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Histone arginine methylation and its dynamic regulation

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1. ABSTRACT

Methylation of histones by protein arginine methyltransferases (PRMTs) is increasingly being found to play an important and dynamic role in gene regulation. In mammals, PRMT1- and CARM1-catalyzed histone asymmetric dimethyl-arginine is involved in gene activation while PRMT5-catalyzed histone symmetric dimethyl-arginine is associated with gene repression. Insight into mechanisms by which histone arginine methylation can be dynamically regulated comes from recent reports demonstrating that conversion of histone methylarginine residues to citrulline by peptidylarginine deiminase 4 (PADI4) leads to transcriptional repression. While the downstream cellular effects of histone arginine methylation remain poorly understood, recent findings indicate that protein arginine methylation, in general, is required for mammalian development and is also likely important for cellular proliferation and differentiation. Given the surge of interest in histone arginine methylation, this review article will focus on recent progress in this area.

2. INTRODUCTION

The fundamental repeating unit of chromatin is the nucleosomal core particle which contains two superhelical turns of approximately 146 base pairs of DNA wrapped around an octamer of two copies each of the core histones H2A, H2B, H3, and H4. Histone tail N-, and in a few cases, C-termini extend from the nucleosomal surface in an unstructured manner and contain a complex array of post-translational modifications that include: phosphorylation, acetylation, ubiquitination, ADP ribosylation, and methylation (1). A growing number of studies, many performed within the last decade, underscore a longstanding proposal in the chromatin field – that these modifications play a critical and active role in regulating many aspects of cell function including gene expression, chromosome dynamics, DNA replication, and DNA repair (2).

With respect to gene expression, histone acetylation and phosphorylation are highly reversible and

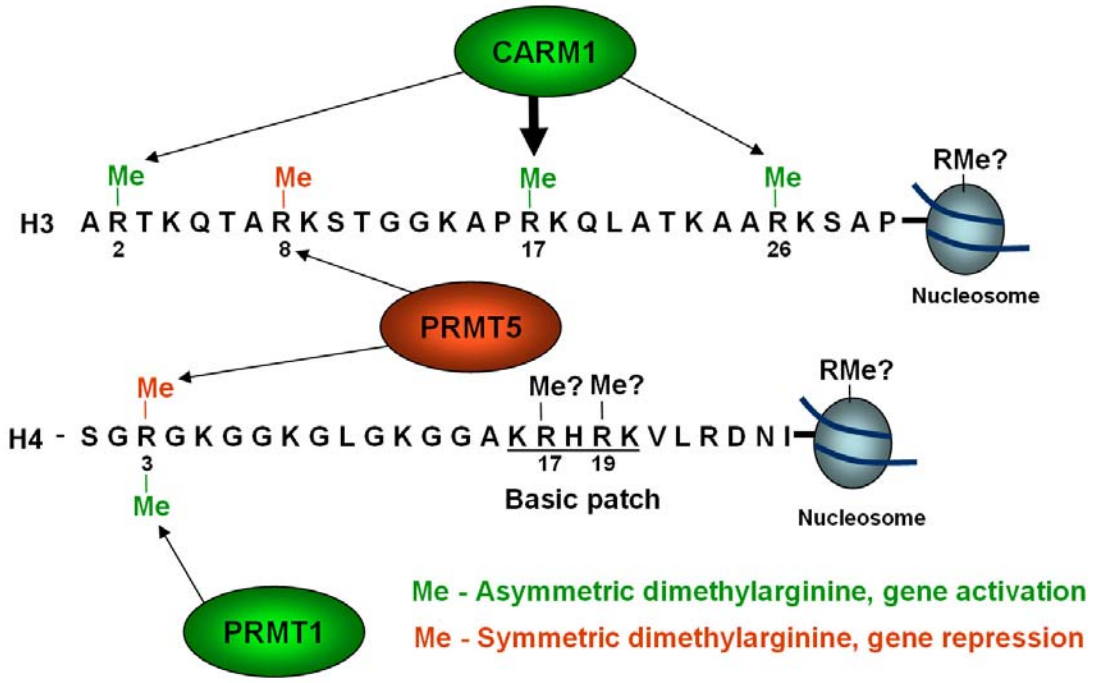

Figure 1. Sites of arginine methylation on histone H3 and H4. CARM1 methylates arginine residues 2, 17, 26, on histone H3 (larger arrowhead indicates H3R17, the major CARM1 target site). PRMT1 methylates arginine 3 on histone H4. PRMT5 methylates arginine 8 on histone H3 and arginine 3 on histone H4. CARM1- and PRMT1-catalyzed asymmetric dimethylarginine modifications activate gene expression (green) while the PRMT5-catalyzed symmetric dimethylarginine modification represses gene activity (red). It remains to be determined whether arginine 17, 19, and 22 on histone H4 and possibly other arginine residues within the histone H3 and H4 core peptide sequence are methylated. The "basic patch" (residues 16 through 20) on the histone H4 tail is involved in regulating higher order chromatin structure.

therefore can be utilized to fine-tune gene expression patterns in response to external stimuli and rapid changes in environmental conditions (3). Histone methylation, on the other hand, has, until recently, been thought to function as more of a long-term epigenetic information storage mechanism involved in faithfully propagating specific gene expression patterns in order to maintain cellular "identity and memory" though both mitotic and meiotic cell divisions (4). While, the role of histone lysine methylation in epigenetic gene regulation is well documented (5-7), the role of histone arginine methylation has, until recently, received considerably less attention. Therefore, the focus of this article will be to review recent progress in this area and to discuss exciting new findings that histone arginine methylation also appears to be dynamically regulated. Interested readers are also directed to other excellent reviews which focus more generally on the role of protein arginine methylation in transcription (8-11).

