FMRP BRIDGES R-LOOPS AND DHX9 THROUGH DIRECT INTERACTIONS

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23   **ABSTRACT**

24   Fragile X Syndrome (FXS) occurs when mutations in the *FMR1* gene cause the absence or
25   dysfunction of FMRP, known mainly as a translation repressor. We recently showed that FXS
26   cells suffer genome-wide DNA double-strand breaks near R-loops under replication stress. The
27   expression of FMRP, and not an FMRP-I304N mutant of the K-homology 2 RNA-binding
28   domain, suppresses the R-loop-induced DNA breakage. These observations led us to
29   hypothesize that FMRP safeguards the genome through promotion of R-loop detection and/or
30   resolution. Here, we demonstrate that FMRP directly binds R-loops through multivalent
31   interactions between the carboxy-terminal intrinsically disordered region and the R-loop sub-
32   structures. We also show that the amino-terminal folded domain of FMRP directly binds DHX9,
33   an R-loop resolvase, in a KH2-dependent manner. The FMRP-DHX9 interaction is recapitulated
34   by co-immunoprecipitation in human cells. Our findings are consistent with a model in which
35   FMRP recruits DHX9 to R-loop forming sites by bridging their interaction through its amino-
36   and carboxy-termini, respectively.

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INTRODUCTION

Fragile X syndrome (FXS) is a neurodevelopmental disorder due to epigenetic silencing or loss-of-function mutations of the *FMR1* gene encoding FMRP (Ciaccio et al., 2017; Sitzmann et al., 2018). FMRP is a nuclear-cytoplasmic RNA binding protein that regulates multiple biological processes of its diverse mRNA substrates, including their maturation in the nucleus, nuclear export, cytoplasmic transport, and ultimately, their translation at the synapse (Banerjee et al., 2018; Sudhakaran et al., 2014; Zhou et al., 2017).

Additionally, studies have suggested that FMRP is also involved in genome maintenance, though its exact role in the nucleus is not clear (Dockendorff and Labrador, 2019). We recently demonstrated that FXS patient-derived cells accumulate genome-wide DNA double strand breaks (DSBs), particularly during replication stress (Chakraborty et al., 2020). We further demonstrated that the DSBs in FXS cells were associated with R-loops, which are three-stranded nucleic acid structures formed during transcription when the nascent RNA stably anneals to the template DNA strand, displacing the non-template DNA strand. Finally, we showed that expression of FMRP, but not the FMRP-I304N mutant, ameliorated the R-loop-induced DSBs. Thus, our work suggested a genome protective role of FMRP by preventing R-loop accumulation during replication-transcription conflict.

The ability of FMRP to participate in multiple processess in the cell is attributed to the presence of multiple domains and their relative 3D-organization. All FMRP splice variants contain two amino (N-)terminal methylated lysine-binding Agenet domains (Age1 and Age2), three K-homology (KH0, KH1, and KH2) RNA binding domains and a carboxy (C-)terminal intrinsically disordered region (C-IDR), which in the case of the predominant isoform 1, contains an RNA binding RGG-box. Additionally, the presence of a nuclear localization signal and a nuclear export signal allows FMRP to shuttle between the nucleus and the cytoplasm, with approximately 4% of FMRP detected in the nucleus (Feng et al., 1997b).

In this study, we investigated how FMRP promotes R-loop resolution by testing if FMRP directly interacts with R-loops and R-loop resolvases. We purified recombinant FMRP and measured its capacity to bind various nucleic acid structures by electrophoretic mobility shift (EMSA) assay. We present evidence that FMRP interacts directly with R-loop structures specifically through its C-IDR. We also present evidence of FMRP interacting with known R-loop regulator proteins in vivo and in vitro, suggesting that FMRP might mediate the interaction between these proteins and R-loop structures. Our study represents a significant advance in the understanding of the mechanisms through which FMRP promotes genome integrity upon replication stress.

RESULTS

We previously showed that FXS patient-derived cells lacking FMRP have elevated genome-wide DSBs near R-loop forming sites when undergoing replication stress by aphidicolin (APH), a DNA polymerase inhibitor (Chakraborty et al., 2020). We proposed that FMRP protects the genome by preventing DSBs during induced replication-transcription conflict. Here we asked whether FMRP alters its expression level and/or its cellular localization in response to APH (Figure 1A). First, the total level of FMRP remained the same with and without APH (Figure 1-figure supplement 1A). However, the nuclear fraction of FMRP increased from 18% in DMSO (vehicle)-treated control cells to 24-36% in APH treatment (Figure 1B). In contrast, GAPDH (cytoplasmic) and Histone H3 (nuclear) controls maintained their respective subcellular localization, with or without APH (Figure 1B). We concluded that FMRP has substantial nuclear fraction in human lymphoblastoids, and it becomes further enriched in the nucleus in response to replication stress. Next, we wanted to visualize the localization of FMRP relative to R-loops. Immunofluorescence microscopy revealed a distinct staining pattern of FMRP, which was distributed in the cytoplasm and at the periphery of the nucleus (Figure 1C & Figure 1-figure supplement 1B-D) in untreated and DMSO-treated cells. Upon induction with APH, FMRP was enriched in the nucleus, particularly at 0.3 μM APH, consistent with the chromatin fractionation experiments. RNA:DNA hybrid signals were also enhanced with APH, as we previously observed with fibroblasts derived from a FXS patient (Chakraborty et al., 2020). Notably, FMRP signals were closely associated with the RNA:DNA hybrid signals, suggesting a potential interaction between the protein and R-loops.

To test the ability of FMRP to directly bind R-loops, we resorted to recombinantly expressing and purifying full length FMRP, the N-terminal folded domain (N-Fold) and the C-terminal intrinsically disordered region (C-IDR) (Figure 2A and Figure 2-figure supplement 1A&C). We then measured their binding affinities for R-loops with and without RNA overhangs and R-loop sub-structures including ssDNA, dsDNA, RNA, and DNA:RNA hybrid (Figure 2B) in an electrophoretic mobility shift assay (EMSA). DNA:RNA hybrids with or without a 5' DNA overhang produced nearly identical results for all proteins and therefore only DNA:RNA without overhang is shown. First, we observed binding between both the N-Fold and C-IDR of FMRP to the R-loop with 5' RNA overhang (Figure 2C&D) and the aforementioned sub-structures of R-loops with varying affinities (Figure 2-figure supplements 2&3). Due to the high propensity of FMRP to aggregate and precipitate at high concentrations (Sjekloca et al., 2009; Sjekloca et al., 2011), it was not feasible to obtain complete binding isotherms and determine the dissociation constants (K_Ds) for some weak FMRP:substrate interactions (Figure 2-figure supplement 2). Of all the tested protein-nucleic acid pairs, the C-IDR and R-loop without overhang showed the highest affinity ($K_D = 4.7 \pm 3.9$ nM, Figure 2E and Figure 2-figure supplement 2). Intriguingly, the interaction was weakened with a 5' RNA overhang to the R-loop ($K_D = 149.1 \pm 9.9$ nM, Figure 2D). Moreover, while the C-IDR showed affinity towards ssDNA and dsDNA in isolation, it barely interacted with the DNA:RNA hybrid or ssRNA (Figure 2H and Figure 2-figure supplement 3). Therefore, we concluded that the C-IDR interacted with R-loops through simultaneous binding to the ssDNA and dsDNA or to the 3D architecture of the entire R-loop structure itself, with the RNA overhang interfering with the interaction. In contrast, the N-Fold bound R-loops with ssRNA overhang more tightly than those without overhang, albeit with still lower affinity than C-IDR (Figure 2F, Figure 2-figure supplements 2&3). Additionally, N-Fold showed affinities for ssRNA and ssDNA, but not

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120 dsDNA nor the DNA:RNA hybrid (Figure 2F and Figure 2-figure supplements 2&3). Therefore,
121 the N-Fold likely interacts with the R-loop through binding with the single stranded segments
122 (RNA or DNA) of the R-loop. Consistent with this interpretation, while the full length FMRP
123 (Figure 2-figure supplements 1D) binds R-loop without overhang with a lower affinity ($K_D =$
124 288.7 ± 4 nM) compared to the C-IDR, it prefers the R-loop with RNA overhang (Figure 2I&J
125 and Figure 2-figure supplements 2&3). We surmised that in the full length protein, the N-Fold
126 interacted with the RNA overhang, thus minimizing the RNA's interference with C-IDR binding
127 to the R-loop. Thus, our results demonstrated that the FMRP binding to R-loops involves
128 multivalent interactions, with N-Fold and C-IDR showing varying affinities to all segments of an
129 R-loop structure. Moreover, these multivalent interactions between FMRP and R-loops are
130 modulated by intra- and inter-molecular cooperative and/or inhibitory effects within FMRP, as
131 well as between FMRP and the R-loop sub-structures.

