Cell

A Chromatin-Dependent Role of the Fragile X Mental Retardation Protein FMRP in the DNA Damage Response

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http://dx.doi.org/10.1016/j.cell.2014.03.040

SUMMARY

Fragile X syndrome, a common form of inherited intellectual disability, is caused by loss of the fragile X mental retardation protein FMRP. FMRP is present predominantly in the cytoplasm, where it regulates translation of proteins that are important for synaptic function. We identify FMRP as a chromatin-binding protein that functions in the DNA damage response (DDR). Specifically, we show that FMRP binds chromatin through its tandem Tudor (Agenet) domain in vitro and associates with chromatin in vivo. We also demonstrate that FMRP participates in the DDR in a chromatin-binding-dependent manner. The DDR machinery is known to play important roles in developmental processes such as gametogenesis. We show that FMRP occupies meiotic chromosomes and regulates the dynamics of the DDR machinery during mouse spermatogenesis. These findings suggest that nuclear FMRP regulates genomic stability at the chromatin interface and may impact gametogenesis and some developmental aspects of fragile X syndrome.

INTRODUCTION

Chromatin is a complex biological entity comprised of DNA wrapped around histone octamers (Wolffe and Guschin, 2000). Posttranslational modifications of histone proteins serve as an interface for various chromatin "readers," which are chromatin-binding proteins that coordinate downstream processes, including the DNA damage response (DDR) and repair events (Costelloe et al., 2006; Downs et al., 2007; Stucki and Jackson, 2006). The mammalian DDR pathway is initiated by the activation of several conserved protein kinases, including ATM and ATR, which are members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family. While ATM is activated by DNA double-strand breaks (DSBs), ATR activity is triggered by stalled replication forks as well as single-strand DNA (Ciccia and Elledge, 2010). Upon activation, ATR phosphorylates histone H2A.X at serine 139 (termed γH2A.X) (Ciccia and Elledge, 2010; Liu et al., 2006; Ward and Chen, 2001) and the breast cancer-associated tumor-suppressor protein BRCA1 at serine 1423 (Gatei et al., 2001; Tibbetts et al., 2000). Both γH2A.X and BRCA1 are important regulators of genomic stability (Celeste et al., 2002; Nagaraju and Scully, 2007).

The fragile X mental retardation protein FMRP is an RNA-binding protein that functions mainly at the neuronal dendrites, where it associates with specific mRNAs and modulates their translation, thus regulating a subset of proteins involved in synaptic function (Bassell and Warren, 2008; Brown et al., 2001). FMRP is critical for metabotropic glutamate receptor (mGluR)-dependent long-term depression, as well as other forms of synaptic plasticity. The lack of FMRP due to *FMR1* gene silencing results in fragile X syndrome, a common form of inherited intellectual disability and one of the leading causes of autism (Bear et al., 2004; Garber et al., 2008; Nelson, 1995; O'Donnell and Warren, 2002; Santoro et al., 2012; Warren and

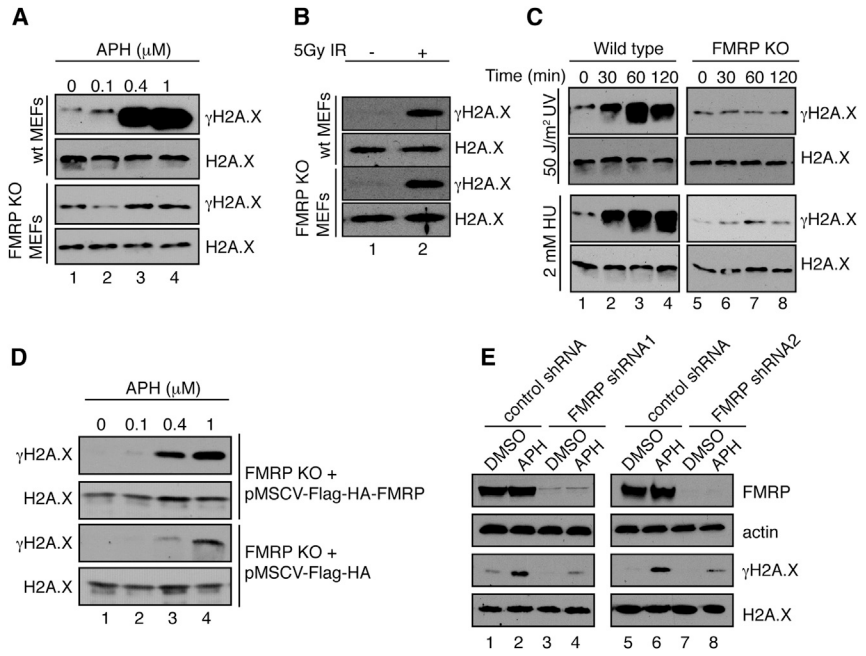

Figure 1. FMRP Modulates Histone H2A.X Phosphorylation Levels in Response to Replication Stress

(A) Wild-type (WT), but not FMRP KO, MEFs exhibited dose-dependent γH2A.X induction in response to APH (lanes 1–4). See also Figures S1A–S1C.

(B) WT MEFs and FMRP KO MEFs exhibited similar degrees of γH2A.X induction (5-fold) in response to 5 Gy of irradiation (lanes 1 and 2).

(C) WT, but not FMRP KO, MEFs exhibited time-dependent γH2A.X induction in response to 50 J/m^2 of UV irradiation or 2 mM of HU (10-fold induction at 60 min posttreatment; compare lanes 1–4 with lanes 5–8).

(D) FMRP KO MEFs reconstituted with WT Flag-HA-FMRP (pMSCV-Flag-HA-FMRP) or vector alone (pMSCV-Flag-HA) were exposed to various concentrations of APH. See also Figure S1D. pMSCV-Flag-HA-FMRP MEFs exhibited more pronounced γH2A.X induction compared with pMSCV-Flag-HA cells (12-fold in Flag-HA-FMRP cells and 4-fold in Flag-HA cells; lanes 1–4).

(E) FMRP RNAi HeLa cells, but not control cells, showed diminished γH2A.X induction in response to APH (3.4-fold and 8-fold, respectively; compare lanes 1/2 with 3/4, and 5/6 with 7/8). See also Figures S1E, S1F, and S2.

Nelson, 1994). Besides cognitive impairment, fragile X males also display macroorchidism (Johannisson et al., 1987; O'Donnell and Warren, 2002) and female *Fmr1* KO mice develop abnormal ovaries (Ascano et al., 2012), indicating an additional germline or gonadal effect of disruption of *Fmr1* expression.

Previous studies demonstrated a wide tissue distribution for FMRP and established it as largely a cytoplasmic protein, with only about 4% FMRP in the nucleus (Feng et al., 1997), where its function remains unknown. However, several reports suggested a potential role for FMRP in the nucleus. Studies in *Xenopus* and zebrafish showed that at 2–3 hr postfertilization, Fmrp is predominantly nuclear (Blonden et al., 2005; Kim et al., 2009; van 't Padje et al., 2005). In addition, Fmrp was found to decorate lampbrush chromosomes in *Xenopus* oocytes (Kim et al., 2009). Furthermore, nuclear FMRP interacting protein (NUFIP) associates with BRCA1 (Cabart et al., 2004), suggesting a potential functional relationship between FMRP and BRCA1 in the nucleus. FMRP has also been found in the PARP complexes, which heavily influence the DDR cascades (Helleday et al., 2005; Isabelle et al., 2010; Kedar et al., 2008). Interestingly, mice lacking the DNA topoisomerase TOP3β, which is part of FMRP-containing messenger ribonucleoprotein particles (mRNPs) and is implicated in neuronal development, display progressive reduction in fecundity and aneuploidy (Kwan et al., 2003; Stoll et al., 2013). The fact that FMRP is present in DDR complexes and is predominantly nuclear in some gametes and early embryos led us to speculate that FMRP might have a novel nuclear function in the DDR during development.

In this study, we provide evidence that FMRP has an important role in the nucleus, where it modulates the replication stress response at the chromatin interface. We show that FMRP regulates H2A.X phosphorylation, BRCA1 focus formation, and accumulation of single-strand DNA intermediates in a chromatin binding-dependent manner, and this nuclear role of FMRP is separable from its well-established role in translational regulation. We extend this nuclear function of FMRP to mammalian meiosis using mouse spermatocytes as a model. We show that FMRP decorates meiotic chromosomes and regulates γH2A.X induction, BRCA1 and ATR recruitment, and resolution of single-strand repair intermediates during meiosis. Taken together, our findings identify FMRP as a chromatin-binding protein and demonstrate that it plays a previously unanticipated role in the DDR at the chromatin interface, which is independent from the canonical role of FMRP in translational regulation.

