

Protein Arginine Methylation in Mammals: Who, What, and Why

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The covalent marking of proteins by methyl group addition to arginine residues can promote their recognition by binding partners or can modulate their biological activity. A small family of gene products that catalyze such methylation reactions in eukaryotes (PRMTs) works in conjunction with a changing cast of associated subunits to recognize distinct cellular substrates. These reactions display many of the attributes of reversible covalent modifications such as protein phosphorylation or protein lysine methylation; however, it is unclear to what extent protein arginine demethylation occurs. Physiological roles for protein arginine methylation have been established in signal transduction, mRNA splicing, transcriptional control, DNA repair, and protein translocation.

Chemistry and Enzymology of Protein Arginine Methylation and Demethylation

Biology relies upon the enlarged repertoire of interactions that occur when proteins are posttranslationally modified. In recent years, it has become clear that methyl groups stand beside phosphate groups as major controlling elements in protein function. A wide variety of methylation (and in some cases demethylation) reactions occur at the side chains of a number of amino acid residues and at protein N and C termini. These modifications generate distinct sets of chemical interactions that play roles in a multitude of regulatory pathways (Clarke and Tamanoi, 2006).

The modification of arginine side chain guanidino groups is quantitatively one of the most extensive protein methylation reactions in mammalian cells (Paik and Kim, 1980; Najbauer et al., 1993). The number of distinct modified proteins is also large (Pahllich et al., 2006). Arginine is unique among amino acids as its guanidino group contains five potential hydrogen bond donors that are positioned for favorable interactions with biological hydrogen bond acceptors. In protein-DNA complexes, arginine residues are the most frequent hydrogen bond donors to backbone phosphate groups and to thymine, adenine, and guanine bases (Luscombe et al., 2001). Specific networks of hydrogen bonds can form with arginine residues and adjacent phosphate groups in RNA loops (Calnan et al., 1991), and the arginine-aspartate two H-bond interaction is especially stable in proteins (Mitchell et al., 1992). Each addition of a methyl group to an arginine residue not only changes its shape, but also removes a potential hydrogen bond donor. Such chemistry could promote the preferential inhibition by methylation of some, but not all, binding partners. For example, arginine methylation of the Sam68 proline-rich motifs can inhibit its binding to SH3, but not WW domains (Bedford et al., 2000). Methylation of arginine residues might also increase their affinity to aromatic rings in cation- π interactions (Hughes and Waters, 2006). Such interactions are seen in the aromatic cage of the SMN tudor domain that likely interacts with the methylated tail of the SmD splicing factor

(Sprangers et al., 2003). Thus, modification of arginine residues in proteins can readily modulate their binding interactions and, thus, can regulate their physiological functions.

Three distinct types of methylated arginine residues occur in mammalian cells. The most prevalent is omega- N^G,N^G -dimethylarginine (Paik and Kim, 1980). Here, two methyl groups are placed on one of the terminal nitrogen atoms of the guanidino group; this derivative is commonly referred to as asymmetric dimethylarginine (ADMA) (Figure 1). Two other derivatives occur at levels of about 20% to 50% that of ADMA (Paik and Kim, 1980). These include the symmetric dimethylated derivative, where one methyl group is placed on each of the terminal guanidino nitrogens (omega- N^G,N^G -dimethylarginine; SDMA) and the monomethylated derivative with a single methyl group on the terminal nitrogen atom (omega- N^G -monomethylarginine; MMA). These three derivatives are present on a multitude of distinct protein species in the cytoplasm, nucleus, and organelles of mammalian cells (Bedford and Richard, 2005). Methylated arginine residues in proteins are often flanked by one or more glycine residues (Gary and Clarke, 1998), but there are many exceptions to this general rule.

The formation of MMA, ADMA, and SDMA in mammalian cells is performed by a sequence-related family of catalytic subunits of protein arginine methyltransferases termed PRMTs (Figure 2). The exact number of genes encoding these catalytic subunits is under current investigation: six genes are known to encode enzymes with well-characterized activities (PRMT1, -3, -4 [CARM1], -5, -6, and -8) and another three genes encode sequence-related proteins with possible or probable methyltransferase activities (PRMT2, -7, -9 [4q31]) (Bedford, 2007). Each PRMT species harbors the characteristic motifs of seven-beta strand methyltransferases (Katz et al., 2003), as well as additional "double E" and "THW" sequence motifs particular to the PRMT subfamily (Cheng et al., 2005). It has also been proposed that the FBXO11 and FBXO10 proteins, which do not harbor these signature motifs, represent a second family of

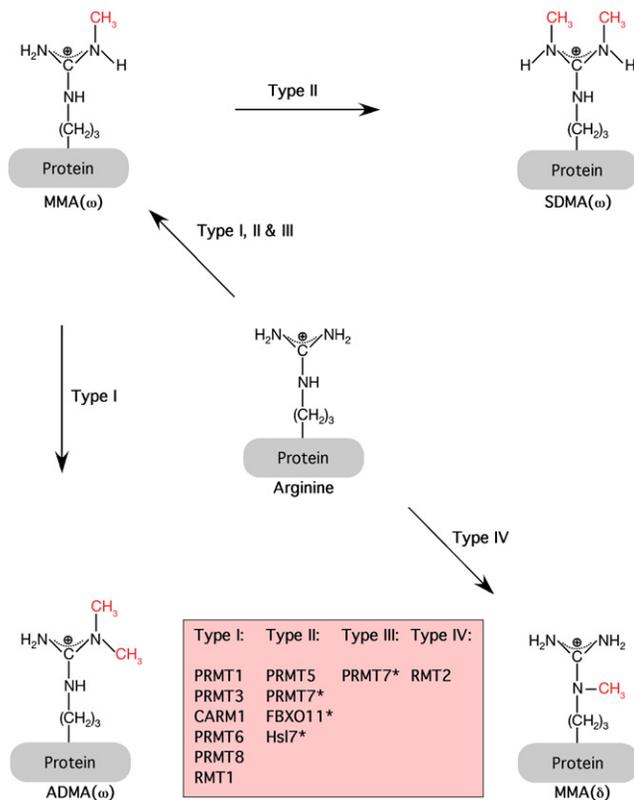


Figure 1. Types of Methylation on Arginine Residues

Types I, II, and III PRMTs generate monomethylarginine (MMA_ω) on one of the terminal (ω) guanidino nitrogen atoms. These two nitrogen atoms are equivalent. The subsequent generation of asymmetric dimethylarginine (ADMA) is catalyzed by type I enzymes, and the production of symmetric dimethylarginine (SDMA) is catalyzed by type II enzymes. Type III PRMTs only monomethylate. Type IV enzyme activity that catalyzes the monomethylation of the internal guanidino nitrogen atom has only been described in yeast. The type of methylation reactions catalyzed by PRMT7, FBXO11, and Hsl7 are still being established and are, thus, marked with an asterisk (see text).

protein arginine methyltransferases; however these activities require validation (Cook et al., 2006; Krause et al., 2007). The gene products with well-characterized activities, generally purified as fusion proteins, catalyze MMA formation; PRMT1, -3, -4 (CARM1), -6 and -8 additionally catalyze ADMA formation, whereas PRMT5 additionally catalyzes SDMA formation. Enzymes that form ADMA are designated “type I”; those that form SDMA are designated “type II” (Gary and Clarke, 1998). To date, no enzyme has been found that forms both ADMA and SDMA derivatives.