132 Next, we investigated the effect on R-loop binding by I304N, an FXS-causing mutant
133 defective in RNA binding and polysome association (De Boulle et al., 1993; Feng et al., 1997a).
134 We recently showed that FMRP-I304N had reduced ability to suppress R-loop-induced DSBs
135 during programmed replication-transcription conflict (Chakraborty et al., 2020). We generated
136 both the full length FMRP and N-Fold containing the I304N substitution (Figure 2A and Figure
137 2-figure supplement 1B&D). While the mutation indeed disrupted the interactions between N-
138 Fold with all substrates tested, it actually enhanced the binding of full length FMRP to R-loop
139 with overhang (Figure 2C,G,I and Figure 2-figure supplements 2&3). These results suggested
140 that although the I304N mutation may weaken the interaction between the N-Fold and substrates,
141 it may also reduce the N-Fold's inhibitory effects on C-IDR binding to R-loops through a long-
142 range intramolecular mechanism. Therefore, the inability of the FMRP-I304N mutant to
143 suppress DSB formation may originate from downstream events (*e.g.*, recruitment of R-loop
144 resolving factors) by the N-Fold, rather than from defective R-loop recognition *per se*, which
145 mainly depends on C-IDR. Therefore, we next tested if FMRP interacts with known R-loop-
146 interacting proteins.

147 To this date, we have observed interaction between FMRP and DHX9, a known R-loop
148 helicase that has been shown to suppress R-loop formation and prevent chromosome breakage
149 (Chakraborty and Grosse, 2011; Cristini et al., 2018). In an *in vitro* binding assay we observed
150 that FMRP directly interacts with recombinantly expressed histidine tagged-DHX9 (Figure
151 3A&B). Moreover, this interaction specifically occurred through the N-Fold domain (Figure
152 3C). Interestingly, the mutant N-Fold-I304N failed to interact with DHX9 (Figure 3D),
153 indicating that the KH2 domain, an integral part of the N-Fold domain organization, assists in the
154 recruitment of R-loop resolving factors. Therefore, we hypothesized that FMRP bridges the
155 interaction between R-loops and R-loop resolving factors, through its C-IDR and N-Fold,
156 respectively. We next asked if FMRP interacts with DHX9 *in vivo.* Using the aforementioned
157 GM06990 lymphoblastoids we first demonstrated co-immunoprecipitation of FMRP and its
158 known interacting protein, FXR1 (FMR1 autosomal homolog 1), as a positive control (Zang et
159 al., 2009) (Figure 3E). We also detected DHX9 interaction with FMRP through co-
160 immunoprecipitation (Figure 3F). In addition, the complex pulled down by anti-DHX9 also
161 comprised of Top IIIβ (Figure 3F), which has been recently implicated in the R-loop suppression
162 by reducing negatively supercoiled DNA behind RNA polymerase II (Yang et al., 2014).

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164 **DISCUSSION**

165   Our work directly builds on our previous report of a genome protective role of FMRP
166 during replication stress-induced R-loop accumulation and DSBs (Chakraborty et al., 2020).
167 Here we augmented this discovery by demonstrating direct interaction between FMRP and R-
168 loops and DHX9. We provided, for the first time, direct evidence that the C-IDR can interact
169 tightly with the R-loop structure. This is a remarkable finding, given that the same C-IDR also
170 has the ability to interact with G-quadruplexes and SoSLIPs that both adopt very different 3D
171 structures than R-loops (Bechara et al., 2009; Santoro et al., 2012; Vasilyev et al., 2015).
172 Previous studies have demonstrated that the formation of R-loops and G-quadruplexes are
173 potentially coupled during transcription (De Magis et al., 2019; Lee et al., 2020). Together with
174 our finding, it appears that FMRP can bind both structures via its C-IDR, thus providing a
175 mechanism for the functional linkage between these nucleic acid strutures.

176   Based on the hierarchy of substrate binding by the FMRP segments we propose the
177 following model (Figure 4). During replication-transcription conflict induced by APH treatment,
178 FMRP binds to R-loops predominantly via its C-IDR, thereby allowing the KH domains to bind
179 the trailing nascent ssRNA, and the N-terminal Agenet domains to presumably interact with
180 methylated histone tails (not depicted) or R-loop resolving factors that contain motifs with
181 methylated arginine or lysine residues. Here, we showed that FMRP interacts with one such R-
182 loop resolving factors, DHX9, through its N-Fold domain. Moreover, the interaction is
183 dependent on a bona-fide KH2 domain, suggesting that mutations in the KH domain may
184 interfere with the Agenet domain's binding to other proteins through intra-molecular
185 interactions. Overall, we propose that FMRP functions as a scaffold that bridges R-loops and R-
186 loop resolving factors, such as DHX9 and Top IIIβ. A recent human interactome analysis in
187 HeLa cells also revealed an interaction between FMRP and the THO-TREX complex, which
188 functions at the interface of transcription elongation and mRNA export (Hein et al., 2015).
189 THOC1, a subunit of the THO/TREX complex was present in the same complex as FMR1,
190 DHX9 and other THOC proteins. Depletion of subunits in the hTHO complex causes DNA
191 damage that is R-loop dependent (Dominguez-Sanchez et al., 2011). Our co-
192 immunoprecipitation experiments also showed an interaction between FMRP and TopIIIβ,
193 whose loss causes R-loop-mediated genome instabilty (Zhang et al., 2019). This result suggests
194 that FMRP forms multiple docking sites for factors that resolve R-loops and ensures proper
195 transcription, RNA processing and export.

196   Modular proteins such as FMRP and DHX9, which contain multiple folded domains
197 interspersed with intrinsically disordered regions, often undergo liquid-liquid phase separation
198 (LLPS), where molecules spontaneously demix from their solvent to form their own microscopic
199 droplets (Banani et al., 2017; Forman-Kay et al., 2018; Holehouse and Pappu, 2018). The C-
200 IDR of FMRP is capable of undergoing LLPS in isolation, in the context of full length, and in the
201 presence of its cognate RNA substrates (Tsang et al., 2019). The multivalent interactions with
202 diverging K_Ds between various FMRP segments, R-loop substructures and R-loop resolving
203 factors (*e.g.*, DHX9) can be the basis for the assembly of a phase-separated, membrane-less foci
204 for resolving R-loops.

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AUTHOR CONTRIBUTION

A.C. and W.F. conceived the study. A.C. and H.H. performed and analyzed the immunostaining experiments. A.C. performed chromatin fractionation and IP experiments with guidance from W.F. L.D. and A.B. designed and performed protein purification for all full length FMRP, FMRPI304N and FMRP domains. L.G. and X.X. performed some preliminary EMSA assays. A.D. designed and performed EMSA assays, purified DHX9 and performed FMRP:DHX9 interaction assay, with P.S. providing experimental guidance and suggestions. A.C., A.B. and W.F. wrote the manuscript with input from all authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this study.

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MATERIALS AND METHODS

Cell line growth and drug treatment conditions. Human EBV transformed lymphoblastoid cell line, GM06990, was grown in RPMI1640 (Corning cell gro), supplemented with GlutaMAX (GIBCO), 15% heat-inactivated FBS (Fetal Bovine Serum, Benchmark), 100 IU/mL penicillin and 100 µg/mL streptomycin (Corning cell gro) at 37°C with 5% CO_2. Cells were treated, at a density of $0.4-0.5x10^6$ cells/ml, with 0.03 µM, 0.3 µM, or 0.6 µM APH (A. G. Scientific), solvent (DMSO, 0.02%, same as the concentration in the APH-treated samples) only, or nothing, for 24 h before harvest.

Co-immunoprecipitation (Co-IP). Approximately $6-7x10^6$ cells were used for each IP reaction. Cells were resuspended in 1 ml IP lysis buffer [25 mM Tris-HCl pH 7.5 / 150 mM NaCl / 1% NP-40 / 1 mM EDTA / 5% glycerol / Halt protease inhibitor cocktail (Thermo scientific) / Halt phosphatase inhibitor cocktail (Thermo scientific)] and incubated on ice for 1 h. Cell lysates were centrifuged at 10,000 rpm for 10 m. Protein concentration in the supernatant was determined using Pierce protein assay reagent (Thermo Scientific). 50 µl of Dynabeads protein G (Invitrogen) per reaction were incubated with 200 µl antibody binding buffer [1X PBS/ 0.02% Tween 20] and 5 µg of anti-FMRP (Biolegend), or anti-DHX9 (Santa Cruz Biotechnology), or IgG (Biolegend) in a rotator for 10 m at room temperature. The immuno-complex was rinsed with 200 µl antibody binding buffer at room temperature, followed by incubation with 500 µg of cell lysate per reaction at 4ºC overnight. After incubation the supernatant was saved as flow-through (FT) and the beads were washed twice with IP lysis buffer without NP-40, saving each wash. 50 µl 2X Laemmli buffer was added to the beads and boiled for elution, before analysis on 8% SDS-PAGE gels and western blotting using anti-FMRP (Cell signaling, 1:500), anti-GAPDH (Thermo scientific, 1:4000) or anti-DHX9 (Santa Cruz Biotechnology, 1:500).

