Methylation regulates the intracellular protein-protein and protein-RNA interactions of FMRP

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Summary

FMRP, the fragile X mental retardation protein, is an RNA-binding protein that interacts with ~4% of fetal brain mRNA. We have recently shown that a methyltransferase (MT) co-translationally methylates FMRP in vitro and that methylation modulates the ability of FMRP to bind mRNA. Here, we recapitulate these in vitro data in vivo, demonstrating that methylation of FMRP affects its ability to bind to FXR1P and regulate the translation of FMRP target mRNAs. Additionally, using double-label fluorescence confocal microscopy, we identified a subpopulation of FMRP-containing small cytoplasmic granules that are distinguishable from larger stress granules. Using the oxidative-stress induced accumulation of abortive pre-initiation complexes as a measure of the association of FMRP with translational components, we have demonstrated that FMRP associates with ribosomes during initiation and, more importantly, that methylation regulates this process by influencing the ratio of FMRP-homodimer-containing mRNPs to FMRP-FXR1P-heterodimer-containing mRNPs. These data suggest a vital role for methylation in normal FMRP functioning.

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Introduction

Protein arginine methylation commonly occurs in arginine–glycine-rich (RG-rich) domains of RNA-binding proteins (Aoki et al., 2002; Christensen and Fuxa, 1988; Cote et al., 2003; Kim et al., 1998; Mears and Rice, 1996; Pelletier et al., 2001; Rho et al., 2001). An eight-member family known as the protein arginine methyltransferases (PRMTs) mediates this post-translational modification. Structural analyses indicate that these enzymes represent a subfamily in the larger superfamily of S-adenosylmethionine-dependent methyltransferases, the members of which methylate RNA, DNA, protein, lipids and small molecules (Martin and McMillan, 2002). Interestingly, each PRMT appears to have both unique and overlapping targets (Frankel et al., 2002). The most prevalent PRMT, PRMT1, represents more than 90% of the class I methylating activity in cells (Tang et al., 2000). PRMTs efficiently methylate peptides containing multiple GRG residues in vitro (Ai et al., 1999; Hyun et al., 2000; Kim et al., 1997) and can methylate proteins containing this consensus sequence in vivo (Cote et al., 2003; Liu and Dreyfuss, 1995). The resulting modified proteins have altered biochemical properties. For example, PRMT activity is required for both the nuclear export and retention of various heterogeneous ribonucleoproteins (hnRNPs) (Nichols et al., 2000; Shen et al., 1998). Similarly, arginine methylation is required for the export of the yeast poly (A)-binding protein Nab2p from the nucleus (Green et al., 2002). Methylation has also been shown to alter the RNA-binding properties of RG-rich proteins such as hnRNP A1 (Kim et al., 1998) and SAM68 (Cote et al., 2003). Finally, several studies have demonstrated that methylating arginine residues in RG-rich regions alters the interactions of SAM68 with the SH_3 domain of p59fyn (Bedford et al., 2000) and the interactions of the spliceosomal Sm protein B/B′ with the survival of motoneuron protein (SMN) (Brahms et al., 2001; Friesen et al., 2001).

FMRP, the fragile X mental retardation protein, harbors three well-characterized RNA-binding domains (Siomi et al., 1993). The domain closest to the C terminus is an RG-rich region containing two copies of the motif RGG. Thus, FMRP is a potential PRMT substrate (Ai et al., 1999). We have recently shown that FMRP is co-translationally methylated by an endogenous methyltransferase (MT) in rabbit reticulocyte lysates, and inhibiting methylation affects its ability to interact with homoribopolymers and with some of its target mRNAs in vitro (Denman, 2002; Denman et al., 2004). Furthermore, the methylation state of FMRP can be modulated by NGF in vivo (Denman et al., 2004). Here, we demonstrate that cellular MT activity does not alter the subcellular localization of FMRP, rather, it regulates its ability to dimerize with FXR1P both in vitro and in vivo. We further demonstrate that methylation differentially affects the ability of specific FMRP target mRNAs to associate with FMRP-containing mRNPs and to be translated; we speculate that these two processes (dimerization and mRNA association) may be linked. Thus, methylation plays a vital role in the normal functioning of FMRP.

Results

FMRP and FXR1P co-translational methylation is inhibited by AdOx in vitro

We have previously reported that FMRP is co-translationally methylated by an endogenous methyltransferase in rabbit reticulocyte lysates (RRL) (Denman et al., 2004). In addition, ^{3}H-methyl incorporation into FMRP was blocked by low concentrations of adenosine-2′,3′-dialdehyde (AdOx). AdOx, which is a direct inhibitor of S-adenosyl-homocysteine hydrolase (SAHH), results in increased levels of S-adenosyl-homocysteine (SAH) that, in turn, leads to product inhibition of the methyltransferases. All of the concomitants of this pathway appear to be present in reticulocytes. SAHH is present in erythrocytes (Schuster and Kenanov, 2005) and is a likely component of RRL, which represents the lysed contents of these cells (Jackson and Hunt, 1983). Class I and class II PRMTs are also found in RRL (Azzouz et al., 2005; Liu and Dreyfuss, 1995). Thus, the above result is consistent with the hypothesis that inhibiting the endogenous SAHH in RRL with AdOx blocks MT-dependent FMRP methylation. One way to test this hypothesis is to demonstrate that AdOx inhibits proteins that are known to be methylated. The fragile X-related protein 1 (FXR1P) has recently been shown to be arginine methylated by a class I PRMT in HeLa cells (Ong et al., 2004). Therefore, we translated both FMRP and FXR1P in the presence of [^{3}H]SAM and in the absence and presence of AdOx. Fig. 1 shows that both proteins incorporated ^{3}H-methyl groups (lane 2), which were not present in the mock reactions (lane 1). The methylated proteins co-migrated with FMRP or FXR1P on western blots. Furthermore, AdOx treatment significantly inhibited ^{3}H-methyl incorporation (lane 3) without affecting protein production, demonstrating that its assimilation depends upon a methyltransferase reaction. To further demonstrate the specific inhibition of AdOx on methylation in RRL, purified histone H3, which can be methylated by PRMT4 and PRMT5 (Boisvert et al., 2005), was incubated with RRL and [^{3}H]SAM and in the absence and presence of AdOx. Once again, the protein was methylated in the presence of [^{3}H]SAM (lane 2) and inhibited by the addition of AdOx (lane 3). Thus, we conclude that the entire AdOx inhibition pathway is present in RRL.

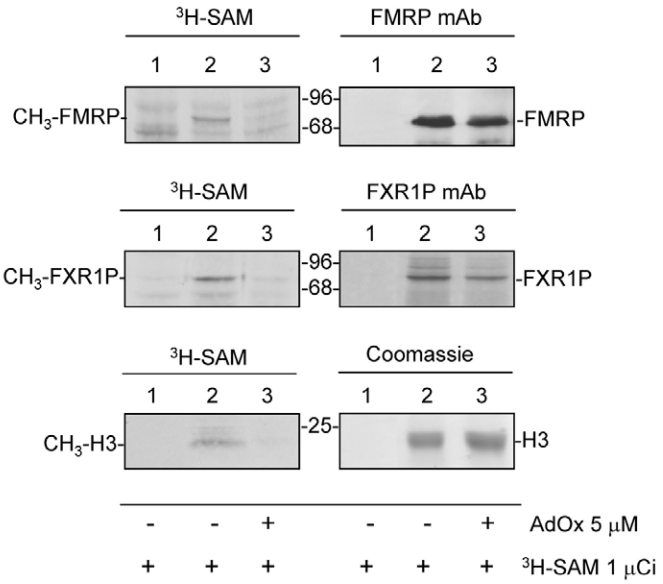

Fig. 1. FMRP and FXR1P are co-translationally methylated by an endogenous MT in RRL. (Top panels) FMRP or (middle panels) FXR1P was produced in RRL in the presence of [^{3}H]SAM and in the presence (lane 3) or absence (lane 2) of 6 μM AdOx, as indicated. Aliquots (20 μl) were resolved by SDS-PAGE and subjected to fluorography, while 5 μl aliquots were blotted and probed with anti-FMRP mAb or anti-FXR1P pAb. Lane 1 shows background methylation in the absence of the plasmids used in the lanes 2 and 3 translations. (Lower panels) Purified histone H3 (2 μg) from calf thymus was incubated with RRL in the presence of [^{3}H]SAM and in the presence (lane 3) or absence (lane 2) of 6 μM AdOx. Aliquots (10 μl) were resolved by SDS-PAGE and subject to fluorography, while 10 μl aliquots were subjected to Coomassie Blue staining.

The methylation state of FMRP affects its protein-protein interactions

Studies of other RNA-binding proteins have demonstrated that methylation alters protein-protein interactions (Bedford et al., 2000; Brahms et al., 2001; Friesen et al., 2001). FMRP is known to homodimerize and can also heterodimerize with its paralogs FXR1P and FXR2P. The interaction region has been delimited to a 34 amino acid stretch that abuts the KH_1 domain of the protein (Bardoni and Mandel, 2002). To address whether methylation alters the ability of FMRP to dimerize the following experiments were performed. Briefly, methylated-FXR1P was produced in RRL (see Fig. 1). Non-methylated-FXRP was produced in *E. coli* in vitro translation lysates. [Prokaryotes do not contain PRMT activity (Gary and Clarke, 1998; Katz et al., 2003)]. Heterodimer formation between RRL-produced biotinylated-FMRP and *E. coli*-produced FXR1P or RRL-produced FXR1P was then measured by affinity capture on SoftLink avidin. Incubations with a mock reaction using RRL, i.e. without FMRP plasmid DNA, were also performed to measure non-specific binding to the resin. Fig. 2A shows that *E. coli*-produced FXR1P does not bind to FMRP (left two panels), whereas RRL-produced FXR1P heterodimerizes (right two panels).

These results indicate that methyl groups on FXR1P appear to be necessary for this interaction. However, *E. coli*-produced FXR1P may also lack other important post-translational modifications, in addition to its methylation, that may affect the comparison to FMRP produced in RRL (Denman, 2002). To address this we produced ^{35}S-labeled FXR1P in RRL in the presence and absence of AdOx. The ability of these proteins to interact with RRL-produced biotinylated-FMRP was measured by quantifying the amount of radiolabeled protein in the bound and unbound fractions as described above. The results, shown in Fig. 2B, reveal that AdOx treatment significantly reduced the ability of FXR1P to form heterodimers in a dose-dependent manner.

