin repeat domain, even in the absence of the G9a catalytic SET domain (42) (Figure 4b). Whether this is physiological or perhaps an alternate pathway that suffices to bring about some methylation in the absence of a pathway that does more linearly couple histone and DNA methylation is unclear.

DNA binding and other target proteins. G9a also has been reported to inhibit the CCAAT/Enhancer-binding protein-β (C/EBP-β) (31). G9a interacts with and methylates C/EBP-β, blocking its ability to activate transcription. Binding is not observed in the absence of a

functional catalytic domain, which suggests product binding; however, this was not formally tested (31). Many other targets for G9a methylation have been identified (29,30); all of which are known to play roles in processes involving chromatin (Figure 4c). It is unclear how many of these substrates can also meet the requirement for product binding to the ankyrin repeats. In general, lysine methylation exerts its effect through modules that bind the methyl marks. As an exception to this rule, G9a methylation appears to block directly the CYDL chromodomain from binding H3K9me3, apparently by altering the chemical properties of a key lysine (30).

Spreading the methyl mark

G9a and GLP are responsible for broad regions of H3K9 methylation up to 4.9 Mb, in what have been termed 'large organized chromatin K9 modifications' (LOCKS), which can cover up to nearly 50% of the genome in some differentiated cell types (66). The finding that there are few LOCKS in embryonic stem cells, and that they grow in number with differentiation is under dispute [see (67), rebuttal in (68)]. However, the existence of LOCKS, their apparent tissue specificity and the presence of G9a/GLP-mediated methylation within LOCKS is not (68). These broad patterns of methylation are at odds with the narrow targeting of G9a and GLP to primarily promoter regions. For example, G9a is found in complex with the CCAAT displacement protein, a transcription factor involved in differentiation and development (8), Blimp-1 (also known as PRDI-BF1), a DNA-binding protein involved in the terminal differentiation of B lymphocytes (5) and is linked to the CtBP co-repressor via an adaptor protein (11). Several DNA-binding zinc-finger proteins also recruit G9a (7,69). Spreading the modification outward from these sites could bring about the methylation of large regions of chromatin adjacent to regions where G9a and GLP are targeted. In spreading, the binding of product [via their ankyrin repeats (33)] could position G9a and GLP to methylate the next neighboring nucleosome, which then can serve as a binding site for enzyme (Figure 5). The cyclic repetition of this process would allow the mark to propagate over great distances. Although this model is attractive, G9a is also targeted to chromatin by non-coding RNAs, which form large nuclear sub-compartments to which genes are recruited during silencing (70–72). Long non-coding RNAs are best recognized for silencing clusters of imprinted genes, but given the volume of non-coding RNAs detected, and their specific expression patterns in development and differentiation [reviewed in (73,74)] it would not be surprising if the formation of G9a-containing LOCKS is a RNA-mediated process. These nuclear sub-structures may distinguish the large swaths of more permanent silencing associated with cell differentiation and imprinting from more localized and reversible domains of G9a- and GLP-mediated silencing.

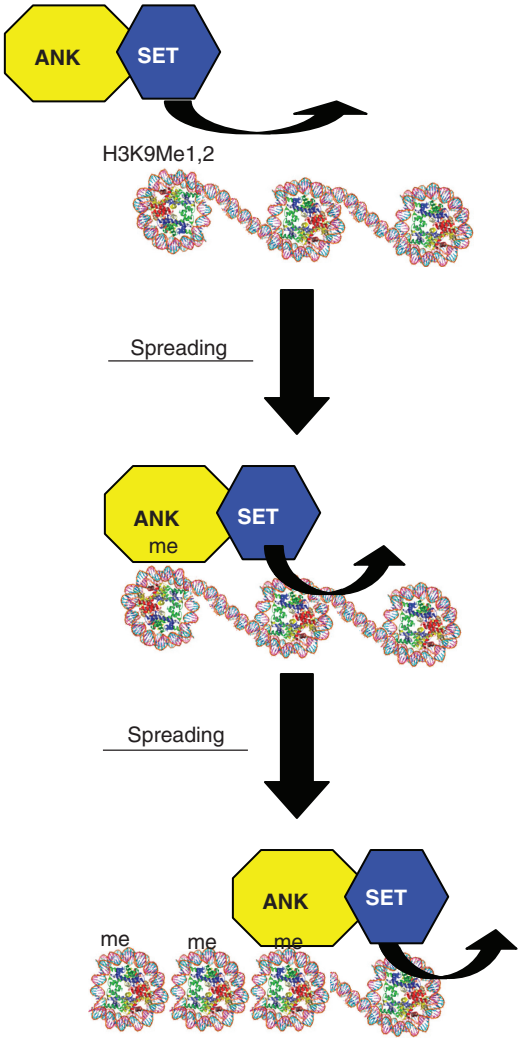

Figure 5. Spreading. Like Su(var3-9)/HP1 and yeast Clr4 (SET and chromodomain), product binding could help propagate the methyl mark by posing the catalytic domain for attack on the next neighboring nucleosome. Repetition of this process allows spreading of the mark from the site of nucleation.

CONCLUSIONS

The web of interactions detailed above will likely benefit from a systems approach like those applied to signaling networks. Catalytic activity, product binding, RNA binding and partner binding will have to be analysed. Of particular interest is defining what non-histone targets of G9a/GLP can be bound as products. It is unknown what governs the switch to co-activation by G9a—whether the presence of another mark like arginine methylation or recruiting a key transcription factor is important. Similarly, it is unclear if DNA methylation follows lysine methylation and HP1 recruitment, or if the two are independent pathways bridged by G9a/GLP acting as a scaffold. Lastly, we ask whether the propagation of the methyl mark occurs by product-binding mediated spreading, a RNA-mediated process, or both.

G9a and GLP provide a tractable system to test hypotheses, as point mutants perturbing catalytic activity and product binding are defined. One unanswered

question that could complicate these experiments is why, despite their apparent heteromeric nature, perturbation of one activity typically has a penetrating effect. In the case of complete knockouts, loss of one member of the complex causes the other to degrade (10), but in the case of more discreet deletions or point mutants, the answer remains enigmatic. Detailed analyses using mutations in both partners will likely be essential.

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