Subcellular fractionation. Cells were grown to a density of $0.4-0.5x10^6$ cells/ml with >90% viability. Cells were treated for 24 h with aphidicolin, DMSO or nothing. Samples were collected as aliquots of approximately $5x10^6$ cells, washed twice with PBS, then frozen for storage. Each thawed aliquot of cells was resuspended in 500 µl Farham's lysis buffer without NP-40 [5 mM PIPES pH 8.0 / 85 mM KCl / Halt protease inhibitor cocktail] and incubated on ice for 2 m. 50 µl of the cell lysate thus prepared was collected as a whole cell extract control and the remaining lysate was spun at 1300 g for 4 m to pellet nuclei. The supernatant served as the crude cytoplasmic fraction. The nuclear pellet was resuspended in 150 µl Farham's lysis buffer and incubated for 20-30 m at 4ºC and served as the nuclear fraction. Equal volume of 2X Laemmli buffer were added and samples were boiled and later sonicated. Approximately $3x10^5$ cell equivalent per fraction was used for electrophoresis on a 12% SDS-PAGE gel, followed by western analysis. Densitometry of autoradiogram was done using ImageJ (https://imagej.nih.gov/ij/) to calculate the percentages of FMRP in the nuclear and cytoplasmic fractions.

Western blot. Whole cell lysates were prepared in lysis buffer [50 mM Tris-HCl pH 7.5 / 0.5 M NaCl / 10 mM $MgCl_2$ / 1% NP-40 / Halt protease inhibitor cocktail / Halt phosphatase inhibitor cocktail] and at least 20 µg of proteins were analyzed by 10% SDS-PAGE before western blotting. The following antibodies were used: anti-FMRP (Biolegend, 1:1000), anti-Histone H3 (Cell Signaling, 1:500) and anti-GAPDH (Thermo scientific, 1:2000).

Immunocytochemistry and microscopy. Approximately $3x10^6$ cells having undergone drug treatment described above were washed twice in PBS before fixing with 500 µl 4%

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275 paraformaldehyde in microfuge tubes. Cells were washed with 500 μl 1X PBS twice, fixed with
276 500 μl 4% paraformaldehyde for 20 m at room temperature, followed by gently washing with 1X
277 PBS three times. Cells were then blocked with 500 μl PBSAT (1% BSA, 0.5% Triton X in 1X
278 PBS), followed by incubation with 100 μl of primary antibody solution for 1 h, washed with
279 PBSAT, and incubation with 100 μl secondary antibody for 1 h. Cells were then washed with
280 PBSAT followed by PBS, and resuspended in mounting media (Prolong Diamond antifade plus
281 DAPI, Invitrogen) before being placed as a drop onto microscope slides. Coverslips were
282 carefully placed on top of the mounted drops and allowed to solidify for 24 h before imaging on
283 Leica STP 800 wide-field fluorescence microscope (for lymphoblasts). Antibodies used for
284 immunostaining include the following: primary antibodies (anti-FMRP, Cell signaling, 1:200 and
285 S9.6, Kerafast, 1:500;) and secondary antibodies (Alex fluor 488, 568, and 647, Invitrogen,
286 1:400). To determine localization of FMRP and R-loop in the nucleus, 3D image stacks were
287 acquired from sixty-one 2D imaging planes with a step size of 0.11 micron using Metamorph.
288 For images shown in Figure 1, a single Z-plane image at approximately the center of the stack
289 was shown for each sample. DAPI was used to create a ROI which was overlayed and colored
290 white to indicate nucleus. Images were adjusted for background and contrast and smoothed
291 using a gaussian blur of 0.7 in Fiji.

292 **Cloning and protein purification.** As previously outlined in Tsang et al (Tsang et al., 2019)
293 and briefly described here, codon optimized full length human FMRP Isoform 1 cDNA was
294 generated by gene synthesis (GeneScript, Inc) and was subcloned into a pET-SUMO vector
295 (Invitrogen). This pET-SUMO-FMRP plasmid was used as a template to generate (i) full length
296 I304N mutant, (ii) FMRP-WT and FMRP-I304N mutant N-Folds (residues 1-455 without and
297 with the I304N substitution, respectively), and (iii) C-IDR (residues 445-632) via QuikChange
298 Site-Directed Mutagenesis (Agilent) for protein expression. The fidelity of these constructs was
299 confirmed by Sanger sequencing (Eurofins Genomics, Louisville, KY). Each construct was
300 transformed into *Escherichia coli* BL21(DE3) Codon Plus Cells (Agilent). Select colonies were
301 inoculated in 50 ml of Luria Broth (LB) medium, before dilution into 1 L fresh LB medium in a
302 Fernbach flask and grown at 37ºC . Protein expression was induced with 1 mM isopropyl-β-D-
303 thiogalactopyranoside (IPTG) at an optical density (600 nm) of ~0.6 and was incubated at 16ºC
304 for 18 h. Cells were harvested by centrifugation at 15,000 rpm for 30 m. The supernatant was
305 carefully discarded, and each cell pellet was stored at -20ºC until ready for protein purification.

306 To begin purification, frozen cell pellets were thawed and re-suspended in 100 ml of lysis buffer
307 containing 100 mM NaCl, 50 mM Na_2PO_4, 200 mM Arginine HCl, 200 mM Glutamic acid, 10%
308 Glycerol, 10 mM β-mercaptoethanol, and 1% CHAPS, pH 7.4, supplemented with DNase I,
309 lysozyme and protease inhibitors (bestatin, pepstatin, and leupeptin). Cells were lysed by
310 sonication and the lysate was subjected to centrifugation at 15,000 rpm for 30 m. The
311 supernatant was loaded onto a 20 ml HisTrap HP column (GE Healthcare) equilibrated in the
312 binding buffer (i.e. same composition as lysis buffer, but without DNase I and lysozyme) and
313 incubated at 4ºC for 30 m. The column was extensively washed three times with 30 ml of the
314 equilibration buffer. SUMO-fusion proteins were eluted using the same equilibration buffer
315 supplemented with 500 mM imidazole, and fractions containing proteins were combined. A 6X-
316 His-tagged Ulp protease was added to cleave the His-SUMO tag at room temperature overnight
317 with rocking. Completion of the Ulp cleavage reaction was confirmed by SDS-PAGE. After
318 cleavage, the protein solution was passed through a 0.2 μm filter to remove any aggregated
319 product, before it was concentrated using a 5 kDa-cutoff Amicon concentrator by centrifugation

320   at 4,000 rpm at room temperature. The concentrated protein solution is again filtered before
321   being loaded onto an equilibrated Superdex 200 size exclusion column (GE Healthcare) to
322   separate the FMRP constructs from the Ulp protease and the His-SUMO fusion tag. Fractions
323   containing pure FMRP proteins were identified by SDS-PAGE and combined for storage at -
324   80ºC.

325   DHX9-His was expressed by transducing 800 ml Tni cell culture in ESF921 serum-free media
326   (Expression Systems) at a density of $1x10^6$ cells/ml with 16 ml baculoviral suspension (generated
327   in Sf9 cells) and grown for 70 h at 27°C with shaking. Cell pellet was resuspended in a lysis
328   buffer containing 50 mM Tris-HCl, pH 7.5, 500 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM
329   DTT, 0.01% NP-40, 2 mM ATP, 4 mM $MgCl_2$, 10 mM Imidazole, cOmplete protease inhibitor
330   cocktail (MilliporeSigma), and 1mM PMSF, with sonication. The lysate was clarified by
331   ultracentrifugation at 40,000 rpm for 45 m. The clarified lysate was incubated with 1 ml Ni-
332   NTA resin (Qiagen) for 1 h, followed by washing the resin with 400 ml wash buffer-A
333   containing 50 mM Tris-HCl, pH 7.5, 1000 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT,
334   0.01% NP-40, 4 mM ATP, 8 mM $MgCl_2$ and 20 mM Imidazole. Protein-bound resin was
335   washed again with 50 ml wash buffer-B containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10%
336   glycerol, 1 mM EDTA, 1 mM DTT, 0.01% NP-40, and 20 mM Imidazole, followed by elution
337   with 10 ml elution buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10% glycerol, 1
338   mM EDTA, 1 mM DTT, 0.01% NP-40, 300 mM Imidazole and cOmplete protease inhibitor
339   cocktail (MilliporeSigma). The elution was subjected to ion exchange purification with
340   equilibrated Hitrap SP HP (1ml) column at a gradient of 100-500 mM KCl. The peak fractions
341   containing the protein were pooled together and purified again with HitrapQ (1 ml) column. The
342   peak fraction was aliquoted, flash frozen with liquid nitrogen and stored at -80ºC. The protein
343   was also evaluated via size exclusion chromatography by loading 400 µl of the Hitrap SP HP
344   purified fraction onto Superdex 200 increase 10/300 GL column (GE Healthcare), and a
345   monodisperse peak was obtained at 11.8 ml elution fraction.

346   **Electrophoretic mobility shift assay (EMSA).** DNA or RNA was labelled at 5′-termini with
347   T4-Polynucleotide kinase (NEB) using γ-P^{32}-ATP as indicated in Figure 2. The oligo sequences
348   are listed in Table S1. R-loops, RNA-DNA hybrids or duplex DNA substrates were generated
349   by annealing the labelled oligonucleotide with the complementary cold oligonucleotides in
350   equimolar ratio, as indicated in Table S2, by gradually decreasing temperature from 95ºC to 4ºC.
351   Prior to binding assays all the substrates were checked by electrophoresis in 5% native TAE
352   (30 mM Tris-acetate, pH 7.4 and 0.5 mM EDTA) polyacrylamide gel.