A clear understanding of how PRMT catalytic polypeptides can be assembled into active complexes with other protein subunits in cells is needed. Although PRMT1, -3, -4 (CARM1), -5, -6, and -8 are active methyltransferases in the absence of other polypeptide species *in vitro*, they might also bind additional partners for their functions *in vivo*. It now appears that interactions between PRMTs and their binding partners (and presumed regulatory subunits) can be transient or permanent (Table 1). It is possible that the species with poorly defined activities require the presence of other subunits for catalytic activity.

An important question under current investigation is whether protein arginine demethylation reactions occur to reverse the effects of the modifications. Initial studies indicated that methyl groups were stable on arginine residues. The apparent absence of protein arginine demethylases suggested that the only way to reverse the effects of the modification would be to degrade the protein to its component amino acids and then make a new unmodified version by protein synthesis. However, two types of enzymes that can remove methyl groups from arginine residues in proteins were recently identified. MMA residues in proteins can be deiminated to citrulline residues by the PAD4 peptidylarginine deiminase (Thompson and Fast, 2006). It is unknown whether the citrulline residue might be converted to an arginine residue to complete the demethylation process. It has been suggested that this enzyme is unlikely to play a physiological demethylation role. Indeed, peptides containing MMA are more slowly deiminated than those containing arginine residues, and those containing ADMA are not deiminated at all. Thus, methylation might, in fact, inhibit a reaction that normally converts arginine residues to citrulline residues (Rajmakers et al., 2007). Additionally, recent work has suggested that a second type of enzyme, the Jumonji domain-containing proteins, which was originally identified as a family of lysine demethylases, can also demethylate arginine residues. Indeed, JMJD6 has been reported to directly regenerate arginine residues from methylated histone species (Chang et al., 2007). It will be important to determine if there are additional enzymes that catalyze similar reactions and to assess the biological significance of each of these demethylation pathways in regulating protein arginine methylation.

The Mammalian PRMTs

In the section below, we describe the conserved family of mammalian PRMTs 1–9, as well as a second family of putative enzymes related to the F box-only proteins (Figure 2). There does not appear to be major redundancy between these enzymes; mouse knockouts display generally clear and dramatic phenotypes (Table 2). The diversity of these enzymes is enhanced by alternative splicing reactions that lead to amino acid sequence variants (Goulet et al., 2007).

The Major Type I Enzyme, PRMT1—Broad Specificity with Multiple Interacting Partners

PRMT1 was the first mammalian protein arginine methyltransferase identified as a single gene product and was initially characterized as a GST-fusion protein expressed in bacterial cells (Lin et al., 1996). Previous purifications of these enzymes from mammalian tissues was complicated by their low abundance and the presence of a multitude of polypeptides in the final preparations, making it difficult to discern which were contaminants and which were truly associated with the enzymatic activity (Gary and Clarke, 1998). PRMT1 is responsible for the bulk (about 85%) of total protein arginine methylation activity in cultured RAT1 fibroblast cells as well as in mouse liver (Tang et al., 2000).

PRMT1 has a very wide substrate specificity, with a preference for methylating arginine residues that are flanked by one or more glycine residues (Gary and Clarke, 1998; Lee and Bedford, 2002). Structural studies have identified three different

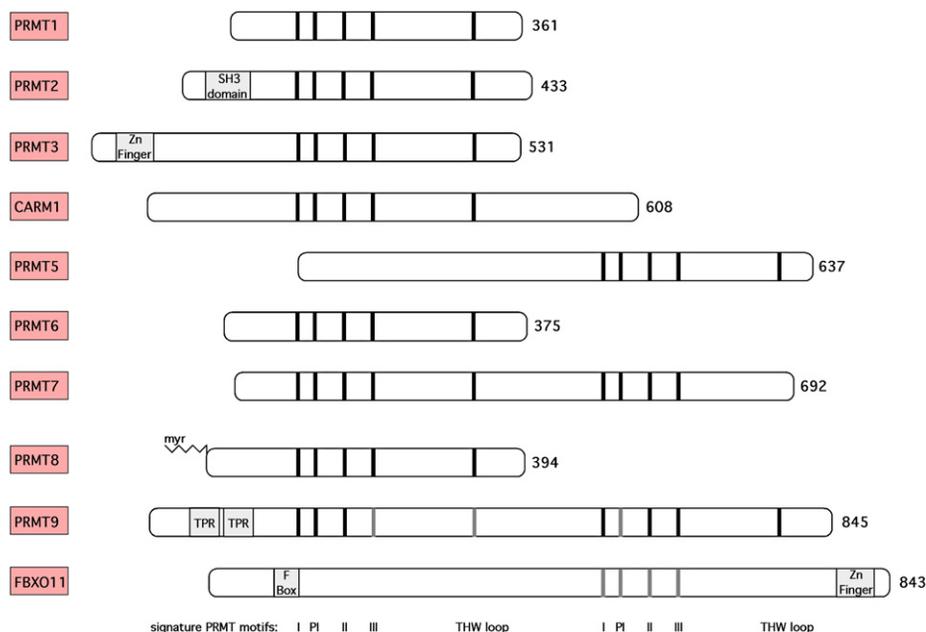


Figure 2. The Protein Arginine Methyltransferase Family

The mammalian PRMT family currently contains nine highly related members; all harbor signature motifs I, post-I, II, and III and the conserved THW loop (in black bars). FBXO11 might have arginine methyltransferase activity, but is not structurally related to the other PRMTs and required “forced” alignments to identify putative signature motifs (in gray bars). Protein domains that might assist in substrate recognition are marked in gray boxes. The accession numbers for the PRMTs are as follows: hPRMT1 (AAF62893), hPRMT2 (AAH00727), hPRMT3 (AAC39837), hCARM1 (NP_954592), hPRMT5 (AAF04502), hPRMT6 (Q96LA8), hPRMT7 (NP_061896), mPRMT8 (DAA01382), hPRMT9 (AAH64403), and mFBXO11 (AAI28480).

peptide-binding channels, suggesting that different PRMT1 substrates might approach the active site from different angles (Zhang and Cheng, 2003). This view is supported by surface-scanning mutational analysis that identified certain mutations that selectively block the binding of distinct substrates (Lee et al., 2007). Recent kinetic studies demonstrated that distal substrate residues influence arginine methylation, and the available evidence suggests a partially processive mechanism (Osborne et al., 2007). The three-dimensional structure of PRMT1 suggests that it is active as a homodimer (Zhang and Cheng, 2003). However, PRMT1 appears to be associated with a number of proteins in the cell (Table 1). Gel filtration analyses of the enzyme in mammalian cell extracts suggest sizes ranging from 275 kDa to 450 kDa; by contrast, the PRMT1 dimer is expected to be 80 kDa (Gary and Clarke, 1998; Hung et al., 2007). This variation could represent the range of different oligomers that are present in the cell.

