Supplementary Information for

FMRP Promotes transcription-coupled homologous recombination via Facilitating TET1-mediated m5C RNA modification Demethylation

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**Supplementary Figure 1.** (a) The top 50 pathways corresponding to undamaged condition from mass spectrometry were categorized into 5 different types: 1) DNA synthesis and repair (green), 2) mRNA related (yellow), 3) tRNA related (red), 4) cell cycle related (purple) and 5) others (grey). (b) Flp-in 293 cells transfected with GFP-TRDMT1 were measured by Western blot. (c) Flp-in 293 cells were transfected with GFP-TRDMT1 with the treatment of 1 mM H₂O₂ for 1 h. Interaction of FMRP and TRDMT1 were detected with Co-IP with or without treatment of EtBr or RNaseH or RNaseA. Anti-GFP antibody was used for pull-down and anti-FMRP was used for detection in WB. (d) FMRP-KO U2OS and Flp-in 293 cells were made with CRISPR-Cas9, and confirmed by Western blot. (e) U2OS-TRE cells were transfected with TA-KR/tetR-KR to induce local oxidative. Then the cells were stained for m5C with an anti-m5C antibody (scale bar: 10 μm). (f) U2OS-TRE cells pretreated with control or the indicated siRNA were measured by Western blot.
Supplementary Figure 2. (a) U2OS-TRE cells transfected with TA-KR plasmid were light irradiated and allowed to recover for 1 h before fixation. Cells were stained with FMRP antibody and represented figures were shown (scale bar: 10 μm). (b) FMRP KO and WT U2OS cells were exposed to 4 Gy IR damage. After 1 h, cells were harvested and analyzed by Western blot. (c) DR-GFP cells were pre-treated with control siRNA or siFMRP. Then the cells were transfected with NLS-I-SceI plasmid to induce DSB. The GFP-positive population was analyzed by flow-cytometry (n = 3, Mean ± SEM). (d) U2OS TRE cells pretreated with siFMRP or control siRNA were transfected with Cas9-sgLMNA-mCherry and LMNA-mClover. The fraction of mClover-positive cells in mCherry positive population was analyzed by flow-cytometry (n = 3, Mean ± SEM). (e) U2OS-TRE cells were co-transfected with TA-Cherry and Flag-FMRP with or without I-SCEI. The cells were then stained with Flag antibody (scale bar: 10 μm). FMRP foci frequency at TA-Cherry was quantified (n=3, 50 cells per replicate, Mean ± SEM). (f) U2OS-TRE cells co-transfected with GFP-FMRP were treated with 4 Gy IR or 1 mM H₂O₂ for 1 h (scale bar: 10 μm). The fraction of cells with FMRP nucleus localization was counted. Statistical analysis was done with the unpaired two-tailed student t-test, ns: not significant; **: p < 0.01; ****: < 0.0001.
Supplementary Figure 3. (a) U2OS-TRE cells were transfected with TA-KR plasmid and HA-RNase H1 WT or D210N mutant. The cells were exposed to light activation for 25 mins and then let to recover for 1 h before fixation and S9.6 staining (scale bar: 10 μm). R-loop foci frequency at TA-KR was quantified (n=3, 50 cells per replicate, Mean ± SEM). 

(b) WT and FMRP KO U2OS-TRE cells were transfected with TA-KR and YFP-MS2 plasmids. The cells were exposed to light activation for 25 mins and then let to recover for different time before fixation. YFP-MS2 foci intensity at TA-KR sites at different time points after light activation was shown (n=15, Mean ± SD). 

(c) U2OS TRE cells pretreated with control siRNA or siFMRP were transfected with TA-KR. The foci intensity of RPA, CSB, TRD51, RAD52, and RAD51 at TA-KR (recover 1 h after light activation) were shown (scale bar: 10 μm) (n≥10, Mean ± SD). Statistical analysis was done with the unpaired two-tailed student t-test, ns: not significant; **: p < 0.01.
Supplementary Figure 4. Clinical and RNA seq gene expression data was downloaded from the cancer genome atlas (TCGA). The median value of FMRP TPM was defined as the cutoff value to divide patients into high expression and low expression groups. RAD51, BRCA1, BRCA2, RPA1, CSB and RAD52 expression in different groups were plotted.
Supplementary Figure 5. (a) The demethylation assay with purified TET1 and FMRP protein was performed with 5mC Tet1 Oxidation Kit. Single-stranded RNA containing m5C were used as substrates. Nucleic acid substrates were extracted by phenol-chloroform method after demethylation assay. The purified substrates were subsequently digested with nuclease P1 and alkaline phosphatase. The resulting ribonucleoside mixture was then subjected to LC-MS analysis. (b) The demethylation assay with purified FMRP protein was performed with 5mC Tet1 Oxidation Kit. Single-stranded RNA containing m5C or m5C RNA:DNA hybrid were used as substrates. Nucleic acid substrates were extracted by phenol-chloroform method after demethylation assay. The purified substrates were subsequently digested with nuclease P1 and alkaline phosphatase. The resulting ribonucleoside mixture was then subjected to LC-MS analysis. (c) Flp-in 293 cells transfected with GFP-FMRP were measured by Western blot. (d) Interaction of FMRP and TET1 detected with Co-IP with or without treatment of benzonase. Flp-in 293 cells were co-transfected with GFP-FMRP and Flag-TET1, treated with 1mM H2O2 for 1 h before co-IP analysis with anti-Flag antibody.
## Supplementary Table 1. Abs used in the study.