To demonstrate a requirement for FMRP methylation in heterodimer formation, biotinylated-methylated-FMRP and biotinylated-non-methylated-FMRP were produced in RRL translation lysates by co-translation in the absence or presence of AdOx, respectively. The ability of these proteins to form heterodimers with RRL-produced FXR1P was then measured. Incubations with RRL mock reactions (with or without AdOx) were also performed to measure non-specific binding. Fig. 2C shows that the AdOx-treated FMRP was much less efficient at

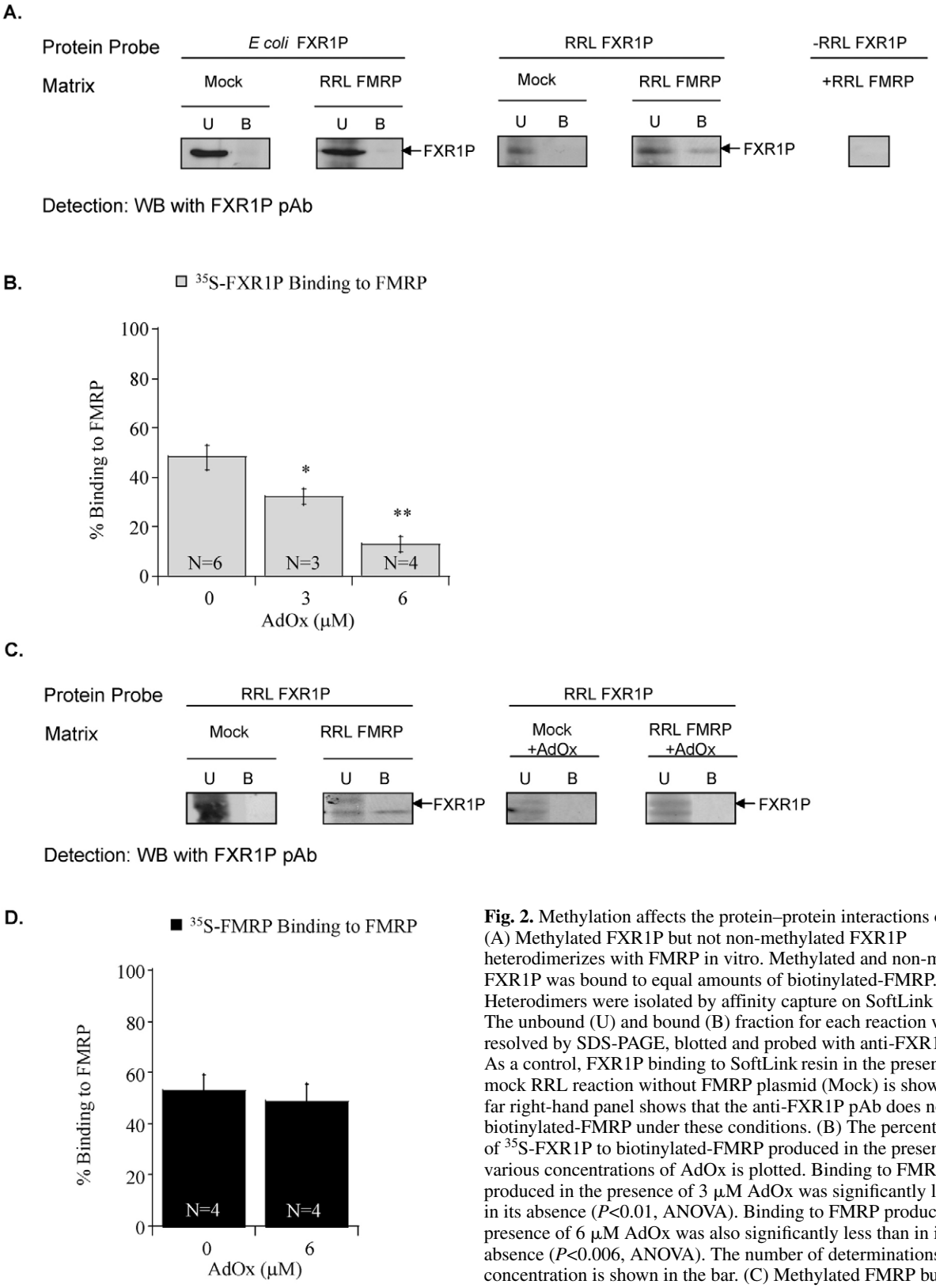

Fig. 2. Methylation affects the protein–protein interactions of FMRP. (A) Methylated FXR1P but not non-methylated FXR1P heterodimerizes with FMRP in vitro. Methylated and non-methylated FXR1P was bound to equal amounts of biotinylated-FMRP. Heterodimers were isolated by affinity capture on SoftLink resin. The unbound (U) and bound (B) fraction for each reaction was resolved by SDS-PAGE, blotted and probed with anti-FXR1P pAb. As a control, FXR1P binding to SoftLink resin in the presence of a mock RRL reaction without FMRP plasmid (Mock) is shown. The far right-hand panel shows that the anti-FXR1P pAb does not detect biotinylated-FMRP under these conditions. (B) The percent binding of ^{35}S-FXR1P to biotinylated-FMRP produced in the presence of various concentrations of AdOx is plotted. Binding to FMRP produced in the presence of 3 μM AdOx was significantly less than in its absence (P<0.01, ANOVA). Binding to FMRP produced in the presence of 6 μM AdOx was also significantly less than in its absence (P<0.006, ANOVA). The number of determinations for each concentration is shown in the bar. (C) Methylated FMRP but not non-methylated FMRP heterodimerizes with FXR1P in vitro. Biotinylated-methylated and biotinylated-non-methylated FMRP produced in RRL was bound to equal amounts of methylated-FXR1P. Heterodimers were isolated and detected as described in A. For controls, FXR1P binding to SoftLink resin in the presence of a mock RRL reactions without FMRP plasmid and with or without AdOx (Mock and Mock+AdOx) is shown. (D) The percent binding of ^{35}S-FMRP to biotinylated-FMRP produced in the absence or presence 6 μM AdOx is plotted. The number of determinations for each concentration is shown in the bar.

forming heterodimers (right two panels) than the untreated control (left two panels).

To determine whether methylation of FMRP affects its ability to homodimerize, we produced ^{35}S-labeled FMRPs in RRL in the presence and absence of AdOx and measured their ability to interact with RRL-produced biotinylated-FMRP. Interestingly, we found that AdOx treatment did not affect the ability of FMRP to form homodimers (Fig. 2D). Finally, in agreement with the data of Laggerbauer et al. (Laggerbauer et al., 2001), we found that the ability of FMRP to heterodimerize with the fragile X KH_2 domain mutant $I_{304}N$ in the absence of AdOx was not significantly different from its ability to homodimerize (not shown).

In addition to an N-terminal dimerization domain, FMRP contains a domain that allows it to interact with polyribosomes. Structure–function studies have shown that this domain is a coil–coil structure and that it is located between the KH_2 domain and the RG-rich region (Siomi et al., 1996). To determine whether methylation affects the ability of FMRP to interact with polyribosomes, the following experiment was performed. Total HeLa cell homogenates from AdOx-treated or untreated cells were spun through 50% sucrose pads. Proteins extracted from the resulting pellets were then blotted and probed with anti-FMRP mAb 2160 and various other antibodies (Fig. 3A). The majority of FMRP and PABP associated with these pellets. The constitutively expressed heat shock protein, Hsp70cp, was also detected, albeit to a lesser degree (Fig. 3A, third panel, lane 1). By contrast, EF-1A and PRMT1 were only observed in total cell lysates. This was true regardless of whether or not the cells were treated with AdOx (lane 4). These data demonstrate that most FMRP associates with dense particles under these conditions.

Treating total cell lysates with EDTA dissociates polyribosomes (Khandjian et al., 1996). Concomitantly, most of the FMRP is lost from the heavy sedimenting fractions of sucrose gradients (Khandjian et al., 2004). However, a small amount of

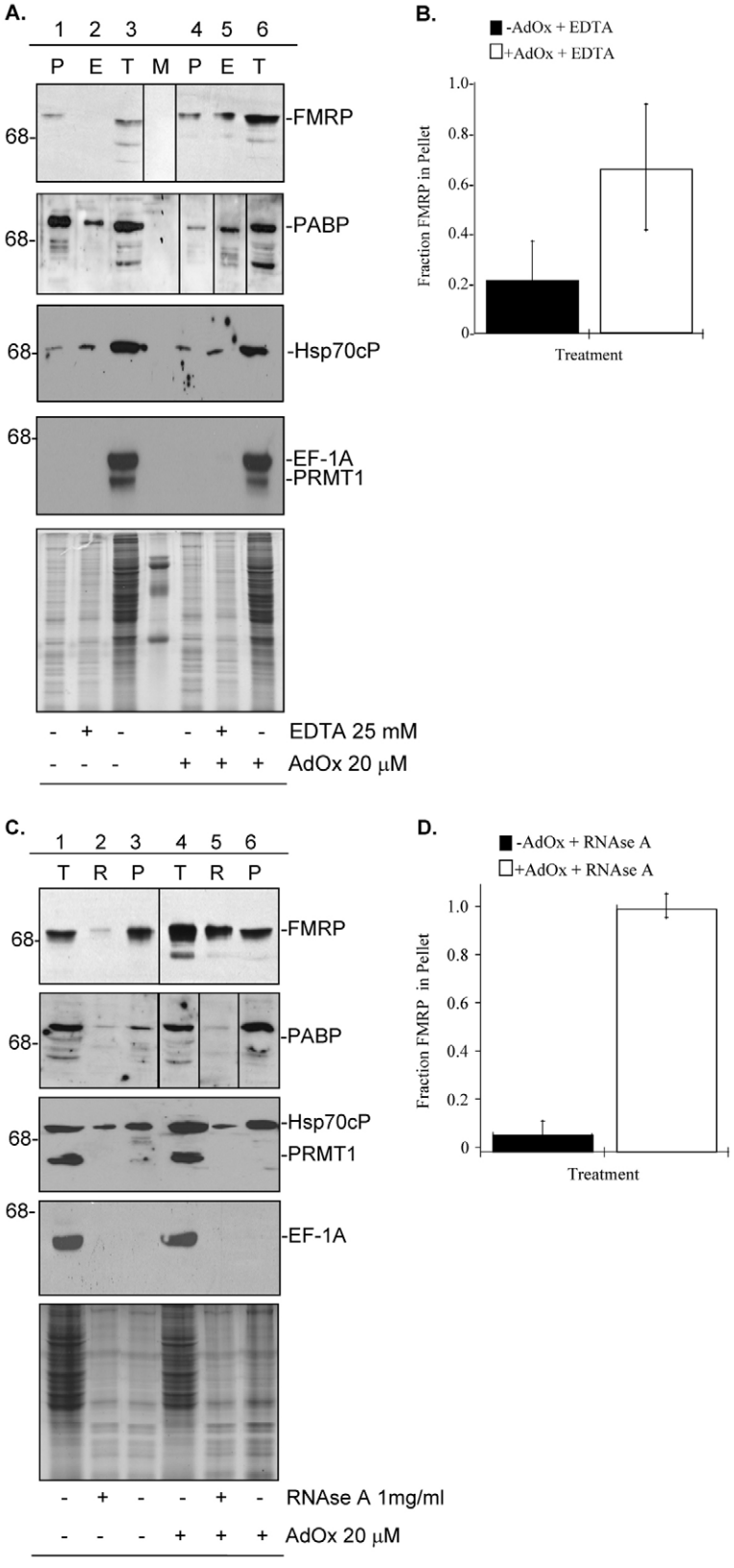

Fig. 3. Methylation enhances the distribution of FMRP in dense granules. (A) Western blot of HeLa cell proteins that pellet through 50% sucrose (lanes 1, 2) or pellets from HeLa cells that were treated for 24 hours with 20 μM AdOx (lanes 4, 5). Proteins in lanes 2 and 4 were from pellets treated with 25 mM EDTA prior to sedimentation. Lanes 3 and 6 are total HeLa cell extracts (20% v/v of the pellet). Blots were probed with anti-FMRP mAb, anti-PABP pAb, anti-Hsp70cp mAb, and anti-EF-1A and anti-PRMT1 mAbs. Boxed lanes are for presentation purposes. The lower panel shows Coomassie staining of the proteins in each fraction. (B) Densitometric analysis of the ratio of FMRP in the pellet fraction of EDTA-treated lysates (E) to FMRP in the pellet fraction of non-treated lysates (P) in the presence or absence of 20 μM AdOx. The results of three sets of cell lysates are presented. (C) Western blot analysis of cell lysates prepared as in A, but treated or not treated with RNAse A prior to sedimentation. Lanes 1 and 4 represent total cell proteins (20% v/v of the pellet), lanes 2 and 5 are pellet proteins treated with RNAse A, lanes 3 and 6 are untreated pellet proteins. Boxed lanes are for presentation purposes. (D) Densitometric analysis of three sets of cell lysates prepared in C.

FMRP still sediments as a high molecular weight species (Ceman et al., 2003; Funakoshi et al., 2003; Mazroui et al., 2003). We also detected only minor amounts of FMRP in the pellet fraction following centrifugation through 50% sucrose after treating cell lysates with EDTA (lane 2). However, pre-treating the cells with AdOx increased the amount of EDTA-resistant FMRP that was found in the pellets (lane 5). Quantitative analysis showed that there was approximately a threefold increase in EDTA-resistant FMRP (Fig. 3B). Moreover, compared with PABP and Hsp70cp, this increase was specific to FMRP.

