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Arginine methylation of RNA-binding proteins regulates cell function and differentiation

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Abstract

Arginine methylation is a post-translational modification that regulates protein function. RNA-binding proteins are an important class of cell function mediators, some of which are methylated on arginine. Early studies of RNA-binding proteins and arginine methylation are briefly introduced, and the enzymes that mediate this post-translational modification are described. We review the most common RNA-binding domains and briefly discuss how they associate with RNAs. We address the following groups of RNA-binding proteins: hnRNP, Sm, Piwi, Vasa, FMRP, and HuD. hnRNPs were the first RNA-binding proteins found to be methylated on arginine. The Sm proteins function in RNA processing and germ cell specification. The Piwi proteins are largely germ cell specific that are also required for germ cell production, as is Vasa. FMRP participates in germ cell formation in *Drosophila* but is more widely known for its neuronal function. Similarly, HuD plays a role in nervous system development and function. We review the effects of arginine methylation on the function of each protein, then conclude by addressing remaining questions and future directions of arginine methylation as an important and emerging area of regulation.

A brief history of RNA-binding proteins and protein arginine methylation

The presence of RNA-binding proteins was first alluded to in 1938, when ribonucleic acid in the cytoplasm was shown to be located on particulate or granular structures (Claude 1938). The ultimate cytoplasmic RNA-binding protein structure, the ribosome, was described in 1962 (Pogo et al. 1962), and perhaps not surprisingly, ribonucleases were among the first RNA-binding proteins to be purified (Robertson et al. 1968). In 1965, three structures containing mRNAs were identified in the cytoplasm of sea-urchin embryos: two contained ribosomes while a third sedimented more slowly than ribosomes and was comprised of RNA-containing particles (Spirin and Nemer 1965). These messenger ribonucleoprotein particles were free mRNA-containing structures described as ‘informosomes’, and their fate depended on the protein content (Spirin 1979). It was hypothesized that the proteins associated with an mRNA during different stages of its lifetime would determine the biogenesis of the RNA — its processing and transport, its existence in temporarily inactive states, and its function as a template for ribosomes (Spirin 1979).

Two years after ribosomes were discovered, the field of arginine methylation initiated when Allfrey, Faulkner, and Mirsky first described the acetylation and methylation of histones and their possible role in the regulation of RNA synthesis (Allfrey et al. 1964). They found that addition of a C¹⁴-methyl group occurred post-translationally, i.e. after the histones were

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synthesized, by observing radiolabeled methyl incorporation in the presence of the translation inhibitor puromycin (Allfrey et al. 1964).

Protein Arginine Methyl Transferases (PRMTs): the enzymes that methylate substrate proteins

Eleven PRMTs have been identified in mammals, although the number of PRMTs present in other organisms differs (Figure 1) (Bachand and Silver 2004; Boisvert et al. 2005; Boulanger et al. 2004; Cook et al. 2006; Gary and Clarke 1998; Gary et al. 1996; Krause et al. 2007; Lee et al. 2005; Yang et al. 2009). The majority of PRMTs recognize a similar motif within proteins: an arginine residue followed by a glycine residue (Krause et al. 2007; Lee and Stallcup 2009). PRMTs are subdivided into four types — type I, type II, type III, and type IV — based on the reactions performed by the enzyme (Figure 1). Type-I, -II, and -III PRMTs perform arginine monomethylation, where a single methyl group is attached to the terminal ω -nitrogen. Types I and II differ in that type-I PRMTs perform an asymmetric dimethylation reaction, attaching two methyl groups to the same nitrogen atom of the arginine residue, while type-II PRMTs perform a symmetric dimethylation reaction, attaching one methyl group to each of the terminal nitrogen atoms. Type-IV PRMTs perform monomethylation only on the δ -nitrogen (Bedford 2007; Krause et al. 2007; Lee and Stallcup 2009). Of the eleven identified mammalian PRMTs, PRMTs 1, 2, 3, 4/CARM1, 6, and 8 are classified as type I, while PRMTs 5, 7, and 9 are classified as type II. PRMT5 plays a key role in germ cell specification, as described in more detail below (Anne et al. 2007; Gonsalvez et al. 2006).

