Cross-talk among epigenetic modifications: lessons from histone arginine methylation

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Abstract
Epigenetic modifications, including those occurring on DNA and on histone proteins, control gene expression by establishing and maintaining different chromatin states. In recent years, it has become apparent that epigenetic modifications do not function alone, but work together in various combinations, and cross-regulate each other in a manner that diversifies their functional states. Arginine methylation is one of the numerous PTMs (post-translational modifications) occurring on histones, catalysed by a family of PRMTs (protein arginine methyltransferases). This modification is involved in the regulation of the epigenome largely by controlling the recruitment of effector molecules to chromatin. Histone arginine methylation associates with both active and repressed chromatin states depending on the residue involved and the configuration of the deposited methyl groups. The present review focuses on the increasing number of cross-talks between histone arginine methylation and other epigenetic modifications, and describe how these cross-talks influence factor binding to regulate transcription. Furthermore, we present models of general cross-talk mechanisms that emerge from the examples of histone arginine methylation and allude to various techniques that help decipher the interplay among epigenetic modifications.

Introduction
The genomic DNA of eukaryotes was once thought to be the ultimate template of inheritance. This view has been challenged in recent years, with epigenetics supporting the idea that heritable changes which influence gene expression may not have anything to do with changes in the DNA sequence [1]. DNA is wrapped around an octamer of proteins that comprises two copies of each of the core histones: H2A, H2B, H3 and H4. This dynamic structure is known as the nucleosome and is the basic unit of chromatin. Euchromatin refers to the decondensed form of chromatin that can be active or inactive, whereas heterochromatin is defined as the compacted silenced state. Hence chromatin is dynamically modulated between transcriptionally repressed and active states in order to regulate gene expression. These states can be altered by PTMs (post-translational modifications) deposited on either the DNA or histones, thus affecting the readout of the underlying DNA sequence [2].

Histones are susceptible to various PTMs including phosphorylation, methylation, acetylation and ubiquitination [3]. The numerous histone modifications identified to date communicate among themselves by influencing the presence of each other or by collaborating to bring about a functional outcome. These communications, referred to as cross-talks, happen in a context-dependent manner revealing that the once-thought strict histone code is actually a complex histone language [4]. Cross-talks can occur on the same histone (cis) or between different histones (trans), and it has been proposed that trans mechanisms could even involve more than one nucleosome [2,3]. The study of these cross-talks has received great attention because the reading of these modifications by different effectors influences gene expression [2], and misinterpretation of these cross-talks by readers may trigger various diseases, including cancer [5–7].

Lately, arginine methylation has attracted much interest owing to its involvement in several cellular processes such as transcription, RNA processing, signal transduction and
Protein arginine methylation catalysed by PRMTs

The mammalian family of PRMTs is shown, consisting of nine members, classified into the three types. All three types of PRMT (I, II and III) can monomethylate arginine (Rme1) on one of the terminal guanidino nitrogen atoms. Type I PRMTs generate asymmetric dimethylation (Rme2a), whereas type II PRMTs generate symmetric dimethylation (Rme2s).

DNA repair. This PTM involves the addition of one or two methyl groups to the guanidino groups of arginine residues resulting in three different methylation states: monomethylated (Rme1), asymmetrically dimethylated (Rme2a) or symmetrically dimethylated (Rme2s) arginine (Figure 1). The methyl groups are deposited by PRMTs (protein arginine methyltransferases). The mammalian family of PRMTs includes nine members which are classified into three types: type I (PRMTs 1, 2, 3, 4, 6 and 8) catalyse the formation of Rme2a, and type II (PRMTs 5 and 7) catalyse the formation of Rme2s. Both types are also able to mediate the formation of Rme1. PRMT7 can only monomethylate some substrates, thus it is also referred to as a type III PRMT. To date, PRMT9 has not been shown to possess enzymatic activity [7]. Histone arginine methylation associates with both active and repressed chromatin states depending on the residue involved and the status of methylation. For example, asymmetric dimethylation of histone H3 Arg2 (H3R2me2a) is associated with transcriptional repression [8–10,21]. ChIP (chromatin immunoprecipitation) analysis both in humans and yeast (Saccharomyces cerevisiae) showed that H3R2me2a is present in heterochromatic regions in yeast and within the middle and 3′-ends of euchromatic genes, whereas H3K4me3 is abundant at the 5′-end of active genes [8,10].