353   *Binding assay*. 1 nM of R-loop, RNA-DNA hybrid, dsDNA, bubble DNA, ssDNA, or RNA
354   substrate was mixed with 1 µl of protein at concentrations indicated in Figure 2, in a buffer
355   composed of 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 µg/ml BSA, 5 mM EDTA, with a final
356   volume of 10 µl. This mixture was incubated 30 m on ice, followed by addition of 10 µl loading
357   buffer composed of 50% glycerol, 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.05% Orange G.

358   *Polyacrylamide gel electrophoresis*. Electrophoretic separation of the protein-bound substrates
359   was carried out by running the mix in 5% native TAE gels, at 110V for 90 m at 4ºC. The gels
360   were vacuum dried for 30 m at 80ºC on a gel dryer and exposed to phosphorimaging screen
361   overnight. Imaging was done using Typhoon molecular imager (Amersham) and bands were
362   quantified using ImageQuant TL 8.0 image analysis software.

363   ***In vitro* protein binding assay (for FMRP protein domains and DHX9-His).** 5 µg DHX9-His

364 was incubated with 10µl Ni-NTA beads in a binding buffer containing 50 mM Tris-HCl, pH 7.5,
365 150 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.01% NP-40, 0.1% Tween-20, 10 mM
366 Imidazole, and 1 µl benzonase (MilliporeSigma) for 1 h, with mild shaking at 4ºC. The
367 supernatant was removed, and beads were washed three times with 200 µl binding buffer. The
368 binding buffer was completely removed and DHX9-His bound Ni-NTA were further incubated
369 for 15 m with 5 µg FMRP (full length)-WT, N-Fold-WT, N-Fold-I304N, or C-IDR (as indicated
370 in the figures) in 20 µl binding buffer. The protein bound resins were spun down and the
371 supernatants were taken out carefully. 5 µl loading buffer was added to supernatants. The resins
372 in each tube was washed three times with 200 µl wash buffer (same buffer with 20 mM
373 Imidazole, and 200 mM KCl, without benzonase). The bound proteins were eluted with 25 µl
374 1X Laemmli buffer. Equal volume of supernatants and the pulldowns were analyzed in 4-15%
375 polyacrylamide gradient gel.

376

377   **Figure Legends**

378

379   **Figure 1. FMRP is enriched in the nucleus upon replication stress. (A)** Subcellular
380   fractionation of FMRP. Western blot showing, whole cell extract (W), cytoplasmic fraction (C)
381   and nuclear fraction (N) of lymphoblastoid cells from unaffected control (NM) with and without
382   replication stress. GAPDH and Histone H3 serve as cytoplasmic and nuclear controls,
383   respectively. Two independent experiments were conducted, and one representative experiment
384   is shown. **(B)** Quantification of FMRP, GAPDH and Histone H3 intensity shows increased
385   percentage of FMRP in the nuclear fraction under APH stress. GAPDH shows minimum
386   occupancy in the nucleus while Histone H3 shows maximum occupancy indicating the purity of
387   fraction. Percentage of nuclear fraction of proteins expressed as the percentage of the band
388   intensity for "N" over that of the sum of "N" and "C" for each condition. Error bars indicate
389   standard error of mean in two independent experiments. One-way ANOVA followed by Tukey's
390   multiple comparison test. *, p = 0.033. **(C)** Co-localization of FMRP and RNA:DNA hybrids.
391   Immunofluorescence images of untreated, DMSO and APH treated NM cells co-stained for
392   RNA:DNA hybrids (cyan), FMRP (magenta) and nucleus (yellow, outlined). Immuno-staining
393   is shown in a single Z-plane. Scale bar, 5 μm**.**

394

395   **Figure 2. FMRP directly binds R-loops *in vitro*. (A)** Schematic representation of FMRP
396   protein domains, indicating the fold region and the C-terminus intrinsically disordered region (C-
397   IDR). The folded FMRP domain also harbor the isoleucine to asparagine mutation at residue
398   304 which causes FXS. **(B)** Nucleic acid structures used in the electrophoretic mobility shift
399   assay (EMSA) to determine binding interaction with FMRP N-Fold domain or FMRP C-IDR.
400   Blue strand represents DNA and red represents RNA, while asterisk indicates P^{32} label at the 5'-
401   end of the DNA or RNA strand. a- R-loop with 5'-RNA overhang (5'-RNA ovh), b- R-loop with
402   no overhang (no ovh), c- Bubble DNA (90 bp), d- RNA:DNA hybrid (no ovh), e- RNA:DNA
403   hybrid (5'-RNA ovh), f- Single-stranded DNA (ssDNA), g- Double-stranded DNA (dsDNA) and
404   h- RNA (30 or 60 bs). **(C&D)** Representative EMSAs for interaction between R-loop with 5'-
405   RNA ovh and the N-Fold and C-IDR domains. Sub., substrates. **(E)** Representative EMSA for
406   interaction between R-loop without ovh and the C-IDR. **(F-H)** Quantification of the percentage
407   of bound nucleic acid substrates at the indicated protein concentrations for N-Fold-WT **(F),** N-
408   Fold-I304N **(G)** and C-IDR **(H). (I)** Representative EMSA for interaction between R-loop with
409   5'- RNA ovh and the full length FMRP with or without I304N mutation. **(J)** Quantification of
410   the percentage of bound nucleic acid substrates at the indicated protein concentrations for
411   FMRP-WT. Note, FMRP-I304N showed nearly identical binding to all nucleic acids except for
412   R-loop (no ovh) and RNA (60 bs). See Figure 2-figure supplements 2&3 for details. The free
413   and bound substrates labeled for **(C)** is true for all EMSA gels.

414

415   **Figure 3. FMRP directly interacts with R-loop resolving factor DHX9. (A)** Purification of
416   Histidine-tagged DHX9 recombinant protein (DHX9-His). **(B-D)** *In vitro* protein binding assays
417   for DHX9-His and full length FMRP **(B)**, FMRP domains **(C)** and N-Fold-WT or N-Fold-I304N
418   **(D)**. **(E)** Co-immunoprecipitation of FMRP by immunoprecipitating with anti-FXR1 monoclonal
419   antibody and immunoblotted for FMRP and FXR1. GAPDH served as negative control. **(F)** Co-
420   immunoprecipitation of FMRP by immunoprecipitating with anti-DHX9 monoclonal antibody

12

421 and immunoblotted for FMRP, DHX9 and TOP IIIβ. GAPDH served as negative control. The
422 black asterisks indicate the lower band of a doublet signal in the "IP-DHX9" lane is the DHX9
423 protein, which is accumulated in the immunoprecipitated complex and absent in the IgG-
424 precipitated control complex ("IP-IgG" lanes).

425

426 **Figure 4. Proposed mechanism of R-loop resolution by FMRP and DHX9.** We hypothesize
427 that FMRP interacts with R-loops, which form as a result of transcription-replication conflict (T-
428 R), through complex arrangement(s) of its N-terminal Fold (N-Fold) domain and C-IDR. We
429 propose that i) the C-IDR directly binds R-loop through the recognition of the triple-stranded
430 structure, probably at the 3'-end in normal cells and the KH domains of N-Fold binds either the
431 displaced ssDNA (not depicted) or the trailing RNA overhang (depicted), ii) the Agenet domain
432 of N-Fold binds R-loop resolving factors, such as DHX9, probably through the C-terminus RGG
433 domain of DHX9 containing methylated arginine residues ("Me"). The isoleucine residue in the
434 KH2 domain ("I304") is important for these interactions. The I304N mutation abolishes the
435 binding to ssDNA, RNA, and DHX9. Through bridging the interactions between nucleic acids
436 and proteins by multivalent interactions, FMRP recruits R-loop resolving factors to the R-loops
437 (iii), allowing replication to proceed normally (iv). In the absence of FMRP, stabilized R-loops
438 suffer from strand breakage, likely in the displaced ssDNA, and result in DSB formation at gene
439 loci involved in neurodevelopment pathways and in transcription factors.

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521
522

523 **SUPPLEMENENTARY INFORMATION**

524 **FIGURE SUPPLEMENTS**

525

526 **Figure 1-figure supplement 1. (A)** Total FMRP level expressed as ratio of FMRP over
527 GAPDH in the whole cell extracts (n=2) from Figure 1A remained nearly constant in all
528 conditions. **(B)** Volumetric 3D reconstruction in MetaMorph of an APH-treated normal cell.
529 Montage representation of 360º rotation of the cell along the X-axis, FMRP in green, S9.6 in red,
530 yellow indicates merge and points of co-localization when rotated vertically along the X-axis.
531 Numbers indicate the angle of rotation. **(C)** Cartoon illustration of a cell with a nucleus (blue) in
532 3D indicating rotation along the X-axis. **(D)** Video attachment of the same cell in Figure 1-
533 figure supplement 1-source data 1.

534

535 **Figure 2—figure supplement 1. Purification of FRMP fragments, their interactions with**
536 **various nucleic acid structures and purification of DHX9. (A-C)** Purification of FMRP
537 protein domains for EMSA. The fusion proteins containing HIS-SUMO-tagged FMRP fragments
538 were subject to Ulp cleavage to remove the tag, followed by FPLC to remove the cleaved HIS-
539 SUMO as well as Ulp itself, as shown for N-Fold-WT (A). The same procedures were applied to
540 the purification of N-Fold-I304N (B) and C-IDR (C). **(D)** Purification of full length FMRP-WT
541 and FMRP-I304N.