RESULTS

Loss of FMRP Compromises Phosphorylation of H2A.X in Response to Replication Stress

In order to determine whether FMRP has a role in the DDR, we analyzed γH2A.X induction in cells that lack FMRP. We first treated wild-type and FMRP knockout (KO) mouse embryonic fibroblasts (MEFs) with increasing concentrations of the replication stress inducer aphidicolin (APH), which largely triggers single-strand breaks, and ionizing radiation, which generates DSBs (Brown and Baltimore, 2003; Rogakou et al., 1998; Zhou and Elledge, 2000). In wild-type but not FMRP KO, MEFs, APH-induced replication stress elicited an ~20-fold induction of γH2A.X (Figure 1A, compare lanes 1–4 of the first and third panels), indicating a requirement for FMRP in the replication stress response. In addition, FMRP KO MEFs showed reduced formation of γH2A.X foci upon treatment with APH as compared to wild-type MEFs (Figures S1A–S1C available online). In contrast, FMRP KO cells showed γH2A.X induction comparable

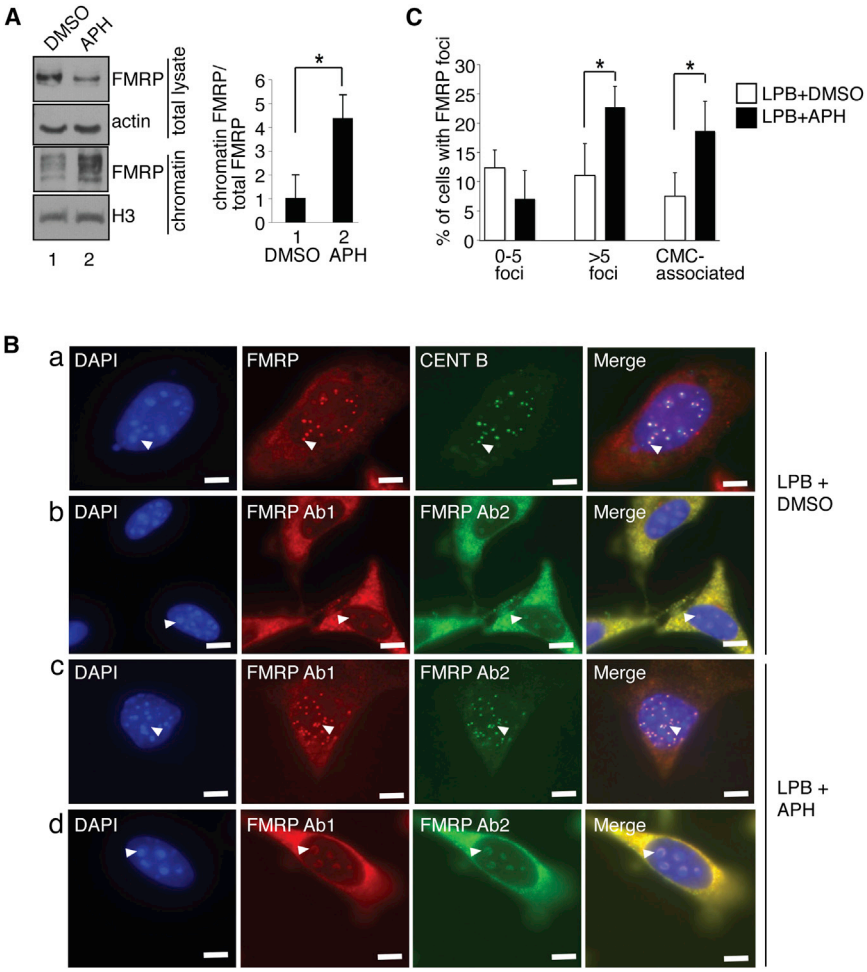

Figure 2. FMRP Chromatin Recruitment in Response to Replication Stress

(A) MEFs were treated with DMSO (lane 1) or APH (lane 2). Chromatin fractions were isolated and western blotted for FMRP. Bar graph, relative ratio of chromatin-associated FMRP to total FMRP. *$p < 0.05$, Student's t test. Data are an average of three independent experiments with SD.

(B) Immunostaining of nuclear FMRP in APH-treated or DMSO-treated MEFs in the presence of LPB. a: FMRP colocalized with CENT B next to CMCs. Arrowheads, representative colocalized FMRP (red) and CENT B (green) foci docked near CMCs. b: Representative FMRP signal (Ab-1: anti-FMRP [Abcam] antibody [red], Ab-2: anti-FMRP [Calbiotech] antibody [green]) enveloping CMCs in LPB-treated MEFs. c: Representative FMRP foci in LPB+APH-treated cells. d: representative FMRP signals enveloping CMCs in LPB+APH-treated MEFs. Arrowheads, selected FMRP foci wrapped around CMCs. Scale bar, 10 μm.

(C) APH treatment resulted in doubling of the number of cells with five or more FMRP foci (>5) or FMRP CMCs. *$p < 0.05$, Student's t test. Data are an average of three independent experiments with SD. See also Figure S3.

to that of the wild-type MEFs in response to ionizing radiation, indicating an intact response to DSB (Figure 1B, lane 2). In sum, FMRP KO MEFs showed distinct responses to different types of DNA damage, i.e., they responded to DSBs similarly to wild-type MEFs, but were defective in their response to replication stress.

To confirm that FMRP KO MEFs are defective in their response to replication stress, we subjected FMRP KO MEFs to additional sources of replication stress agents, including hydroxyurea (HU) and UV irradiation. In both cases, FMRP KO MEFs failed to show a time-dependent increase of the γH2A.X level as compared to wild-type MEFs (10-fold induction at 60 min posttreatment; Figure 1C, compare lanes 1–4 with lanes 5–8 of the upper and lower panels). Importantly, FMRP KO MEFs reconstituted with a FLAG-HA epitope-tagged, wild-type FMRP (Flag-HA-FMRP) conferred a more robust γH2A.X response to increasing concentrations of APH compared with the Flag-HA vector alone (Figures 1D and S1D; 12-fold induction in Flag-HA-FMRP cells as compared to 4-fold induction in Flag-HA only cells). This was not a MEF-cell-specific effect, since reduction of FMRP in HeLa cells by RNAi also resulted in a compromised induction of γH2AX in response to replication stress (Figure 1E). In addition to H2A.X phosphorylation regulation, loss of FMRP also affected another ATR-dependent, replication response-specific phosphorylation event: phosphorylation of BRCA1 at Ser-1423 (Tibbetts et al., 2000; Figures S1E and S1F). Consistent with the potential role of FMRP in the replication stress response, FMRP RNAi knockdown HeLa cells reconstituted with Flag-HA vector alone, but not tagged wild-type FMRP (Flag-HA-FMRP), were more sensitive to replication stress in the clonogenic survival assay (Figures S2A and S2B), and FMRP KO MEFs were also more sensitive to replication stress compared to wild-type MEFs (Figure S2C). These findings are in line with previous reports describing a prosurvival role of FMRP (Jeon et al., 2011, 2012; Liu et al., 2012). Taken together, the above findings link FMRP to replication stress-induced DDR and indicate that FMRP may be part of the ATR-dependent signaling pathway.

FMRP Is Recruited to Chromatin in Response to Replication Stress

Many proteins that function in the DDR are recruited to chromatin in response to DNA damage, where they participate in the DDR events (Bostelman et al., 2007; Conde et al., 2009; Krum et al., 2010; Pei et al., 2011; Wakeman et al., 2012; Wysocki et al., 2005). We therefore investigated the possibility that the FMRP may function in the replication stress response through recruitment to chromatin. By chromatin fractionation, we detected association of FMRP with chromatin, and this association was elevated by ~4-fold upon APH treatment (Figure 2A, compare lanes 1 and 2). Although biochemical fractionation allows detection of FMRP association with chromatin, direct

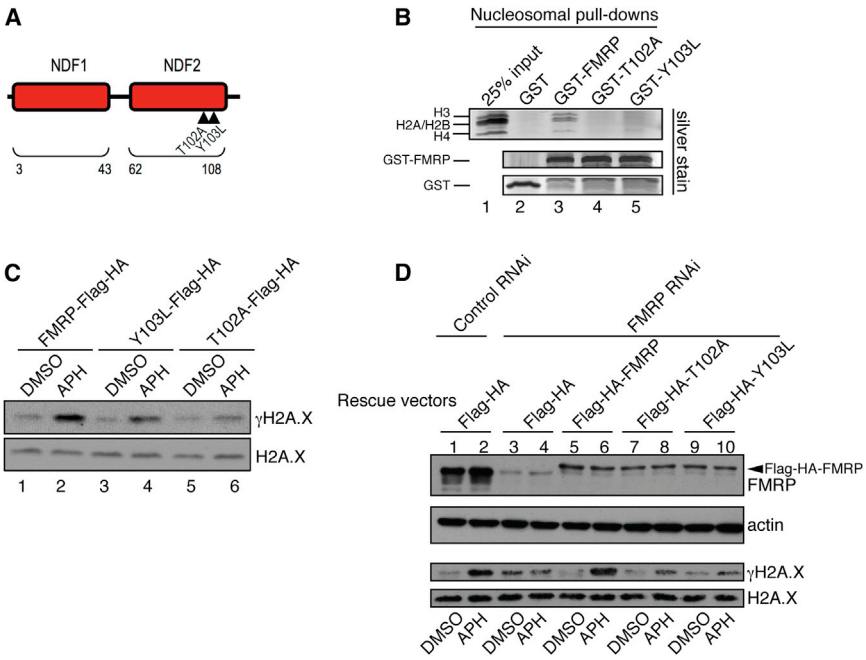

Figure 3. FMRP Docking to Chromatin Is Essential for FMRP-Dependent Modulation of γH2A.X Levels in Response to Replication Stress

(A) Diagram of Agenet$_{FMRP}$. Mutations T102A and Y103L are demarcated by triangles. See also Figure S4.