Treating cell lysates with RNAse A releases mRNPs from polyribosomes and following such treatment the majority of FMRP sediments faster than 80S monosomes (Ceman et al., 2003). We also treated lysates from HeLa cells grown in the absence or presence of AdOx with RNAse A and fractionated them as described above. The results, shown in Fig. 3C,D were similar to those found after EDTA treatment, namely that AdOx treatment specifically increased the amount of FMRP that pelleted through 50% sucrose (compare lanes 2 and 5).

FMRP is found in small cytoplasmic granules

As only dense objects sediment through 50% sucrose, the proteins found after EDTA or RNAse A treatment are assumed to be a type of granule (Funakoshi et al., 2003). To determine whether FMRP-containing granules could be visualized under normal cell culture conditions, HeLa cells were immunostained with anti-FMRP mAb and examined by fluorescence confocal microscopy. As has been previously demonstrated in HeLa cells (Khandjian, 1999; Mazroui et al., 2002), most of the FMRP immunostaining was diffusely distributed throughout the cytoplasm. However, punctate FMRP immunostained particles were also present in some of the cells. Fig. 4A shows a 0.5 μm *z*-series montage through a single granule-laden cell. FMRP immunostained granules were distributed throughout the cytoplasm. Based on their appearance and disappearance within the montage, the particles are ~0.3-1.0 μm^3. These results are not unique to HeLa cells; we have observed similar granules in several other cultured cell lines (not shown).

It has been suggested that FMRP may be a component of stress granules (Mazroui et al., 2002). The constituents of stress granules are well characterized (Anderson and Kedersha, 2002) and two core components are the polyadenylation-binding protein (PABP) and the T-cell internal antigen 1 (TIA-1). Therefore, we next assessed HeLa cells using double-label fluorescence confocal microscopy to determine whether the FMRP-containing granules colocalized with either PABP or TIA-1. HeLa cells were treated with AdOx to inhibit methylation and, subsequently, immunostained with anti-FMRP mAb and anti-PABP pAb, or anti-FMRP mAb and anti-TIA-1 pAb. As a control, untreated HeLa cells were also immunostained by these antibodies. Fig. 4B shows that the number of FMRP-containing granules/cell was not markedly affected by treatment with AdOx. However, the total number of cells containing such granules increased approximately twofold following AdOx treatment ($P<0.001$, by ANOVA), providing confirmatory evidence for the western blotting data in Fig. 3. More importantly, it was evident that these FMRP-immunostaining granules did not colocalize with either PABP or TIA-1 (see also Fig. 5A below).

Oxidative stress results in FMRP recruitment into stress granules

The fact that both PABP and TIA-1 exhibit diffuse non-granular immunostaining suggests that inhibiting HeLa cell methylation with AdOx does not represent a significant stress to the cells. To conclusively demonstrate that the small cytoplasmic FMRP-containing granules we observe under normal cell culture conditions were not stress granules, HeLa cells were treated with sodium arsenite, a known stress granule inducer. The cells were then co-immunostained with anti-FMRP mAb and anti-PABP pAb. As shown in Fig. 5A (top panel), a fraction of the cells exhibit small cytoplasmic FMRP-containing granules in the absence of arsenite, but these granules lacked PABP. Treating the cells with AdOx prior to immunostaining had no effect on the localization of either protein, nor did it affect their subcellular distribution (second panel). However, in the presence of arsenite (third panel) virtually all of the cells contained much larger cytoplasmic granules ~1.5-3.0 μm^3 that co-immunostained with anti-FMRP and anti-PABP antibodies. Further characterization of these granules showed that in addition to FMRP and PABP these particles also contained TIA-1 (not shown). Quantitative measurements of the percent colocalization of these particles demonstrated that ~87-89% of the FMRP-containing granules colocalized with TIA-1 and PABP in arsenite-treated cells (Table 1). These data differ significantly from FMRP-containing granules absent arsenite treatment (where ~10-14% of the granules colocalized with TIA-1 or PABP), reaffirming the idea that these are two distinct populations of particles.

To define the role methylation plays in stress granule formation, the following experiments were performed. First, HeLa cells were pre-treated for 24 hours in the presence of AdOx. Subsequently, they were treated with sodium arsenite to induce stress granule formation. The results of this experiment clearly show that this treatment regime resulted in the accumulation of FMRP into granules that did not immunostain with anti-PABP pAb (Fig. 5A, lower panel). Analysis of the frequency and distribution of these particles showed that there was a twofold increase in the number of FMRP-granules per cell following AdOx treatment and that the FMRP granules that did not colocalize with PABP were significantly smaller than the average size of all granules (Table 2). Thus, methylation alters the recruitment of FMRP into larger stress granules.

To further explore the role methylation plays in the response of FMRP to oxidative stress, HeLa cells were probed with anti-FMRP mAb and a polyclonal antibody (anti-mRG) directed to aDMA that recognizes human FMRP (Denman et al., 2004). As shown in Fig. 5B (top panel), the majority of the anti-mRG staining localized in HeLa cell nuclei. However, weaker cytoplasmic anti-mRG staining was also observed. Importantly, in the naïve control cells, this staining was not associated with FMRP-containing granules, indicating that the epitope recognized by this antibody was either not present or masked. By contrast, arsenite treatment (third panel) resulted in granules that stained positively with anti-mRG pAb; ~75% of the FMRP colocalized with the anti-mRG staining. This indicates that one or more proteins within these granules (possibly FMRP) harbor exposed aDMA residues. Finally, AdOx pre-treatment substantially reduced the amount of anti-mRG pAb staining in cells (second and fourth panels) and also

substantially reduced the number of mRG-staining arsenite-induced granules that colocalized with FMRP to ~14%.

All stress granules are not created equal

The above results indicate that the loss of methyl arginine does not prevent FMRP from being recruited into stress granules, but does alter the balance between FMRP that associates with stress granule markers and FMRP that does not. To determine whether this was related to the differences observed between FMRP homodimerization and FMRP-FXR1P heterodimerization (Fig. 2), we examined the effect AdOx treatment had on the recruitment of FXR1P into stress granules. In naïve cells, FXR1P and FMRP were found in small granules that were distributed throughout the cytoplasm (Fig. 6A, top panel). Quantitative analyses of these granules showed that ~20% of the FXR1P granules colocalized with FMRP, and pre-treating the cells with AdOx reduced the colocalization tenfold (Table 1). Interestingly, when the distribution of FXR1P and FMRP in the arsenite-induced granules was examined, we found that, in contrast to FMRP and TIA-1 or FMRP and PABP, which exhibited 87-89% colocalization, only 74% of FMRP-containing granules contained FXR1P (Fig. 6A, third panel). This difference was statistically significant (Table 1). This result implies that not every FMRP stress granule

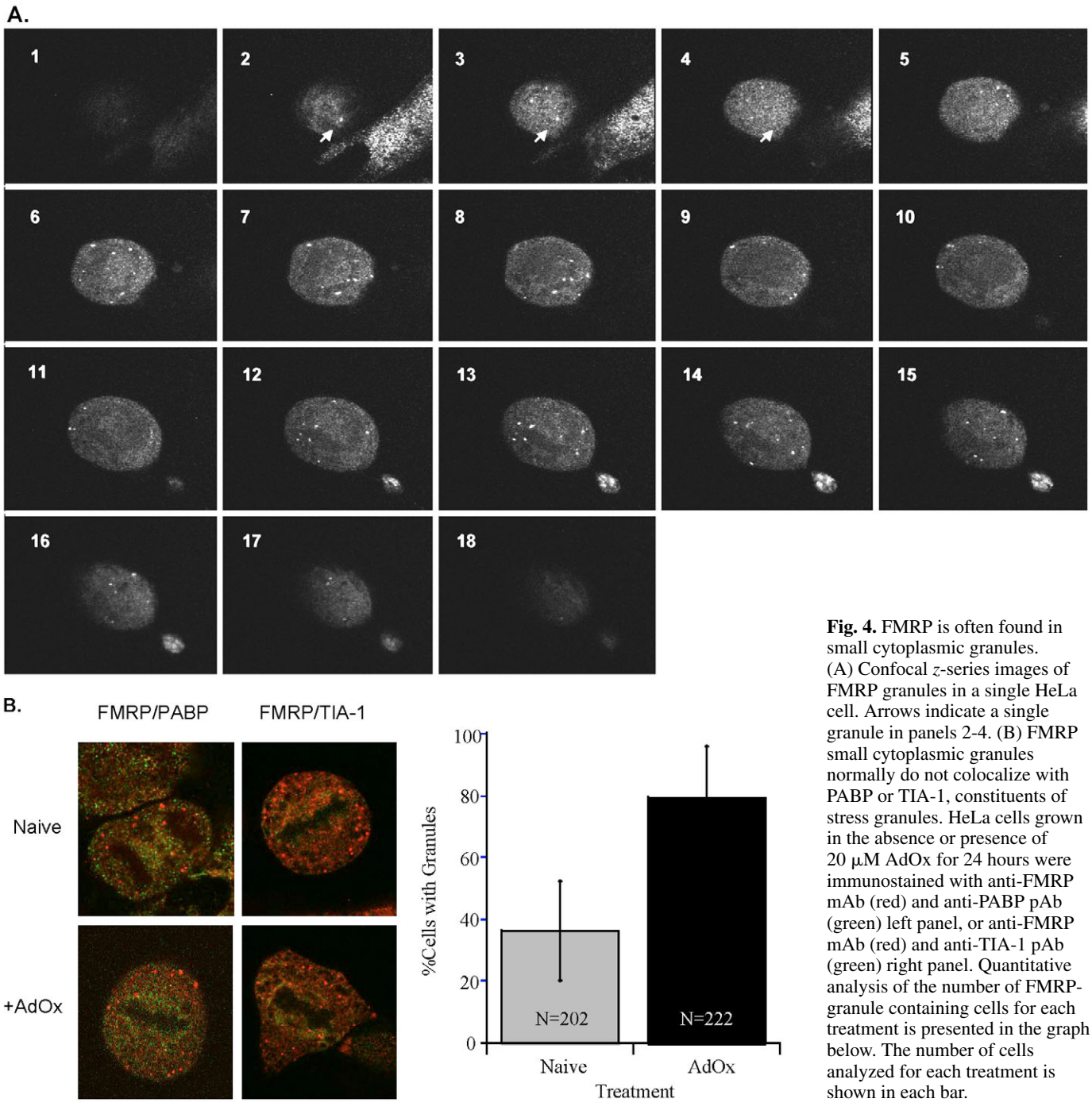

Fig. 4. FMRP is often found in small cytoplasmic granules. (A) Confocal *z*-series images of FMRP granules in a single HeLa cell. Arrows indicate a single granule in panels 2-4. (B) FMRP small cytoplasmic granules normally do not colocalize with PABP or TIA-1, constituents of stress granules. HeLa cells grown in the absence or presence of 20 μM AdOx for 24 hours were immunostained with anti-FMRP mAb (red) and anti-PABP pAb (green) left panel, or anti-FMRP mAb (red) and anti-TIA-1 pAb (green) right panel. Quantitative analysis of the number of FMRP-granule containing cells for each treatment is presented in the graph below. The number of cells analyzed for each treatment is shown in each bar.