3. ROLE OF HISTONE ARGININE METHYLATION IN GENE REGULATION

3.1. Protein arginine methyltransferases catalyze histone arginine methylation

Protein arginine methyltransferases (PRMTs) transfer the methyl group from S-adenosylmethionine (AdoMet) to the terminal guanidino nitrogens of arginine residues generating monomethyl-arginine, symmetric dimethyl-arginine, and asymmetric dimethyl-arginine. To date, three mammalian protein arginine methyltransferases have been found to catalyze histone methylation: type I arginine methyltransferases CARM1 (cofactor associated arginine methyltransferase) and protein arginine methyltransferase 1 (PRMT1) generate monomethyl-arginine and asymmetric dimethyl-arginine derivatives, while type II arginine methyltransferase PRMT5 (also called JBP1, for Jak kinase binding protein 1) generates monomethyl-arginine and symmetric dimethyl-arginine derivatives (12, 13). CARM1 methylates arginines 2, 17 and 26 and one or more of four arginines (128/129/131/134) at the C-terminus of histone H3, and histone H2A (14). PRMT1 methylates arginine 3 of histone H4 and to a lesser extent arginine 3 of histone H2A, which contains an identical N-terminal sequence SGRGK (15, 16). PRMT5 methylates arginine 8 of histone H3 and arginine 3 of histone H4 (13). A summary of known histone methylarginine sites on histone H3 and H4 is shown in Figure 1. Whether other arginine sites, such as R17 and R19 within what is known as the "basic patch" in H4, or any arginine residues within the histone globular domains are methylated, remains to be established (see Figure 1).

Arginine methyltransferases are involved in regulating the transcriptional response on many different levels, such as through the modification of sequence-specific transcription factors (e.g. STAT1, (10)), general

transcriptional coactivators (e.g. p300/CBP, (10)), histones (17, 18), elongation factors (e.g. SPT5, (19), mRNA export factors (e.g. Npl3, (20)), and splicing machinery (e.g. hnRNP proteins, (21)). Here, we will focus on summarizing current progress in studies of histone arginine methylation and its role in gene regulation. While this modification will undoubtedly be found to function in a myriad of chromatin-templated events, particular attention will be paid to its best characterized role as a nuclear hormone receptor coactivator of gene transcription in both *in vivo* and *in vitro* settings (see below).

3.2. Transcriptional activation through nuclear hormone receptors

Development, cell proliferation, and cell responses to environmental signals are all orchestrated by coordinated patterns of gene transcription. In eukaryotes, transcriptional regulation results from the ability of transcriptional activators and repressors to recruit chromatin remodeling complexes and basal transcriptional machinery to specific promoters. Efficient regulated transcription requires the presence of sequence-specific activators that bind DNA regulatory elements within the promoters.

One well-studied example of site-specific activators is the family of nuclear receptors (NR). These receptors are typically composed of a DNA-binding domain and a ligand-binding domain. Ligands include steroid and thyroid hormones, retinoic acid, vitamin D and many others (for current reviews see (22, 23)). Upon ligand binding, NRs are activated and regulate expression of the target genes by recognizing DNA-regulatory elements called hormone response elements (HREs) in their promoters and recruiting coactivators (or corepressors). Many, if not most, of the identified cofactors are involved in regulation of chromatin structure by either covalently modifying histones or remodeling the chromatin template (for review see (24)). Examples of the histone-modifying activities include the p160 and p300 family of histone acetylases (HATs), whereas Mediator and human Brg ATP-remodeling complexes belong to the latter category (for review see (25)). Recruitment of these cofactors to the gene regulatory elements results in increased chromatin template accessibility for the basal transcriptional machinery, co-recruitment of other coactivators, direct interactions with the basal machinery, and initiation of transcription. Often, the coactivators act synergistically, resulting in the amplification of the output transcriptional signal. The most studied coactivators for the NRs are HAT proteins of the p300 family (including two related proteins p300 and CREB binding protein CBP) and p160 family (including three related proteins SRC-1, GRIP1/TIF2, and pCIP/RAC3/ACTR/AIB1/TRAM1) (25). p160 coactivators can associate directly with the NR activation domain, acetylate HRE proximal histones, and serve as a binding platform for other coactivators such as p300/CBP and the recently identified CoCoA (coiled-coil coactivator) resulting in their recruitment to the HRE (25), (26).

3.3. Role of CARM1 and PRMT1 in NR-mediated transcriptional activation

The role for histone arginine methylation in transcriptional activation was originally identified in the context of NR-mediated transcriptional activation. In a two-hybrid screen for novel proteins binding to the p160 coactivator GRIP1, Stallcup and coworkers identified a novel protein with homology to protein arginine methyltransferases and demonstrated that it can methylate histone H3 *in vitro* (12) - hence the name: CARM1, coactivator-associated arginine methyltransferase 1. In this breakthrough paper, CARM1 was shown to coactivate NR-mediated transcription, likely, at least in part through histone methylation. CARM-1 recruitment to the promoter occurs in response to hormone stimulation and results in the site-specific methylation of histone H3 R17 *in vivo* (27). These findings provide an important extension to an emerging paradigm – that covalent modification of histone proteins plays a pivotal role in governing the output of gene expression (see Figure 2).

In the original paper describing CARM1, the authors also noted the ability of the related methyltransferase, PRMT1, to preferentially methylate histone H4 *in vitro* (12). Shortly thereafter, PRMT1 was biochemically purified as a novel histone H4-specific methyltransferase that stimulates HAT activity of p300 and acts as a coactivator for NR-mediated transcription (28). PRMT1 specifically methylates R3 of histone H4 both *in vitro*, and *in vivo* (28), (16). Added complexity and potential diversification of biological readout, is provided by the findings that, although histone H4R3 can be both monomethylated and asymmetrically dimethylated *in vitro*, only monomethylation was detected in mass spectroscopic analyses using bulk histone H4 isolated from 293 cells (16). These findings suggest that the activity of arginine-directed histone methyltransferases, similar to lysine methylation that can exist in mono-, di- and tri-methylated states, may be highly regulated (for example, by the presence of a cofactor or regulatory subunits that remain undefined).