(B) GST-FMRP or GST-FMRP carrying mutations in Agenet$_{FMRP}$ (GST-T102A and GST-Y103L) were incubated with isolated nucleosomes. Pull-down material was run on gradient gels followed by silver staining. A complete set of core nucleosomal histones, including H3, H2A, H2B, and H4, were detected in WT, but not mutant, FMRP-mediated pull-downs (compare lanes 3–5). See also Figure S5A.

(C) WT FMRP (lanes 1 and 2) triggered more pronounced γH2A.X induction in FMRP KO MEFs in response to APH (12.8-fold) as compared with FMRP mutants (4-fold and 3-fold γH2A.X for Y103L and T102A mutants respectively; lanes 3–6). See also Figure S1D.

(D) FMRP RNAi in HeLa cells abolished γH2A.X induction in response to APH as compared with control RNAi (compare lanes 1/2 with lanes 3/4). Cotransfection with constructs expressing WT but not mutant forms of FMRP restored the induction of γH2A.X in FMRP RNAi cells in response to APH (compare lanes 5/6 with lanes 7/8 and 9/10). The slower-migrating band (in lanes 5–10) is Flag-HA-FMRP (indicated by an arrowhead).

visualization of FMRP in the nucleus is problematic due to the low level of nuclear FMRP (Figure S3A). However, it is possible to raise nuclear FMRP levels by using leptomycin B (LPB), which inhibits nuclear protein export (Tamanini et al., 1999). As shown in Figure 2B, in the presence of LPB, we detected FMRP foci in the vicinity of pericentromeric domains (chromocenters [CMCs]), which are easily recognizable in the mouse nuclei as large DAPI-positive domains (Figure 2B, a and b). Consistently, FMRP staining overlapped with the centromeric protein B (CENT B) signal, which marks pericentromeric heterochromatin (Figure 2B, a, arrowheads). In some cases, FMRP formed larger structures wrapped around the CMCs (Figure 2B, b, arrowheads). The number of cells with FMRP foci as well as CMC-associated FMRP domains increased 2-fold after APH treatment (Figures 2B, c and d, and 2C). In addition, we observed colocalization of FMRP and γH2A.X in MEFs treated with LPB (Figures S3B and S3C). Although the significance of FMRP colocalization with CENT B, CMCs, and γH2A.X foci requires further investigation, the above data nevertheless indicate that FMRP accumulates at specific chromatin domains and this accumulation can be increased upon replication stress, supporting our biochemical data (Figure 2A).

FMRP Binds Chromatin via Its N-Terminal Agenet Domain, and This Interaction Is Critical for FMRP Function in the DDR

What is the molecular basis for the observed chromatin association of FMRP? FMRP contains an N-terminal Agenet domain (Agenet$_{FMRP}$), which is a double-tudor domain that belongs to the Royal family of chromatin-binding proteins (Maurer-Stroh et al., 2003; Ramos et al., 2006). Interestingly, the Agenet domain was recently shown to bind histone substrates methylated at various lysine residues (Adams-Cioaba et al., 2010; Sabra et al., 2013). This led us to hypothesize that FMRP might target chromatin through its Agenet domain. Agenet$_{FMRP}$ consists of two adjacent Tudor domains, termed N-terminal domain of FMRP1 (NDF1) and N-terminal domain of FMRP2 (NDF2) (Ramos et al., 2006; Figure 3A). NMR studies identified residues T102 and Y103 on the surface of NDF2 as important for binding trimethylated lysine (Ramos et al., 2006; Figure 3A). Mutating T102 and Y103 to A and L, respectively (T102A and Y103L), significantly compromised FMRP binding to native nucleosomes isolated from HeLa cells (Figure 3B, compare lane 3 with lanes 4 and 5), indicating that Agenet$_{FMRP}$ is required for FMRP association with nucleosomal substrates. We next explored the possibility that methyl-lysine recognition is involved in FMRP binding to chromatin. We used a panel of recombinant *Xenopus* histones carrying methyl-lysine analogs at various positions (Simon et al., 2007) in in vitro binding reactions with Agenet$_{FMRP}$. Agenet$_{FMRP}$ did not show a significant interaction with unmethylated histone H3, but bound histone H3 containing methyl-lysine analogs at several positions (Figure S4A). Full-length FMRP also bound methylated, but not unmethylated, histone H3 (data not shown).

We next carried out microscale thermophoresis (MST) (Jerabek-Willemsen et al., 2011; Wienken et al., 2010) in order to understand the dynamics of Agenet$_{FMRP}$ binding to various histone methylation marks. Consistent with the biochemical binding data, we found that Agenet$_{FMRP}$ exhibited higher affinity for histone H3 carrying lysine methylation mimics, including H3Kc79me2 (K_d 135 ± 28 nM; Figure S4B) and H3Kc27me1 (K_d 102 ± 11 nM; Figure S4C) as compared with unmethylated H3 (K_d 1063 ± 136 nM; Figure S4D). Both the biochemical and

MST data suggest that $Agenet_{FMRP}$ preferentially binds methylated histone H3, but does not display significant methyl site specificity in vitro. Importantly, $Agenet_{FMRP}$ mutations that abolish FMRP binding to native chromatin (Figures 3A and 3B) also interfered with $AgenetKHKH_{FMRP}$ (Agenet and two adjacent nucleic acid binding domains) binding to the in vitro assembled methylated MLA nucleosomes (H3Kc79me2; Figure S5A, compare lanes 3–5). Collectively, these data demonstrate that $Agenet_{FMRP}$ is necessary and sufficient for FMRP binding to chromatin, which might involve a sequence-independent methyl-lysine recognition function of $Agenet_{FMRP}$.

FMRP Binding to Chromatin Is Required for FMRP-Dependent Modulation of γH2A.X Levels in Response to Replication Stress

We next carried out genetic complementation experiments to investigate potential functional roles of FMRP chromatin association in the DDR. FMRP KO MEFs were reconstituted with wild-type or mutant forms of FMRP (T102A and Y103L), which are compromised in their ability to bind nucleosomes. Wild-type FMRP (Figure 3C, lanes 1 and 2) was more effective than the mutant forms of FMRP (Figures 3C, lanes 3–6, and S1D, which shows comparable expression of wild-type and mutant FMRP proteins) in conferring the induction of H2A.X phosphorylation in the mouse FMRP KO MEF cells in response to APH treatment (12.8-fold γH2A.X increase with the wild-type FMRP and 4- and 3-fold γH2A.X increase with the Y103L and T102A mutants, respectively). Similar results were obtained with HeLa cells in which the endogenous FMRP was inhibited by RNAi, and which were then complemented with either the wild-type FMRP or the FMRP Agenet domain mutants. As shown in Figure 3D, wild-type FMRP conferred a significantly higher level of γH2A.X response (9-fold induction, compare lanes 5 and 6, third panel from the top) than the Agenet point mutants (T102A and Y103L; 3-fold γH2A.X induction, compare lanes 7 and 8, and lanes 9 and 10, third panel from the top). These findings suggest that the recruitment of FMRP to chromatin is critical for FMRP-dependent regulation of H2A.X phosphorylation.