Histone H3 arginine residues

On histone 3, Arg2 is the most characterized arginine residue to date, and its dimethylation states have been shown to participate in several cross-talks. One of the most studied cross-talks is between H3R2me2a and H3K4me3 (trimethylation of histone H3 Lys4). These two modifications are catalysed in humans by PRMT6 [8,9,19] and the COMPASS (complex proteins associated with Set1) complex [20] respectively. Experiments from different groups have shown that H3K4 can be trimethylated only in the absence of H3R2me2a [8–10,21]. ChIP (chromatin immunoprecipitation) analysis both in humans and yeast (Saccharomyces cerevisiae) showed that H3R2me2a and H3K4me3 are mutually exclusive. H3R2me2a is present in heterochromatic regions in yeast and within the middle and 3′-ends of euchromatic genes, whereas H3K4me3 is abundant at the 5′-end of active genes [8,10].

H3R2me2a interferes with the binding of the WD40-domain-containing protein WDR5 (a subunit of the methyltransferase COMPASS complex) to the H3 tail. The prevention of WDR5 binding in turn disrupts the activity of the catalytic component of the complex, MLL (mixed-lineage leukaemia) 1 protein, necessary to trimethylate H3K4 [9,22]. The same mechanism has been observed in S. cerevisiae, where H3R2me2a regulates the activity of Set1 (yeast orthologue of human MLL1) by modulating the binding of the COMPASS subunit Spp1 [10]. Spp1 recognizes H3K4me2 (dimethylation of H3K4) through its PHD (plant homeodomain) finger only when H3R2me2a is absent, in order to stimulate H3K4me3 by Set1.

Interplay between histone arginine methylation and other epigenetic modifications

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Whether or not arginine methylation can be enzymatically reversed is still under investigation. An enzyme, JMJD6 (Jumonji domain-containing 6), was reported to demethylate both H3R2me2a and H4R3me2a [14]. This finding, however, was disputed by another study which showed that JMJD6 is a lysine hydroxylase [15]. Although it is not clear whether or not arginine methylation can be reversed, methylation of this residue can be blocked by deimination, a reaction catalysed by a family of enzymes called PADs or PADIs (peptidyl arginine deiminases). This modification converts an arginine residue into citrulline by the removal of one of the two terminal amino groups and, as a result, the new residue can no longer be targeted by PRMTs. To date, two different PADIs have been shown to exert this activity on histones. PAD4 can remove the amino group from Arg7, Arg9, Arg17 and Arg26 in histone H3, and also Arg1 in histones H4 and H2A [16,17]. Recently, it was shown that PAD12 could also convert Arg26 of histone H3 into citrulline in vivo [18].
Although H3R2me2a by PRMT6 and H3K4me3 by MLL1 inhibit the deposition of each other [8,9,19], it is reasonable that both modifications coexist within cells [19], perhaps for a short period of time. Structural and crystallographic analysis of many H3K4me3-binding proteins showed that, when H3K4me3 and H3R2me2a coexist on the same peptide, the latter modification blocks the recognition of H3K4me3 by many proteins. The double chromodomains-containing CHD1 (chromodomain helicase DNA-binding protein 1) [23,24], the Tudor-domain-containing JMD2A, the aforementioned WD40-containing WDR5 and several PHD-containing proteins PHF2 (PHD finger protein 2), ING (inhibitor of growth) 2, BPTF (bromodomain PHD finger transcription factor), DATF1 (death-associated transcription factor 1) [9,19,25], TFIID (transcription factor IID) subunit TAF3 [26,27] and ING4 [28] all show a marked decrease or total loss of H3K4me3 recognition when H3R2me2a is present. However, the effect of H3R2me2a on the binding of ING2 and BPTF to H3K4me3 has not been clearly established; some authors showed a decrease in the binding [19,25], whereas others did not observe any inhibition [26]. Furthermore, the recruitment of some proteins by this part of histone H3 is insensitive to this cross-talk, for instance RAG2 (recombination activating gene 2) [19,29] and Pygo [30]. These findings suggest that H3R2me2a blocks the recruitment of H3K4me3 readers selectively and hence regulates specific downstream events on chromatin.