RNA-binding domains

RNA-binding proteins contain one or, more commonly, multiple RNA-binding domains to facilitate specific association with RNAs. Some common RNA-binding domains include the RNA-binding domain (RBD, also known as the RNA recognition motif (RRM)); K-homology (KH) domain, named for its identification in hnRNP K (Siomi et al. 1993); RGG domain, named for its amino acid composition (Kiledjian and Dreyfuss 1992); Sm domain, found in Sm proteins, and first identified with sera from a serum lupus erythematosus patient (Sm-Stephanie Smith) (Benito-Garcia et al. 2004; Tan and Kunkel 1966); and zinc finger, named for a characteristic functional domain (Lu et al. 2003). The following motifs have also been shown to bind RNA: double stranded RNA-binding domain (Ramos et al. 2000); cold-shock domain (Murzin 1993); Pumilio/FBF (PUF) domain (Wang et al. 2002); PAZ (Ma et al. 2004); and PIWI domains (Ma et al. 2005). For more extensive reviews on RNA-binding domains, please see Lunde et al. (2007) and Glisovic et al. (2008).

In RNA-protein interactions, a preference for amino acids arginine and lysine, which have basic side chains, is almost universally acknowledged (Ciriello et al. 2010; Ellis et al. 2007; Jones et al. 2001; Kim et al. 2003; Lejeune et al. 2005; Treger and Westhof 2001). Methylation of arginine could result in the loss of a hydrogen bond that forms with RNA or it may sterically hinder the association between RNA and protein. Alternatively, methylation could enhance an association by making the arginine more “hydrophobic”, thereby facilitating stacking with the RNA bases (reviewed in Bedford and Richard (2005)).

Arginine methylation of ribonucleoproteins and its functional consequences

Heterogeneous nuclear ribonucleoproteins (hnRNPs)

hnRNP A1 was one of the first RNA-binding proteins to be described as arginine-methylated (Rajpurohit et al. 1992). The hnRNPs contain about 65% of the total N^G, N^G-dimethylarginine found in the cell nucleus (Boffa et al. 1977). During transcription, pre-mRNA associates with at least 20 nuclear proteins, collectively referred to as the hnRNPs (Liu and Dreyfuss 1995). Methylation of hnRNP A1 reduces its ability to bind single stranded nucleic acids, and increases its sensitivity to trypsin digestion (Rajpurohit et al. 1994). hnRNP A1 is methylated on four arginines in a region that contains multiple RGG sequences interspersed with phenylalanines (Kim et al. 1997). Methylation of hnRNP A1 was predicted to lock the protein into a nonspecific binding mode by preventing the formation of arginine-dependent hydrogen bonds, as described in other systems (Calnan et al. 1991; Najbauer et al. 1993). A different role for arginine methylation of hnRNPs was identified in studies in yeast, whereby arginine methylation facilitated nuclear export (Shen et al. 1998) (Figure 2). In fact, methylation of yeast hnRNP protein Hrp1p had no effect on RNA-binding; however, RNA-binding to Hrp1p inhibited its methylation, supporting a model where hnRNP methylation occurs prior to RNA-binding (Valentini et al. 1999). Methylation in this model is thought to modulate protein-protein interactions that facilitate nuclear export (Figure 2).

hnRNP A2 is also methylated on arginines in its RGG domain, which plays an important role in cellular localization. hnRNP A2 usually resides in the nucleus, although deletion of the RGG domain results in cytoplasmic localization. When methylation was blocked, hnRNP A2 was similarly localized to the cytoplasm, suggesting that methylation of the RGG domain directs nuclear localization (Nichols et al. 2000) (Figure 2).