CARM1 cooperates with PRMT1 and p300/CBP in NR-mediated transcriptional activation (29), (30)). All three proteins are dependent on p160 for their NR coactivatory function (29). CARM1 and PRMT1 associate with the same domain of p160 through a homologous region shared between the two methyltransferases (31). Unlike CARM1, both PRMT1 and p300/CBP can also associate with NRs directly. Nevertheless, their function as NR coactivators still depends on their ability to bind p160, suggesting that they act as secondary rather than primary coactivators. Notably, CARM1 can also cooperate with other types of NR primary coactivators. For example,β-catenin can directly bind to steroid hormone-activated androgen receptor and serve as its primary coactivator. CARM1 can associate and synergize with β-catenin to activate androgen receptor dependent transcription (32).

Another class I arginine methyltransferase, PRMT2, was also shown to function as a coactivator for NR estrogen alpha (33). Substrate specificity of PRMT2 has not yet been determined, but it remains an interesting

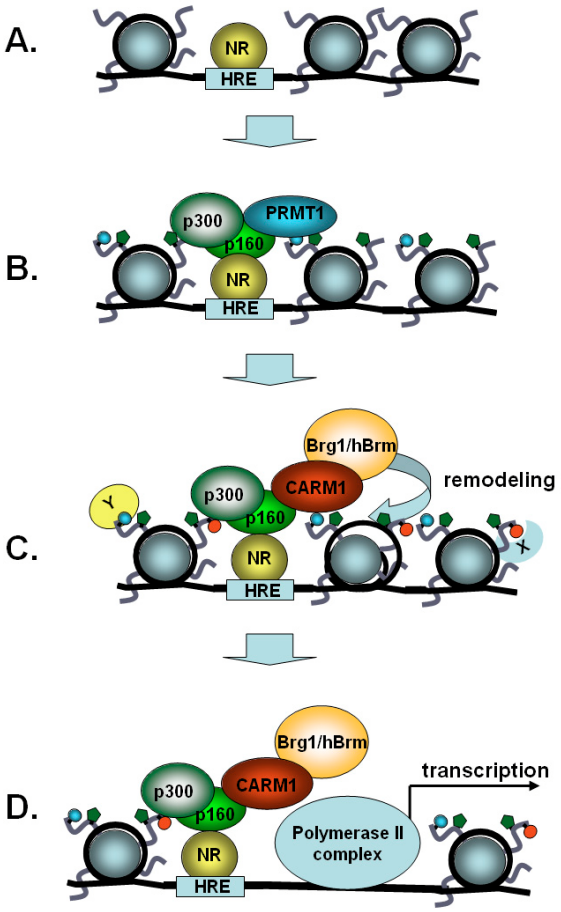

Figure 2. Role of PRMT1 and CARM1 in nuclear receptor-mediated transcriptional activation. A. Recruitment of the ligand-activated nuclear receptor (NR) to the hormone response element (HRE). B. Association of the primary coactivator p160 with NR leads to the corecruitment of secondary activators like PRMT1 and p300. Methylation of H4R3 by PRMT1 (blue circle = methyl modification) stimulates acetyltransferase activity of p300 (green pentagon = acetyl modification), but acetylation of H4 by p300 inhibits H4 R3 methylation by PRMT1. C. Acetylation of histone H3 by p300 (green pentagon = acetyl modification) enhances CARM1 methylation of H3R17 (red circle = methyl modification). CARM1 associates with Brg1/hBrm remodeling complex and stimulates its ATPase activity. Histone tails methylated on arginines may also serve as a binding platform for the yet unknown downstream effectors (X and Y). D. Assembly of the coactivatory complex on HRE, histone modifications, and chromatin remodeling lead to the activation of transcription of nuclear hormone-dependent genes.

possibility that histone-directed methyltransferase activity and NR-mediated coactivatory function are general properties of the class I arginine methyltransferases.

3.4. Role of CARM1 and PRMT1 in signal transduction

The role of histone arginine methylation in transcriptional regulation extends well beyond NR-mediated gene activation. For example, YY1 (Yin Yang 1) directs specific recruitment of PRMT1 and methylation of H4R3 (34), whereas CARM1 cooperates with β-catenin in the context of LEF/TCF mediated activation (32). Histone arginine methylation by CARM1 and PRMT1 is also involved in DNA damage-induced p53-dependent activation of the *GADD45* gene (18). These findings suggest that gene regulation by histone arginine methylation is involved in mediating rapid and reversible responses in signal transduction pathways like hormonal induction, Wnt signaling, and DNA damage.

3.5. Interplay between CARM1 and ATP-dependent chromatin remodeling machinery

Recently, a CARM1-associated protein complex was biochemically purified from mammalian cells. Analysis of the associated polypeptides revealed that CARM1 associates with the Brg1-containing SWI/SNF-like chromatin remodeling complex, providing a link between ATP-dependent remodeling machinery and arginine methylation (see step 3 in Figure 2 and (35)). Interestingly, PRMT5, an arginine methyltransferase involved in transcriptional repression, is also found associated with the human SWI/SNF-like complexes (see below and (36)). In the context of a multi-subunit Brg1-containing complex, but not when present alone, CARM1 is able to methylate nucleosomal histones, suggesting that its histone methyltransferase activity can be redirected from free histones to nucleosomes by associated proteins. Reciprocally, CARM1 stimulates the ATPase activity of Brg1 (35). These results suggest that reciprocal and synergistic regulation of methyltransferases can result in the coordination of methylation and remodeling events on the chromatin template. Ample precedent exists for changes in site and substrate specificity when chromatin-modifying enzymes are examined in recombinant form as compared to more native complexes (37), (38). However, Roeder and coworkers demonstrated that CARM1 and PRMT1 can directly modify nucleosomal histones in an *in vitro* defined setting (18). Nevertheless, the enzymatic activity of CARM1, and many, if not all, other arginine methyltransferases is likely to be regulated *in vivo* by the association with other enzymatic activities such as Brg1.