More recent studies showed that H3R2 can also be symmetrically dimethylated in vivo, a reaction catalysed in mammals by PRMT5 and PRMT7 [12,13]. Using ChIP-seq analysis, Yuan et al. [13] showed that H3R2me2s co-localizes with H3K4me3 throughout the mouse genome and this overlap is conserved in many eukaryotes. Another study showed that in human cells H3R2me2s, unlike its asymmetric form, is present specifically at the −1 nucleosome in gene promoters together with H3K4me3 [12]. The apparent co-localization between H3R2me2s and H3K4me3 raised the hypothesis that these two modifications might influence the deposition of each other. Indeed, yeast Set1 and other COMPASS subunits are required for H3R2me2s, in a way that seems to be dependent on H3K4me3. Consistent with this result, mutation of H3K4 to alanine impairs the deposition of H3R2me2s, suggesting that COMPASS regulates simultaneously methylation of Arg2 and at Lys4 [13]. Additionally, the presence of H3R2me2s enhances H3K4 methylation by facilitating the recruitment of the human COMPASS core subunit WDR5 [12]. This recruitment is PRMT5-dependent, and both PRMT5 and WDR5 co-immunoprecipitate [31]. Once both modifications are deposited, further evidence proposes that these two marks function together to influence the recruitment of effector molecules to chromatin. Ramon-Maiques et al. [29] and Yuan et al. [13] showed that, in vitro, the PHD-containing protein RAG2 binds with higher affinity to peptides that are concurrently modified with H3R2me2s and H3K4me3 than to peptides carrying single modifications [13,29]. Hence, the cross-talk between H3R2me2s and H3K4me3 constitutes one of the rare examples in which two modifications first influence the deposition of each other and then together control downstream factor-binding events.

Interestingly, H3R2me2s is also found at locations distant to the transcription start sites, where it overlaps with high levels of H3K4me1 and H3K4me2 [12]. A similar overlap has also been observed between H3R2me2a and these two Lys4-methyl marks, but this co-localization occurs within the body of genes [10]. Therefore it would be of interest to determine whether cross-talk exists between the two H3R2 dimethyl states and H3K4me1/H3K4me2 that is dependent on the genomic location of nucleosomes.

Methylation of H3R2 is also involved in a cross-talk with the unmodified version of Lys4 (H3K4me0). H3K4me0 is recognized by the AIRE (autoimmune regulator protein) through its PHD domain to activate gene expression [32]. Addition of one methyl group to H3R2 inhibits severely the binding of AIRE to the H3 tail, while dimethylation of H3R2 abrogates it completely [25,33–35]. The same effect is observed when Arg2 is mutated to alanine or lysine, which is consistent with the fact that an unmodified arginine residue is required for this interaction [32,34,35]. In line with the above observations, overexpression of PRMT6 reduced activation of AIRE target genes [34].

Another well-documented cross-talk of Arg2 is the one involving its non-methylated state (H3R2me0) and DNA methylation. UHRF1 (ubiquitin-like with PHD and ring finger domains 1), a PHD and tandem Tudor-domain-containing protein, recognizes H3R2me0. This protein acts as a transcriptional repressor by maintaining CpG methylation and heterochromatin formation [36–38]. H3R2me1 decreases UHRF1 binding to the histone tail, an effect amplified by dimethylation of H3R2, as shown by structural analysis [36,38,39] and in vitro binding assays [37]. ChIP experiments also showed that promoters of UHRF1 target genes are devoid of H3R2me2s [37]. UHRF1 can also bind H3K9me3 through its Tudor domain [36,37,40], and the binding is stronger when both H3K9me3 and H3R2me0 are present [40].