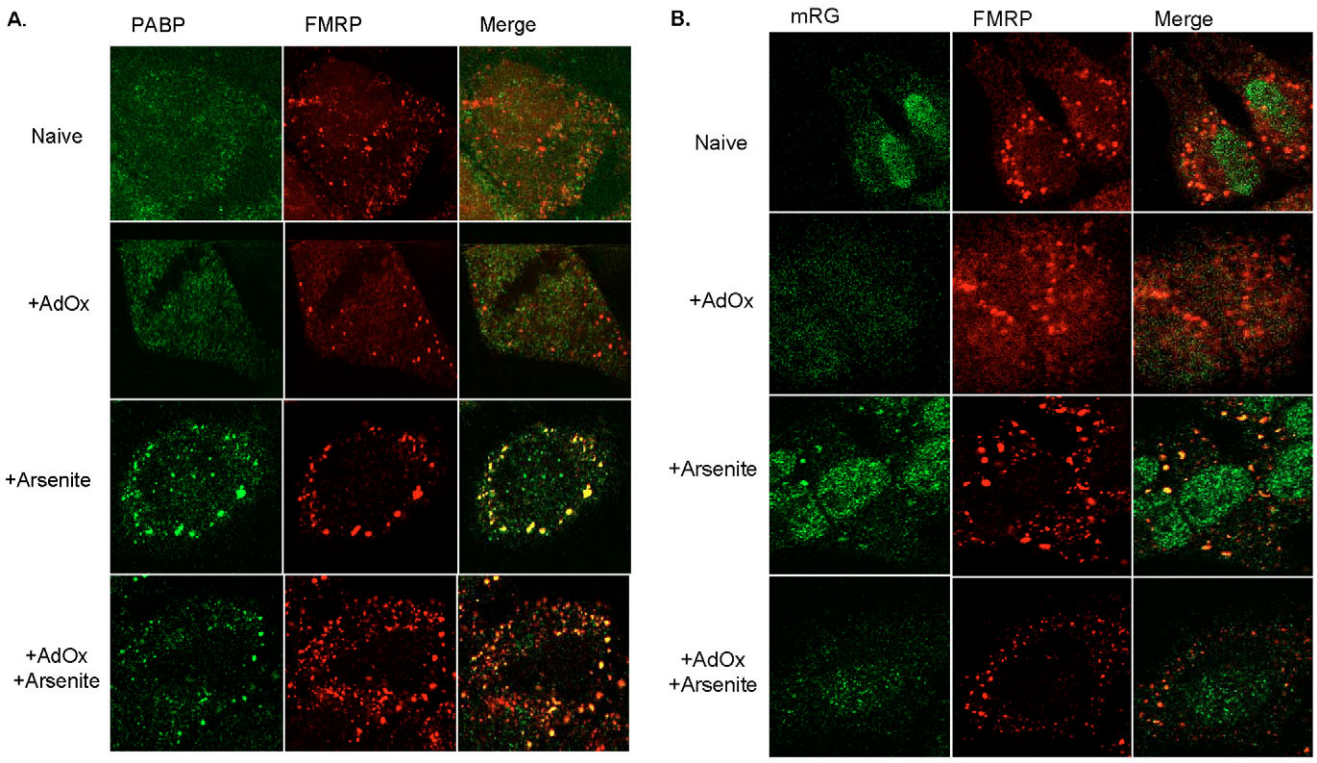

Fig. 5. Arsenite induces the accumulation of FMRP in stress granules. (A) HeLa cells grown in the absence of treatment (naive), the presence of 20 μM AdOx for 24 hours, the presence of 0.5 mM sodium arsenite for 30 minutes, or pre-treated with 20 μM AdOx for 24 hours and then treated with 0.5 mM sodium arsenite for 30 minutes were immunostained with anti-PABP pAb (green) and anti-FMRP mAb (red) as in Fig. 3. (B) FMRP small cytoplasmic granules lack asymmetric dimethyl-arginine. HeLa cells treated as in A were immunostained with anti-mRG pAb (green) and anti-FMRP mAb (red).

contains FXR1P. More importantly, AdOx treatment, before the addition of arsenite significantly reduced the amount of FXR1P in FMRP-containing granules (Fig. 6A, fourth panel and Table 1). This large effect was not observed when FMRP and PABP or FMRP and TIA-1 were examined. As these results are consistent with the hypothesis that the incorporation of FXR1P into stress granules depends on its methylation, we investigated this effect further. Specifically, the distribution of FXR1P and PABP in stress granules was determined in the absence and presence of AdOx (Fig. 6B). In the presence of sodium arsenite, FXR1P accumulated in large granules containing PABP. As with the FMRP and PABP-containing granules formed in the presence of arsenite, treatment with AdOx prior to the induction of stress granules resulted in a

Table 1. Effect of AdOx on the distribution of FMRP-cytoplasmic and FMRP-stress granules

Type	AdOx*	FMRP/TIA-1	FMRP/PABP†	FMRP/methyl‡	FMRP/FXR1P	FXR1P/PABP
Cytoplasmic	–	10.4 (1.5) *n*=258	13.6 (2.2) *n*=65	4.6 (1.0) *n*=412	21.4 (3.4) *n*=1552	ND¶
Cytoplasmic	+	6.0 (1.4) *n*=249	13.0 (3.6) *n*=121	1 (0) *n*=707	2.9 (0.6)** *n*=1495	ND
Stress§	–	87.3 (0.1) *n*=1055	89.4 (2.5) *n*=1391	76.1 (13.8) *n*=644	73.9 (3.7)*** *n*=1091	88.0 (2.0) *n*=1224
Stress	+	74.6 (2.5)** *n*=730	74.6 (1.9)** *n*=1308	14.1 (3.8)** *n*=1187	48.9 (7.2)** *n*=862	65.8 (2.3)** *n*=2048

*HeLa cells were incubated in the absence (–) or presence (+) of 20 μM AdOx for 24 hours.

†Granules were defined using Image J software. The mean percent co-localization between FMRP and TIA-1 (column 3), FMRP and PABP (column 4), FMRP and aDMA (column 5), FMRP and FXR1P (column 6), and FXR1P and PABP (column 7) is shown. Numbers in parenthesis are s.d. *n*, the total number of granules analyzed for each immunostained pair under each condition is presented below the mean values. Double asterisks indicate the co-localization of a particular protein pair is significantly altered by AdOx treatment (**P<0.001, ANOVA). Triple asterisks indicates co-localization is significantly different from FMRP/TIA-1 and FMRP/PABP (***P<0.0001, ANOVA).

‡Asymmetric dimethylation was determined from immunostaining with anti-mRG pAb.

§Stress granules were induced by treatment with 0.5 mM sodium arsenite for 30 minutes. Induction always followed AdOx treatment.

¶ND, not determined.

Table 2. Distribution of FMRP granules and FXR1P granules in HeLa cells following arsenite treatment

Granule type	Pre-treatment*	Number of cells	Granules/cell	Granule size†	Non-co-localized granule size‡
FMRP	–AdOx	46	84 (4)	361 (21)	114 (18)¶
FMRP	+AdOx	85	167 (13)§	353 (8)	232 (15)¶
FXR1P	–AdOx	45	112 (2)	347 (5)	140 (20)¶
FXR1P	+AdOx	70	162 (6)§	335 (5)	205 (16)¶

*HeLa cells were not treated or treated with 20 μM AdOx for 24 hours. Subsequently, stress granules were induced using sodium arsenite as in Table 1. The cells were then fixed and immunostained with either anti-FMRP and anti-PABP, or anti-FXR1P and anti-PABP as described.

†Average area in arbitrary units of all FMRP-containing granules following arsenite treatment from confocal images as in Fig. 5A was computed using the measure particles function of Image J. Average area in arbitrary units of all FXR1P-containing granules following arsenite treatment from confocal images as in Fig. 6B was similarly computed. Numbers in parentheses are s.d.

‡PABP co-localizing granules were manually masked in Adobe PhotoShop and the area of the remaining non-co-localized granules was computed as described above.

§Number of FMRP granules per cell in the presence of AdOx is statistically different than in its absence ($P<0.001$, ANOVA). Number of FXR1P granules per cell in the presence of AdOx is statistically different from in its absence ($P<0.003$, ANOVA).

¶Non-colocalized FMRP granules are significantly smaller than the average granule size ($P<0.0003$) for cells in the absence of AdOx and ($P<0.0006$) for cells in the presence of AdOx by ANOVA. Non-colocalized FXR1P granules are significantly smaller than the average granule size ($P<0.0005$) for cells in the absence of AdOx and ($P<0.01$) for cells in the presence of AdOx (ANOVA).

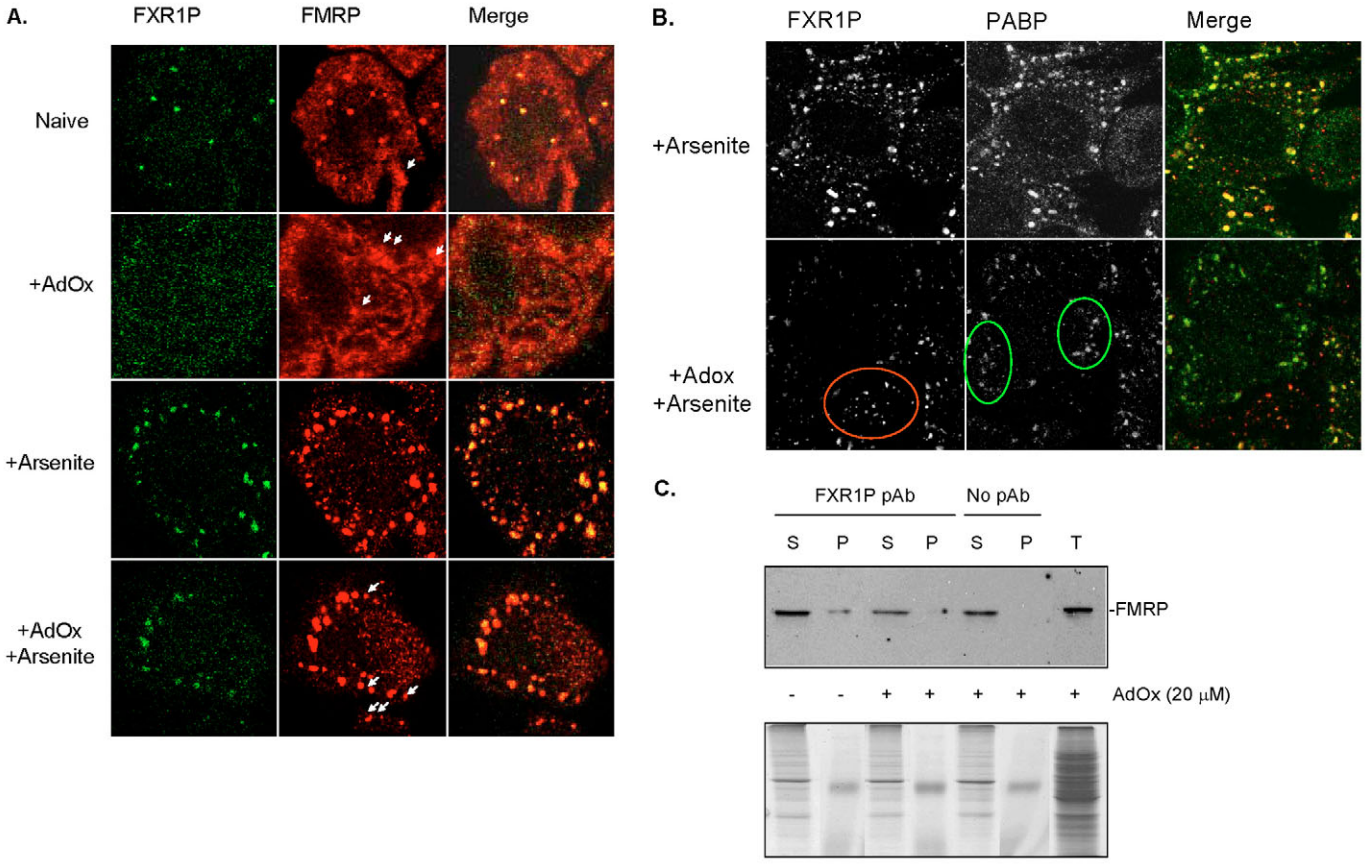

Fig. 6. (A) Stress granules do not have uniform composition. HeLa cells grown in the absence of treatment (naive), the presence of 20 μM AdOx for 24 hours, or the presence of 0.5 mM sodium arsenite for 30 minutes, or pre-treated with 20 μM AdOx for 24 hours and then treated with 0.5 mM sodium arsenite for 30 minutes were immunostained with anti-FXR1P pAb (green) and anti-FMRP mAb (red) as in Fig. 3. Arrows show granules that do not contain FXR1P. (B) AdOx alters the distribution of FXR1P-containing granules. HeLa cells grown in the presence of 0.5 mM sodium arsenite for 30 minutes, or pre-treated with 20 μM AdOx for 24 hours and then treated with 0.5 mM sodium arsenite for 30 minutes were immunostained with anti-FXR1P pAb (red) and anti-PABP (green). The area outlined in red shows a cluster of granules lacking PABP, while the areas outlined in green show clusters of granules lacking FXR1P. The grayscale images of FXR1P and PABP immunostaining alone highlight the effect of AdOx. (C) AdOx treatment results in a loss of FMRP from FXR1P immunoprecipitates. HeLa cells were treated with 20 μM AdOx for 24 hours and then immunoprecipitated with anti-FXR1P pAb as described. The resulting supernatant (S) or immunoprecipitate (P) fractions were resolved by SDS-PAGE and then probed with anti-FMRP mAb. As controls, untreated HeLa cells were subject to immunoprecipitation with anti-FXR1P pAb and AdOx-treated cells were run through the immunoprecipitation protocol without anti-FXR1P pAb.

significant decrease in the colocalization of FXR1P and PABP (Table 1). Furthermore, the FXR1P non-colocalizing granules were significantly smaller than the average size of all of the granules, Table 2. These data demonstrate that FXR1P forms bona fide stress granules and that conditions that result in the hypo-methylation of cellular proteins negatively impact the recruitment of FXR1P into stress granules. This observation is consistent with our results on the effect of AdOx on FMRP-FXR1P heterodimerization in vitro.