FMRP Mutants Defective in Supporting H2A.X Phosphorylation Are Not Compromised in Their Ability to Modulate Translation-Dependent AMPAR Trafficking

FMRP has a well-documented role in regulating activity-dependent synaptic translation of a specific subset of mRNAs, which is important for the maintenance of synaptic plasticity (Bassell and Warren, 2008; Bear et al., 2004; Brown et al., 2001; O'Donnell and Warren, 2002). Previous studies showed that a reduction of FMRP in dendrites leads to an excessive internalization of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) subunit GluR1 (Nakamoto et al., 2007), which is a critical process for the maintenance of synaptic plasticity. This finding provided the foundation for the mGluR theory of fragile X syndrome (Bear et al., 2004). We asked whether the chromatin-binding-defective FMRP point mutants were also compromised in their ability to dampen AMPAR internalization. As expected, immunofluorescence staining showed that FMRP KO neurons exhibited less AMPAR signal remaining on the surface and more internalized AMPAR signal relative to wild-type neurons (Figure S5B, compare panels 1 and 2). Quantitatively, the ratio of internalized to total AMPARs was increased in neurons isolated from *Fmr1* KO mice as compared with wild-type neurons (Figure S5C, compare boxplots 1 and 2). Importantly, the FMRP chromatin-binding-defective mutants were able to rescue this AMPAR trafficking defect similarly to the wild-type FMRP (Figures S5B, panels 3–5, and S5C [compare boxplot 1 with boxplots 2 and 3–5]). These findings indicate that the newly identified role of FMRP in the DDR is mechanistically distinct from its canonical function in modulating synaptic strength.

FMRP Patient Mutant R138Q Is Defective in Mediating DDR Events, but Retains Normal Translation-Dependent AMPAR Internalization

Recently, a novel FMRP sequence variant, R138Q, was found in a developmentally delayed male without the typical CGG-repeat expansion in the 5′ UTR of the *FMR1* gene (Collins et al., 2010). Because the R138Q mutation lies near the extreme C terminus of $Agenet_{FMRP}$ (Figure S6A), we investigated whether this patient mutation affects FMRP nucleosomal binding. As shown in Figure 4A, the FMRP R138Q mutant failed to bind native nucleosomes (compare lanes 3 and 4) as well as recombinant H3Kc79me2 nucleosome (Figures 4B, S5A [lane 6], and S6B, which shows comparable levels of wild-type and R138Q recombinant proteins used for the binding assays). Importantly, the R138Q mutant also failed to confer γH2A.X induction in the FMRP KO MEFs in response to replication stress (Figures 4C [compare lanes 1–6 with lanes 7–12] and S6C, which shows comparable levels of expression of wild-type and R138Q reconstituted in the FMRP KO MEF cells). In addition to the γH2A.X defect, the R138Q FMRP mutant did not effectively support the formation of BRCA1 foci and phosphorylation of BRCA1 at Ser-1423 in FMRP KO MEFs in response to APH treatment as compared with wild-type FMRP (Figures 4D–4G and S6D). In addition, we observed an increased incidence of single-strand DNA intermediates (as indicated by RPA32 staining) in FMRP KO MEFs rescued with the R138Q mutant as compared with wild-type FMRP, suggesting a repair defect (compare Figures 4H and 4I; quantification in Figures 4J and 4K). Importantly, RPA32 staining associated with CMCs was also increased in FMRP KO MEFs complemented with R138Q, suggesting a possible functional significance of FMRP targeting to CMCs in the context of the DDR (Figures 4H and 4I, bottom, arrows). FMRP KO MEFs reconstituted with the R138Q mutant were also more sensitive to increasing concentrations of HU as compared with wild-type FMRP reconstituted cells in the clonogenic survival assay (Figure S6E). In contrast, the FMRP R138Q mutant functioned similarly to wild-type FMRP in suppressing excessive AMPAR internalization in FMRP KO neurons (Figure S6F [compare panels 3 and 4] and S6G [compare boxplots 3 and 4]). Taken together, these results suggest the tantalizing possibility that abrogation of this newly identified nuclear function of FMRP in the DDR may lead to a DDR-dependent clinical phenotype.

FMRP Is Loaded onto Chromosomes during Male Meiosis and Regulates Placement of γH2AX

The above findings provide strong support for a role of FMRP in the DDR via its association with chromatin. However, the

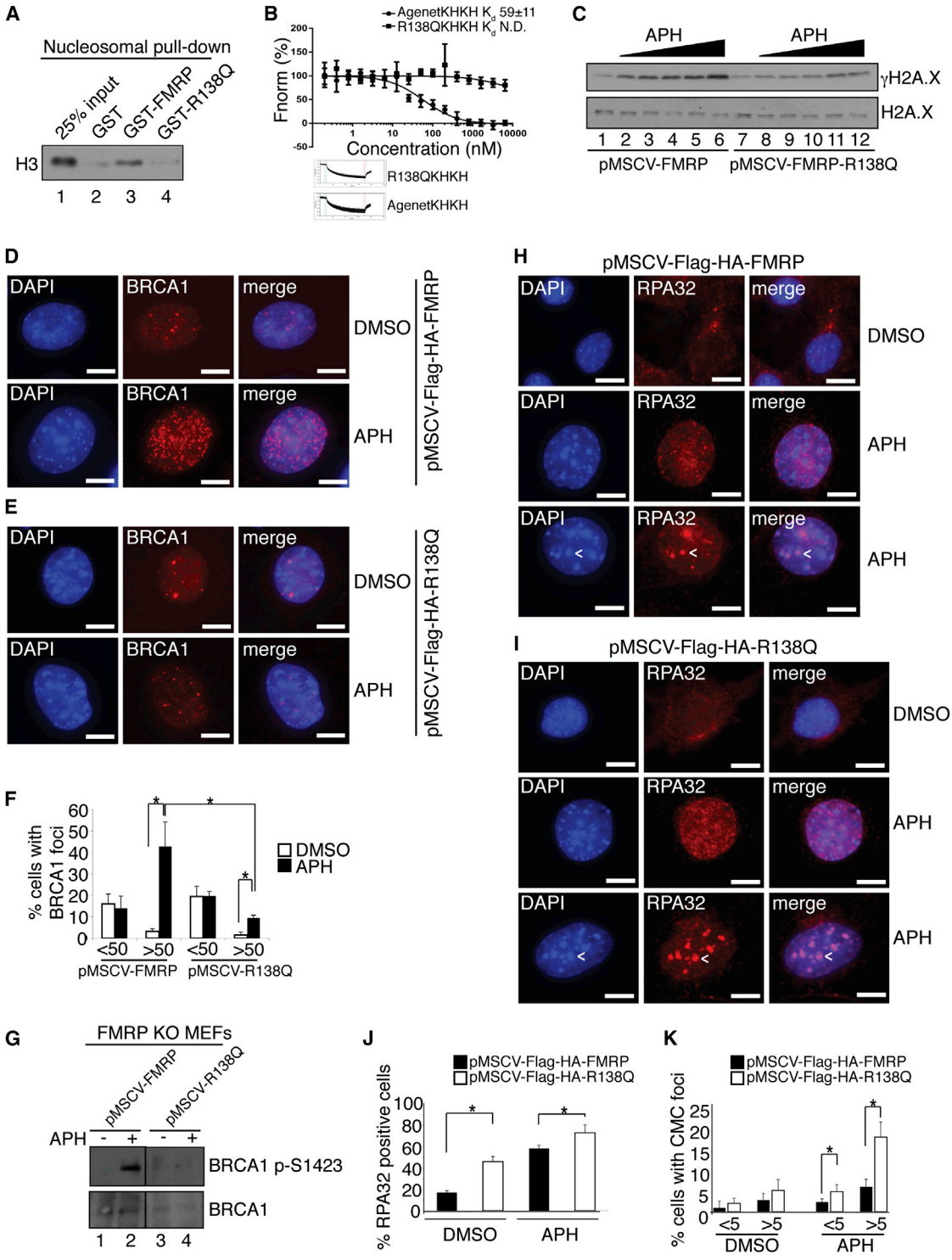

Figure 4. Patient Mutant R138Q Is Defective in γH2A.X Induction and BRCA1 Foci Formation and Promotes Excessive RPA Retention on Chromatin

(A) Unlike WT FMRP, the R138Q FMRP mutant failed to bind nucleosomes in vitro (compare lanes 3 and 4).

(B) Equilibrium binding analysis using recombinant MLA nucleosomes dimethylated at H3K79 and WT AgenetKHKH (K_d = 59 nM) or R138QKHKH (binding not detected). See also Figures S5A and S6B.

(C) FMRP KO MEFs rescued with WT FMRP, but not the R138Q FMRP patient mutant, exhibited a dose-dependent γH2A.X response triggered by APH (0.05 μM, 0.1 μM, 0.3 μM, 0.5 μM, 1 μM). See also Figure S6C.

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biological significance of this finding was unclear. In this regard, mammalian meiosis represents perhaps the most relevant biological process in which extensive DNA damage and recombinogenic events normally occur. In wild-type meiotic cells, DSBs are generated during prophase by the topoisomerase-like enzyme SPO11, and form sites for homologous recombination and crossing over. DSBs accumulate γH2A.X and recruit many components of the somatic DDR machinery, including ATR and BRCA1. Repair then occurs in a highly regulated fashion, accompanied by pairing of homologous chromosomes (synapsis) and recombination between homologs (Blanco-Rodríguez, 2012; Garcia-Cruz et al., 2009; Turner et al., 2004, 2005). Importantly, in addition to defects in synaptic signaling in neurons, male fragile X patients exhibit macroorchidism and *Fmr1* KO mouse ovaries display premature follicular overdevelopment (Ascano et al., 2012; Turner et al., 1975, 1980). Meiotic germ cells are therefore a relevant biological context in which to analyze the association of FMRP with chromatin in the DDR in vivo.