Two Minor Type I Enzymes with More Defined Cellular Localization—PRMT 3 and PRMT8

PRMT3 is located exclusively in the cytosol and has a zinc finger domain that appears to anchor it to its substrates (Frankel and Clarke, 2000). A major methyl-accepting substrate in mammalian cells is the S2 protein of the small ribosomal subunit; much of PRMT3 is, in fact, associated with ribosomes via an interaction between its zinc finger domain and the S2 protein (Swiercz et al., 2007).

PRMT8 is an unusual catalytic subunit for two reasons. Although its amino acid sequence is closely related to PRMT1, it has a relatively narrow tissue distribution, being limited mainly

to brain (Lee et al., 2005a). It is also the only PRMT known to be membrane associated; PRMT8 is attached to the plasma membrane via N-terminal myristoylation (Lee et al., 2005a). The in vitro activity of the full-length recombinant enzyme is low; however, removal of the N-terminal domain by truncation or proteolysis results in a large activation (Sayegh et al., 2007). The N-terminal region contains two proline-rich sequences that can bind a number of SH3 domains, including that of PRMT2. It is possible that binding of proteins to this N-terminal domain can result in physiological PRMT8 activation and/or changes in its cellular location. Recently, a number of PRMT8-binding proteins were identified in cultured cells of probable neuronal origin (Pahlich et al., 2008).

Type I Enzymes with Restricted Substrate Specificities—CARM1/PRMT4 and PRMT6

CARM1/PRMT4 can be distinguished from PRMT1 because it catalyzes the methylation of only a few distinct substrates (Lee and Bedford, 2002). It binds the steroid receptor coactivators (SRC-1-3) and has clear transcriptional coactivator activity itself, thus its name—the coactivator associated arginine methyltransferase 1 (CARM1) (Chen et al., 1999). As it was the fourth arginine methyltransferase described, it is also referred to as PRMT4. CARM1 loss is not compatible with life (Yadav et al., 2003). In cells, CARM1 forms a complex with ATP-remodeling (SWI/SNF) factors (Xu et al., 2004). CARM1 recruitment to transcriptional promoters feeds into the “histone code,” resulting in elevated levels of H3R17 (histone H3 Arg-17) and H3R26 methylation, which are associated with transcriptional activation. It also methylates other transcriptional coactivators and a subset

Table 1. Interacting Protein Partners of Mammalian PRMTs

PRMT Species	Interacting Species
PRMT1	poly(ADP-ribose) polymerase and NF-kappaB
	Sam68 and the MLL complex
	Btg1 and Tis2/Btg2
	intracytoplasmic domain of the type I interferon receptor
	hCAF1
PRMT2	RB
	PRMT8 (via SH3 domain)
PRMT3	DAL-1/4.1B
	Zn-finger domain – binds 40S rpS2
CARM1/PRMT4	p160 family coactivators
	SWI/SNF complex
	Flightless-1
PRMT5	nucleolin ^a
	hlws1 transcription elongation factor
	Histone-binding protein COPR5
	LIM protein AJUBA ^a
	SWI-SNF
	methylosome complex with pICln and MEP50
	Blimp1
	DAL-1/4.1B tumor suppressor
	RNA polymerase II FCP phosphatase
	SPT4/SPT5 transcription elongation factor ^a
	PRMT7
PRMT7	CTCFL testis-specific factor
	PRMT5
PRMT8	Ewing Sarcoma protein
	PRMT1
	SH3 domains of PRMT2, Fyn, Plc γ , and P85

^a PRMT5-interacting species that may be compromised by the use of FLAG-tagged fusion proteins (Nishioka and Reinberg, 2003).

of splicing factors (Cheng et al., 2007; Feng et al., 2006). The CARM1 crystal structure was solved by two groups, providing insight into its mechanism of action (Troffer-Charlier et al., 2007; Yue et al., 2007). A comparison of the *apo* and *holo* states of CARM1 reveals AdoHcy-induced conformational changes of the cofactor pocket, which likely generate an access channel for the target arginine (Yue et al., 2007). Structural analysis reveals that the CARM1 N-terminus assumes a PH domain-like fold (Troffer-Charlier et al., 2007).

PRMT6 is a nuclear enzyme characterized by its specificity for distinct methyl-accepting substrates and by its automethylation (Frankel et al., 2002). PRMT6-specific substrates include the nuclear scaffold protein HMGA1a/b (Miranda et al., 2005; Sgarra et al., 2006), DNA polymerase beta (El-Andaloussi et al., 2007), the HIV Tat protein (Boulangier et al., 2005), and histone H3 (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008). PRMT6 is the major PRMT responsible for histone H3R2 methylation, and it has a clearly defined role in antagonizing the MLL-complex-dependent methylation of the Lys-4 residue. Kinetic

Table 2. Phenotypes of Mice Lacking Protein Arginine Methyltransferases

Gene	Phenotype(s)
<i>Prmt1</i>	embryonic lethal, but ES cells can survive
	trapping mutant generates a hypomorphic allele
<i>Prmt2</i>	mice are viable
	mouse embryo fibroblasts have increased activity of NF-kappaB and are more resistant to apoptosis than wild type cells
<i>Prmt3</i>	mice are viable, but mutant embryos are slightly smaller
	trapping mutant generates a hypomorphic allele
<i>Carm1/Prmt4</i>	newborns small and die shortly after birth
	T cell development blocked in thymus
	lipid metabolism altered in embryos

studies have shed light on how the enzyme catalyzes the dimethylation of arginine residues (Lakowski and Frankel, 2008). The use of a peptide substrate with a single arginine residue (WGGYSRGGYGGW) demonstrated that the monomethylated derivative is recognized with higher affinity and methylated more rapidly than the unmodified derivative. These results suggest a distributive, rather than processive, mechanism. It remains unknown if this also occurs for protein substrates or for substrates with multiple methylatable arginine residues.