To demonstrate that the AdOx-induced loss of FXR1P from stress granules was related to the inability to form FXR1P-FMRP heterodimers, HeLa cells were treated with AdOx as above. Subsequently, the cells were lysed and immunoprecipitated with anti-FXR1P pAb, and the amount of co-immunoprecipitating FMRP was monitored by western blotting with anti-FMRP mAb. HeLa cells that were not exposed to AdOx were used as a control. As shown in Fig. 6C, FMRP was found in the FXR1P immunoprecipitates in the absence of AdOx. In AdOx-treated immunoprecipitates, however, it was clearly reduced, recapitulating the in vitro data in Fig. 2.

Methylation alters the translation of the target mRNAs of FMRP

If the dimerization properties of FMRP and its recruitment into abortive pre-initiation complexes under oxidative stress are affected by methylation, then one would expect corresponding changes in the expression of its target mRNAs. To investigate this, HeLa cells were treated or not treated with AdOx and then immunoprecipitated with anti-FMRP mAb. Semi-quantitative RT-PCR of total RNA extracted from the resulting pellets was then performed using primers for FMRP target mRNAs and control mRNAs (Sung et al., 2004; Sung et al., 2003). As an additional control, total RNA from the treated or non-treated cells was also amplified. Fig. 7A (top panel) shows FMR1 mRNA levels in the immunoprecipitates from AdOx-treated and untreated cells. Surprisingly, FMR1 mRNA, although expressed to the same extent as in the untreated cells, was nearly undetectable in immunoprecipitates from AdOx-treated cells. EF-1A mRNA, another FMRP target mRNA (Sung et al., 2003), was also equally expressed in AdOx-treated and untreated cells (Fig. 7A, second panel). As expected, we found EF-1A mRNA in the FMRP immunoprecipitates and again its levels were reduced in AdOx-treated cells. Finally, we previously showed that FMRP binds Tip60a mRNA and appears to negatively regulate its translation (Dolzhanskaya et al., 2003). Tip60a mRNA is weakly expressed in HeLa cells and likewise was found in low amounts in FMRP immunoprecipitates. Interestingly, there was little difference in the amount of Tip60a mRNA associated with FMRP in AdOx-treated and untreated cells (Fig. 7A, third panel). By contrast, βAPP mRNA and dynamin A1 mRNA, which do not bind to FMRP (Denman, 2003) were found in neither immunoprecipitate, although they were robustly expressed in both the AdOx-treated and untreated cells (Fig. 7A, fourth and fifth panels). These results, which suggest that the loss of methyl groups have a differential effect on the formation of particular FMRP-mRNPs, recapitulate previously observed differences in RNA binding in vitro (Denman, 2002) (Fig. 7B).

If these effects are bona fide, one might expect corresponding changes in the steady-state levels of expressed proteins, given the role of FMRP in translational regulation (Denman et al., 2004; Sung et al., 2003). To investigate this, we compared the protein expression of FMRP targets, FMR1 and EF-1A, with that of dynamin A1, a representative non-binding mRNA, in the presence and absence of AdOx by western blotting. (Tip60a protein expression was excluded from this analysis because, although the message is weakly expressed in HeLa cells, we could not detect a protein product.) Fig. 7C shows that FMRP levels rise nearly two-fold in AdOx-treated cells, while EF-1A levels rise much more modestly (~1.3-fold). Dynamin A1 levels, however, remain unchanged.

Discussion

Heterogeneous ribonucleoproteins (hnRNPs) (Kzhyshkowska et al., 2001; Liu and Dreyfuss, 1995; Nichols et al., 2000) and other RNA-binding proteins that contain RG-rich regions are known substrates of the protein arginine methyltransferases (Cote et al., 2003; Lee and Bedford, 2002; Mears and Rice, 1996; Pelletier et al., 2001). This includes the recent demonstration that R_{445} in FXR1P is asymmetrically dimethylated by an unknown class I PRMT in vivo (Ong et al., 2004) and that mouse Fmrp is methylated by an unknown class I PRMT in vivo (Stetler et al., 2006). We have shown that FMRP is methylated by an endogenous methyltransferase in RRL (Denman et al., 2004). This is not surprising, as RRL is a known source of PRMTs that can methylate RG-rich proteins such as the hnRNPs (Liu and Dreyfuss, 1995). Here, we have extended this analysis by showing that the MTs in RRL methylate bona fide PRMT substrates and that they can be effectively inhibited by AdOx.

Protein arginine methylation is known to play a role in regulating transcription, RNA splicing and in RNA/protein import/export from the nucleus. The diversity of target proteins containing methyl-arginine (Boisvert et al., 2003; Najbauer and Aswad, 1990) suggests that arginine methylation plays a role in many cellular processes, while the diversity in the number (eight) and class of enzymes (three) that generate this modification suggest that the process must be tightly and specifically controlled. Because arginine methylation is mediated by multiple PRMTs and occurs in numerous proteins, there is a corresponding diversity in responses to it. Examples of arginine methylation affecting the balance of nuclear versus cytoplasmic protein localization have been reported (Green et al., 2002; Nichols et al., 2000; Shen et al., 1998; Smith et al., 2004). Likewise, arginine methylation can also influence protein–protein interactions (Bedford et al., 2000). Finally, accessory proteins that complex with various PRMTs often modify the outcome of arginine methylation. For example, Dal-1/4.1B, a brain-specific member of the 4.1 family of proteins, inhibits PRMT3- and PRMT5-dependent methylation (Jiang et al., 2005; Singh et al., 2004). By contrast, PRMT5, in association with Brg1 complexes actively methylates histone H3 at arginine 8, and thereby negatively regulates the expression of tumor suppressor genes (Pal et al., 2004).

As methylating FMRP altered its ability to interact with RNA in vitro (Denman, 2002), we decided to investigate whether it also affected some of the other properties of this protein. Blocking methylation with AdOx altered the cellular methylation state of HeLa cell proteins (Fig. 5B; see Fig. S1 in the supplementary material). However, under these conditions, the distribution of FMRP between the nucleus and

cytoplasm was not different from naïve cells. Thus, methylation does not appear to significantly affect the rate of import/export of FMRP. These results are consistent with the established mechanism of FMRP shuttling. Previous work has shown that the ability of FMRP to shuttle between the nucleus and cytoplasm is leptomycin B-dependent, indicating that it uses the Crm1/exportin 1 nuclear export pathway (Henderson and Eleftheriou, 2000; Tamanini et al., 1999a). By contrast, the yeast proteins Npl13, Hrp1p and Nab2p, which facilitate the bulk of mRNA export from the nucleus and whose methylation affects their localization, do not use this pathway (Neville and Rosbash, 1999). Likewise, Sam68, which exhibits methylation-dependent changes in its subcellular distribution, also does not use this pathway (Reddy et al., 1999).

In contrast to the lack of change in subcellular distribution, we showed that methylation plays a well-defined role in modulating the interactions of FMRP with other proteins. Specifically, we demonstrated that inhibiting methylation reduced the ability of FMRP to heterodimerize with FXR1P in vitro, while under the same conditions its ability to homodimerize was substantially unaffected (Fig. 2). Methylated residues on both FMRP and FXR1P are apparently required for maximal interaction. Thus, although the region mediating both interactions in FMRP is the same (Schenck et al., 2001), our data indicate that the determinants used to homodimerize differ from those used to bind FXR1P. Because of the differential effect methylation has on dimerization it will be important to examine the interaction of FMRP with its other protein partners

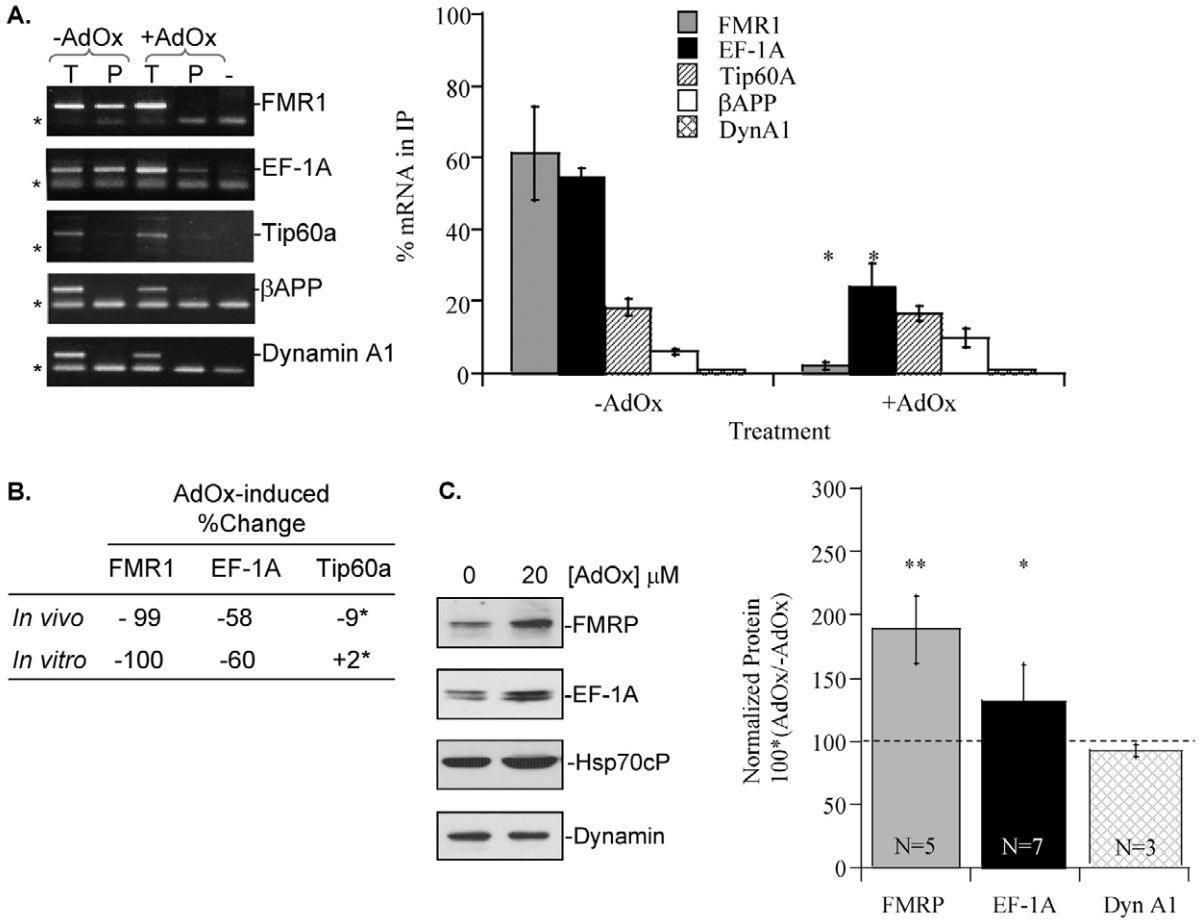

Fig. 7. Methylation affects the ability of FMRP to bind specific mRNAs and regulate their translation. (A) Semi-quantitative RT-PCR of mRNAs associated with HeLa cell FMRP immunoprecipitates (IP) in the absence of AdOx, and following treatment of the cells with 20 μM AdOx for 24 hours prior to immunoprecipitation. Primer pairs for FMR1 mRNA, EF-1A mRNA, Tip60a mRNA, βAPP mRNA and dynamin A1 were used to amplify each message from total HeLa cell RNA (T) and the FMRP IP (P) for each treatment. The five panels (left) show representative results for each message. The specific amplicons are labeled; non-specific amplicons and primer-dimers are marked by an asterisk. The graph (right) shows the percentage of the mRNA associated with the FMRP immunoprecipitate for AdOx-treated and non-treated cells. Values for three independent immunoprecipitations are shown. Asterisks indicate significant decreases in AdOx-treated cells (P<0.004 by ANOVA). (B) Comparison of the percent change in mRNAs associated with FMRP in AdOx-treated versus non-treated cells with the differences in in vitro binding of the mRNAs in the presence and absence of AdOx [results taken from Denman (Denman, 2002)]. (C) Western blot analysis of HeLa cell proteins from cells treated with 0 or 20 μM AdOx for 24 hours. Duplicate blots (left) were probed simultaneously with anti-FMRP mAb and anti-dynamin mAb, or anti-EF-1A mAb and anti-Hsp70cp mAb. The graph (right) shows the ratio of AdOx-treated to non-treated protein expression normalized to Hsp70cP. The number of determinations for each protein is shown in the bar. Double asterisk indicates that the AdOx-treated expression level is significantly different from the control (P<0.004, ANOVA); the single asterisk indicates that the AdOx-treated expression level is significantly different from the control (P<0.06, ANOVA).