Recently, a provocative theory, the “regulated nucleosome mobility” hypothesis, has been put forward suggesting that ATP-dependent remodeling complexes may function to facilitate release of DNA from the lateral surface of the nucleosome thereby permitting the addition (and subtraction) of histone modifications in the core histone-fold domain (38). Covalent modifications in the globular core may then function in ways that are distinct from those that occur in the histone tail domains. To our knowledge arginine methyl marks within the globular domains of histones have yet to be identified. However, the recent results with lysine methylation suggest that they may be identified in the future (witness, for example, K79 methylation in histone H3) (39), (40).

3.6. PRMT5 is a transcriptional repressor

Methylation of histones by CARM1 and PRMT1, class I arginine methyltransferases, has been linked to

transcriptional activation (27, 41). However, class I enzymes might also function as transcriptional repressors by modifying non-histone substrates, as evidenced by the destabilization of the p300/CBP interaction with CREB upon methylation by CARM1, a subject further examined later in this review (42). In contrast, histone methylation by the class II arginine methyltransferase, PRMT5, has to date been shown to be associated with transcriptional repression (13, 36). PRMT5 was purified as a component of a large multi-subunit complex containing mSin3/HDAC histone deacetylase and Brg1/hBrm chromatin remodeling complex. Both Brg1/hBrm-containing PRMT5 complex and recombinant PRMT5 methylate R8 of histone H3 and R3 of histone H4 and display an *in vitro* preference for methylating hypo- over hyperacetylated histones H3 and H4 (13, 36).

Chromatin immunoprecipitation studies demonstrated that PRMT5 is recruited to tumor suppressor genes ST7 and NM23, and to c-Myc target gene *cad*, coinciding with histone methylation and transcriptional repression (13, 36). Thus, PRMT5 is involved in transcriptional repression of genes involved in control of cell proliferation. It seems likely that PRMT5 might act antagonistically to CARM1 and PRMT1, as it decreases transcriptional competence of the chromatin template. Furthermore, PRMT1 and PRMT5 appear to target the same arginine residue:H4R3 (Figure 1). It remains to be addressed in future studies whether the same genes may be antagonistically regulated by both enzymes.

3.7. Multiple roles for arginine methyltransferases in gene regulation

Arginine methyltransferases, through their enzymatic activity directed at histones, site-specific transcription factors, coregulators, splicing factors, elongation factors, and possibly basal transcriptional machinery, may influence and integrate changes in chromatin structure, transcriptional initiation, elongation, transcript processing, and nuclear organization. One elegant example of such multilevel regulation is methylation of p300/CBP by CARM1, which results in the destabilization of its interaction with CREB and repression of the CREB-dependent transcription (42). Thus, CARM1 activates NR-dependent genes not only by histone methylation of the HRE-containing promoters, but also by redirecting its synergistic regulator p300/CBP from the cAMP signaling pathway into the hormone-mediated pathway.

The contribution of arginine methyltransferases to NR-mediated transcription is likely not restricted to their enzymatic activity. Notably, CARM1 has its own autonomous transcriptional activation domain located at the C-terminus of the protein, which contributes to the full coactivatory properties of the protein (43). Furthermore, arginine methyltransferases can influence other regulators co-recruited to the promoter as evidenced by CARM1's ability to stimulate remodeling activity of its associated Brg1 complex (35). However, it remains presently unclear whether this property is dependent or independent of its methyltransferase activity.

3.8. How does histone arginine methylation activate and repress transcription?

An important part of gene expression regulation by histone-modifying activities is the interplay or cross-talk between different modifications, whereby the presence of one modification can either promote or prevent other modifications in either the same or different histones (44). Association and co-recruitment of CARM1 and PRMT1 with HAT activities suggest that such cross-talk may exist between histone arginine methylation and lysine acetylation. Studies with recombinant histones as substrates revealed that H4R3 methylation stimulates H4 acetylation by p300, acetylation of H4 inhibits H4R3 methylation by PRMT1 (28), and prior acetylation of histone H3 by p300 enhances CARM1 methylation of H3R17 (41). In contrast to interplay between acetylation and methylation of H3 by CARM1, acetylation of K9 and K14 of H3 interferes with H3R8 methylation by PRMT5. These results suggest that histone methylation by PRMT1 may stimulate acetylation by p300, which, in turn, may enhance methylation by CARM1, whereas prior acetylation inhibits methylation of the H3 tail by the repressive arginine methyltransferase, PRMT5.

Once the steady-state balance of arginine methylation is achieved, it remains unclear what downstream mechanisms trigger gene activation or inactivation. In the case of histone acetylation and histone methylation, effector binding proteins such as bromodomain and chromodomain-containing proteins, have been identified that "read" their cognate covalent marks in a context-dependent fashion. We refer to effector-binding:histone interactions as being part of a general "trans" mechanism as compared to "cis" mechanisms defined more by inherent changes in higher-order chromatin structures (45). Alternatively, histone modifications can also prevent the binding of proteins or protein complexes, as exemplified by the disruption of nucleosome remodeling and deacetylase (NuRD) complex binding by histone H3 lysine 4 methylation (46).