Sm proteins

Sm proteins constitute the core of the spliceosomal small nuclear ribonucleoprotein (snRNP) particle, playing an essential role in splicing of pre-mRNA. The seven Sm proteins are translated in the cytoplasm and assemble into a ring structure on the Sm site of the small nuclear RNA to form a snRNP core-Sm structure (Will and Luhrmann 2001). Although formation of the snRNP core can occur spontaneously in vitro, this process is highly regulated and dependent on the survival of motor neuron (SMN) complex in vivo (Meister et al. 2001; Pellizzoni et al. 2002); the SMN protein, a component of the SMN complex, will be discussed in more detail below. Three of the Sm proteins, SmD1, SmD3 and SmB, are post-translationally methylated by PRMT5 on arginine residues in their C-terminal tails (Brahms et al. 2000) (Figure 2). Methylation increases the affinity of the Sm proteins for the SMN complex, providing a potential regulatory mechanism in snRNP assembly (Brahms et al. 2001; Friesen et al. 2001; Selenko et al. 2001). Although it is not yet known how methylation of SmD1 modulates function (Gonsalvez et al. 2008), the RG-rich tail of the SmD3 protein is required for snRNP assembly and optimal nuclear import in cultured human cells (Friesen et al. 2001; Girard et al. 2004; Narayanan et al. 2004). Symmetric dimethylation of the SmD3 protein is not required for snRNP assembly and nuclear transport (Khusial et al. 2005), although it may enhance these activities.

Arginine methylation mediates direct protein-protein interactions, as demonstrated by the interaction of methylated Sm proteins with the Tudor domain of the SMN protein (Côté and Richard 2005). The Tudor domain is a conserved domain of ~60 amino acids that was initially found in proteins that associate with nucleic acids (Ponting 1997; Selenko et al. 2001). The Tudor domain of SMN interacts with the arginine-glycine-rich tails of the Sm

proteins (Selenko et al. 2001). Methylation of SMN by PRMT5 prevents the mis-assembly of Sm proteins to non-target RNA, and also prevents Sm protein aggregation (Chari et al. 2008; Pellizzoni et al. 2002).

Arginine methylation of RNA-binding proteins in germ cells

Sm proteins

In addition to their role in the spliceosome, Sm proteins have a novel, non-splicing function in the specification of germ cells (Barbee et al. 2002). In *Drosophila*, arginine methylation of the SmB protein by the *Drosophila* ortholog of PRMT5 is essential for germ cell formation, migration, and differentiation (Gonsalvez et al. 2006). In addition, localization to the pole plasm of both SmB and SmD3 requires methylation (Anne 2010a) (Figure 3). Methylation of a subset of the arginine residues on SmB is essential for anchoring the polar granules at the posterior cortex of the oocytes, whereas methylation of another subset of arginines controls germ cell migration during embryogenesis. Thus, arginine methylation is crucial for directing the subcellular localization of SmB (Anne 2010a).