The above work describes well-characterized cross-talk mechanisms of H3R2, but recent evidence indicates that H3R2 methylation may be involved in additional cross-talks. For example, a recent report linked dNTMT (Drosophila N-terminal methyltransferase), an enzyme involved in histone H2B N-terminal methylation, to an H3R2me2a-mediating PRMT dART8 (Drosophila arginine methyltransferase 8) in Drosophila melanogaster [41]. The authors suggest that H3R2me2a inhibits histone H2B N-terminal methylation, but the precise mechanism of this possible cross-talk needs to be elucidated. Another potential cross-talk is the one between H3R2 methylation and H3T3ph (histone H3 Thr3 phosphorylation). This is proposed on the basis of the fact that H3T3ph disrupts UHRF1 binding to H3R2me0 [37,40] and influences the recognition of H3K4me3 by some binding factors, similarly to dimethylated H3R2 [25,33,34,37].

Apart from Arg2, histone H3 can also be methylated at Arg9, Arg17 and Arg26, and all three residues are involved
in modification cross-talks. However, in general, their cross-talk mechanisms are less studied in comparison with those involving H3R2. Pal et al. [42] showed that H3R8me2s is catalysed by PRMT5, and this methylation is inhibited by the presence of H3K9ac and H3K14ac (acetylation of histone H3 Lys9 and Lys14 respectively) [42]. Reciprocally, H3R8me2s decreases the deposition of H3K9ac [42]. Methylation on this same lysine residue by the histone methyltransferase G9a is almost completely disrupted by the presence of H3R8me2 (both symmetric and asymmetric), as shown by in vitro experiments [43]. H3R8me2 also inhibits HP1 (heterochromatin protein 1) γ binding to H3K9me2 and me3 [44]. Additionally, Southall et al. [21] found that H3R8me2 reduces MLL1 activity towards H3K4 by 30%, but the precise mechanism underlying this cross-talk remains to be determined.

Regarding H3R17, Daujat et al. [45] showed that H3K18ac and, to a lesser extent, H3K23ac promotes methylation of this arginine residue by PRMT4 [45], a result confirmed later by two other studies [46,47]. More recently, Wu and Xu [48] have indirectly linked H3R17me2a to H3K4me3. During this cross-talk, PAF1c (RNA polymerase-associated factor 1 complex) acts as a methyl arginine reader by binding to methylated Arg17 and subsequently recruits MLL1 through an interaction with Ash2L in order to trigger H3K4me3 deposition. Another study showed that R17me2a and R26me2a, together with pan-H3ac, reduce the binding of the NuRD (nucleosome remodelling and deacetylase complex) and TIF1 (translation initiation factor 1) co-repressors to histone H3 [49]. On the basis of the above findings, a reasonable sequence of events would be that H3K18ac and H3K23ac are deposited first to stimulate Arg17 methylation (and probably R26me2a), which then inhibits the recruitment of co-repressors to chromatin and enhances trimethylation of H3K4 to activate gene expression.

A rather unique cross-talk of histone arginine methylation is the one with citrullination because this mechanism involves the same amino acid residue. For example, deiminated arginine residues can no longer be methylated by PRMTs, whereas arginine methylation blocks its own citrullination. This citrullination is followed by disengagement of RNA polymerase II from the promoter and transcriptional repression [16,17]. In contrast, oestrogen-induced citrullination of H3R26 by PADI2 facilitates oestrogen-receptor-mediated gene transactivation [18]. H3Cit26 strongly co-localizes with H3K27ac in MCF-7 cells, raising the possibility for a cross-talk between these two modifications [18].

The cross-talk between arginine methylation and citrullination influences downstream chromatin events [6]. The authors showed that H3R8 methylation reduces slightly the binding of HP1α to H3K9me3, whereas deimination of the same residue upon oestrogen stimulation of PADI4 completely abrogates HP1α recruitment. It was proposed that the difference in HP1α-binding affinity to H3K9me3 in the context of H3R8me or H3Cit8 may allow for a gradual activation of the genes targeted by the HP1α repressor during oestrogen induction [6].