Little progress has been made in characterizing the downstream molecular effectors responsible for specifically recognizing histone arginine methyl marks and translating them into a meaningful biological readout. However, it is well established, , that protein arginine methylation can alter specific protein-protein interactions between transcription factors and their regulators, as in the case of p300/CBP methylation by CARM1 (42). An intriguing candidate for histone arginine methyl-binding domain is the Tudor domain, which is structurally related to a chromodomain (47) and present in many proteins with chromatin-related functions, many of them repressors (e.g. RBBP1, ESET, Polycomb-like). SMN protein Tudor domain has been found to specifically recognize symmetrically dimethylated arginine residues of small nuclear riboproteins (48). We speculate that the Tudor domain may serve as a module for recognition of histones symmetrically dimethylated by PRMT5, and mediate PRMT5-dependent transcriptional repression. Alternatively, methylation of arginine residues may facilitate or antagonize the binding of effectors that engage other nearby covalent marks in what have been

formally described as histone modification "cassettes" (44). For example, arginine methylation may affect binding of bromo- or chromodomain proteins to the nearby acetylated or methylated lysine residues. Determining which histone tail-effector interactions are indeed affected by arginine methylation represents a major challenge for future studies.

4. ROLE OF PRMT1 AND CARM1 IN MAMMALIAN DEVELOPMENT AND CELLULAR DIFFERENTIATION

While the above studies indicate that PRMT-catalyzed histone arginine methylation plays an important role in gene regulation, little is known about how this regulatory modification effects downstream biological and cellular events. Important insight into the more general role of PRMTs in cell function comes from analysis of PRMT1 and CARM1-null mice. Pawlak and coworkers (49) originally identified PRMT1 in an *in vitro* screen for mutations in developmentally-regulated genes. The mutation was generated by insertion of the U3BGeo gene trap retrovirus into the second intron of the PRMT1 gene, thus creating an essentially null mutation. The resulting PRMT1-B-galactosidase fusion protein was found to be strongly induced by day E7.5 along the middle of the neural plate and in the forming head fold. Analysis of total cellular RNA from wild type embryos and adults found that, while PRMT1 expression appeared most abundant in the developing central nervous system, it was also expressed at lower levels in all embryonic and adult tissues examined. Embryos homozygous for the PRMT1-null mutation were found to implant into the uterus, but failed to develop beyond the early egg cylinder stage with the embryonic portions of the conceptuses being growth retarded. However, PRMT1 does not appear to be essential for cell function as PRMT1-null ES cells are viable.

With respect to CARM1, Yadav *et al.*, (50) found that embryos with a targeted disruption of this enzyme are small and die perinatally. The investigators then generated a CARM1 -/- mouse embryonic fibroblast cell line to study the effect of loss of CARM1 on protein methylation. Results showed that, as predicted from *in vitro* studies, two known CARM1 substrates, PABP1 and p300, were not methylated in the CARM1 -/- cell line compared to wild type controls. Interestingly however, histone H3R17 asymmetric dimethyl-arginine was largely retained in the mutant cell line. This result is at odds with *in vitro* studies (14) and suggests that other PRMTs may compensate for methylation of H3R17 in CARM1 -/- mouse embryonic fibroblast.

In addition to embryonic development, several reports also suggest that PRMT-catalyzed protein methylation plays an important role in cellular differentiation For example, nerve growth factor (NGF)-specific signal transduction was found to activate PRMT1 leading to changes in protein methylation during neuronal cell differentiation (51). During erythroid differentiation, PRMT1 also appears to be required for erythroid colony outgrowth and inhibition of PRMT activity blocks erythroid maturation without affecting expansion of progenitor cells (52). With respect to CARM1, Kim *et al.*, (53) reported that this PRMT likely plays a significant role in promoting the differentiation of early thymocyte progenitor cells. The investigators characterized the CARM1 -/- immature thymus phenotype and found that loss of CARM1 significantly inhibited thymus cell proliferation. Further, flow cytometric analysis revealed that a T cell developmental block occurred at the CD44+CD25- stage in CARM1 -/- thymi. CARM1 also appears to play a key role in muscle differentiation. Chen *et al.* (54) found that CARM1 and GRIP-1 cooperatively stimulate the activity of myocyte enhancer factor-2C (MEF-2C) and are recruited to the endogenous muscle creatine kinase promoter. Importantly, they also found that inhibition of CARM1 inhibited muscle differentiation and abrogated the expression of key transcription factors.

5. DYNAMIC REGULATION OF HISTONE ARGININE METHYLATION

The above collective findings indicate that PRMT-catalyzed histone arginine methylation plays a key role in cell signaling and gene regulation, and that protein arginine methylation in general is important for embryonic development and cellular differentiation. The potential importance of this modification in cell function is highlighted by the observation that protein arginine methylation may eventually rival other covalent modifications, such as phosphorylation, in the number potential target substrates (55). However, when compared to phosphorylation, protein methylation has received considerably less attention as a signaling molecule. This is likely due, at least in part, to the suspicion that protein methylation may be more static or irreversible. If this suspicion is correct, it then becomes difficult to imagine how protein methylation might play a significant role in the regulation of dynamic and reversible signaling events. The belief the protein methylation is "permanent" has been supported by circumstantial evidence, such as the observation that the amine bond becomes more stable following protein methylation (requiring 12 ATP molecules for catalysis), thus making it less likely that this modification could undergo rapid turnover (10). This idea was also supported by older experimental findings showing that the slow turnover rate of bulk histones was similar to that of methylated histones (56, 57). However, recent evidence has emerged suggesting that histone methylation is dynamically regulated at specific gene loci and at specific developmental time points.