(CYFIP1, CYFIP2, NuFIP and 82-FIP) in the future (Bardoni et al., 2003a; Bardoni et al., 2003b; Schenck et al., 2003). Interestingly, although the protein dimerization sites of FMRP are located in N-terminal sequences it is the C-terminal region that is methylated (Stetler et al., 2006) (R.B.D., unpublished). Thus, the effect of this modification on FXR1P binding must be propagated over a relatively large primary sequence space. However, a precedent for this exists. Laggerbauer et al. have shown that the $I_{304}N$ mutation in the KH_2 domain of FMRP negatively affects its ability to homodimerize (Laggerbauer et al., 2001). Similarly, Ceman et al. have demonstrated that the $I_{304}N$ mutation in FMRP reduces its ability to be recognized by an antibody whose epitope is located within the FMRP/FXR1P interaction region (Ceman et al., 2001).

The nature of the particles formed by FMRP and the role they play in cellular metabolism has been of great interest. The initial sucrose gradient sedimentation studies showing that FMRP associates with polyribosomes as a mRNP (Corbin et al., 1997; Tamanini et al., 1996) (reviewed by Sung and Denman, 2001) implied a role in translational regulation. This has subsequently been confirmed experimentally (Dolzhanskaya et al., 2003; Laggerbauer et al., 2001; Li et al., 2001; Mazroui et al., 2002; Sung et al., 2003). However, the growing list of FMRP-interacting proteins (Denman, 2003) suggests that other types of FMRP-containing particles form in vivo too. For example, FMRP-containing mRNA transport granules have been elegantly visualized in differentiated PC12 cells (De Diego Otero et al., 2002), in differentiated human SY5Y cells (Villace et al., 2004) and, most recently, in mouse brain (Aschrafi et al., 2005). Additionally, FMRP has recently been found associated with components of the RISC complex (Jin et al., 2004). Finally, studies by Mazroui et al. have demonstrated that heterologously expressed FMRP forms distinctly sedimenting mRNA-containing granules that resemble endogenous stress granules observed in HeLa cells following heat shock (Mazroui et al., 2002).

The studies presented here add to our understanding of FMRP-containing granules. First, we showed that although the inhibitor AdOx produced widespread effects on cellular protein methylation it did not produce a stress response under the conditions we used. Nevertheless, we identified a population of small FMRP-containing granules in cultured cells grown under normal growth conditions. We performed double-label immunostaining with FMRP and PABP, and FMRP and TIA-1 mAbs to examine the composition of these particles in more detail. Our results showed that these granules exhibited virtually no overlap in PABP or TIA-1 immunostaining, confirming that they were not stress granules.

Because these small FMRP-granules do not colocalize with PABP it is possible that they are related to processing bodies, sites of mRNA degradation (Kedersha et al., 2005). However, they may be distinct entities that are devoid of mRNA. Preliminary experiments indicate the former is more likely (Y.-J. Sung and R.B.D., unpublished). In addition, the failure of the anti-mRG pAb to recognize these particles is consistent with a lack of methyl-arginine (Fig. 5B). Nevertheless, we also demonstrated that FMRP associated with larger cytoplasmic granules when the cells were subject to oxidative stress. These particles, which are authentic stress granules as they contain both PABP and TIA-1, appear to be the major repository for FMRP under these conditions.

Importantly, we found that methylation was not absolutely required for recruiting FMRP into these large granules. However, pre-treating cells with AdOx before stress granule induction resulted in a small, but significant, accumulation of smaller FMRP-containing granules that were distinguishable from stress granules by their lack of colocalization with PABP or TIA-1 (Tables 1 and 2). Based on the fact that the majority of FMRP dimers in cultured cells appear to be homodimers (Tamanini et al., 1999b), and the observation that methylation was required for heterodimerization with FXR1P, we hypothesized that the FMRP found in stress granules following AdOx pre-treatment was primarily in the form of FMRP homodimers. Indeed, we demonstrated that AdOx significantly reduced the numbers of large granules containing FMRP and FXR1P under stress conditions, and that there was a corresponding loss of FMRP from FXR1P immunoprecipitates (Fig. 6).

Besides protein–protein interactions, we also examined the effect AdOx had on the ability of FMRP to interact with mRNA. Previous in vitro studies have demonstrated that target mRNA binding was differentially affected by methylation. Specifically we showed that the binding of FMR1 mRNA to FMRP was more sensitive to its methylation state than was the binding of EF-1A mRNA (Denman, 2002). These data were recapitulated when we examined the association of each message with FMRP-containing mRNPs in the presence of AdOx (Fig. 7A). In this case, FMR1 mRNA was nearly absent from FMRP-containing mRNPs, whereas EF-1A mRNA was moderately affected. Furthermore, the altered binding was reflected in changes in the steady-state expression of each protein. Based on these data, it is tempting to speculate that there may be an inter-relationship between the dimerization state of FMRP and its ability to bind a particular mRNA that is regulated by methylation. Obviously, much work needs to be done to further such a hypothesis.

Finally, these data provide additional support for the recent model we proposed for the role post-translational methylation plays in the normal cellular function of FMRP (Denman et al., 2004). One other piece of data that has a bearing on this model is the observation that PRMTs have been detected in distal neurites of PC12 cells and in the processes of brain neurons (Dolzhanskaya et al., 2005). These data are in accordance with the well-established role that PRMTs play at the periphery of other cell types (Abramovich et al., 1997; Chen et al., 2004). Taken in conjunction with the findings that NGF stimulation results in (1) increased PRMT activity in differentiating PC12 cells (Cimato et al., 2002) and (2) alterations in the methylation state of FMRP (Denman et al., 2004), these new data are consistent with the hypothesis that receptor-stimulated FMRP-mRNP remodeling may occur via changes in its methylation state.

Materials and Methods

Proteins and antibodies

Histone H3 was purchase from Roche. PRMT1 (ab7027) was obtained from AbCam. FMRP mAb-2160, which recognizes an epitope in the N-terminus of human FMRP, and normal mouse serum were purchased from Chemicon. FXR1 (Y-19) pAb were obtained from Santa Cruz. Symmetric dimethylarginine pAb (SYM11), asymmetric dimethylarginine pAb (ASYM24), EF-1A (CBP-KK1) mAb and dynamin A1 (HUDY-1) mAb were purchased from UpState. Hsp70cP mAb (HSP-820) was obtained from StressGen. Dr John Aletta (SUNY, Buffalo) generated asymmetric dimethylarginine pAb (mRG). GST-PABP(1-182) and GST-PABP(462-633) pAbs, which recognize N-terminal and C-terminal residues of PABP (Imataka

et al., 1998), respectively, were gifts from Dr Henri Tiedge (SUNY, Brooklyn). TIA-1 mAb was a gift from Dr Ivan Jeanne Weiler (University of Illinois at Urbana-Champaign). HRP-conjugated secondary antibodies were purchased from KPL and Santa Cruz. Alexa Fluor-conjugated secondary antibodies were purchased from Molecular Probes.

Buffers

The buffer used in the in vitro dimerization experiments was RNA-binding buffer (buffer 1): 50 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$ and 150 mM NaCl (Sung et al., 2003). TMK Buffer is 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ and 25 mM KCl, as previously described (Denman, 1997).

Cell culture

HeLa cells were grown at 37°C in 5% CO_2 and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. In some cases, the cells were treated with 10-40 μM adenosine-2′,3′-dialdehyde (AdOx), or with sodium arsenite and harvested as indicated. The effectiveness of AdOx treatment was optimized by treating the cells with various concentrations of AdOx (0-50 μM) and measuring decreased reactivity to anti-ASYM24 and anti-SYM11 pAbs by western blotting. At 20 μM AdOx, more than 90% of the total reactivity of each antibody was lost (see Fig. S1 in the supplementary material). Cell viability, as measured by the Alamar Blue assay (Currie et al., 1997) showed that the treatments had no effect on the viability of the cells at these concentrations.

Gene expression in cultured cells

Western blotting was performed as described (Sung et al., 2003). To detect human FMRP expression, FMRP mAb-2160 was used at a 1:10,000 dilution coupled with a 1:10,000 dilution of HRP-conjugated goat anti-mouse secondary antibody. HSP-820 mAb and dynamin A1 mAb were used at a 1:5000 dilution. PRMT1 mAb was used at a dilution of 1:2000. Both PABP pAbs were used at dilutions of 1:1000. mRG pAb was used at a dilution of 1:1500. Blots were blocked for 1 hour at room temperature in PBS supplemented with 3% non-fat dry milk and probed overnight in fresh buffer with the corresponding primary antibody at 4°C. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce). HRP-conjugated goat-anti-mouse secondary antibody was used at a 1:5000 dilution. FXR1 (Y-19) Ab was used at a 1:100 dilution. Blots were blocked and probed as above. HRP-conjugated bovine-anti-goat secondary antibody was used at a 1:2000 dilution.

For immunostaining, cells were grown on poly-L-lysine coated coverslips (10^4 cells per coverslip) in the presence or absence of 10-40 μM AdOx and in the presence or absence of 0.5 mM sodium arsenite as indicated. The cells were fixed in 2% paraformaldehyde for 10 minutes and washed with PBS and then blocked in (RPMI1640 base medium, 0.05% saponin, 0.1% sodium azide, 2% goat serum) for 30 minutes at room temperature. Subsequently, the cells were stained with antibodies to FMRP (1:500), FXR1P (1:50), TIA-1 (1:100), PABP (1:100) or mRG (1:150) for 1 hour. This was followed by a 30 minute incubation with Alexa Fluor secondary antibodies (1:500 dilution) at room temperature. Finally, the coverslips were washed in RPMI1640 base medium, 1% goat serum and mounted in buffered glycerol. Fluorescence was detected with a NIKON PCM 2000 dual laser-scanning confocal microscope (Nikon, Melville, NY). Images were acquired at 40× magnification.

Protein colocalization measurements were performed by counting single and double stained granules of representative images (Thomas et al., 2004) using the Analyze Particles feature in the Image J suite of programs (http://rsb.info.nih.gov.80/ij/). Data for each protein set was tabulated and statistically analyzed in Microsoft Excel.