We used a mouse *Fmr1* KO model to investigate whether FMRP is associated with chromatin and the DDR during mammalian meiosis. All mouse experiments were approved by the animal care and use committee at the appropriate institution. We first asked whether FMRP is present in the germ cell nucleus during meiosis. We performed immunostaining on chromosome spreads of adult male spermatocytes in meiotic prophase. Strikingly, we identified distinct FMRP puncta on condensed pachytene-stage chromosomes (Figure 5A). These puncta were aligned along the chromosomes, as visualized by costaining for the synaptonemal complex (SC) component SYCP1. FMRP puncta were not found on the chromosomes in *Fmr1* KO cells, confirming the specificity of the antibody staining (Figure S7A). We conclude that FMRP is present in the nucleus during meiotic pachytene and is localized on or near the chromatin at this stage.

In wild-type meiotic cells, γH2A.X accumulates throughout the nucleus during the leptotene and zygotene stages of prophase concomitantly with DSB formation, but is removed from the chromosomes as repair proceeds and is absent from the autosomes by the pachytene stage. In males, the X and Y chromosomes retain γH2A.X during pachytene because these two chromosomes lack homologs and cannot fully synapse, and repair is delayed (Handel and Schimenti, 2010). Analogously, in mutants with defective repair and synapsis machinery, γH2A.X and other components of the DDR pathway are retained at unrepaired regions on the autosomes (Turner et al., 2005). We asked whether deposition of γH2A.X during meiotic prophase was impaired in *Fmr1* KO cells. *Fmr1* KO spermatocytes exhibited two distinct defects in γH2A.X accumulation: (1) reduced deposition of γH2A.X during the leptotene stage, and (2) inappropriate retention of γH2A.X on autosomes during pachytene (Figure 5B). This phenotype was not the result of delayed or impaired formation of DSBs, since there was no difference in SPO11 staining between wild-type and KO cells (Figure S7B). These defects were evident in only a subset of cells (Figure 5C), perhaps explaining the preserved fertility of the *Fmr1* KO males.

Fmr1 Mutant Mice Exhibit Defective Chromosome Synapsis and Defective Resolution of Single-Strand Intermediates during Meiotic Prophase

In wild-type meiotic cells, the RAD51 homolog DMC1 associates with the single-strand intermediates produced during DSB repair and facilitates invasion of the homologous chromosome, allowing recombination (Pittman et al., 1998; Schwacha and Kleckner, 1997; Yoshida et al., 1998). This process occurs during the zygotene stage and is largely complete by pachytene, by which time most DMC1 has dissociated from the chromosomes. Successful strand invasion catalyzed by DMC1 is required to proceed with repair and crossing over, including recruitment of the MLH1/MLH3 heterodimer during middle to late pachytene (Moens et al., 2002; Pittman et al., 1998; Yoshida et al., 1998). To determine whether single-strand intermediates were resolved in meiotic cells in the absence of FMRP, we costained pachytene nuclei with DMC1 and MLH1. We found that *Fmr1* KO midpachytene spermatocytes inappropriately retained high levels of DMC1 on the chromosomes (Figures 6A and 6B), associated with reduced recruitment of MLH1 (Figures 6A, 6C, and 6D). These findings suggest that resolution of single-strand DNA repair intermediates is delayed in meiotic germ cells in the absence of FMRP, resulting in impaired crossover formation.

Consistent with a failure to repair DNA breaks, we found that BRCA1 and ATR were also inappropriately retained on the chromosomes in pachytene spermatocytes. BRCA1 and ATR were restricted to the unpaired X and Y chromosomes in wild-type spermatocytes, but were present on regions of the autosomes in *Fmr1* KO spermatocytes (Figures 7A–7C). BRCA1 and ATR staining on the sex chromosomes was also

(D and E) BRCA1 foci formation in FMRP KO MEFs rescued with WT FMRP (D) in response to APH was more pronounced as compared with FMRP KO MEFs rescued with the R138Q FMRP patient mutant (E). See also Figure S6D.
(F) Forty percent of FMRP KO MEFs rescued with WT FMRP exhibited >50 BRCA1 foci per cell upon APH treatment, compared with 10% in MEFs rescued with the R138Q patient mutant.
(G) BRCA1 S1423 phosphorylation in FMRP KO MEFs rescued with WT FMRP in response to APH was more pronounced as compared with rescue with the R138Q FMRP patient mutant (compare lanes 2 and 4).
(H and I) RPA32 foci formation in FMRP KO MEFs rescued with WT FMRP in response to APH was less pronounced as compared with FMRP KO MEFs rescued with the R138Q patient mutant (compare middle panels in (H) and (I). Note the accumulation of a subset of RPA32 foci at CMCs (arrowheads, lower panels).
(J and K) Quantification of total (J) and CMC-associated (K) RPA32 foci in FMRP KO MEFs rescued with WT FMRP and R138Q patient mutant in response to APH. The percentage of cells positive for RPA32 increased from 10% to 50% upon APH treatment after rescue with WT FMRP, and from 40% to 70% after rescue with the R138Q mutant. Note increased numbers of RPA32-positive cells in the case of R138Q mutant rescue MEFs even in the absence of APH treatment.
(K) Seventeen percent of R138Q mutant rescue MEFs and 6% of WT FMRP rescue MEFs had more than five CMC-associated RPA32 foci upon APH treatment.
*$p < 0.05$, Student's t test. Data are an average of three independent experiments with SD. Scale bars, 10 μm.
See also Figure S6D.

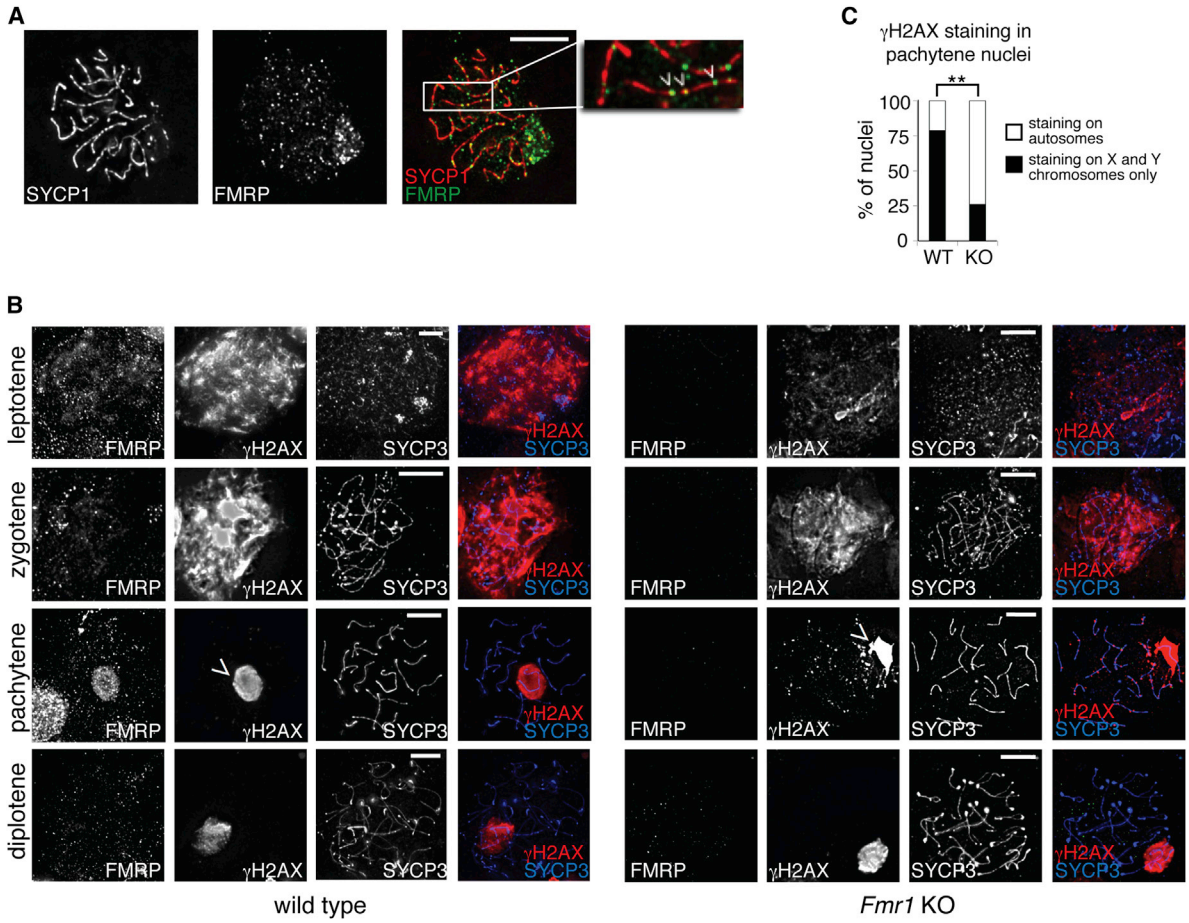

Figure 5. FMRP Is Present on Meiotic Chromosomes and Regulates Placement of γH2A.X

Immunofluorescence staining was performed on spread chromosomes from adult male primary spermatocytes, and cells were imaged by deconvolution microscopy.