5.1. Histone arginine methylation is dynamically regulated both globally and at gene-specific loci

With the development of site-specific antibodies directed at histone modifications, including methyl-arginine, chromatin immunoprecipitation (ChIP) assays have grown popular as a means to evaluate histone modification dynamics at specific gene loci (58, 59). Recently, Metivier *et al.*, (60) utilized ChIP analyses to investigate cofactor and histone modification dynamics on the estrogen-responsive pS2 gene following estradiol treatment. During the initial phase of each pS2 transcriptional cycle, histone-modifying cofactors such as PRMTs and HATs are recruited to the promoter by estrogen receptor alpha (ER-alpha) where they then

methylate and acetylate nearby histones. PRMT and HAT levels then begin to decline following release of ER-alpha from the promoter. Interestingly, a contemporaneous loss of histone methyl-arginine (specifically H3R17me2a and H4R3me2a) and H3 acetylation also occurs following ER-alpha disengagement. With respect to histone acetylation, the rapid loss of histone H3 acetyl marks could be accounted for by the observed increasing levels of HDACs on the pS2 gene following ER-alpha release. However, the rapid loss of H3R17 and H4R3 methylation provided early evidence that an enzymatic activity might exist that is able to remove the PRMT-catalyzed arginine methyl mark.

At the global level, recent reports indicate that histone arginine methylation appears to be dynamically regulated during oogenesis and preimplantation development (61). A longstanding assumption in the epigenetics field is that the DNA and histone modifications are "reset" during gametogenesis and preimplantation development in order to facilitate reprogramming of the genome during the early stages of embryogenesis (62). This prediction is supported by DNA methylation studies which show that 5-methyl cytosine levels are dramatically decreased during germ cell and preimplantation development (63). With this observation in mind, a "proof of principle" study was carried out to investigate the possibility that specific histone modifications are also reset during this time period (61). Results showed that histone modifications, such as methylation of H3K9 and H3K4, appear to be relatively stable 'epigenetic' marks during early stages of mouse development. However, other modifications, such as methylation of H3R17 and H4R3 and acetylation of histone H4, appear to be dynamically regulated; with levels of these modifications decreasing during metaphase II of meiosis and during metaphase of early mitotic cell divisions. The rapid loss of staining for the H3R17 and H4R3 methyl modifications suggest that enzymatic activities exist within the mouse oocyte are capable of removing methyl-arginine modifications from histones. Also, given the putative link between histone arginine methylation and acetylation, these findings suggest a synergistic coupling of histone demethylation and deacetylation in oocytes and early embryos. While the functional significance for the global resetting of histone arginine methylation marks is unclear, a role in regulating gene expression in the egg and early embryo seems likely.

5.2 . Identification of an enzyme activity capable of removing arginine-based methyl marks from histones

In addition to methylation, protein arginine can also be converted to citrulline by a family of enzymes called peptidylarginine deiminases (PADIs). To date, five PADI isoforms (1, 2, 3, 4 and 6) have been identified in human and mouse genomes (see (64) for review). PADIs are thought to regulate a diversity of cellular functions via a calcium-dependent deimination reaction. For example, PADI2 likely regulates the myelination of axons by catalyzing the citrullination of myelin basic protein (65), (66). PADI6 expression is restricted to the oocyte and preimplantation embryo (67) where it is mainly localized to a unique keratin-containing structure called the egg cytoplasmic sheets. PADI4 was originally identified in granulocytes differentiated from HL-60 cells after retinoic acid (RA) or dimethylsulfoxide (DMSO) treatment (68). Upon calcium ionophore treatment, histones become citrullinated by PADI4 in treated granulocytes, indicating that citrullination represents a new kind of post-translational histone modification (69, 70). The function of histone (or non-histone) citrullination is largely unknown.

The findings that histone H3R17 and H4R3 methyl modifications are rapidly removed by factors within the oocyte cytoplasm (61) and that PADI6 was abundantly expressed in the egg cytoplasm (67), lead to the following hypothesis - in addition to arginine, PADIs are also able to convert methyl-arginine residues to citrulline in cellular histones. Recent evidence (71, 72) lend strong support to this hypothesis. Utilizing a variety of biochemical and immunocytological approaches, a concomitant loss of methyl-arginine and appearance of citrulline on histone H3 at arginine 17 and on histone H4 at arginine 3 was observed following PADI4 activation in both *in vitro* and *in vivo* settings. In support of these findings, methylamine, a predicted product of "demethylimination" is released from the reactions when methyl-arginine-containing histones are used as substrate. Assuming that demethylimination is reversible, it remains unclear if other enzymatic activities, or possibly histone replacement are required to convert citrullinated residues back to arginine.

Given that methylation of NR target promoters by CARM1 and PRMT1 enhances gene activity, it seemed reasonable to speculate that PADI4 targets methyl-arginine residues on the promoters of NR-target genes for citrullination and that this activity affects transcription, likely in a repressive fashion. Tests of this hypothesis have recently been provided (71). Consistent with the hypothesis that PADI4 functions as a transcriptional repressor, a wild type PADI4, but not catalytically inactive mutants, represses estrogen-induced transcriptional activation of an ERE-reporter gene. In support of the hypothesis that PADI4 represses transcription by converting histone methyl-arginine residues to citrulline on specific promoters, kinetic ChIP analyses, using histone H3 and H4 methyl-arginine and citrulline-specific antibodies, found that levels of histone methyl-arginine and citrulline are inversely correlated on the endogenous pS2 gene in MCF-7 cells following estrogen stimulation. These findings support a model whereby arginine methyltransferases are first recruited to the pS2 gene (along with other coactivators and transcription factors) and methylation of the promoter then leads to transcriptional activation. Subsequent recruitment of PADI4 to the promoter would then allow for the conversion of methyl-arginine residues to citrulline thereby inducing conformational changes in the nucleosome leading to transcriptional repression (Figure 3). In keeping with the concept that arginine "demethyliminases" are repressive in the above setting, we point out that a lysine-directed "demethylase" has recently been reported that is generally repressive in nature (see below).