The Oskar protein is important in the formation of the pole plasm in *Drosophila* oocytes (Ephrussi et al. 1991), and it is critical that Oskar expression is restricted to the posterior of the oocyte (Ephrussi and Lehmann 1992) (Figure 3). To accomplish this, the *oskar* mRNA is kept in a translationally-inactive state while being transported to the posterior of the oocyte, where Oskar is expressed in later stages of development (Ephrussi et al. 1991; Kim-Ha et al. 1991; Martin and Ephrussi 2009; St Johnston 2005). Interestingly, SmD3 associates with *oskar* mRNA to facilitate localization of the mRNA, an interaction that could be regulated by methylation of SmD3. In a set of experiments using a GFP-tagged SmD3 protein that displays hypomethylated arginine residues, *oskar* mRNA localization was altered, but Oskar expression was unaffected (Gonsalvez et al. 2010). When the methylated residues of SmD3 were substituted from arginine to lysine, there was partial rescue of *oskar* mRNA localization in a genetic background including hypomethylated SmD3-GFP, suggesting that methylation of SmD3 does not affect *oskar* mRNA localization (Gonsalvez et al. 2010). In addition, loss of Dart5, the methyltransferase responsible for SmD3 methylation, does not result in altered *oskar* mRNA localization (Gonsalvez et al. 2010). Interpretation of this data is complicated by the fact that expression of SmD3 with R-to-K substitutions was noticeably reduced, suggesting that those arginine residues may play a role in protein stability. One possible explanation is that methylation of SmD3 plays a limited role in *oskar* mRNA localization and that the hypomethylated SmD3-GFP also has a gain-of-function mutation. Thus, changes in methylation may have very subtle effects on *oskar* mRNA localization, such as the mislocalization noted upon temperature shift in a strain expressing both hypomethylated SmD3-GFP and SmD3 with R-to-K substitutions (Gonsalvez et al. 2010). Finally, hypomethylation of SmD3 is accompanied by a decrease in SmB methylation, suggesting coupled methylation of the SmB and SmD3 proteins (Gonsalvez et al. 2010). Further work is necessary to determine the precise functions of SmD3 methylation.

Previous models suggested that Oskar recruits Vasa, which recruits Tudor to polar granules. New data suggest that Oskar recruits both Vasa and Valois to polar granules (Anne 2010b) (Figure 3). Vasa then recruits Tudor, either indirectly via RNA interactions or directly via interactions between Tudor and an RG-rich region of Vasa (Anne 2010b). Additional evidence to either further support or disprove this model is necessary.

P-element-induced wimpy testes (PIWI) proteins

PIWI was first discovered in *Drosophila*, where its mutation abolished germline stem cell division, leading to severe defects in spermatogenesis and depletion of germline stem cells in fly ovarioles (Lin and Spradling 1997). PIWI proteins have now been identified in other

organisms including worms, mice, and humans (Cox et al. 1998; Deng and Lin 2002; Kirino et al. 2009; Kuramochi-Miyagawa et al. 2004; Qiao et al. 2002; Sasaki et al. 2003). PIWI proteins are part of the Argonaute family of proteins, and contain PAZ, MID, and PIWI domains (Cox et al. 1998). Accordingly, PIWI proteins are required for processing of 26–30 nucleotide, non-coding RNAs named PIWI-interacting RNAs (piRNAs) and the subsequent targeting of piRNAs to transposon-encoded RNAs (reviewed in more detail in Saxe and Lin (2011) and Siomi et al. (2011)). PIWI proteins play key roles in maintaining the integrity of the genome and epigenetic regulation. Since they are primarily expressed in germ cells, they are critically important in germline determination and gametogenesis (Saxe and Lin 2011; Siomi et al. 2011). During *Drosophila* oogenesis, the piRNA pathway is required for normal maintenance of microtubule organizing centers, which is required for proper *oskar* RNA localization (Khurana and Theurkauf 2010) (Figure 3). PIWI is also associated with DICER-1 and dFMRP, cytoplasmic components of the microRNA machinery (FMRP will be discussed in more detail below). Expression of these components is important in the formation of pole cells, which are the precursors to germline stem cells (Megosh et al. 2006). The mouse PIWI protein, MIWI, associates with mRNAs and piRNAs in polysomes, suggesting that MIWI-bound piRNAs might also regulate the translation or stability of specific mRNAs (Grivna et al. 2006).