(A) Pachytene stage nucleus showing FMRP puncta along the chromosomes. SYCP1 marks the full length of the autosomes during the pachytene stage. Inset shows FMRP puncta (green) aligned along SYCP1-stained chromosomes (red). See also Figure S7A.

(B) γH2A.X and FMRP staining in WT (left) and *Fmr1* KO (right) primary spermatocyte nuclei at leptotene, zygotene, pachytene, and diplotene stages of meiotic prophase. SYCP3 accumulates on chromosomes beginning in leptotene and is present along their full length during pachytene. In *Fmr1* KO cells, accumulation of γH2A.X is delayed in the leptotene stage. At the pachytene stage, γH2A.X is restricted to the sex chromosomes (arrowheads) in WT cells, but remains at some autosomal locations in *Fmr1* KO cells. Scale bars, 10 μm.

(C) Percentage of cells retaining γH2A.X outside of the sex chromosomes in WT and KO pachytene spermatocytes. **$p < 0.01$, Fisher's exact test. See also Figure S7B.

discontinuous in *Fmr1* KO spermatocytes, but continuous in wild-type cells. Similar to the defects in γH2A.X deposition, DMC1 retention, and MLH1 recruitment, these BRCA1 and ATR localization phenotypes varied between cells: some KO cells exhibited autosomal BRCA1 and ATR staining, whereas others resembled wild-type cells (Figure 7C).

Because failure to resolve DSBs and to form interhomolog crossovers is also associated with defective synapsis, we next asked whether *Fmr1* KO spermatocytes also displayed synapsis defects. SYCP3, a lateral element of the SC, assembles on unpaired chromosomes during early prophase, whereas SYCP1, a central element of the SC, assembles only on synapsed chromosomes (Fraune et al., 2012). We found that whereas wild-type pachytene nuclei had continuous SYCP1 staining along the chromosomes, many *Fmr1* KO nuclei had discontinuous SYCP1 staining, indicating that SC formation was not complete (Figure 7D; Bishop et al., 1992; Pittman et al., 1998; Yoshida et al., 1998). Taken together, these findings suggest that resolution of single-strand repair intermediates, crossing over, and subsequent pairing of homologous chromosomes during meiotic prophase are incomplete in a subset of spermatocytes lacking FMRP.

Histone H3K79 Methylation Plays a Role in the Recruitment of FMRP to Chromatin In Vivo

As described above, both the $Agenet_{FMRP}$ and a full-length FMRP bind histone substrates in a methyl-lysine-dependent manner (Figure S4 and data not shown). However, it remains

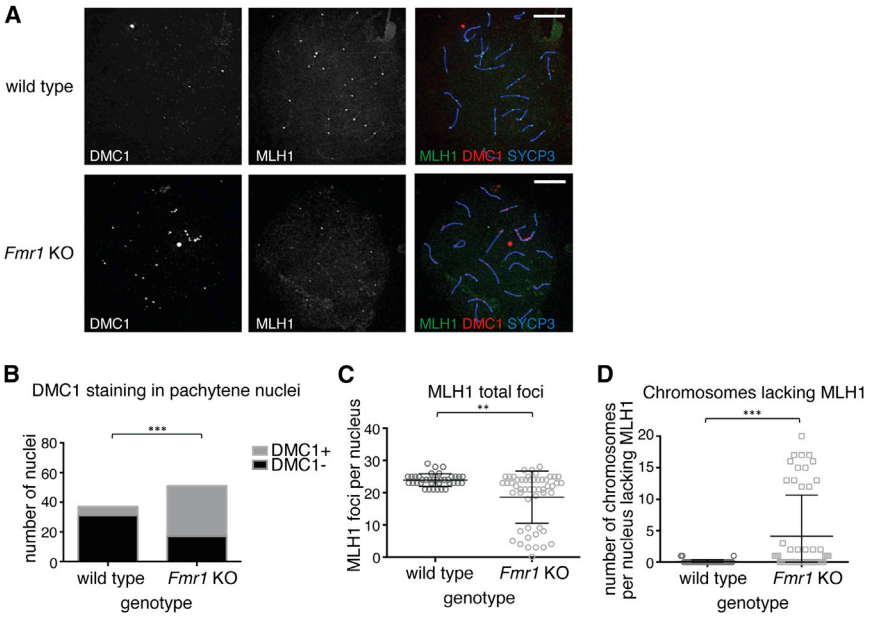

Figure 6. *Fmr1* KO Spermatocytes Exhibit DNA Repair Defects and Delayed Resolution of Single-Strand Intermediates at the Pachytene Stage

Staining of chromosome spreads was performed as in Figure 5.

(A) Costaining of DMC1, MLH1, and the SC component SYCP3, showing retention of DMC1 and reduction of MLH1 in *Fmr1* KO cells at midpachytene.

(B) Numbers of WT and KO cells positive for DMC1 staining at midpachytene. ***$p < 0.0001$, Fisher's exact test.

(C) MLH1 foci per midpachytene nucleus in WT and KO. **$p < 0.01$, Mann-Whitney U test.

(D) Number of chromosomes per midpachytene nucleus lacking MLH1 foci. In WT cells, there is at least one MLH1 focus per chromosome. ***$p < 0.0001$, Mann-Whitney U test. Scale bars, 10 μm.

unclear whether FMRP binds methyl histones with some specificity in vivo and which methyltransferases are necessary for FMRP chromatin recruitment. Dot1, the H3K79 methyltransferase, has been shown to play a role in yeast meiosis (Ontoso et al., 2013; San-Segundo and Roeder, 2000). In addition, recent reports demonstrated an increase in H3K79me2 and H3K79me3 levels in mouse spermatocytes in pachytene, with H3K79me3 specifically enriched at the sex chromosomes and centromeres (Ontoso et al., 2014). As a first step toward understanding the role of histone methylation in FMRP recruitment, we generated mice conditionally lacking DOT1L (*Dot1L* cKO; Figures S7C–S7E; Bernt et al., 2011), the only known mammalian H3K79 methyltransferase, in the germ cells, and stained meiotic spreads for FMRP. We found a small but significant reduction in the number of chromatin-associated FMRP puncta in the *Dot1L* cKO. This effect was especially evident on the X and Y chromosomes, where FMRP is particularly abundant during pachytene (Figures 7E and 7F). Importantly, similar to FMRP KO MEFs, *Dot1L* mutant MEFs exhibited reduced γH2A.X foci formation in response to APH (Figure S1C, right) as well as an increased sensitivity to increasing concentrations of APH compared with wild-type MEFs (Figure S2C). We conclude that methylated H3K79 might function in the same DDR pathway as FMRP and help to recruit or retain FMRP at chromatin associated with DNA damage repair intermediates.

DISCUSSION

We have identified FMRP as a chromatin-binding protein and uncovered a novel and unanticipated function for FMRP in the nucleus, where it regulates the DDR. In addition, we uncovered a biological role for the DDR function of FMRP during mammalian spermatogenesis. We provide strong evidence that the Agenet domain binds histone H3 in a methylation-dependent manner, without displaying an overt preference toward a specific methyl-lysine site. Conceivably, however, the binding specificity could be enhanced in vivo. Our preliminary data consistently showed that the histone H3K79 methyltransferase DOT1L is important for FMRP chromatin association during meiosis, suggesting that H3K79 methylation may play a role in FMRP chromatin targeting in vivo. Our current data do not exclude the possibility that FMRP may also be capable of binding other methylated targets, such as nucleic acids.

This newly identified function of FMRP in the replication stress response appears to be independent of the classical role of FMRP in maintaining synaptic plasticity via translational regulation. Instead, nuclear FMRP may function in the DNA repair pathways through chromatin association. Our finding is consistent with published observations, including the report that FMRP interacts with poly(ADP-ribose) glycohydrolase (PARG) and poly (ADP-ribose) polymerase (PARP), which are major modulators of genomic stability (Gagné et al., 2005; Isabelle et al., 2010, Ciccia and Elledge, 2010; Haince et al., 2007). The fact that the DNA topoisomerase TOP3β is present in FMRP-containing mRNPs, is involved in neuronal development and genomic stability, and contributes to germ cell development suggests yet another intriguing connection between FMRP and the DDR. It is interesting to note that similar to FMRP, TOP3β is also associated with XY bivalents during pachytene (Kwan et al., 2003). We speculate that FMRP performs a docking function to regulate the chromatin accessibility of DDR proteins. Although the detailed molecular mechanisms of FMRP-dependent DDR await further clarification, our data on the connection of FMRP with chromatin in the DDR represent an important advance in our understanding of FMRP function.