5.3. Identification of a histone lysine demethylase

The above observations demonstrate that histone methyl-arginine residues can be functionally "demethylated" by PADI activity. Might histone lysine methyl marks also be dynamically regulated, and if so, by what mechanisms (73)? An older literature suggest that the answer is probably "yes". As

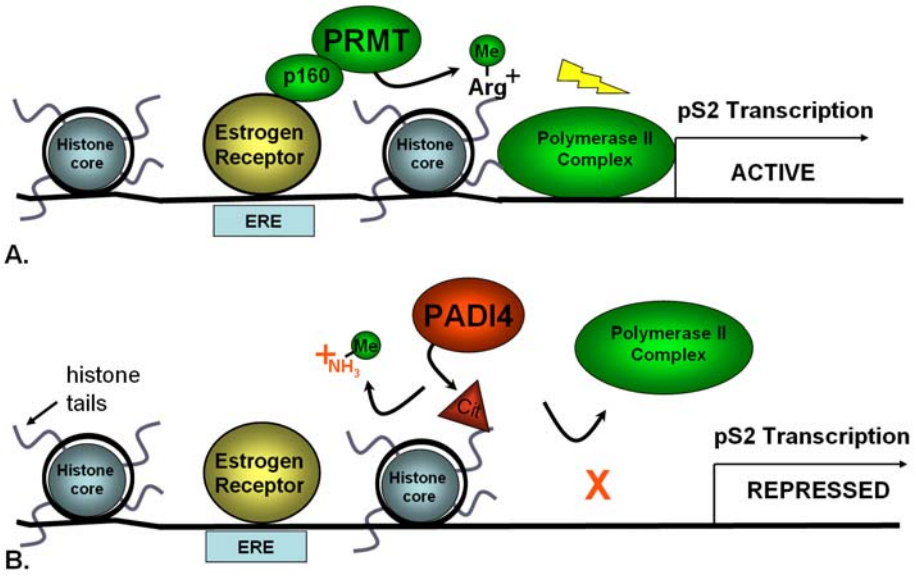

Figure 3. Model of antagonistic regulation of transcription by PRMT1 and PADI4. A. Following recruitment by estrogen receptor to the pS2 gene promoter, PRMT1 methylates histone H4R3 leading to chromatin remodeling and increased template accessibility. Basal transcriptional machinery (RNA polymerase II complex) then associates with chromatin template and activates transcription. B. Subsequently, following recruitment to the promoter, PADI4 then catalyzes the conversion of methylarginine (MeArg) to citrulline (Cit) releasing methylamine ($MeNH_3$). Citrullination of histone methylarginine residues decreases the ability of transcriptional machinery to bind to chromatin template thereby repressing transcription. PRMT1 - Protein arginine methyltransferase 1. CARM1 – Coactivator-associated arginine methyltransferase 1. pS2 gene – estrogen-responsive.

early as 1972, Borun, Pearson, and Paik concluded that up to one third of all protein methylysine groups are exchanged during the HeLa cell cycle (74). Paik and Kim partially purified a protein lysine demethylase activity ~ 16 fold from rat kidney tissue extracts and predicted that epsilon-alkyllysinase (a metabolic enzyme that can demethylate free epsilon-N-methyl-L-lysine) was the enzyme responsible for demethylating histone methyl-lysine residues (75). Until quite recently, however, the molecular identity of any putative histone lysine demethylase remained unknown.

In an elegant recent breakthrough publication, Shi and colleagues (76) presented convincing evidence that, LSD1 (named for Lysine Specific histone Demethylase), a nuclear amine oxidase homologue, represents a bona fide histone lysine demethylase. LSD1 was originally identified as member of a number of co-repressor complexes (77). Biochemical and enzymological analysis of LSD-1 found that this enzyme is highly specific and targets only histone H3K4 for demethylation. Interestingly, LSD1 requires FAD as an electron acceptor for the reaction, and tri-methyl groups at Lys4 are not a suitable substrate for the enzyme, likely a direct consequence of the amino-oxidase function of this enzyme. In order to demonstrate that LSD-1-catalyzed histone lysine demethylation is involved in gene regulation, RNA interference and chromatin immunoprecipitation analyses were used to show that genes which are normally repressed by LSD-1 activity became derepressed. Results show that reduction of LSD-1 levels by RNAi resulted in increasing levels of H3K4me2 on these promoters, a result predicted for a demethylase that is specific for this "active" methylation site in H3.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Growing results demonstrate that histone methyl-arginine and methyl-lysine are dynamically regulated *in vivo*, providing several new chapters to the enzymology and reversibility of what was recently regarded as a possibly more "static" indexing system for the epigenome (78). Much more work is needed to assert a uniform verdict on the "dynamic" versus "static" question regarding either arginine- or lysine-based methylation. At this point, only a very limited number of enzymes and target genes have been examined in any detail, although it seems likely, if not certain, that both lists will lengthen considerably in the near future.

Many intriguing questions remain in all corners of the emerging work being reported on histone arginine methylation and its regulation. For example, the recent finding that histone H4R3 can be also symmetrically dimethylated by PRMT5 (13) indicates that three methyl states may exist at this residue; an asymmetric dimethyl-arginine "on" state, a symmetric dimethyl-arginine "off" state, and an, as yet to be functionally defined, monomethyl-arginine state. Does the monomethyl modification poise the arginine residue for subsequent dimethylation or does this derivative possess an entirely different function? Another important question that remains to be answered is whether the symmetric and asymmetric dimethyl-arginine modifications regulate similar sets of genes or whether their regulatory pathways are non-overlapping.