In vitro methylation assay

In vitro methylation of FMRP was performed in rabbit reticulocyte lysates during coupled transcription translation as described (Denman et al., 2004). [^{3}H]SAM (2 μCi) was added to 25 μl reaction mixtures and incubated for 90 minutes at 37°C. In some cases, 6 μM AdOx was also added to the reaction to inhibit endogenous MT activity (Denman, 2002). Aliquots (10 μl) of each reaction (20%) were resolved on SDS-polyacrylamide gels. The gels were fixed, in 30% methanol and 10% acetic acid overnight. After removing the fixing solution, the gels were soaked in En^{3}Hance for 1 hour, then water for 30 minutes, and then dried and subjected to fluorography as described (Cimato et al., 2002). Aliquots (5 μl) of each reaction were used in western blot analyses to confirm the production of the protein of interest.

Dimerization assay

Unlabeled or ^{35}S-labeled proteins were produced using a TNT rabbit reticulocyte coupled transcription-translation system (RRL) (Sung et al., 2000), or in the *E. coli* Expressway system (Invitrogen) according to the manufacturer's protocols. In some cases, adenosine-2′,3′-dialdehyde (AdOx) was added directly to translation reaction mixtures to a concentration of 5-10 μM as indicated. In addition, some proteins were also biotinylated by including Transcend tRNA (Promega) in the reaction (Denman, 1998; Sung et al., 2000). Dimerization reactions were performed with 10 μl of biotinylated-protein and 10-20 μl of target protein in 50 μl of buffer 1. The reactions were incubated on ice for 1 hour and the resulting heterodimers isolated by affinity capture on SoftLink avidin resin. The unbound (U) and bound (B) fraction for each reaction was resolved by SDS-PAGE, blotted and probed with FXR1P pAb or subject to autoradiography. Control reactions in which the biotinylated-protein was omitted were also performed to determine background binding to SoftLink resin. Dimerization was quantified using IPLab Gel software. The percent binding was calculated as $[Bound_{Intensity}/[Bound_{Intensity} + (2\times Unbound_{Intensity})] \times 100$; this corrects for load differences between the bound and unbound fractions. Background binding was calculated in the same way for the control reactions and the difference of the two numbers is presented.

Polyribosome and granule isolation

HeLa cells were grown in the absence or presence of 10-40 μM AdOx as indicated. Twenty-four hours later, the cells were harvested and lysed. Equal amounts of each extract were treated or not treated for 5 minutes with 25 mM EDTA at 4°C to dissociate polyribosomes (Sung et al., 2003), or treated or not treated for 20 minutes at 4°C with 1 mg/ml RNAse A (Funakoshi et al., 2003). Polyribosomes and granules were subsequently sedimented by centrifugation through 50% sucrose pads. Proteins extracted from the pellets were then blotted and probed with various antibodies.

Immunoprecipitation and Immunoprecipitation-RT-PCR of FMRP associated mRNAs

HeLa cells, not treated or treated with 20 μM AdOx for 24 hours were immunoprecipitated with anti-FXR1P pAb, as described (Sung et al., 2003). The supernatant and immunoprecipitate fractions were subject to western blotting with anti-FMRP as described above.

For immunoprecipitation-RT-PCR, HeLa cells, not treated or treated with 10-40 μM AdOx for 24 hours, were subject to immunoprecipitation with anti-FMRP mAb 2160 as described (Sung et al., 2003). Total RNA was extracted from total cells and the FMRP immunoprecipitates using 1 ml of TRI-Reagent. The final RNA pellets were dissolved in 25 μl of DEPC-treated H_2O and 1 μg was used to prepare first-strand cDNA. cDNAs were amplified using primers for FMRP target messages [Fmr1 mRNA (5′GGCTAGAAGCTTTCTGGA, 5′ACGTGGAGGAGGCTTCA-AAGGAAA), EF-1A mRNA (5′GGCTAGAAGCTTTCTGGA, 5′GTGAAT-GGAGTACCCTAA) and Tip60a mRNA (5′AACCATCTTGGAAGATCCTGA, 5′GTCTTTGGGAGTGAAGTGCAG)] or control messages [dynamin A1 (5′ATGCTTCTCATCGACATTGAGTTGGCTA, 5′CACGAACGCTTCCCTTT-TGAGCTGGTTA) or βAPP mRNA (5′ATGCTGCCCGGTTTGGCACTG, 5′TCCAACTAAGCAGCGGTAGGG)]. A range of cDNA concentrations and cycles was used to ensure the amplifications were linear (Sung et al., 2004).

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References

Abramovich, C., Yakobson, B., Chebath, J. and Revel, M. (1997). A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *EMBO J.* **16**, 260-266.

Ai, L. S., Lin, C. H., Hsieh, M. and Li, C. (1999). Arginine methylation of a glycine and arginine rich peptide derived from sequences of human FMRP and fibrillarin. *Proc. Natl. Sci. Counc. Repub. China B* **23**, 175-180.

Anderson, P. and Kedersha, N. (2002). Stressful initiations. *J. Cell Sci.* **115**, 3227-3234.

Aoki, K., Ishii, Y., Matsumoto, K. and Tsujimoto, M. (2002). Methylation of *Xenopus* CIRP2 regulates its arginine- and glycine-rich region-mediated nucleocytoplasmic distribution. *Nucleic Acids Res.* **30**, 5182-5192.

Aschrafi, A., Cunningham, B. A., Edelman, G. M. and Vanderklish, P. W. (2005). The fragile X mental retardation protein and group I metabotropic glutamate receptors regulate levels of mRNA granules in brain. *Proc. Natl. Acad. Sci. USA* **102**, 2180-2185.

Azzouz, T. N., Pillai, R. S., Dapp, C., Chari, A., Meister, G., Kambach, C., Fischer, U. and Schumperli, D. (2005). Toward an assembly line for U7 snRNPs: interactions of U7-specific Lsm proteins with PRMT5 and SMN complexes. *J. Biol. Chem.* **280**, 34435-34440.

Bardoni, B. and Mandel, J.-L. (2002). Advances in understanding of fragile X pathogenesis and FMRP function, and in identification of X linked mental retardation genes. *Curr. Opin. Genet. Dev.* **12**, 284-293.

Bardoni, B., Castets, M., Huot, M.-E., Schenck, A., Adinolfi, S., Corbin, F., Pastore, A., Khandjian, E. W. and Mandel, J.-L. (2003a). 82-FIP, a novel FMRP (Fragile X Mental Retardation Protein) interacting protein, shows a cell cycle-dependent intracellular localization. *Hum. Mol. Genet.* **12**, 1689-1698.

Bardoni, B., Willemsen, R., Weiler, I. J., Schenck, A., Severijnen, L.-A., Hindelang, C., Lalli, E. and Mandel, J.-L. (2003b). NUFIP1 (nuclear FMRP interacting protein

1) is a nucleocytoplasmic shuttling protein associated with active synaptoneurosomes. *Exp. Cell Res.* **289**, 95-107.

Bedford, M. T., Frankel, A., Yaffe, M. B., Clarke, S., Leder, P. and Richard, S. (2000). Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. *J. Biol. Chem.* **275**, 16030-16036.

Boisvert, F.-M., Cote, J., Boulanger, M.-C. and Richard, S. (2003). A proteomic analysis of methylated protein complexes. *Mol. Cell Proteomics* **2**, 1319-1330.

Boisvert, F.-M., Chenard, C. A. and Richard, S. (2005). Protein interfaces in signaling regulated by arginine methylation. *Sci. STKE* doi: 10.1126/stke.2712005re2.

Brahms, H., Meheus, L., de Brabandere, V., Fischer, U. and Luhrmann, R. (2001). Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein. *RNA* **7**, 1531-1542.

Ceman, S., DiMarco, S. and Warren, S. T. (2001). A single amino acid substitution in the FMR protein (I304N) results in reduced phosphorylation and an altered conformation. *Am. J. Hum. Genet.* **69**, 348.

Ceman, S., O'Donnell, W. T., Reed, M., Patton, S., Pohl, J. and Warren, S. T. (2003). Phosphorylation influences the translation state of FMRP-associated polyribosomes. *Hum. Mol. Genet.* **12**, 3295-3305.

Chen, Y.-F., Zhang, A. Y., Zou, A.-P., Campbell, W. B. and Li, P.-L. (2004). Protein methylation activates reconstituted ryanodine receptor-Ca^{2+} release channels from coronary artery myocytes. *J. Vasc. Res.* **41**, 229-240.

Christensen, M. E. and Fuxa, K. P. (1988). The nucleolar protein, B-36, contains a glycine and dimethylarginine-rich sequence conserved in several other nuclear RNA-binding proteins. *Biochem. Biophys. Res. Commun.* **155**, 1278-1283.

Cimato, T. R., Tang, J., Xu, Y., Guarnaccia, C., Herschman, H. R., Pongor, S. and Aletta, J. M. (2002). Nerve growth factor-mediated increases in protein methylation occur predominantly at type I arginine methylation sites and involve protein arginine methyltransferase 1. *J. Neurosci. Res.* **67**, 435-442.

Corbin, F., Bouillon, M., Fortin, A., Morin, S., Rousseau, F. and Khandjian, E. W. (1997). The fragile X mental retardation protein is associated with poly(A)+ mRNA in actively translating polyribosomes. *Hum. Mol. Genet.* **6**, 1465-1472.

Cote, J., Boisvert, F.-M., Boulanger, M.-C., Bedford, M. T. and Richard, S. (2003). Sam68 RNA binding protein is an in vivo substrate for protein arginine N-methyltransferase 1. *Mol. Biol. Cell* **14**, 274-287.

Currie, J. R., Chen-Hwang, M.-C., Denman, R. B., Smedman, M., Potempska, A., Ramakrishna, N., Rubenstein, R., Wisniewski, H. M. and Miller, D. L. (1997). Reduction of histone cytotoxicity by the Alzheimer beta-amyloid peptide precursor. *Biochim. Biophys. Acta* **1355**, 248-258.

De Diego Otero, Y., Severijnen, L.-A., van Cappellen, G., Schrier, M., Oostra, B. and Willemsen, R. (2002). Transport of fragile X mental retardation protein via granules in neurites of PC12 Cells. *Mol. Cell. Biol.* **22**, 8332-8341.

Denman, R. B. (1997). Ribozyme and antisense RNAs inhibit coupled transcription translation by binding to rabbit polyribosomes. *Biochem. Biophys. Res. Commun.* **230**, 226-231.

Denman, R. B. (1998). A general method for isolating targets of RNA and DNA binding proteins. *Promega Notes* **67**, 5-8.

Denman, R. B. (2002). Methylation of the arginine-glycine-rich region in the fragile X mental retardation protein FMRP differentially affects RNA binding. *Cell. Mol. Biol. Lett.* **7**, 877-883.

Denman, R. B. (2003). Deja vu all over again: FMRP binds U-rich target mRNAs. *Biochem. Biophys. Res. Comm.* **310**, 1-7.

Denman, R. B., Dolzhanskaya, N. and Sung, Y.-J. (2004). Regulating a translational regulator: mechanisms cells use to control the activity of the fragile X mental retardation protein. *Cell. Mol. Life Sci.* **61**, 1714-1728.

Dolzhanskaya, N., Sung, Y.-J., Merz, G., Brown, W. T., Nolin, S., El Idrissi, A., Dobkin, C. and Denman, R. B. (2003). Elevated nuclear Tip60a and NF-kBp65 levels in fragile X syndrome results from altered mRNA binding to FMRP. In *Current Topics in Peptide and Protein Research*, *vol. 5*, pp. 201-220. Trivandrum, India: Research Trends.

Dolzhanskaya, N., Dobkin, C., El Idrissi, A., Bauchwitz, R. P. and Denman, R. B. (2005). Protein arginine methylation in fragile X mouse models: a cause of phenotypic diversity in fragile X syndrome? In *The Molecular Basis of Fragile X Syndrome* (ed. Y.-J. Sung and R. B. Denman), pp. 245-265. Trivandrum, India: Research Signposts.

Frankel, A., Yadav, N., Lee, J., Branscombe, T. L., Clarke, S. and Bedford, M. T. (2002). The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* **277**, 3537-3543.

Friesen, W. J., Massenet, S., Paushkin, S., Wyce, A. and Dreyfuss, G. (2001). Smn, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets. *Mol. Cell* **7**, 1111-1117.