Importantly, DDR events such as γH2A.X induction and ATR/BRCA1 signaling heavily influence meiosis, specifically crossover formation and synapsis (Turner et al., 2004, 2005). Defects in synapsis can lead to chromosome nondisjunction, resulting in impaired gamete development or the generation of aneuploid gametes and developmental defects in the resulting embryo (Handel and Schimenti, 2010). Our findings suggest that the

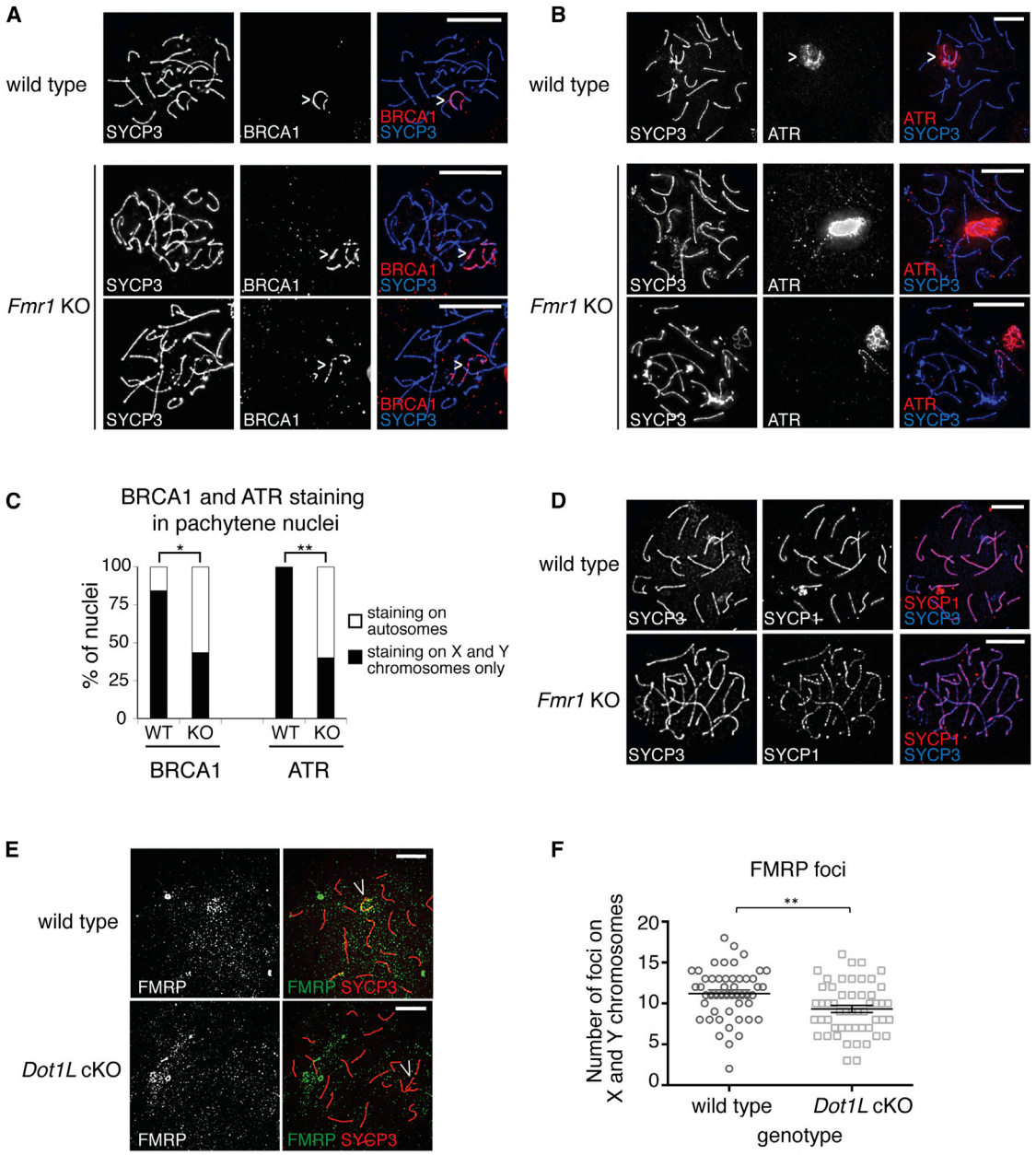

Figure 7. Abnormal BRCA1 and ATR Loading and Synapsis Defects in *Fmr1* KO Spermatocytes

(A) Sample images of BRCA1 staining in pachytene spermatocytes in WT and KO animals. In WT, BRCA1 staining is continuous and restricted to the sex chromosomes (arrowhead). In KO, it is discontinuous and frequently present on the autosomes. SYCP3 marks the chromosomes.

(B) Sample images of ATR staining in pachytene spermatocytes in WT and KO animals. In WT, ATR staining forms a cloud around the sex chromosomes (arrowhead) and is absent from the autosomes. In KO, ATR staining is retained in puncta on the autosomes and sometimes coats a complete autosome (bottom panels). SYCP3 marks the chromosomes.

(C) Percentage of cells that retained BRCA1 or ATR outside of the sex chromosomes in WT and KO spermatocytes. *$p < 0.05$; **$p < 0.01$, Fisher's exact test.

(D) Costaining of lateral (SYCP3) and central (SYCP1) elements of the SC shows discontinuous SYCP1 staining in *Fmr1* KO cells, indicating defective SC formation.

(E and F) Methylated H3K79 helps to recruit FMRP to chromatin.

(E) Staining of FMRP in pachytene spermatocyte spreads from WT and *Dot1L* cKO mutants. Chromosome-associated FMRP signal is reduced in cKO cells, especially near the X and Y chromosomes. SYCP3 marks the chromosomes.

(F) Quantitation of X- and Y-chromosome-associated FMRP foci. **$p < 0.01$, unpaired t test. Scale bars, 10 μm.

See also Figures S1C and S7C–S7E.

rate of germline chromosomal instability among *Fmr1* KO mice or fragile X patients at sites outside the fragile X locus may be elevated. This hypothesis is supported by a recent study that described increased rates of DNA damage and apoptosis in spermatocytes of *Fmr1* KO mice (Tian et al., 2013). In addition, low FMRP levels were correlated with spermatogenesis defects in patients with maturation arrest (Tian et al., 2013). Thus, our findings provide a potential molecular mechanism for the DNA damage, apoptosis, and spermatogenesis defects observed in mice and patients lacking FMRP.

Interestingly, in yeast *Dot1* mutants, meiotic cells exhibit increased levels of unrepaired DNA damage and proceed through sporulation to produce mature spores with poor viability (San-Segundo and Roeder, 2000). In mouse spermatocytes, DOT1L chromatin loading and H3K79 methylation are dynamically regulated during meiosis. In particular, H3K79me3 and DOT1L protein accumulate at the sex chromosomes, and H3K79me3 accumulates at centromeres during the pachytene stage (Ontoso et al., 2014). Our finding that FMRP is depleted at the sex chromosomes in *Dot1L* conditional mutants supports an interaction between FMRP and methylated histones during meiosis, and raises the possibility that H3K79 methylation may be important for FMRP chromatin association in vivo. Interestingly, the Tudor domain of Survival Motor Neuron protein (SMN), which carries a methyl-lysine interacting surface similar to that of the FMRP Agenet domain (Ramos et al., 2006), was recently shown to interact with H3K79me1/2 in a DOT1L-dependent manner (Sabra et al., 2013)

Macroorchidism is a hallmark of fragile X syndrome, but little is known with respect to its etiology. Malformed spermatids have been observed in both human fragile X patients and *Fmr1* KO mice, suggesting a defect in sperm development (Slegtenhorst-Eegdeman et al., 1998; Johannisson et al., 1987). Adult male patients carrying the full fragile X repeat expansion produce sperm that carry a contracted premutation but never the full expansion (Reyniers et al., 1993), implying that sperms carrying a full mutation are selected against at a premeiotic stage, allowing only those with a contracted *FMR1* repeat to reach maturity (Bächner et al., 1993; Malter et al., 1997). Our finding that spermatocytes lacking FMRP exhibit defects in chromosome synapsis during meiotic prophase lends support to this model, and suggests a mechanism for this effect.