The *Drosophila* PIWI protein Aubergine (Aub) and the mouse PIWI proteins MILI and MIWI have been shown to contain symmetric dimethylated arginines (sDMA) (Chen et al.; Kirino et al. 2009; Vagin et al. 2009). A third murine PIWI protein family member, MIWI2, exists and can also be found in a complex with PRMT5, its cofactor WDR77, and a number of Tudor-domain containing proteins; its methylation status, however, remains undetermined (Kirino et al. 2009; Vagin et al. 2009). Binding of Tudor-domain proteins to PIWI proteins appears to be regulated by their methylation status, as determined by studies with MILI and MIWI (Chen et al. 2009; Vagin et al. 2009). Association of MIWI with Tudor-domain proteins is required for normal MIWI localization (Vagin et al. 2009), suggesting that methylation regulates the formation of piRNA-containing granules. Although loss of functional TDRD1, a Tudor-domain containing protein, does not change the ability of MILI to associate with piRNAs in embryonic testes (Reuter et al. 2009), the total amount of piRNAs was severely decreased (Vagin et al. 2009), underscoring an important role of TDRD1 in the piRNA pathway. In fact, TDRD1 is necessary for spermatogenesis (Chuma et al. 2006).

A novel, gonad-specific *Drosophila* Tudor protein, Vreteno (Vret), was recently found to associate with PIWI and Aub, regulating their stability and localization (Zamparini et al. 2011). It is interesting to note that the amino acid sequence of the Vret Tudor domains suggests that they have structural differences compared to the Tudor domains of other proteins (Zamparini et al. 2011), so whether or not methylation affects the interaction of Vret with other Piwi proteins needs to be studied. For a comprehensive review of the role of Tudor-domain containing proteins with PIWI function, please see Siomi et al. (2010).

Murine Vasa Homolog

The murine Vasa homolog (MVH), also referred to as DDX4, is a highly conserved DEAD box RNA helicase that is found throughout the animal kingdom, and has been linked to germline development in organisms ranging from nematodes to humans (Raz 2000). Vasa was first described in *Drosophila*, where it was shown to be required maternally for the formation of polar granules and germ cells (Hay et al. 1988). Accordingly, Vasa expression is exclusively restricted to the germ cell lineage (Hay et al. 1988; Lasko and Ashburner 1988), as is true for MVH (Leroy et al. 1989). Loss of MVH expression causes a deficiency in the proliferation and differentiation of spermatocytes with a consequent loss of male fertility (Tanaka et al. 2000). Vasa is very similar to eukaryotic initiation factor-4A (Lasko

and Ashburner 1988), a helicase that is key for translation initiation (Rogers et al. 2002). A proposed function for Vasa is that it regulates translation of target mRNAs involved in germ cell establishment, such as *oskar* and *nanos*, and in oogenesis via *gurken* (Johnstone and Lasko 2004; Liu et al. 2003; Tinker et al. 1998; Tomancak et al. 1998) (Figure 3).

Mass spectrometry analysis of MVH isolated from mouse testes revealed both symmetric and asymmetric dimethylation: the former of which is mediated by PRMT5 (Kirino et al. 2010). MVH associates with PIWI family members MILI and MIWI, and Tudor proteins TDRD1 and TDRD6 (Kirino et al. 2010). Vasa may also be involved in the biogenesis and or function of piRNAs because it is present in pi-bodies and piP-bodies (Aravin et al. 2009; Shoji et al. 2009). In contrast to its association with the PIWI protein Aub, association of Vasa with Tudor does not depend on arginine methylation nor does arginine methylation of Vasa affect its localization in the pole plasm (Kirino et al. 2010). Thus, it is unclear how arginine methylation affects Vasa function.

Role of arginine methylation of RNA-binding proteins in neuronal development

Fragile-X mental retardation protein (FMRP)

The FMR1 gene was cloned in 1991 in an effort to identify the genetic basis of Fragile-X syndrome (Verkerk et al. 1991). FMR1 encodes FMRP, which was determined to have the domain structure of an RNA-binding protein. FMRP was subsequently found to bind ~4% of brain mRNAs (Ashley et al. 1993; Brown et al. 2001) and regulate their translation (Brown et al. 2001).