A Well-Characterized Type II Enzyme—PRMT5

PRMT5 appears to be the major type II mammalian enzyme activity. It likely functions with multiple binding partners, but the identification of these partners has been greatly complicated by a technical problem caused by the α FLAG antibody recognizing PRMT5 (Nishioka and Reinberg, 2003). Nevertheless, PRMT5 seems to function in several types of complexes in both the cytoplasm and the nucleus. In the nucleus, binding to COPR5 (cooperator of PRMT5) can result in the preferential methylation of H4R3 over H3R8 (Lacroix et al., 2008). Moreover, COPR5 binding appears to be responsible for PRMT5 transcriptional corepressor activities. Like CARM1, PRMT5 can complex with hSWI/SNF ATP-dependent chromatin remodeling proteins, and in this context it functions as a transcriptional coactivator (Dacwag et al., 2007). It also associates with regulators of transcriptional elongation (Liu et al., 2007). In the cytoplasm, PRMT5 is involved in snRNP biogenesis through its ability to methylate a number of Sm proteins (Neuenkirchen et al., 2008). In these modifications, PRMT5 might function in conjunction with PRMT7 (Gonsalvez et al., 2007; see below).

Additional Members of the Seven- β Strand PRMT Family with No or Poorly Characterized activities—PRMT2, PRMT7, and PRMT9(4q31)

In comparison to the well-described type I and II PRMTs, no activity has been shown for PRMT2 or PRMT9(4q31); the evidence for PRMT7 activity remains controversial. It is possible that these species function only when complexed to additional polypeptide subunits; alternatively, the specific substrates for these enzymes have not been identified.

PRMT2 is of interest because it is a clear coactivator of gene expression much like PRMT1 and CARM1, and this activity

appears to rely upon the integrity of the methyltransferase domain. PRMT2 is a coactivator of both the androgen receptor and the estrogen receptor α (Meyer et al., 2007). In addition, PRMT2 can promote apoptosis and inhibit NF- κ B transcription by blocking I κ B- α nuclear export (Ganesh et al., 2006). Interestingly, its SH3 domain binds the PRMT8 N-terminal domain (Sayegh et al., 2007).

PRMT7 has been described as having type III activity (Figure 1) toward arginine-containing peptides (Miranda et al., 2004) or type II activity toward peptide and protein substrates (Lee et al., 2005b). Neither of these activities is particularly robust. Indirect evidence correlates PRMT7 activity with either resistance or sensitivity to DNA-damaging agents (Gros et al., 2006; Verbiest et al., 2008) and sensitivity of the kidney to damage caused by certain antibiotics (Zheng et al., 2005). The physical interaction of the CTCFL protein with PRMT7 also suggests a role in imprinting control region (ICR) DNA methylation (Jelincic et al., 2006). Both PRMT5 and PRMT7 are involved in the methylation of the C-terminal tails of Sm proteins, and both are required for normal snRNP biogenesis in human cells (Gonsalvez et al., 2007). This result opens the possibility that a PRMT5/PRMT7 heterodimer could be the active core of the enzyme complex involved in this Sm protein methylation reaction. PRMT7 might also play a role in embryonic stem cell (ESC) pluripotency, as its expression is lost when ESCs are induced to differentiate, much like the pluripotency marker OCT4 (Buhr et al., 2008). More work is needed to establish PRMT7 interacting partners and to characterize its enzymatic activities.

PRMT9(4q31) was identified based on homology with other family members as a product of a gene on human chromosome 4q31 (Lee et al., 2005a). We refer to this protein with the human chromosomal location because the PRMT9 designation has also been used for the product of the *FBXO11* gene in humans on chromosome 2p16 (Cook et al., 2006) (see below). PRMT9(4q31) has not been biochemically characterized to date. Interestingly, it contains two tetratricopeptide repeats (TPR), motifs which often mediate protein-protein interactions (Bedford, 2007).

Are F Box-Only Family Members Protein Arginine Methyltransferases?

Two F box-only family members have been suggested to be protein arginine methyltransferases (Cook et al., 2006; Krause et al., 2007). Notably, neither appear to belong to the family of seven- β strand methyltransferases that includes PRMT1-9 (Katz et al., 2003). The first, FBXO11/PRMT9(2p16), was identified from a “forced” alignment of its amino acid sequence to the PRMT family (Cook et al., 2006). The identification of type II methylation activity (Cook et al., 2006) is questionable as the HeLa-cell-expressed FLAG-tagged fusion protein preparation was likely to have been contaminated with PRMT5 (Nishioka and Reinberg, 2003). In an independent study, no activity was reported for the human or worm FBXO11 proteins (Fielenbach et al., 2007). A second F box-only protein, FBXO10, has been tentatively identified as PRMT11, but no biochemical characterization has been done (Krause et al., 2007). Further work is needed to conclusively determine whether F box-only proteins can be protein arginine methyltransferases.

Biological Roles of Arginine Methylation

Protein substrates for PRMTs make up an increasingly large fraction of the proteome. As new substrates are characterized, a broadening spectrum of PRMT-regulated cellular processes has been realized. Importantly, PRMT knockout mice provided a key resource for the *in vivo* validation of many of these *in vitro* findings.

Transcriptional Coactivators

PRMTs can methylate and regulate transcription factors, other coactivators, and histones. Their ability to methylate histones provides a direct route for input into the epigenetic regulation of gene expression. The arginine methylation of coactivators (e.g., p300, CBP, and SRC3) provides an indirect mechanism to impact a gene's epigenetic state by regulating the competence of these histone acetyltransferases. The first evidence implicating PRMTs in transcriptional regulation came with the identification of CARM1 as a nuclear receptor coactivator (Chen et al., 1999). In transient transfection assays, CARM1 and PRMT1 synergistically enhanced steroid hormone receptor-mediated reporter gene activation (Bedford and Richard, 2005). Strong synergy between these two PRMTs is further supported by siRNA-mediated single and double knock-downs and subsequent transcriptome analysis, which unmasked a group of STAT5-controlled genes whose expression is downregulated only when both CARM1 and PRMT1 are removed (Kleinschmidt et al., 2008). Although PRMTs were initially identified as estrogen and androgen receptor coactivators, they are now emerging as coregulators of a large number of transcription factors including p53, YY1, NF- κ B, PPAR γ , RUNX1, and E2F1 (Yadav et al., 2008; Zhao et al., 2008). Thus, PRMT1 and CARM1 function as general transcriptional coactivators, much like p300/CBP. Because they coactivate a large number of transcription factors, one would expect there to be a unifying mechanism that would explain this phenomenon, such as histone methylation, which in turn recruits methyl-binding effector proteins that facilitate transcription. To date, no such methylarginine-binding effectors have been described. However, transcription factor-specific mechanisms have been identified. RUNX1, for example, is methylated by PRMT1, and this methylation triggers the dissociation of the transcriptional repressor SIN3A, thereby promoting RUNX1 transcriptional activity (Zhao et al., 2008).