**Histone H4 arginine residues**

All known cross-talk mechanisms described for arginine methylation on H4 involve Arg3, and specifically its dimethylated (asymmetric or symmetric) forms (Table 1). Earlier studies have shown by in vitro and in vivo work that H4R3me2a by PRMT1 facilitates p300-mediated histone H4 acetylation, leading to nuclear-receptor-dependent transcription [50,51]. More recent work validated this cross-talk by demonstrating that the tumour-suppressor gene p53 recruits PRMT1, p300 and PRMT4 to activate transcription in a stepwise manner. During this p30-mediated activation, H4R3me2a is initially deposited and then stimulates the acetylase activity of p30 towards H4 Lys5, Lys8 and Lys12 [46]. Additionally, ChIP analysis in chicken erythroleukaemia 6C2 cells in which PRMT1 was knocked down showed that loss of H4R3me2a across the β-globin locus is accompanied by a vast reduction in the levels of H4K5ac, H4K8ac and H4K12ac [52]. The reverse relationship between these two modifications differs because H4 acetylation inhibits PRMT1-mediated methylation of H4R3 [51,53]. An in vitro study, however, showed that H4 acetylation of any of Lys5, Lys8, Lys12 or Lys16 stimulates the methylease activity of the yeast PRMT1 orthologue (Hmt1p) towards H4R3. Despite this discrepancy, it was shown that mutation at Arg3 or Lys8 results in a similar phenotype in yeast, suggesting that modifications at these two residues function within the same pathway [54].

Two studies showed that H4R3me2a is also involved in an arginine methylation trans-histone cross-talk. Li et al. [55] demonstrated that H4R3me2a catalysed by PRMT1 facilitates subsequent acetylation of H3K9 and H3K14, and recruitment of the transcriptional machinery to activate expression of the globin genes. In vitro experiments provide a direct link for this cross-talk, as H4R3me2a is a binding surface for the co-activator PCAF (p300/CREB [cAMP-response-element-binding protein]-binding protein)-associated factor), which possesses H3K9/H3K14 acetyltransferase activity. In the second study, Huang et al. [52] showed that loss of H4R3me2a by PRMT1 knockdown leads to localized induction of H3K9me2 and global increase of H3K27me3 over the β-globin locus. Whether the interplay between H4R3me2a and methylated H3K9/H3K27 is direct remains to be determined.

The symmetrically dimethylated form of H4R3 is mediated by PRMT5 and PRTM7, and is implicated in transcriptional repression. A recent study demonstrated a trans-histone cross-talk involving H4R3me2s and H3K4me. MLL4, a methyltransferase related to MLL1, which can also trimethylate H3K4, binds to unmodified or asymmetrically modified H4R3 through a tandem PHD domain. However, H4R3me2s strongly impairs MLL4 recruitment and downstream H3K4 methylation, inhibiting neuronal differentiation [56]. In another study, Feng et al. [53] tested whether H4 acetylation affects PRMT5-mediated H4R3me2s in a manner similar

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<td>Transcriptional repression</td>
<td>H. sapiens; X. laevis; in vitro; M. musculus</td>
<td>PRMT5; PRMT7</td>
<td>[59,60,62]</td>
</tr>
<tr>
<td>H4R3me2s</td>
<td>H4K20me3</td>
<td>Mod1→Mod2</td>
<td>Transcriptional repression</td>
<td>H. sapiens</td>
<td>PRMT5</td>
<td>[61]</td>
</tr>
</tbody>
</table>
Mechanisms of modification cross-talk

General themes emerge from the cross-talks of histone arginine methylation reviewed above, which are applicable across all epigenetic modifications. Cross-talks among
modifications employ mechanisms that can be divided into two major categories, each comprising two subcategories. The first category includes histone modifications that take part in a sequential cross-talk, during which one modification either promotes (sequential positive) or inhibits (sequential negative) the deposition of another modification (Figures 2A and 2B respectively). The second category of mechanisms comprises modifications that function in a combinatorial manner during which two or more modifications existing simultaneously regulate together the binding of effector molecules. These epigenetic modifications can work synergistically, for example by recruiting together a specific reader (Figure 2C), or antagonistically, where one modification blocks reader binding to the other modification (Figure 2D). These two general mechanisms appear to apply for all modification cross-talks regardless of whether they occur within the same histone (cis) or between histones (trans).