Two groups independently identified the RGG box of FMRP as the high-affinity RNA-binding site, recognizing intramolecular G-quadruplexes present in brain mRNAs, including the FMR1 mRNA (Darnell et al., 2001; Schaeffer et al. 2001). G-quadruplex-like structures were subsequently found in a number of FMRP-associated mRNAs (Brown et al., 2001; Darnell et al., 2001; Menon and Mihailescu 2007; Muddashetty et al. 2007; Todd et al., 2003; Westmark and Malter 2007; Zalfa et al., 2007). It was later found that the RGG box of FMRP is methylated on four arginine residues (Stetler et al., 2006), and in vitro methylation inhibited RNA binding to the RGG box. Additional work suggested that depending on which RGG box arginines were methylated, methylation might inhibit binding of some RNAs yet have no effect on other RNAs (Blackwell et al. 2010) (Figure 4).

A recent paper by Jennifer Darnell and colleagues shed new and unexpected light on both the RNAs bound by FMRP and the effect of FMRP-binding on translation regulation (Darnell et al. 2011). Using high-throughput sequencing of RNAs identified after UV-cross-linking, followed by immunoprecipitation (HITS-CLIP), 842 RNAs were identified associated with FMRP. Of these RNAs, 24% were also found in an earlier study of FMRP-associated brain mRNAs (Brown et al. 2001). What was unexpected in the Darnell study was that 66% of the FMRP-mRNA-binding sites were within the coding sequence of the mRNAs, including multiple sites within the same coding sequence (Darnell et al. 2011), suggesting very little, if any, sequence specificity. This is in sharp contrast to the results of this group and others showing that FMRP binds intramolecular G-quadruplexes in both in vitro experiments and in bona fide RNAs (Brown et al. 2001; Darnell et al. 2001; Darnell et al. 2004; Didiot et al. 2008; Menon and Mihailescu; Muddashetty et al.; Muddashetty et al. 2011; Schaeffer et al. 2001; Todd et al.; Westmark and Malter 2007; Zalfa et al.).

One way to reconcile these disparate findings is to propose that RNA binding by FMRP is determined by the localization of FMRP within the cell (Eberhart et al. 1996; Kim et al. 2009; Tamanini et al. 1999). Nuclear FMRP might recognize specific motifs that would not

have been evident in the recent study because only polyribosomal FMRP was analyzed (Darnell et al. 2011). We propose that FMRP binds its RNA cargoes in the nucleus, where it recognizes and is recruited to RNAs bearing specific motifs like the G-quadruplex, U-rich sequences, or kissing complex-like structures (Brown et al. 2001; Chen et al. 2003; Darnell et al. 2005; Darnell et al. 2001) (Figure 5B). Nuclear FMRP might be competent to bind a subset of RNAs because it is not post-translationally modified or associated with proteins that block or modulate RNA binding. Translation of the bound RNAs begins after export to the cytoplasm, but the associated FMRP remains bound to both the RNA and ribosomes, moving along the RNA as translation proceeds through the coding sequence (as evident in the HITS-CLIP experiment).

HuD

Like FMRP, the RNA-binding protein HuD plays a key role in nervous system development and function (Deschênes-Furry et al. 2006), although how HuD does this is distinct from FMRP (Figure 4). HuD is a member of the Hu family of RNA-binding proteins, which is directly involved in the development of paraneoplastic encephalomyelitis and sensory neuropathy syndromes (Dalmau et al. 1990; Musunuru and Darnell 2001; Szabo et al. 1991). These syndromes result from the expression of neuronal proteins on tumor cells that trigger the development of autoimmune neurological diseases. The antibody produced was termed anti-Hu based on the name of the patient from which it was isolated (Graus et al. 1986; Musunuru and Darnell 2001); anti-Hu was subsequently used to identify and characterize the proteins that it would bear its name.

Members of the Hu family have 3 RNA-binding domains and share a high degree of homology with the ELAV protein (Szabo et al. 1991). Hu family members are implicated in mRNA stabilization, cell-cycle regulation, and neurite extension (reviewed extensively in Deschênes-Furry et al. (2006)). HuD is among the few known proteins that stabilize transcripts in the cytoplasm (Wang and Tanaka Hall 2001). HuD is also an essential regulator of neuronal differentiation and survival (Anderson et al. 2001; Antic and Keene 1997; Aranda-Abreu et al. 1999; Deschênes-Furry et al. 2003; Kasashima et al. 1999; Perrone-Bizzozero and Bolognani 2002; Wakamatsu and Weston 1997).