Transcriptional Corepressors

The synergy between PRMT5 and E2F1 in *Ccne1* corepression provided the first demonstration for PRMT repressor activity (Fabrizio et al., 2002). PRMT5 has since been identified as a general transcriptional repressor that functions with numerous transcription factors and repressor complexes, including BRG1 and hBRM (Pal et al., 2003), Blimp1 (Ancelin et al., 2006), and Snail (Hou et al., 2008). The mechanism by which PRMT5 mediates transcriptional repression remains to be elucidated. However, this enzyme, once recruited to a promoter, symmetrically dimethylates H3R8 and H4R3 (Pal et al., 2004). Importantly, the H4R3 site is a major target for PRMT1 methylation (ADMA) and is generally regarded as a transcriptional activating mark. Thus, both H4R3me2s (repressive; me2s indicates SDMA modification) and H4R3me2a (active; me2a indicates ADMA modification) marks are produced *in vivo*. No effector has yet been

identified that will bind to these isolated histone methyl marks. However, the RAG2-PHD finger binds H3R2me2sK4me3 slightly better than either H3R2me2aK4me3 or H3K4me3 (Ramon-Maiques et al., 2007), suggesting that arginine methylation sites on the histone tails can be recognized in combination with other marks. The specificity of PRMT5 for H3R8 and H4R3 can be altered by its interaction with COPR5 (Lacroix et al., 2008), and this could perhaps play an important role in determining PRMT5 corepressor status. Indeed, the general assumption that PRMT5 functions as a transcriptional repressor is not cut and dry, as there are at least two examples where it functions as a coactivator. First, reduced PRMT5 levels (by RNAi) results in decreased IL-2 expression (Richard et al., 2005), and second, PRMT5 is required for MyoD-induced muscle differentiation, in particular for *Myog* transcription (Dacwag et al., 2007).

PRMT6 also feeds into the epigenetic code and possesses transcriptional repressor activities. It is the primary enzyme responsible for H3R2 methylation in mammalian cells (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008). PRMT6 can strongly methylate H3K4me1 and H3K4me2 peptides and weakly methylate a H3K4me3 peptide (Iberg et al., 2008). However, prior methylation of the H3R2 site essentially prevents the MLL1 complex from methylating H3K4 (Hyllus et al., 2007). H3K4 methylation is found at the 5' end of active genes and is responsible for recruiting chromatin-remodeling enzymes to establish and maintain a transcriptionally active state. The effectors that bind H3K4me3 harbor methyl-specific binding domains, including PHD, Chromo, and Tudor domains. H3R2 methylation blocks the ability of most of these domains to bind the histone H3 N-terminal tail in *in vitro* assays (Iberg et al., 2008). In cells, WDR5 chromatin binding is sensitive to PRMT6 expression levels (Guccione et al., 2007; Hyllus et al., 2007; Iberg et al., 2008). Thus, PRMT6 functions as a transcriptional repressor by blocking the recruitment of transcriptional activators to the methylated H3K4 mark (Figure 3). PRMT6 also methylates the N-terminal tails of histones H4 and H2A (their first 5 residues are the same) at R3 (Hyllus et al., 2007); however, the role of methylation at these sites is unknown.

PRMT7, which has been reported to deposit the H4R3me2s mark, is a key player in DNA methylation of the imprint control region (ICR) (Jelinic et al., 2006). CTCFL binds ICR through its zinc finger region. The CTCFL N-terminal end interacts with the PRMT7 C-terminal end, and the CTCFL/PRMT7 complex is proposed to methylate H4R3, thereby facilitating the recruitment of the DNA methyltransferases Dnmt3a and Dnmt3b. This finding provides the first link between genomic imprinting and arginine methylation.

mRNA Splicing

The vast majority of PRMT substrates are associated with RNA (Pahlich et al., 2006). Thus, arginine methylation has been implicated in all aspects of RNA metabolism, including mRNA transcription, splicing, transport, translation, and turnover. Direct evidence for a role of arginine methylation in splicing first came from studies showing that *in vitro* splicing reactions could be blocked using pan SDMA-specific antibodies (Boisvert et al., 2002). In addition, splicing efficiency is reduced in hypomethylated nuclear extracts. CARM1, which deposits an ADMA mark, has also been clearly identified as a regulator of alternative

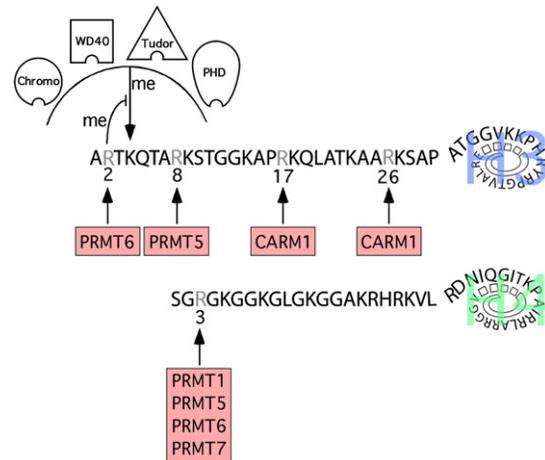


Figure 3. Arginine Methylation of Histone H3 and H4 N-Terminal Tails

The sites of PRMT-mediated methylation on the histone tails are noted. H3R2 methylation antagonizes the docking of a number of effector proteins, including those containing chromo, PHD, Tudor, and WD40 domains. H3R2 methylation structurally impedes the binding of H3K4me3 recognition domains and also prevents H3K4 methylation by the MLL1 complex. The first 5 residues of histones H2A and H4 are identical, and H2A is also methylated at the R3 position.

splicing (Cheng et al., 2007; Ohkura et al., 2005). CARM1 methylates the splicing factors CA150, SAP49, SmB and U1C. Of note, CA150 methylation generates a docking motif for the SMN Tudor domain. The use of splicing reporter assays demonstrates that CARM1 promotes exon skipping in an enzyme-dependent manner.