The idea of a functional involvement of FMRP in the DDR is especially appealing given recent evidence pointing to FMRP as a prosurvival protein. The absence of FMRP promotes apoptosis (Jeon et al., 2011) and telomere erosion in fragile X patients, which is a major hallmark of genomic instability (Jenkins et al., 2008). In addition, fragile X patients have been reported to display a lower incidence of cancer (Schultz-Pedersen et al., 2001), whereas an increase in FMRP levels promotes tumor metastasis (Lucá et al., 2013). Lastly, given that the loss of FMRP function leads to a common form of intellectual disability and autism, it is tempting to speculate that the role of FMRP in the DDR might represent a novel, previously unappreciated contributing factor in the development of fragile X syndrome. Interestingly, a forward genetic screen in *Drosophila* identified 26 missense mutations in the N terminus of dFMRP that affect axonal development (Reeve et al., 2005). Some of these mutations are localized to the dFMRP Agenet domain and are predicted to impact the ability of dFMRP to bind chromatin. It has been suggested that the Agenet domain may also play a role in the translation-independent function of FMRP in synaptic signaling (Deng et al., 2013). Therefore, it remains to be determined whether, in addition to its role in germ cell meiosis reported here, this nuclear function of FMRP also affects neuronal development, and whether the loss of FMRP has any DDR-related consequences in patients with fragile X syndrome.

EXPERIMENTAL PROCEDURES

Native Nucleosome Binding Reactions

Reactions were performed in the presence of binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100) using 100 ng of glutathione S-transferase (GST) fusion proteins and 5 μg of native nucleosomes isolated from HeLa cells at +4°C, and rotated for 2 hr before addition of glutathione agarose beads (GE Healthcare). Beads were washed four times with binding buffer. Three independent experiments were performed.

MLA Nucleosome Binding Reactions

Mononucleosomes were prepared as described previously (Lu et al., 2008). The reactions were performed similarly to native nucleosome binding reactions, but using 2 μg of MLA nucleosomes. Three independent experiments were performed.

γH2A.X Induction Rescue Experiments

HeLa cells were transfected with FMRP small hairpin RNA (shRNA) or control scramble shRNA. Scramble shRNA was cotransfected with empty backbone vector (POZ-Flag-HA). FMRP shRNA was cotransfected with Flag-HA vector alone or with rescue vectors expressing either wild-type or mutant forms of FMRP (Flag-HA-FMRP, Flag-HA-T102Y, or Flag-HA-Y103L). At 3 days post-transfection, cells were treated with DMSO or APH (0.5 μM) for 24 hr and then lysed in SDS sample buffer. Samples were subjected to western blotting. FMRP KO MEF rescue experiments were performed identically to HeLa rescue experiments, except that rescue constructs were introduced into cells using the pMSCV-Flag-HA viral system. Three independent experiments were performed.

FMRP Chromatin Recruitment Experiments

Chromatin fractionation experiments were adopted from Méndez and Stillman (2000). Briefly, after 1 μM APH treatment of MEFs for 24 hr, chromatin was isolated by resuspending cells in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 3 mM EDTA, 0.5% Triton X-100, and protease inhibitor cocktail [Roche]) and nuclei were collected by low-speed centrifugation (4 min, 1,300 × *g*), washed once in buffer A, and lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitor cocktail [Roche]). Insoluble chromatin was collected by centrifugation (4 min, 1,700 × *g*), washed again in buffer B, and centrifuged again. The final chromatin pellet was resuspended in Laemmli buffer, sonicated, and boiled for 15 min. Total protein lysate for determination of total protein levels was aliquoted from cells still resuspended in buffer A. All procedures were performed at +4°C. Three independent experiments were performed.

Immunofluorescence Experiments

MEFs were treated with 10 ng/ml of LPB for 24 hr in the presence or absence of 0.5 μM APH. Cells were then fixed with ice-cold methanol, stained with the antibodies of interest, and mounted using DAPI mounting medium (Vectashield). MEFs were counted according to the number of nuclear FMRP foci or large CMC-associated FMRP domains (CMCs) after LPB+DMSO or LPB+APH treatment (24 hr). When Bethyl anti-BRCA1 and anti-RPA32 rabbit antibodies were used for staining, cells were extracted with CSK buffer (10 mM HEPES pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 30 min at room temperature and then fixed in 4%

paraformaldehyde (PFA) for 10 min, followed by washes in PBS and immunostaining. A total of 100 cells were counted in three independent experiments.

Preparation of Meiotic Chromosome Spreads

Male *Fmr1*^{KO}/Y and +/Y or *Dot1L* $^{\Delta/\Delta}$ and *Dot1L*$^{fl/+}$ littermates were sacrificed at 7 weeks of age. At least two individuals of each genotype were used for each experiment. The spread preparation protocol was modified from Peters et al. (1997). Testes were immersed in Dulbecco's modified Eagle's medium and the tunicae were removed. The separated tubule suspension was spun for 8 min at 1,000 × *g*, and cells were resuspended in 1 ml hypo-buffer (30 mM Tris-HCl pH 8.2, 50 mM sucrose pH 8.2, 17 mM sodium citrate) and incubated for 7 min at room temperature. The cell suspension was split into five tubes and spun for 8 min at 1,000 rpm. It was then resuspended in 170 μl 0.1 M sucrose and dropped onto the slides, and allowed to spread for 2–3 hr. Slides were prepared with 1% PFA with 0.1% Triton X-100, pH 9.2. For staining, slides were blocked in 3% BSA for 1 hr, incubated with primary antibody in 1% BSA overnight at 4°C, and then incubated with secondary antibody in 1% BSA for 1 hr at room temperature. Imaging was performed on a DeltaVision Elite deconvolution imaging system (Applied Precision) at 60× or 100× magnification. Stacks were compressed and analyzed using ImageJ software. The morphology of SYCP3-stained chromosomes was used to determine the stage of prophase.

For further details regarding the materials and methods used in this work, see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.03.040.

AUTHOR CONTRIBUTIONS

R.A. and Y.S. designed the study and cowrote the manuscript. R.A. analyzed the data, performed histone pull-downs, cloning, mutagenesis, in vitro binding experiments, microscale thermophoresis, FMRP RNAi experiments, DNA damage experiments, MEF reconstitution experiments, survival assays, chromatin recruitment experiments, and immunofluorescence experiments. B.J.L. performed experiments using *Fmr1* KO and *Dot1L* cKO mice, performed meiotic chromosome spreads and immunostaining, analyzed data, and cowrote the manuscript. M.N.K. performed AMPAR internalization experiments and data analysis, and contributed to the writing of the manuscript. A.B. performed BRCA1 rescue experiments. S.C., M.A., and C.X. performed in vitro binding assays with MLA histones, histone peptides, FMRP, and Agenet. J. Murn developed FMRP KO rescue cell lines. S.P. analyzed MST data. M.D.S., K.J.A, and A.S. designed and assembled MLA nucleosomes. C.R.V. developed *Dot1L* mutant MEFs and commented on the manuscript. T.G.K. and J. Min supervised histone peptide binding assays. W.F. and R.E.K. supervised nucleosome binding and commented on the manuscript. D.C.P. and S.T.W. supervised experiments and contributed to the writing of the manuscript. Y.S. supervised and directed the studies.

ACKNOWLEDGMENTS

We thank S. Elledge and B. Yankner for advice and helpful suggestions; A. Ciccia for help with DNA damage experiments; D. Reinberg and P. Voigt for help with nucleosomal preparations; J. Griesbach, A. Lazic, and S. Duhr from NanoTemper Technologies, and K. Yamagata, Y. Zheng, and X. Shang for help with microscale thermophoresis and kinetic data analysis; and E. Greer, A. Alekseyenko, and G. Shanower for logistical support. We also thank J. Mowrey, S. Ceman, S. Keeney, and U. Fischer for reagents; M. Bear for *Fmr1* KO mice; S. Armstrong for *Dot1L*$^{fl/fl}$ mice; K. Luger, G. Narlikar, S. Ceman, and U. Fischer for helpful comments; and M. Goodheart, D. Cooper, E. Derby, L. Elow, K. Igarashi, L. Kolinski, L. Pomponi, and M. Schuck for technical assistance. We also thank our collaborators who contributed to this project but whose work was not included in the manuscript: B. Ren, U. Wagner, and the Ren laboratory for FMRP genome-wide analysis; K. Zhao and his group for H3K79me2 genome-wide analysis; A. Vaquero for Suv39h double-null MEFs; and G. Schotta and T. Jenuwein for Suv4-20h double null MEFs. This work was supported by NIH grants NCI118487 and MH096066 to Y.S., National Research Training Grant AG00222-7 to R.A., NRSA grant HD075591 and a Hope Funds for Cancer Research postdoctoral fellowship to B.J.L., and HHMI funding to D.C.P., and in part by NIH grants HD020521 and HD024064 to S.T.W. Y.S. is an American Cancer Society Research Professor, and is a cofounder of Constellation Pharmaceuticals and a member of its scientific advisory board.

Received: May 31, 2013
Revised: February 4, 2014
Accepted: March 20, 2014
Published: May 8, 2014

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