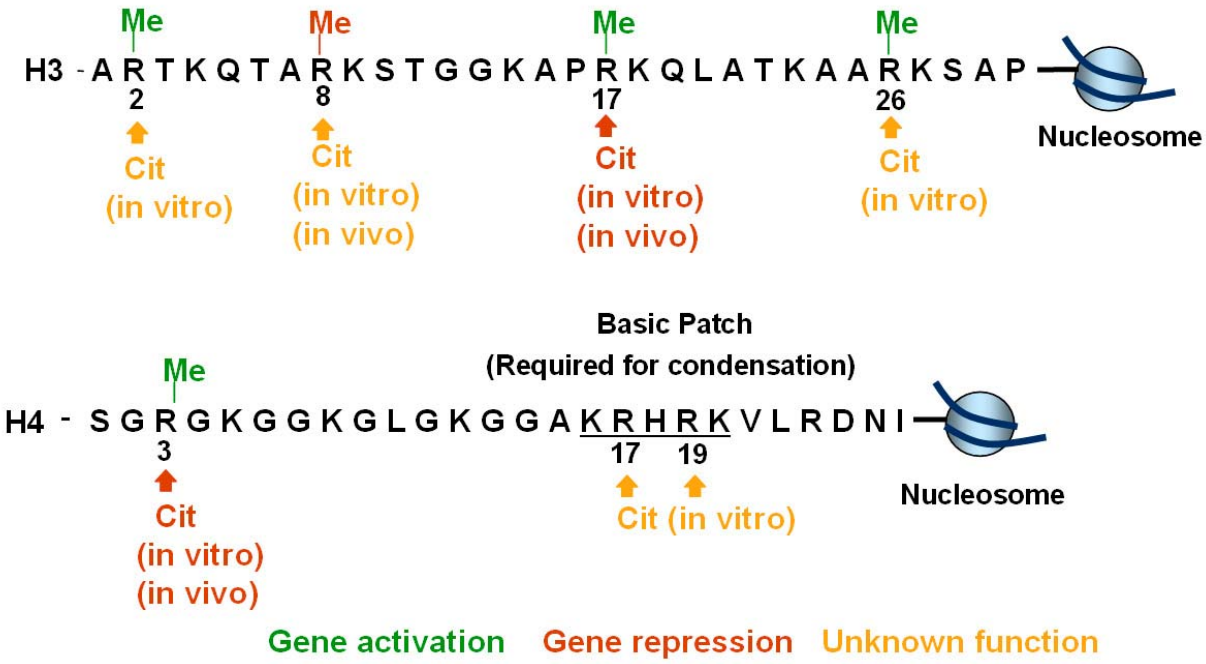

Figure 4. Sites on the histone H3 and H4 tail that are citrullinated. Histone H3 arginine 8 and arginine 17 and histone H4 arginine 3 are citrullinated *in vivo*. Histone H3 arginine 2, 8, 17, and 26 and histone H4 arginine 3, 17, and 19 are citrullinated *in vitro*. Citrullination at histone H3R17 and H4R3 is associated with gene repression. For reference, methylarginine sites (Me) involved in either gene activation (green) or gene repression (red) have been included. The histone H4 "basic patch" (residues 16-20) plays a critical role in regulating higher order chromatin structure and gene expression.

Whether other arginine sites in other histones, such as Arg17 and Arg19 in H4 (see Figure 4), are methylated and/or citrullinated as part of chromatin structure is also not known. Here, we point out that the base of the H4 tail, including what is known as the "basic patch" in H4, is known to play a critical role in governing a chromatin-higher order structure that plays an important role on gene expression (79).

Another important issue that remains to be resolved regarding histone citrullination is whether this modification can be converted back to arginine, either by histone replacement, histone clipping, or possibly by an as yet to be identified enzymatic activity (73). Evidence suggesting that citrulline can be converted back to arginine comes from temporal ChIP analyses of the pS2 gene (72) which shows that histone H3 citrullination increases following estradiol treatment and then decreases significantly after treatment. This rapid decrease in H3 citrullination suggest that the citrulline mark is somehow lost and might be converted back to arginine (or replaced).

How these modifications regulate gene activity is also unclear. Here, lysine methylation will likely guide the way for experiments aimed at searching for effector modules that "read" the arginine-methyl marks. As with lysine methylation, we suspect that the "methylation state" of histone arginine residues will have functional importance and thus increase the regulatory options for the cell. Along this line, we look forward to more mass spectrometric analyses aimed at better documenting the relative amounts of monomethyl-arginine, dimethyl-arginine (symmetric and asymmetric) and citrulline in cellular histones isolated from various sources and physiological states. As was the case with lysine methylation, the generation of immunological reagents directed at these marks will have to deal with this inherent complexity as well as potential cross-reacting epitopes from other similar sites in histone and non-histone substrates. Epitope "disruption" issues will remain a concern for all antibody-based studies as the complexity of the "histone code" increases with neighboring marks and cross-talk (44). Thus, we recommend a multifaceted approach as researchers enter into these new and exciting waters.

In many ways, the selective recruitment of methylases and "demethylases/demethyliminases" to highly regulated (e.g. hormone-regulated genes, see Figure 3 above) gene promoters follows closely the paradigm already well established for histone acetylation. We look forward to future studies that will investigate other genes targeted by these enzyme activities, as well as other non-histone substrates. Using histone acetylation as a guide, we suspect that arginine- and lysine-based methylation, and the enzyme systems responsible for governing their steady-state balance, will extend well outside of the realm of histone and chromatin biology.

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