Nuclear/Cytoplasmic Shuttling

The first identified biological role for arginine methylation involved nucleocytoplasmic shuttling in yeast (Shen et al., 1998). RNA-binding proteins (RBPs), like snRNPs and hnRNPs, account for over 60% of the ADMA found in the nucleus (Bedford and Richard, 2005). These RBPs contain GAR motifs and PGM (proline-glycine-methionine) motifs, which are the prime sites of PRMT1 and CARM1 methylation, respectively (Cheng et al., 2007; Najbauer et al., 1993). Importantly, many of these RBPs shuttle between the nucleus and the cytoplasm, and two members (Npl3 and Hrp1) of this protein family are trapped in the nucleus in cells lacking Hmt1/Rmt1, the primary Type 1 PRMT in yeast (Shen et al., 1998). In mammalian cells, the inhibition of methylation of the high molecular weight forms of FGF2 results in a decrease in their nuclear accumulation (Pintucci et al., 1996), and this is also the case for Sam68 (Bedford and Richard, 2005). The *Xenopus laevis* RNA-binding protein CIRP2 is methylated by XIPRMT1, and XIPRMT1 overexpression forces hypermethylated CIRP2 out of the nucleus (or blocks its return), resulting in its cytoplasmic accumulation (Aoki et al., 2002). Finally, although the methylated form of the adenovirus 100K protein is exclusively nuclear, methylation inhibitors prevent 100K nuclear accumulation (Iacovides et al., 2007). Interestingly, some hypomethylated proteins accumulate in the nucleus and others in the cytoplasm. The mechanism(s) by which arginine methylation regulates nucleocytoplasmic shuttling has not been elucidated.

DNA Repair

In higher eukaryotes, DNA double strand breaks (DSBs), induced by ionizing radiation or during replication, are repaired through homologous recombination repair (HRR) or non-homologous end joining (NHEJ). The mammalian Mre11/Rad50/NBS1 (MRN) complex plays a critical role in HRR. Using methyl-specific antibodies, the Richard laboratory isolated methylated protein complexes from HeLa cell extracts and, in so doing, identified the components of the MRN complex (Boisvert et al., 2003). Mre11 is the only protein in this complex that harbors a GAR motif, and follow-up studies indicated that this region of Mre11 was indeed methylated by PRMT1 *in vitro* and *in vivo* (Boisvert et al., 2005a). Further investigation showed that the GAR motif regulates the 3' → 5' exonuclease activity of Mre11 on dsDNA. In cells treated with a global methyltransferase inhibitor followed by the DNA-damaging agent etoposide, few γ H2AX foci formed, and Mre11 was not recruited to DSBs, implying that methylation regulates MRN relocalization to DSBs (Boisvert et al., 2005b). Indeed, the methylated MRN GAR motif, on its own, can colocalize with γ H2AX foci (Dery et al., 2008). Like Mre11, 53BP1 is also involved in DSB repair. 53BP1 contains a GAR motif that is methylated by PRMT1 *in vivo*; however, methylation does not increase after DNA damage and a 53BP1 GAR motif mutant showed normal foci formation (Adams et al., 2005; Boisvert et al., 2005c).

DNA polymerase β (Pol- β) is implicated in base excision repair. Pol- β harbors two functional domains—an N-terminal lyase domain and a C-terminal domain that contains the nucleotidyltransferase activity of the polymerase. Recent findings indicate that the Pol- β lyase domain can associate with PRMT6 and PRMT1 (El-Andaloussi et al., 2006, 2007). These two PRMTs play distinct, nonredundant roles in regulating Pol- β function. PRMT6 methylates Pol- β R83 and R152 and augments its polymerase activity by enhancing its DNA binding and processivity (El-Andaloussi et al., 2006). PRMT1 methylates R137, which in turn blocks the Pol- β -PCNA interaction (El-Andaloussi et al., 2007). As yet, there is no evidence that the levels of arginine methylation on histone tails are altered, either in the vicinity of DNA damage or as part of a more global DNA-damage response.

Signal Transduction

Arginine methylation plays very clear roles in the nucleus, where it impacts transcription and splicing. However, the functions that arginine methylation plays in conveying information from the cell surface, through the cytoplasm, and into the nucleus have remained elusive, particularly because it appears to be a stable mark. There are, nevertheless, a number of findings that point toward a role for PRMTs in signal transduction. PRMT8, owing to its myristoylation and plasma membrane association, could impact a signaling pathway near its start (Lee et al., 2005a). In addition, PRMT8 harbors a proline-rich domain that can interact with SH3 domains, suggesting that it could serve as a signaling hub (Sayegh et al., 2007). PRMT1 binds the cytoplasmic region of the type I interferon receptor and has also been implicated in NGF receptor signaling (reviewed in Bedford and Richard, 2005). PRMT activities are also regulated by the T cell antigen receptor signaling pathway (Blanchet et al., 2005). Recent findings indicate that insulin treatment of cultured myotubes promotes PRMT1 accumulation in the membrane fraction (Iwa-

saki and Yada, 2007), thus providing the first evidence that arginine methylation might be involved in insulin signaling and glucose metabolism. Recently it was reported that PRMT1 plays a role in nongenomic estrogen signaling (Le Romancer et al., 2008). In this process, the estrogen receptor (ER) forms a multi-protein cytoplasm complex that activates a number of downstream signaling molecules, including PKC, ras, Akt, and MAPK. Estrogen-ER triggers the rapid, reversible receptor methylation by PRMT1, which in turn facilitates ER binding to Src, FAK, and p85. Thus, PRMT1 recruitment by ER occurs in both the cytoplasm and the nucleus, generating two distinct complexes: a signal transducing component and an epigenetic-modulating component. Finally, arginine methylation can act as an inhibitory modification when found in the proximity of Akt phosphorylation sites. Specifically, the methylation of FOXO1 by PRMT1 prevents a key phosphorylation event by Akt and promotes the nuclear localization of this transcription factor (Yamagata et al., 2008). Thus, when a methylation motif is superimposed on an Akt motif, phosphorylation is inhibited.

The Regulation of Arginine Methylation

Most PRMTs are active as recombinant proteins, suggesting that these enzymes might also be constitutively active in cells unless regulated by posttranslational modifications or through interactions with other proteins and protein complexes. In addition, PRMT activity can be restricted by subcellular compartmentalization. Finally, enzymes that counteract PRMT activity can reverse or block substrate methylation. Indeed, all these methods of PRMT regulation have been reported; however, their relative importance remains to be established.

First, PRMT-binding proteins can regulate methyltransferase activity. These binding proteins have the capacity to inhibit, activate, or change the substrate specificity of PRMTs. The related proteins, BTG1 and TIS2/BTG2, bind PRMT1 and stimulate its activity toward selected substrates (Lin et al., 1996). hCAF1, a BTG1-binding protein, also regulates PRMT1 activity (Robin-Lespinasse et al., 2007). Binding of the tumor suppressor DAL-1 inhibits PRMT3 enzymatic activity, both in *in vitro* reactions and in cell lines (Singh et al., 2004). CARM1 is found in the nucleosomal methylation activator complex (NUMAC), a complex of at least 10 proteins (Xu et al., 2004). Within NUMAC, CARM1 acquires the ability to methylate nucleosomal histone H3, whereas recombinant CARM1 preferentially methylates free histone H3. Likewise, when associated with COPR5, PRMT5 changes its balance of activity from H3R8 toward H4R3 (Lacroix et al., 2008). PRMT5 also complexes with the hSWI/SNF chromatin remodelers, BRG and BRM, and this association enhances PRMT5 methyltransferase activity (Pal et al., 2004). CTCFL (a protein that associates with the imprinting control region) binding has also been reported to elevate PRMT7 activity (Jelinic et al., 2006).