542

543 **Figure 2—figure supplement 2.** Binding affinity of the FMRP fragments for the nucleic acid
544 substrates were calculated as dissociation constants (K_Ds in nM) averaged from two independent
545 EMSA experiments.

546

547 **Figure 2—figure supplement 3.** Representative EMSA for all proteins and nucleic acids. #
548 denotes that SDS/PK was not added in this sample.

549

550

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552

553   **Table S1.** List of all oligonucleotides for making substrates for EMSA experiments.

554

Name	Size	Sequence
D1	90 nt	5′-CATTGCATATTTAAAACATGTTGGATCCCACGTTGCATGCTGATAGCCTACTAGAGCTGTATGAATTCAAATGACCTCTTATCAAGTGAC -3′
D2	90 nt	5′-GTCACTTGATAAGAGGTCATTTGAATTCATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGGGATCCAACATGTTTTAAATATGCAATG-3′
D3	30 nt	5'- GGCTTAGAGCTTAATTGCTGAATCTGGTGC-3'
D4	60 nt	5′-ACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC -3'
D5	30 nt	5′-GGGTGAACCTGCAGGTGGGCAAAGATGTCCCAGCAAGGCACTGGTAGAATTCGGCAGCGT -3'
R1	60 nt	5′-GUGCUACGAUGCUAGUCGUAGCUCGGGAGUGCACCAGAUUCAGCAAUUAAGCUCUAAGCC- 3'
R2	30 nt	5′-GCACCAGAUUCAGCAAUUAAGCUCUAAGCC -3'

555

556   "nt", nucleotide.

557   **Table S2.** Scheme for generating substrates for EMSA experiments.

558

R-loop with 5′-RNA overhang	D1+D2+R1
R-loop with no-RNA overhang	D1+D2+R2
RNA-DNA hybrid with 5′-RNA overhang	D3+R1
RNA-DNA hybrid with no RNA overhang	D3+R2
dsDNA	D4+D5
Bubble DNA	D1+D2
ssDNA	D1
RNA (60 nt)	R1
RNA (30 nt)	R2

559

560

Figure 1

A

B

C
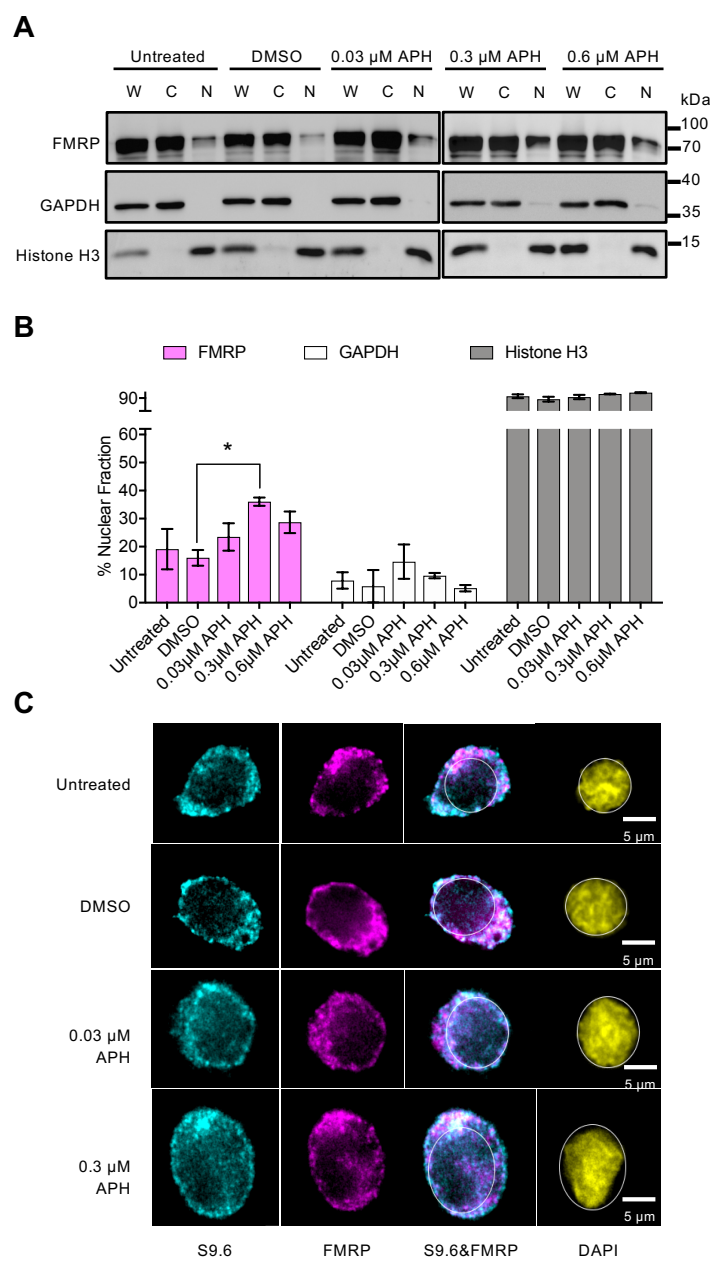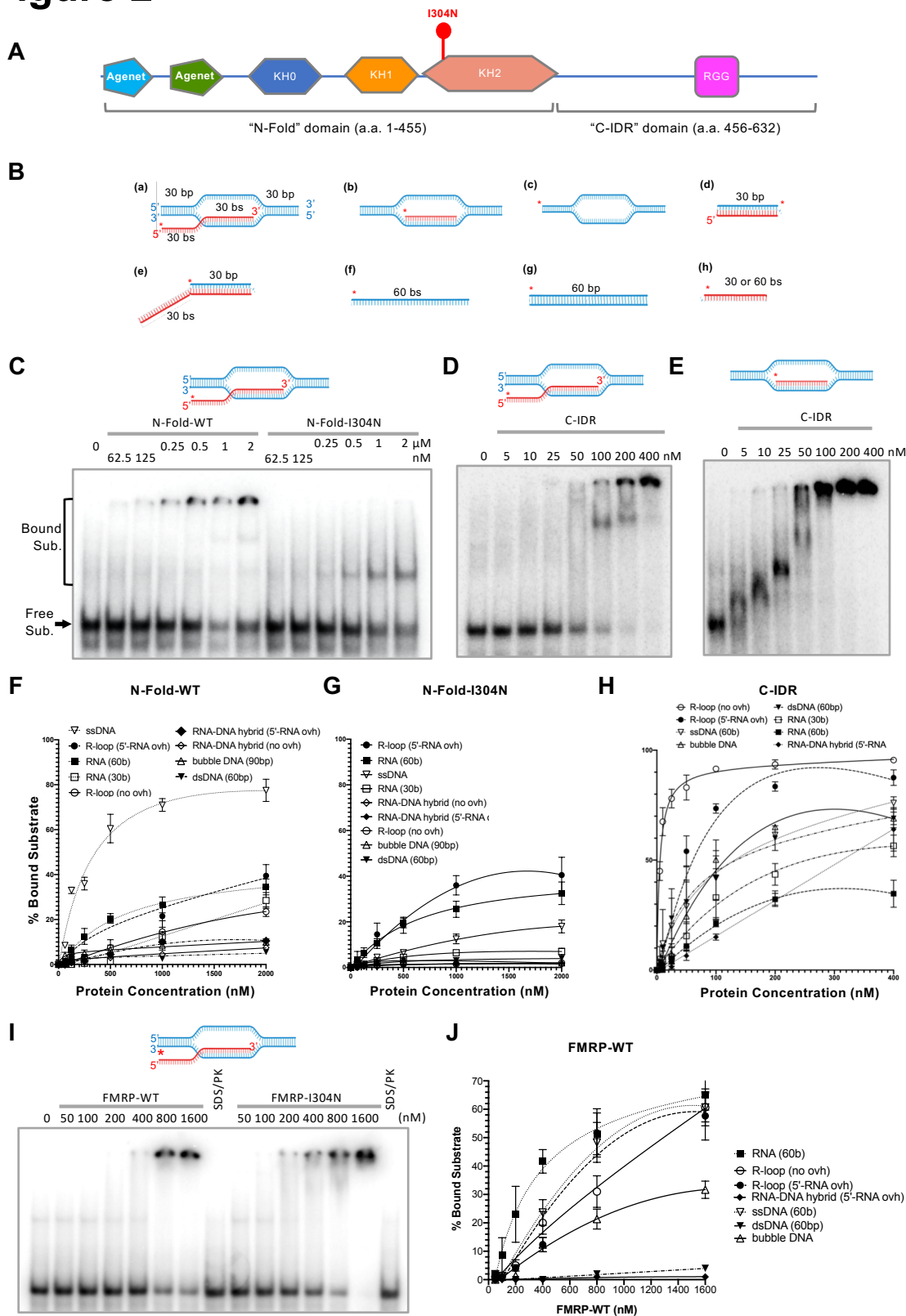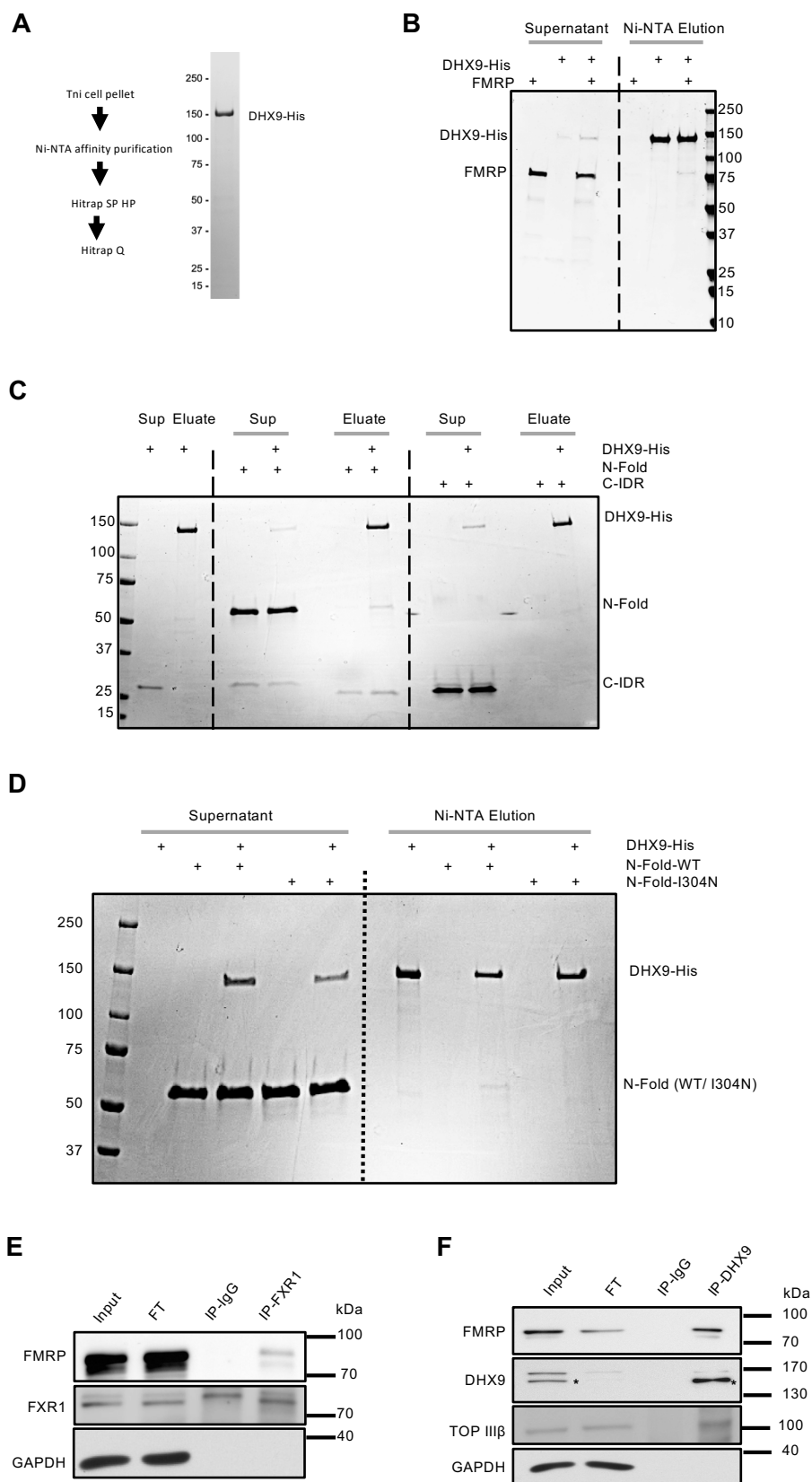
Figure 2