Hu proteins recognize and bind to AU-rich elements (ARE) found within the 3'-untranslated regions (UTRs) of approximately 5% of human genes (Bakheet et al. 2003; Pascale et al. 2005) and are directly implicated in RNA turnover (Bakheet et al.; Bakheet et al. 2003; Chung et al. 1996; Liu et al. 1995). HuD is methylated on arginine; inhibiting methylation increased the half-life of an associated mRNA, maintaining PC12 cells in the proliferative, undifferentiated state (Fujiwara et al. 2006). It is hypothesized that mRNA decay was abrogated because unmethylated HuD forms a tighter complex with its bound mRNA. Recently, HuD was shown to interact with SMN through its Tudor domain (Hubers et al. 2011). Methylation of HuD by PRMT4/CARM1 reduced its interaction with *p21^{cip1/waf1}* mRNA and *GAP-43* RNA, but not with other mRNA targets of HuD (Hubers et al. 2011). Loss of interaction with *p21^{cip1/waf1}* mRNA led to cell cycle exit. Thus, methylation of HuD regulates the switch between proliferation and differentiation in PC12 cells (Fujiwara et al. 2006; Hubers et al. 2011).

Perspective

The hnRNPs, Sm proteins, PIWI proteins, Vasa, FMRP, and HuD all illustrate the importance of arginine methylation in modulating protein function. Methylation enhances the association of Sm and PIWI proteins for Tudor domain-containing proteins, and modulates association with RNAs, as demonstrated in studies of hnRNPA1, FMRP, and

HuD. Thus, arginine methylation adds a layer of regulation to these important molecular interactions.

In the case of post-translational modifications such as phosphorylation, the protein can move between a modified and unmodified form easily. This is also true for lysine acetylation and methylation (Bannister and Kouzarides 2011). Past work on arginine methylation suggests that methylated arginine residues cannot be returned to their previous, unmodified state. Instead, they can go from methylated arginine to citrulline, but not back to unmodified arginine (Bedford and Richard 2005). This may not be universal, though, as a histone arginine demethylase, JMJD6, was recently described (Chang et al. 2007) and can return methylated histone arginines to their previous unmodified state. Although the enzymatic activity of JMJD6 has come into question (Webby et al. 2009), it does suggest that methylation could be reversible, and encourages continued research into identifying arginine demethylases.

Many methylated proteins have multiple methylation sites that may be substrates of more than one PRMT. This raises two questions: how do the individual methylation sites correspond to specific protein functions, and how do PRMTs maintain specificity for these methylation reactions? The location of the methylation site required for binding of Tdrkh to MIWI has been identified (Chen et al., PNAS 2009), but it is unclear how Tudor proteins identify binding sites in other proteins, especially those with multiple Tudor domains. Although the sites of methylation for FMRP have been identified (Stetler et al. 2006) and PRMT1 has been identified as a PRMT capable of methylating the protein in cells (Blackwell et al. 2010), additional PRMTs have been shown to methylate FMRP in vitro (Dolzhanskaya et al. 2006) and might also be capable of methylating one or more sites within FMRP in vivo. Methylation of FMRP has been shown to affect RNA association (Blackwell et al. 2010), and the RGG box is required for association with the RNA-binding protein Yb1/p50 (Blackwell and Ceman 2011), suggesting that methylation may also affect protein interactions. It is possible that methylation of FMRP affects binding of both RNAs and protein or, alternatively, that only RNA binding is affected and RNA binding is a prerequisite for protein binding (or vice versa). Thus, future studies are required to determine which PRMTs are responsible for the *cellular* methylation reactions, and the precise function for each methylation event. In addition, PRMTs are differentially expressed across development (Ikenaka et al. 2006) and PRMT levels fluctuate in a cell cycle-dependent fashion (Kim et al. 2010), therefore, additional understanding of the effects of arginine methylation on protein function, combined with a greater understanding of when, where, and how PRMT activity is regulated, would allow the prediction of which RNA-protein complexes are likely to be prevalent during development.