Second, PRMT activity will likely be regulated by posttranslational modifications. Some evidence shows that PRMT1, -4, -6 and -8 are automethylated; however, the functional consequence of this methylation remains unknown (Sayegh et al., 2007). In addition, CARM1 can be phosphorylated during mitosis by an as yet unidentified kinase (Higashimoto et al., 2007). This

phosphorylation event prevents CARM1 homodimerization and decreases its PRMT activity.

Third, adjacent posttranslational modifications can mask arginine methylation motifs. Histone N-terminal tails are rich in various posttranslational modifications and provide a number of examples of this phenomenon. There is an interplay between H3K9 lysine acetylation and PRMT5-mediated H3R8 arginine methylation: H3K9ac blocks H3R8me (Pal et al., 2004). The existence of an H3R8me2a or me2s mark will in turn block G9a-mediated H3K9 methylation (Rathert et al., 2008). In addition, PRMT6-mediated H3R2 methylation is inhibited, but not totally blocked, by H3K4me3 (Guccione et al., 2007; Iberg et al., 2008). Interestingly, H3K4me1 and H3K4me2 peptides are just as good a substrate for PRMT6 as the unmethylated peptide (Iberg et al., 2008). H3K4 methylation is also blocked by prior H3R2 methylation (Hyllus et al., 2007). It is very likely that similar regulatory scenarios play out on nonhistone PRMT substrates, where diverse signaling pathways may be impacted.

Fourth, arginine residues, within the context of a protein, can be converted to citrulline by deimination. The core histones H2A, H3, and H4 comprise a major group of deiminated proteins (Nakashima et al., 2002). Peptidyl arginine deiminases (PADs) catalyze the deimination of arginine, but not MMA or DMA, to citrulline (Rajmakers et al., 2007). Thus, PADs are not demethylases. However, these enzymes might carry out a preemptive strike on key sites of arginine methylation, thereby preventing subsequent methylation. Indeed, PADs can block methylation on an arginine residue by converting it to citrulline (Thompson and Fast, 2006).

Lastly, the first arginine demethylase was recently reported (Chang et al., 2007). This enzyme, JMJD6, a Jumonji domain-containing protein, demethylates H3R2me2 and H4R3me2. Of note, it appears that JMJD6 can demethylate both the symmetric and asymmetric forms of H4R3me2 (Chang et al., 2007). Further work is required in this field, especially the characterization of additional proteins in the jumonji-only family of enzymes, of which JMJD6 is a member. The possibility also exists that other types of arginine demethylases have yet to be discovered.

Methylarginine-Regulated Protein-Protein Interactions

Arginine methylation facilitates the interaction of GAR and PGM motifs with Tudor domains. The symmetric dimethylation of SmB by PRMT5 is required for an interaction with the Tudor domains of SMN, SPF30, and TDRD3 (Cote and Richard, 2005). The asymmetric dimethylation of CA150 by CARM1 also provides a docking site for the SMN Tudor domain (Cheng et al., 2007). Thus, motifs harboring either ADMA or SDMA residues bind a subset of Tudor domain-containing proteins. It is likely that a conserved aromatic “cage” in Tudor domains is the methylarginine-binding pocket (Sprangers et al., 2003), as is the case for other methyl-binding domains (Taverna et al., 2007). Tudor domain-containing proteins such as JMJD2a, 53BP1, and PHF20 also bind methyllysine motifs (Kim et al., 2006); thus, there are two distinct classes of methyl-binding Tudor domains. However, the propensity of Tudor domains to bind either methylarginine or methyllysine motifs cannot be predicted from their primary sequence, and thus, their binding selectivity must be determined empirically.

Arginine methylation can also negatively regulate protein-protein interactions. The methylation of arginine residues adjacent to a proline-rich motif can block the binding to SH3, but not WW, domain protein modules (Bedford et al., 2000). A second example of arginine methylation blocking a protein-protein interaction is the CARM1-mediated modification of the p300 GRIP1-binding domain (Lee et al., 2005c). Finally, recent structural studies have revealed that histone H3R2 forms critical interactions with an array of H3 N-terminal tail-binding domains (Taverna et al., 2007). PRMT6-mediated H3R2 methylation completely blocks the interaction of WDR5 with histone H3 (Couture et al., 2006). Moreover, this modification reduces the binding affinity of the CHD1 double chromodomains, 4-fold relative to H3K4me3 alone (Flanagan et al., 2005). Further work has demonstrated that, as a rule, H3R2 methylation blocks the binding of most H3 N-terminal tail-binding domains (chromo, PHD, WD40, and Tudor) (Iberg et al., 2008) (Figure 3). Future studies are needed to determine if there are any protein domains that can preferentially interact with the H3R2me mark.

Arginine Methylation and Disease

PRMTs are ubiquitously expressed (except for PRMT8); they are found in both the nucleus and the cytoplasm and they methylate a host of cellular proteins. Thus, deregulation of these enzymes or their substrates will likely be implicated in the pathogenesis of a number of different diseases. Clearly, PRMTs are not only epigenetic regulators, as free methylarginine regulates nitric oxide synthase, viral proteins are heavily methylated, and genetics implicates a prominent “reader” of methylarginine marks with spinal muscular atrophy. In this section, we begin to scratch the surface of what will likely develop into a very active field of research.

Cancer

Initial studies linking the PRMTs to cancer were circumstantial, but we are now gaining a better mechanistic understanding of how deregulated PRMT function might transform cells. Both PRMT1 and CARM1 are general transcriptional coactivators, and as such, their aberrant expression or altered regulation likely affects many transcriptional pathways. *PRMT1* mRNA levels have been reported to be higher in a panel of breast cancer cell lines than in normal controls (Goulet et al., 2007). The first concrete evidence that PRMT1 plays a role in oncogenesis came from studies demonstrating that mouse primary hematopoietic cells that are transduced with the MLL-EEN gene fusion product display enhanced self-renewal abilities and can form compact CFU-GEMM-like colonies in vitro (Cheung et al., 2007). The portion of EEN that is fused to MLL harbors a SH3 domain that can bind the proline-rich molecule Sam68. Sam68 is a well-characterized PRMT1 substrate. When either Sam68 or PRMT1 are fused directly to MLL in place of EEN, these fusions also can activate MLL oncogenic properties, implying that methylated Sam68 is a crucial player here (Cheung et al., 2007). Interestingly, CARM1 does not possess this ability.