Figure 3

Figure 4

Figure 1—figure supplement 1

Figure 1—figure supplement 1. (A) Total FMRP level expressed as ratio of FMRP over GAPDH in the whole cell extracts (n=2) from Figure 1A remained nearly constant in all conditions. **(B)** Volumetric 3D reconstruction in MetaMorph of an APH-treated normal cell. Montage representation of 360º rotation of the cell along the X-axis, FMRP in green, S9.6 in red, yellow indicates merge and points of co-localization when rotated vertically along the X-axis. Numbers indicate the angle of rotation. **(C)** Cartoon illustration of a cell with a nucleus (blue) in 3D indicating rotation along the X-axis. **(D)** Video attachment of the same cell in Figure 1—figure supplement 1—source data 1.

Figure 2—figure supplement 1

A

B   **C**   **D**

Figure 2—figure supplement 1. Purification of FRMP fragments, their interactions with various nucleic acid structures and purification of DHX9. (A-C) Purification of FMRP protein domains for EMSA. The fusion proteins containing HIS-SUMO-tagged FMRP fragments were subject to Ulp cleavage to remove the tag, followed by FPLC to remove the cleaved HIS-SUMO as well as Ulp itself, as shown for N-Fold-WT (A). The same procedures were applied to the purification of N-Fold-I304N (B) and C-IDR (C). (**D**) Purification of full length FMRP-WT and FMRP-I304N.
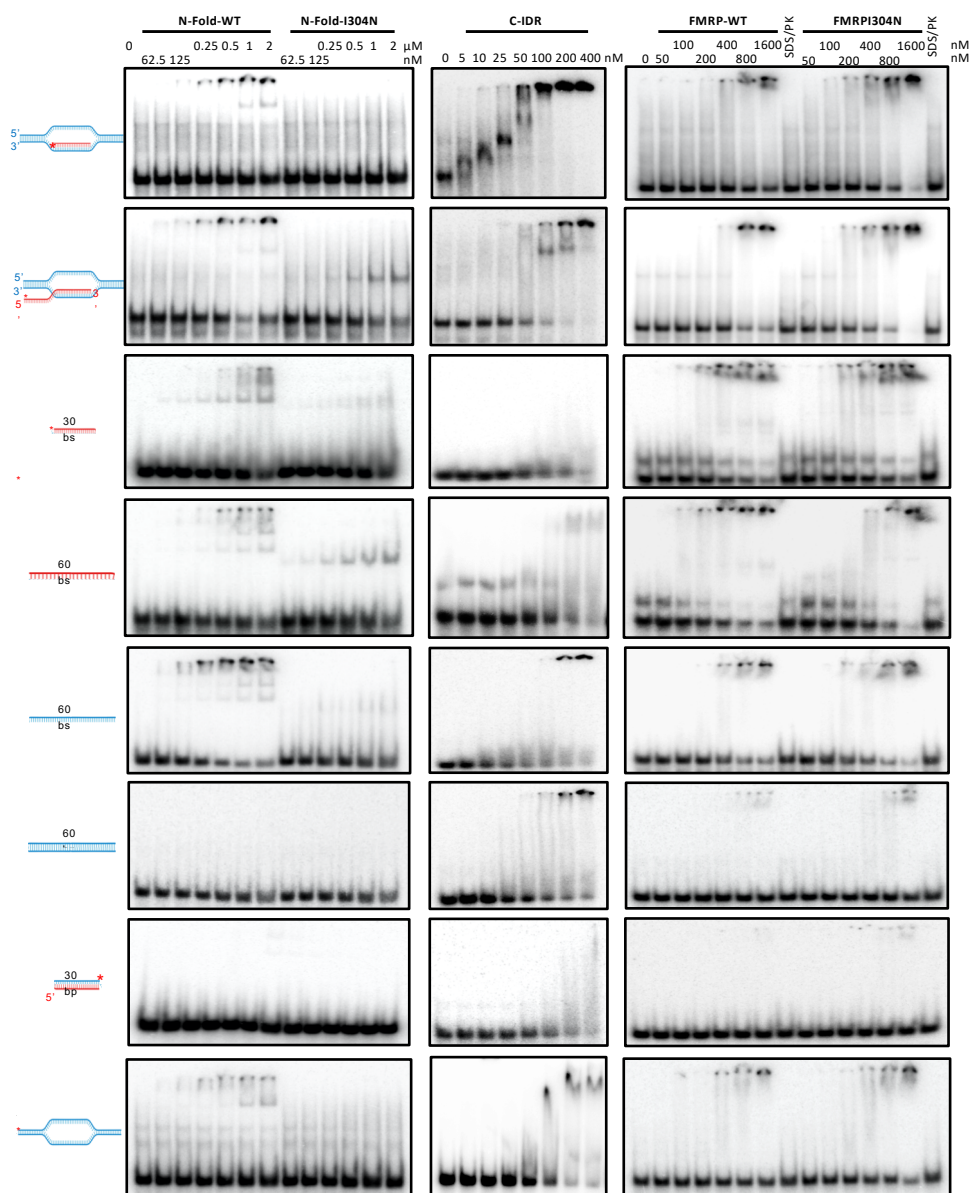
Figure 2—figure supplement 2