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Abbreviations

FMRP	Fragile-X mental retardation protein
PIWI	P-element-induced wimpy testes
PRMT	protein arginine methyl transferase
SMN	survival of motor neurons

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Figure 1. Arginine methylation reactions performed by PRMTs

A side chain of arginine is depicted, and the resulting methylation additions applied to it are indicated in red. Identified type I-IV PRMTs from *Trypanosoma brucei* (Fisk et al. 2009), *Saccharomyces cerevisiae* (Gary et al. 1996; Niewmierzycka and Clarke 1999), *Schizosaccharomyces pombe* (Bachand and Silver 2004), *Caenorhabditis elegans* (Takahashi et al. 2011; Yang et al. 2009), *Drosophila melanogaster* (Boulanger et al. 2004), and mammals (Krause et al. 2007) are presented. See text for a complete description of the PRMT types and their specific reactions.

Figure 2. Model for role of arginine methylation on RNA-binding protein function

hnRNPs are shown in pink and the Sm proteins are shown in green. Methylation of hnRNP A2 facilitates nuclear import; methylation of hnRNP A1 locks it into a non-specific RNA-binding mode. SmD1, SmD3 and SmB are methylated by PRMT5 (Brahms et al. 2000). Methylation increases the affinity of the Sm proteins for the SMN complex in the cytoplasm (Brahms et al. 2001; Friesen et al. 2001; Selenko et al. 2001). Curled lines represent RNA, unless otherwise noted. Arrows signify movement. Cyt -cytoplasm and Nuc- nucleus.

Figure 3. Impact of methylation on pole plasm formation during *Drosophila* oogenesis
oskar RNA (red) is held in a translationally inactive state while being transported to the posterior of the oocyte. SmD3 and proper posterior microtubule depolymerization, via the piRNA pathway, are required for normal *oskar* mRNA localization. By stage 10 of oogenesis, Oskar (Osk) is localized at the posterior pole, and is required for localization of Tudor (Tud), Vasa, and Valois (Val). SmB and SmD3 are methylated by Dart5 (red), which also localizes to the pole, and methylated SmB/D3 interacts with Tudor. Additional mRNAs besides *oskar*, *gurken*, and *nanos*, also localize to the pole, but are not indicated in the diagram. White regions indicate nurse cells; curled lines represent *oskar* mRNA. Dart 5 is also known as Capsuleen.

Figure 4. Methylation-dependent activities of RNA-binding proteins FMRP and HuD in neurons FMRP is shown in blue, HuD is shown in brown. FMRP enters the nucleus to bind mRNAs (Kim et al. 2009). In its unmethylated form, FMRP binds RNAs containing a G-quadruplex structure (sc1-like RNA, black). Upon activation, PRMT1 (yellow) methylates FMRP, rendering it unable to bind G-quadruplex-containing structures, but still able to bind other RNAs, like AATYK (green) (Blackwell et al. 2011), which participate in neurite outgrowth (Tomomura et al. 2007). PRMT1 is modulated by cell cycle (Kim et al. 2010) and is required for neurite outgrowth (Miyata et al. 2008). Thus, methylation may direct the differentiation program of neurons by modulating the identity of FMRP-associated RNAs. Methylation of HuD decreases mRNA half-life by reducing its affinity for HuD (Fujiwara et al. 2006). Curled lines represent RNA, unless otherwise noted. Arrows signify movement. Cyt, cytoplasm; Nuc, nucleus; //, indicates distance down the neuronal process to the site of translation.