CARM1 levels are elevated in castration-resistant prostate cancer (Hong et al., 2004; Majumder et al., 2006), as well as in aggressive breast tumors that also express high levels of the oncogenic coactivator AIB1 (El Messaoudi et al., 2006). Importantly, CARM1 methylates AIB1, thereby regulating its activity

and stability (Feng et al., 2006; Naeem et al., 2007). CARM1 recruitment to ER α -regulated promoters relies on the presence of AIB1, and CARM1 is essential for estrogen-induced proliferation of the MCF-7 breast cancer cell line (Frietze et al., 2008).

The normal developmental process of epithelial-mesenchymal transition (EMT) plays an important role in tumor progression. As a consequence of EMT, cancer cells can migrate and invade much more efficiently, and this process likely plays an active role in the metastatic spread of a tumor. A hallmark of EMT is the loss of E-cadherin expression; E-cadherin expression is actively repressed by the transcription factor SNAIL. A recent report demonstrated that PRMT5 interacts with SNAIL through a bridging molecule (AJUBA) (Hou et al., 2008). Importantly, siRNA-mediated PRMT5 knockdown results in elevated E-cadherin expression. These data indicate that PRMT5 is a critical SNAIL corepressor. In addition, PRMT5 overexpression promotes anchorage-independent cell growth (Pal et al., 2004), supporting the notion that PRMT5 might be an oncoprotein. Consistent with this idea, PRMT5 levels are elevated in gastric cancer (Kim et al., 2005) and in lymphoma and leukemia cells (Pal et al., 2007).

Cardiovascular Disease

Nitric oxide (NO), which is synthesized from L-arginine by nitric oxide synthase (NOS), plays multiple roles in the cardiovascular system, the immune system, and as a neurotransmitter in the brain. ADMA and MMA, but not SDMA, are endogenously produced amino acids that inhibit NOS (reviewed in Bedford and Richard, 2005). These free methylarginine species are generated by the proteolysis of methylated proteins. Thus, PRMTs are responsible for generating NOS inhibitors in vivo. To maintain a steady pool size, free methylarginines are metabolized by dimethylarginine dimethylaminohydrolase (DDAH) enzymes. An imbalance in this pool, by PRMT or DDAH dysfunction, might increase cardiovascular risk. Recent studies of *Ddah1* knockout mice provide genetic support for this hypothesis (Leiper et al., 2007). These knockout mice accumulate ADMA and display reduced NO signaling, which manifests itself in vascular pathophysiology, including elevated blood pressure.

Viral Pathogenesis

A number of different viral proteins have been characterized as PRMT substrates, including the herpes simplex virus 1 nuclear regulatory protein ICP27; the hepatitis C virus protein NS3; the Epstein-Barr virus nuclear antigen 2 (EBNA2); adenovirus E1B-AP5 and L4-100K; and the HIV-1 proteins Rev, Tat, and the nucleocapsid protein (reviewed in Bedford and Richard, 2005). Each of these methylated viral proteins contains GAR-motifs that are targeted for methylation by either PRMT1 or PRMT6. Studies using global methyltransferase inhibitors have revealed that methylation contributes to maximal virus infectivity in an HIV-1 infection cell culture model (Willemsen et al., 2006) and is critical for productive adenovirus infection (Iacovides et al., 2007). Thus, the methylation of viral proteins may provide a means by which they can enhance their complexity and expand their repertoire of interacting partners and in the process possibly disrupt normal cellular functions.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive disease resulting from the loss of *SMN1* gene function. SMA is

among the leading genetic causes of infant death, with a prevalence of ~ 1 in 6000 live births. SMN acts as a molecular chaperone for arginine-methylated proteins that participate in RNA metabolism. The SMN protein contains a Tudor domain that is a well-characterized methylarginine-dependent binding module. This Tudor domain can bind both ADMA and SDMA motifs and interacts with a number of PRMT5 and CARM1 substrates (Cheng et al., 2007; Tadesse et al., 2008). Point mutations within the SMN Tudor domain have been identified in SMA patients, establishing a clear link between the methyl-binding requirements of SMN and SMA pathogenesis (CUSCO et al., 2004).

Challenges in Analyzing PRMTs

It is remarkable how much we have learned about protein arginine methyltransferases from studying the properties of recombinant PRMTs. However, these species, generally expressed as tagged fusion proteins, can have different catalytic and substrate recognition properties than the endogenous protein and can lack the necessary accessory subunits. The fact that PRMTs often form cellular complexes with other polypeptides that can affect their activities (Table 1) also emphasizes the need for caution in interpreting results from studies with recombinant fusion proteins. This situation could be further complicated because some protein-protein interactions might be permanent and some might be transitory. Purifications of PRMTs from cells and tissues should result in the retention of at least the more tightly bound partners (Hung et al., 2007). However, little progress has been made in characterizing what are probably multiple endogenous complexes of each PRMT.

Several approaches can be used to distinguish the MMA, ADMA, and SDMA PRMT products (Pahlich et al., 2006). Chromatographic methods can detect very small amounts (subfemtomole) of radiolabeled derivatives. The use of high-capacity, high resolution cation exchange chromatography columns based on sulfonate crosslinked polystyrene resins allows the near-baseline resolution of the three major mammalian arginine derivatives: MMA, ADMA, and SDMA (Branscombe et al., 2001). For additional confirmation, it is possible to do thin-layer chromatography on each of the radiolabeled peak fractions (Branscombe et al., 2001; Lakowski and Frankel, 2008). However, it is more difficult to use thin-layer chromatography as an initial separation step of crude hydrolysates because the resolution is much more limited than in ion-exchange chromatography and the presence of other radiolabeled materials can be mistaken for methylated arginine residues. Recent advances in mass spectrometry instrumentation and techniques have opened a new window in the nonisotopic analysis of enzyme products (Hsieh and Tam, 2006; Pesavento et al., 2008). In many cases, it is now possible to detect monomethyl and dimethylarginine derivatives, and it is possible to distinguish symmetric from asymmetric dimethylation by breakdown products (Brame et al., 2004; Gehrig et al., 2004).

Emerging Themes

A number of areas in the PRMT field will likely receive attention in the near future. These include the structural determination of the PRMTs in complexes with their interacting partners, substrates, and small molecule inhibitors. Also of interest will be studies that

address the integration of signal transduction and arginine methylation pathways. Specifically, how is PRMT activity regulated by posttranslational modifications such as phosphorylation, acetylation, ubiquitination, and trans- or automethylation? We also clearly need to gain a better understanding of the enzyme(s) that demethylate arginine residues. Finally, only three mammalian proteins (SMN, SPF30, and TDRD3) have been demonstrated to bind methylarginine motifs through their Tudor domains. The quest for additional methylarginine-binding modules and proteins will likely be a high priority for a number of research groups over the coming years.

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