K_D of FMRP domains for nucleic acid substrates

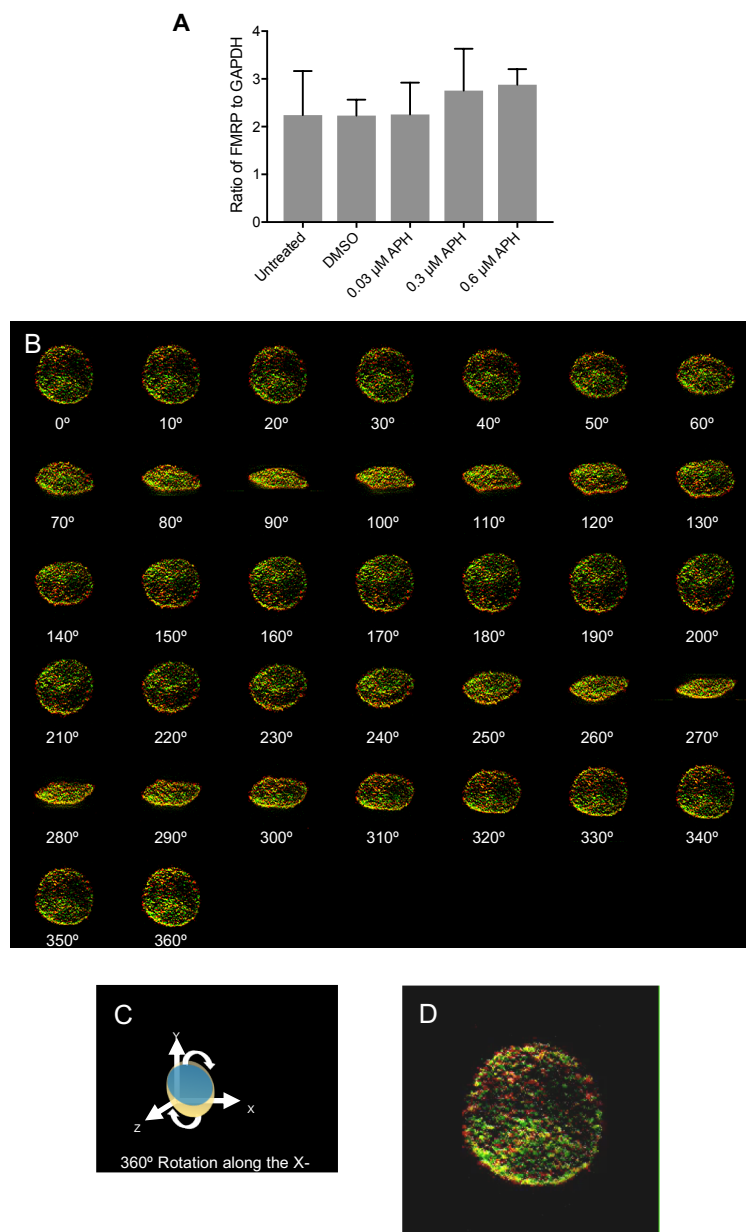| Substrate	FMRP-WT	FMRP-I304N*	N-Fold-WT	N-Fold-I304N	C-IDR
R-loop (no ovh)	288.7 ± 4	213.7 ± 4 .7	NA	NA	4.7 ± 3.9
R-loop (5' ovh)	NA	NA	322 ± 3.9	NA	149.1 ± 9.9
DNA bubble	NA	NA	NA	NA	NA
dsDNA (60 bp)	NA	NA	NA	NA	56.8 ± 7.1
RNA (30 bs)	NA	NA	NA	NA	360.2 ± 4.7
RNA (60 bs)	113.6 ± 6	311.6 ± 3.7	615.9 ± 2.2	NA	NA
ssDNA (60 bs)	NA	NA	380.9 ± 5.7	NA	79.6 ± 9.3
RNA-DNA hybrid	NA	NA	NA	NA	NA

Figure 2—figure supplement 2. Binding affinity of the FMRP fragments for the nucleic acid substrates were calculated as dissociation constants (K_Ds in nM) from EMSA experiments in Figure 2.

Figure 2—figure supplement 3

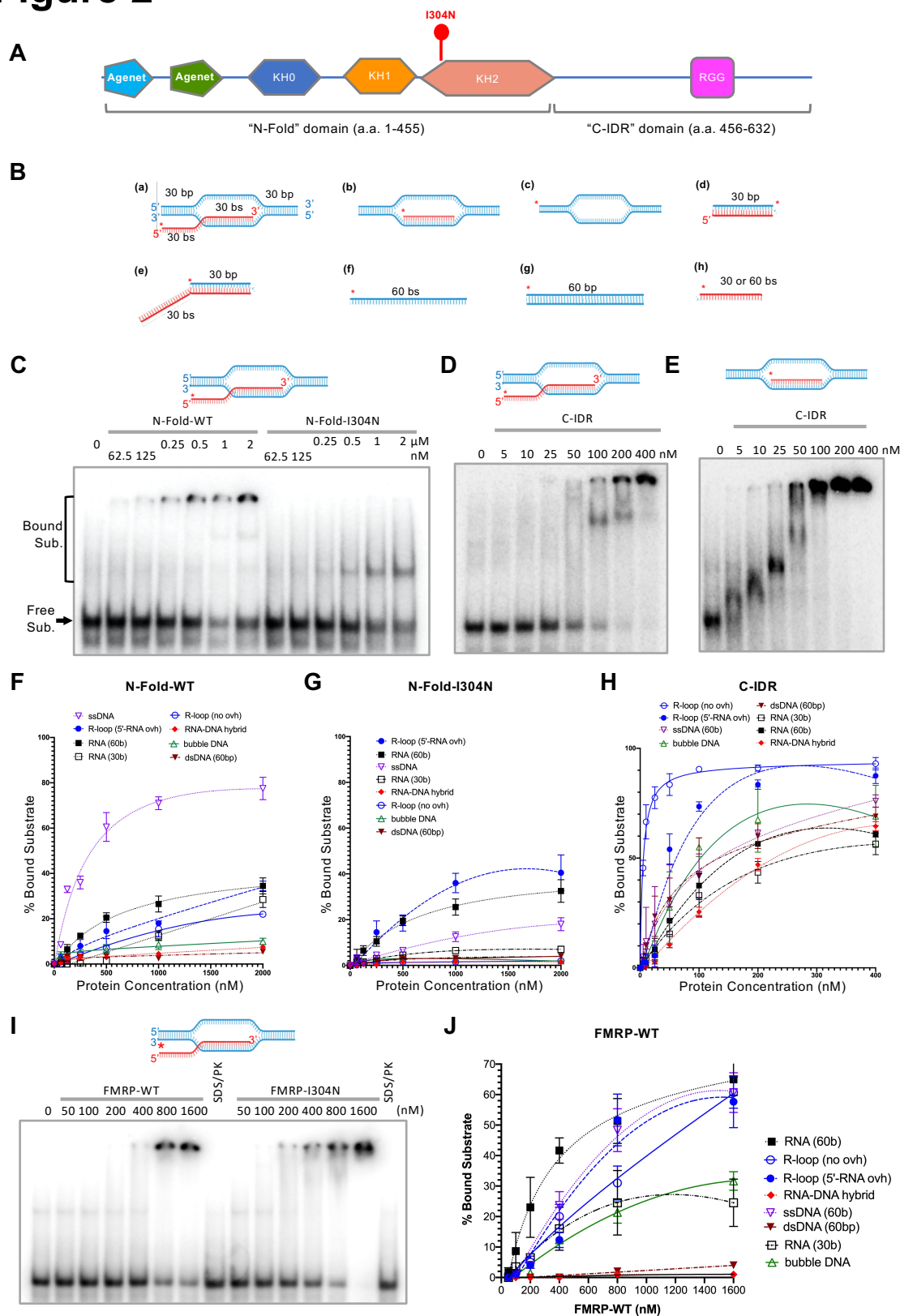**Figure 2—figure supplement 3.** Representative EMSA for all proteins and nucleic acids.
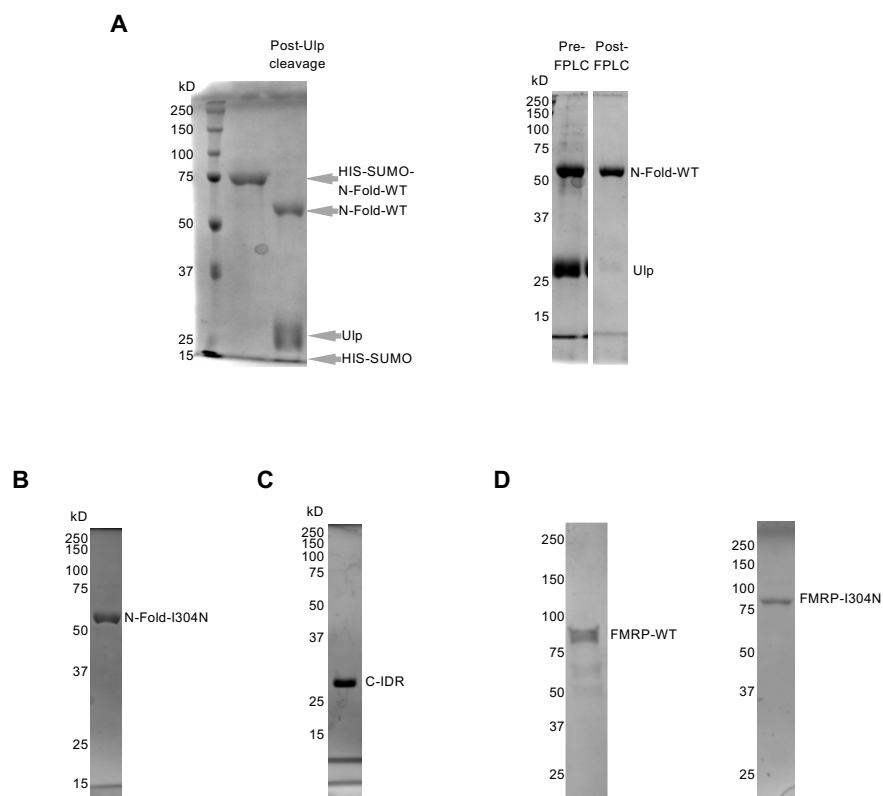
Figure 1—figure supplement 1