Funakoshi, T., Kobayashi, S., Ohashi, S., Sato, T.-A. and Anzai, K. (2003). Isolation and characterization of brain Y-box protein: developmentally regulated expression, polyribosomal association and dendritic localization. *Mol. Brain Res.* **118**, 1-9.

Gary, J. D. and Clarke, S. (1998). RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.* **61**, 65-131.

Green, D. M., Marfatia, K. A., Crafton, E. B., Zhang, X., Cheng, X. and Corbett, A. H. (2002). Nab2p is required for poly(A) RNA export in *Saccharomyces cerevisiae* and is regulated by arginine methylation *via* Hmt1p. *J. Biol. Chem.* **277**, 7752-7760.

Henderson, B. R. and Eleftheriou, A. (2000). A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp. Cell Res.* **256**, 213-224.

Hyun, Y. L., Lew, D. B., Park, S. H., Kim, C. W., Paik, W. K. and Kim, S. (2000). Enzymic methylation of arginyl residues in -gly-arg-gly- peptides. *Biochem. J.* **348**, 573-578.

Imataka, H., Gradi, A. and Sonenberg, N. (1998). A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.* **17**, 7480-7489.

Jackson, R. J. and Hunt, T. (1983). Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. In *Methods in Enzymology*, *Vol. 96* (ed. S. Fleischer and B. Fleischer), pp. 50-75. San Diego, CA: Academic Press.

Jiang, W., Roemer, M. E. and Newsham, I. F. (2005). The tumor suppressor DAL-1/4.1B modulates protein arginine N-methyltransferase 5 activity in a substrate-specific manner. *Biochem. Biophys. Res. Comm.* **329**, 522-530.

Jin, P., Zarnescu, D. C., Ceman, S., Nakamoto, M., Mowrey, J., Jongens, T. A., Nelson, D. L., Moses, K. and Warren, S. T. (2004). Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nat. Neurosci.* **7**, 113-117.

Katz, J. E., Dlakic, M. and Clarke, S. (2003). Automated identification of putative methyltransferases from genomic open reading frames. *Mol. Cell. Proteomics* **2**, 525-540.

Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fitzler, M. J., Scheuner, D., Kaufman, R. J., Golan, D. E. and Anderson, P. (2005). Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell Biol.* **169**, 871-884.

Khandjian, E. (1999). Biology of the fragile X mental retardation protein, an RNA-binding protein. *Biochem. Cell Biol.* **77**, 331-342.

Khandjian, E. W., Corbin, F., Woerly, S. and Rousseau, F. (1996). The fragile X mental retardation protein is associated with ribosomes. *Nat. Genet.* **12**, 91-93.

Khandjian, E. W., Huot, M.-E., Tremblay, S., Davidovic, L., Mazroui, R. and Bardoni, B. (2004). Biochemical evidence for the association of fragile X mental retardation protein with brain polyribosomal ribonucleoparticles. *Proc. Natl. Acad. Sci. USA* **101**, 13357-13362.

Kim, S., Merrill, B. M., Rajpurohit, R., Kumar, A., Stone, K. L., Papov, V. V., Schneiders, J. M., Szer, W., Wilson, S. H., Paik, W. K. et al. (1997). Identification of N(G)-methylarginine residues in human heterogeneous RNP protein A1: Phe/Gly-Gly-Gly-Arg-Gly-Gly-Gly/Phe is a preferred recognition motif. *Biochemistry* **36**, 5185-5192.

Kim, S., Park, G. H. and Paik, W. K. (1998). Recent advances in protein methylation: enzymatic methylation of nucleic acid binding proteins. *Amino Acids* **15**, 291-306.

Kzhyshkowska, J., Schutt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H. and Dobner, T. (2001). Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1. *Biochem. J.* **358**, 305-314.

Laggerbauer, B., Ostareck, D., Keidel, E.-M., Ostareck-Lederer, A. and Fischer, U. (2001). Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum. Mol. Genet.* **10**, 329-338.

Lee, J. and Bedford, M. T. (2002). PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. *EMBO Rep.* **3**, 268-273.

Li, Z., Zhang, Y., Ku, L., Wilkinson, K. D., Warren, S. T. and Feng, Y. (2001). The fragile X mental retardation protein inhibits translation via interacting with mRNA. *Nucleic Acids Res.* **29**, 2276-2283.

Liu, Q. and Dreyfuss, G. (1995). In vivo and *in vitro* arginine methylation of RNA binding proteins. *Mol. Cell. Biol.* **15**, 2800-2808.

Martin, J. and McMillan, F. (2002). SAM (dependent) I AM: the S-adenosylmethionine-dependent methyltransferase fold. *Curr. Opin. Struct. Biol.* **12**, 783-793.

Mazroui, R., Huot, M.-E., Tremblay, S., Filion, C., Labelle, Y. and Khandjian, E. W. (2002). Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression. *Hum. Mol. Genet.* **11**, 3007-3017.

Mazroui, R., Huot, M.-E., Tremblay, S., Boilard, N., Labelle, Y. and Khandjian, E. W. (2003). Fragile X Mental Retardation protein determinants required for its association with polyribosomal mRNPs. *Hum. Mol. Genet.* **12**, 3087-3096.

Mears, W. E. and Rice, S. A. (1996). The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines in vivo methylation. *J. Virol.* **70**, 7445-7453.

Najbauer, J. and Aswad, D. (1990). Diversity of methyl acceptor proteins in rat pheochromocytoma (PC12) cells revealed after treatment with adenosine dialdehyde. *J. Biol. Chem.* **265**, 12717-12721.

Neville, M. and Rosbash, M. (1999). The NES-Crm1p export pathway is not a major mRNA export route in Saccharomyces cerevisiae. *EMBO J.* **18**, 3746-3756.

Nichols, R. C., Wang, X. W., Tang, J., Hamilton, B. J., High, F. A., Herschman, H. R. and Rigby, W. F. (2000). The RGG domain in hnRNP A2 affects subcellular localization. *Exp. Cell Res.* **256**, 522-532.

Ong, S.-E., Mittler, G. and Mann, M. (2004). Identifying and quantifying in vivo methylation sites by heavy methyl SILAC. *Nat. Methods* **1**, 119-126.

Pal, S., Viswanath, S. N., Erdjument-Bromage, H., Tempst, P. and Sif, S. (2004). Human SWI/SNF-associated PRMT5 methylated histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. *Mol. Cell. Biol.* **24**, 9630-9645.

Pelletier, M., Xu, Y., Wang, X., Zahariev, S., Pongor, S., Aletta, J. M. and Read, L. K. (2001). Arginine methylation of a mitochondrial guide RNA binding protein from Trypanosoma brucei. *Mol. Biochem. Parasitol.* **118**, 49-59.

Reddy, T. R., Xu, W., Mau, J. K., Goodwin, C. D., Suhasini, M., Tang, H., Frimpong, K., Rose, D. W. and Wong-Staal, F. (1999). Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. *Nat. Med.* **5**, 635-642.

Rho, J., Choi, S., Seong, Y. R., Choi, J. and Im, D.-S. (2001). The arginine-1493 residue in QRRGRTGR1493G motif IV of the hepatitis C virus NS3 helicase domain is

essential for NS3 protein methylation by the protein arginine methyltransferase 1. *J. Virol.* **75**, 8031-8044.

Schenck, A., Bardoni, B., Moro, A., Bagni, C. and Mandel, J.-L. (2001). A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. *Proc. Natl. Acad. Sci. USA* **98**, 8844-8849.

Schenck, A., Bardoni, B., Langmann, C., Harden, N., Mandel, J.-L. and Giangrande, A. (2003). CYFIP/Sra-1 controls neuronal connectivity in Drosophila and links the Rac1 GTPase pathway to the fragile X protein. *Neuron* **38**, 887-898.

Schuster, S. and Kenanov, D. (2005). Adenine and adenosine salvage pathways in erythrocytes and the role of S-adenosylhomocysteine hydrolase. A theoretical study using elementary flux modes. *FEBS J.* **272**, 5278-5290.

Shen, E. C., Henry, M. F., Weiss, V. H., Valentini, S. R., Silver, P. A. and Lee, M. S. (1998). Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes Dev.* **12**, 679-691.

Singh, V., Miranda, T. B., Jiang, W., Frankel, A., Roemer, M. E., Robb, V. A., Gutmann, D. H., Herschman, H. R., Clarke, S. and Newsham, I. F. (2004). DAL-1/4.1B tumor suppressor interacts with protein arginine N-methyltransferase 3 (PRMT3) and inhibits its ability to methylate substrates in vitro and in vivo. *Oncogene* **23**, 7761-7771.

Siomi, H., Siomi, M. C., Nussbaum, R. L. and Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. *Cell* **74**, 291-298.

Siomi, M. C., Zhang, Y., Siomi, H. and Dreyfuss, G. (1996). Specific sequences in the fragile X syndrome protein FMR1 and the FXR proteins mediate their binding to 60S ribosomal subunits and the interactions among them. *Mol. Cell. Biol.* **16**, 3825-3832.

Smith, W. A., Schurter, B. T., Wong-Staal, F. and David, M. (2004). Arginine methylation of RNA helicase A determines its subcellular localization. *J. Biol. Chem.* **279**, 22795-22798.

Stetler, A., Winograd, C., Sayegh, J., Cheever, A., Patton, E., Zhang, Z., Clarke, S. and Ceman, S. (2006). Identification and characterization of the methyl arginines in the fragile X mental retardation protein Fmrp. *Hum. Mol. Genet.* **15**, 87-96.

Sung, Y.-J. and Denman, R. B. (2001). RNA binding properties of the fragile X syndrome mental retardation protein FMRP. *Recent Res. Dev. Biophys. Biochem.* **1**, 109-123.

Sung, Y.-J., Conti, J., Currie, J. R., Brown, W. T. and Denman, R. B. (2000). RNAs that interact with the fragile X syndrome RNA binding protein FMRP. *Biochem. Biophys. Res. Commun.* **275**, 973-980.

Sung, Y.-J., Dolzhanskaya, N., Nolin, S. L., Brown, T., Currie, J. R. and Denman, R. B. (2003). The fragile X mental retardation protein FMRP binds elongation factor 1A mRNA and negatively regulates its translation in vivo. *J. Biol. Chem.* **278**, 15669-15678.

Sung, Y.-J., Weiler, I.-J., Greenough, W. T. and Denman, R. B. (2004). Selectively enriched mRNAs in rat synaptoneurosomes. *Mol. Brain Res.* **126**, 81-87.

Tamanini, F., Meijer, N., Verheij, C., Willems, P. J., Galjaard, H., Oostra, B. A. and Hoogeveen, A. T. (1996). FMRP is associated to the ribosomes via RNA. *Hum. Mol. Genet.* **5**, 809-813.

Tamanini, F., Bontenkoe, C., Bakker, C. E., van Unen, L., Anar, B., Willemsen, R., Yoshida, M., Galjaard, H., Oostra, B. A. and Hoogeveen, A. T. (1999a). Different targets for the fragile X-related proteins revealed by their distinct nuclear localizations. *Hum. Mol. Gen.* **8**, 863-869.

Tamanini, F., Van Uen, L., Bakker, C., Sacchi, N., Galjaard, H., Oostra, B. A. and Hoogeveen, A. T. (1999b). Oligomerization properties of fragile-X mental-retardation protein (FMRP) and the fragile-X-related proteins FXR1P and FXR2P. *Biochem. J.* **343**, 517-523.

Tang, J., Kao, P. N. and Herschman, H. R. (2000). Protein-arginine methyltransferase I, the predominant protein-arginine methyltransferase in cells, interacts with and is regulated by interleukin enhancer-binding factor 3. *J. Biol. Chem.* **275**, 19866-19876.

Thomas, M. G., Martinez Tosar, L. J., Loschi, M., Pasquini, J. M., Correale, J., Kindler, S. and Boccaccio, G. L. (2004). Staufen recruitment into stress granules does not affect early mRNA transport in oligodendrocytes. *Mol. Biol. Cell* **16**, 405-420.

Villace, P., Marion, R. M. and Ortin, J. (2004). The composition of Staufen-containing RNA granules from human cells indicates their role in the regulated transport and translation of messenger RNAs. *Nucleic Acids Res.* **32**, 2411-2420.