H3R2 is one of the few histone residues whose dimethylation states employ all four mechanisms of cross-talk. For example, H3R2me2s is involved in a sequential positive mechanism because it recruits the co-activator WDR5 through its WD40 domain, which subsequently enhances H3K4me3 deposition [12,13] (Figure 2A). In contrast, the alternative dimethylated form of this residue, H3R2me2a, is involved in a sequential negative mechanism because it inhibits the recruitment of Set1/MLL1 complex components and, as a result, blocks H3K4me3 [8–10,22] (Figure 2B). H3R2 methylations are also involved in cross-talks utilizing combinatorial mechanisms. H3R2me2s together with H3K4me3 enhances the recruitment of the PHD finger of RAG2 leading to V(D)J recombination and transcriptional activation [29] (Figure 2C). H3R2me2s is a unique methylation because it first uses a sequential cross-talk to lay down H3K4me3 and then together with H3K4me3 affect factor binding via a combinatorial mechanism. Finally, a combinatorial antagonistic cross-talk is observed between H3R2me2a and H3K4me3 (Figure 2D). For example, TFIIID is recruited by H3K4me3 to initiate transcription, but when H3R2me2a is deposited on the same tail, this prevents the binding of TFIIID to chromatin [26].

All cross-talks involving histone arginine methylation described to date can be classified in one of these categories (Table 1). These mechanisms could also apply to non-histone proteins (i.e. p53) that possess numerous modifications including arginine methylation. In addition, these mechanisms could also be relevant for cross-talks that may occur between histone modifications and modification on other chromatin-bound proteins such as polymerases, transcription factors, modifying enzymes and remodellers [4].

**Concluding remarks**

Determining how epigenetic modifications cross-talk with each other has attracted much interest in recent years because of the increasing evidence that deregulation of PTM deposition is key for the appearance of several diseases including cancer [5–7]. Recent developments in sequencing and MS techniques make the discovery and characterization of modification cross-talks more achievable. Methods that involve next-generation sequencing (i.e. ChIP-seq and bisulfite-seq) provide genome-wide profiles of histone and DNA modifications [61,62]. Such genome-wide profiles can infer the interplay among epigenetic modifications on the basis of the overlap of their distributions [10,12,13]. In addition, ETD (electron transfer dissociation) and ECD (electron capture dissociation) allow the analysis of long peptides (middle-down MS) or full histones (top-down MS), helping to discover PTMs co-occurring on individual histone molecules [63]. Coupling MS to SILAC (stable isotope labelling by amino acids in cell culture) leads to the analysis of PTM deposition kinetics [64], which could point towards possible cross-talks. Moreover, SNAP (SILAC nucleosome affinity purification) identifies cross-talks among histone modifications and DNA methylation occurring in the context of the nucleosome [65]. All of these MS methodologies provide unambiguous detection of amino acid modifications and offer hypotheses about their interactions. However, other biochemical techniques are also useful and utilized to elucidate modification cross-talks. Immunoassay-based methodologies such as GPS (Global Proteomic Screen in S. cerevisiae) can decipher histone modification cross-talks in budding yeast relying on the use of the recently developed histone point-mutant collections [66]. Protein arrays can also be used to detect cross-talks employing a combinatorial mechanism in a high-throughput manner [67].

So far, most of the efforts for elucidating cross-talks involving histone arginine methylation have been placed on two histones, H3 and H4. However, methylated arginine residues have been detected on other histones including Arg11 and Arg29 on H2A [68], and Arg37 on CenH3, the non-canonical histone present at centromeric chromatin [69]. Hence, it can be predicted that cross-talks involving arginine residues of histones other than H3 and H4 will be exposed after further investigation. Furthermore, cross-talks involving arginine monomethylation are not as well characterized as the ones involving dimethylated states. It is not clear whether this is due to monomethylation being mainly a transient state or because this modification has not been studied extensively. Trans-histone cross-talks involving arginine methylation are also scarcely reported [52,55,56]. However, the recent methodology established by Reinberg and colleagues to demonstrate that the two copies of the same histone found within one nucleosome can possess different modifications [70] creates new prospects for discovering trans-histone cross-talks. It might not be surprising that future studies employing this methodology will show that current cross-talks thought to be occurring in cis within the same histone molecule, are actually occurring in trans between the two sister histones.

Finally, it remains unclear whether arginine methylation can be actively removed by a demethylase. However, several independent studies demonstrated that arginine methylation levels change quickly under certain conditions, pointing towards the existence of an enzyme involved in this task.
Identification of such a demethylase, which will prove the dynamic nature of this modification, will open new questions for the cross-talk mechanisms involving arginine methylation.

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