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Figure 2

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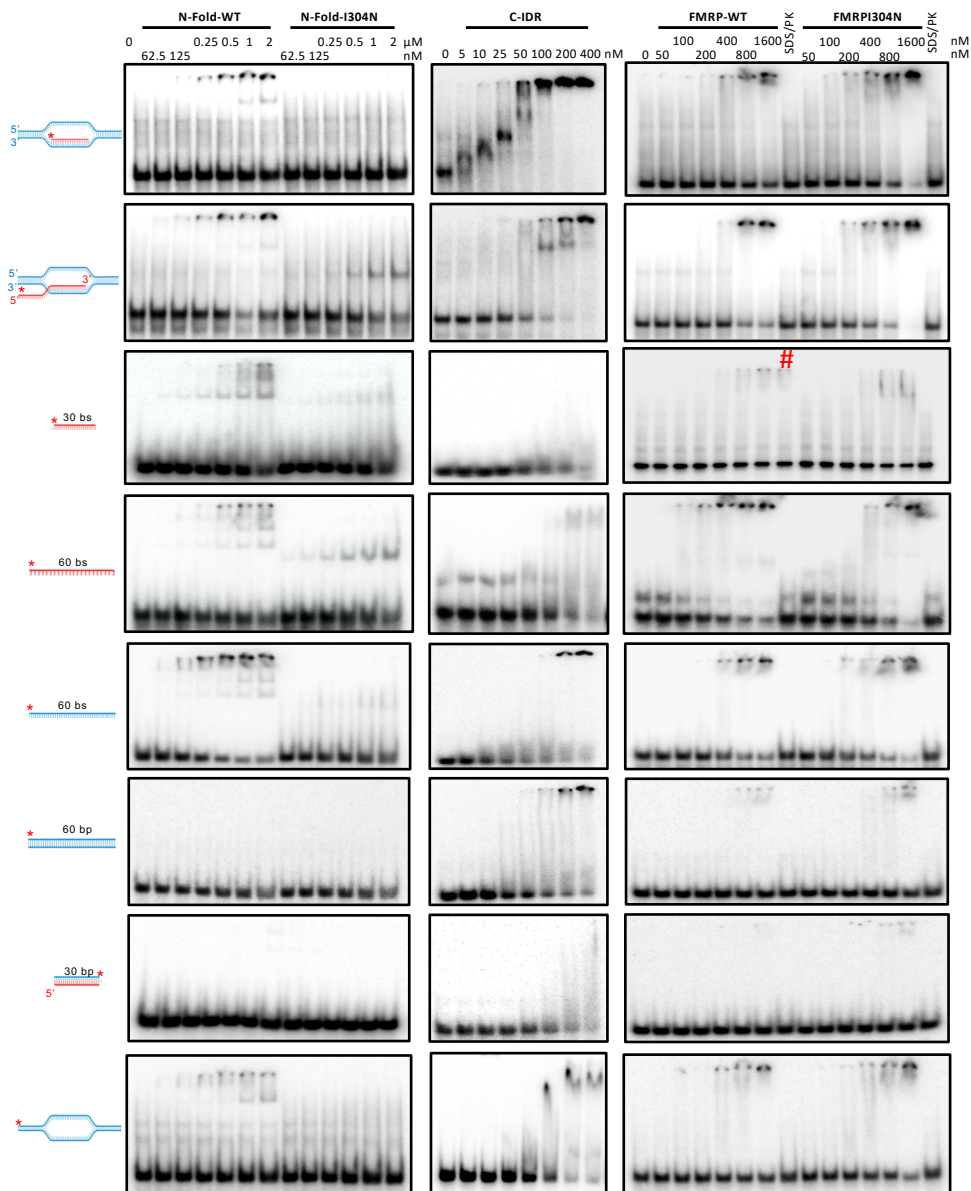
Figure 2—figure supplement 2

K_D of FMRP domains for nucleic acid substrates

Substrate	FMRP-WT	FMRP-I304N*	N-Fold-WT	N-Fold-I304N	C-IDR
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R-loop (5' ovh)	NA	NA	322 ± 3.9	NA	149.1 ± 9.9
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RNA-DNA hybrid	NA	NA	NA	NA	NA

Figure 2—figure supplement 2. Binding affinity of the FMRP fragments for the nucleic acid substrates were calculated as dissociation constants (K_Ds in nM) averaged from at least three EMSA experiments in Figure 2.
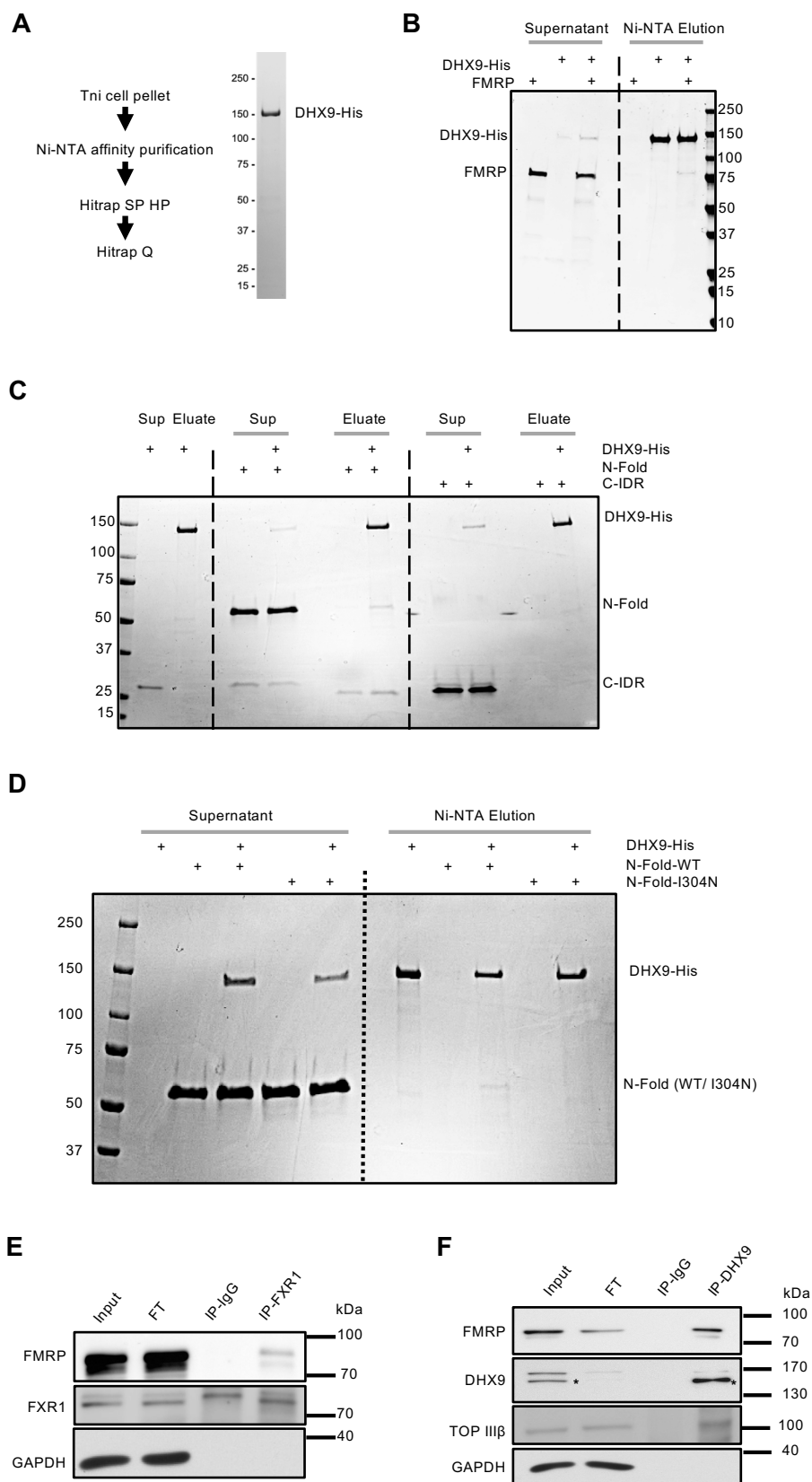
Figure 2—figure supplement 3

Figure 2—figure supplement 3. Representative EMSA for all proteins and nucleic acids. # denotes that SDS/PK was not added in this sample.

Figure 3

Figure 4