| Antibody | Species | Clone, Catalog no. | Company                    |
|----------|---------|--------------------|----------------------------|
| TRDMT1   | Mouse monoclonal | D-9, sc-365001 | Santa Cruz Biotechnology |
| m5C      | Mouse monoclonal | 33D3, ab10805   | Abcam                      |
| FMRP     | Rabbit polyclonal| ab17722          | Abcam                      |
| RAD51    | Rabbit polyclonal| ab63801          | Abcam                      |
| S9.6     | Mouse monoclonal | ENH001           | Kerafast                   |
| GFP      | Mouse monoclonal | 11814460001      | Roche                      |
| Myc-tag  | Mouse monoclonal | 9E10, ab32       | Abcam                      |
| Flag     | Mouse monoclonal | M2, IB13026      | Eastman Kodak             |
| γH2AX, ser139 | Mouse monoclonal | JBW301, 05–636 | EMD Millipore             |
| BRCA1    | Rabbit polyclonal| C-20, sc-642     | Santa Cruz Biotechnology   |
| p-ATM    | Rabbit monoclonal| S1981, D25E5     | Cell Signaling Technology  |
| p-CHK2   | Rabbit monoclonal| T68, C13C1       | Cell Signaling Technology  |
| β-Actin  | Mouse monoclonal | 8H10D10          | Cell Signaling Technology  |
Supplementary Materials and Methods

Immunoprecipitation and LC/MS analysis of GFP-TRDMT1

GFP-TRDMT1 stably expressed Flp-in 293 cells were treated with or without 2 mM H₂O₂ for 3 h before harvest as damaged and undamaged conditions. Wild-type 293 cells were used as control. The cell noodles were made in liquid nitrogen and then cryogenically milled into micron-sized particles to maximize the efficiency of solvent extraction of proteins. Two lysis buffer (20 mM HEPES, 0.5% TritonX-100, and protease inhibitors) with different salt concentrations (150 mM or 300 mM NaCl) were used to capture the protein interactions while preserving the extraction efficiency(1). Affinity purification of GFP-TRDMT1 from the whole cell lysates was carried out by an anti-GFP nanobody (2) coupled to magnetic dynabeads (Thermo, Cat# 14302D). After protein reduction and alkylation, the immunoprecipitation samples under each condition were run on a 4–12% SDS-PAGE gel using a short gradient. The whole region of each sample was cut and in-gel digested with trypsin as previously described (3). After proteolysis, the peptide mixtures were desalted and analyzed with a nano-LC 1200 coupled to a Q Exactive™ Orbitrap™ mass spectrometer (Thermo Fisher). The peptides were loaded onto a picocolumn (C18, 3 μm particle size, 300 Å pore size, 50 μm × 10.5 cm; New Objective) and eluted using a 60 min LC gradient: 7% B–12% B, 0–3 min; 12% B–40% B, 3–50 min; 40% B–100% B, 50–53 min; 100% B, 53–60 min; mobile phase A consisted of 0.1% formic acid (FA) in LC/MS water, and mobile phase B consisted of 0.1% FA in 100% acetonitrile. The QEx instrument was operated in the data-dependent mode, where the top 10 most abundant ions (mass range 350–1,500, charge state 2 - 6) were fragmented by high-energy collisional dissociation (normalized collision energy 30). The target resolution was 60,000 for MS and 7,500 for MS/MS analyses. The quadrupole isolation window was 2.0 Th and the maximum injection time for MS/MS was set at 100 ms. After MS analysis, the data was searched by Maxquant (4) for identification and label-free quantification. The mass accuracy was specified as 10 and 20 p.p.m. for MS and MS/MS, respectively. Other search parameters included cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. A maximum of three trypsin missed-cleavage sites was allowed.

Plasmids

The TA-KR, tetR-KR, TA-Cherry, and tetR-Cherry on pBroad3 plasmids, pCMV-NLS-I-Scel, Myc-CSB, pEGFP-RAD52 (5), GFP-TRDMT1 (6), HA-RNaseH WT, and HA-RNaseH D210N plasmids(7) used for the DART system have been described. Flag-FMRP (Cat# 48690), Flag-FMRP I304N (Cat# 48692), and Flag-TET1 (Cat# 49792) were purchased from Addgene. The FMRP gene was then subcloned to the pEGFP-C3 plasmid (Clontech/Takara Bio USA; Mountain View, CA, USA) linked by EcoRI and PstI for imaging experiments. For CRISPR-Cas9 knockout, the sgRNA sequence was integrated into the PX-330 plasmid (provided by Feng Zhang). The Myc-tagged FMRP mutations were subcloned via overlapping PCR to the pLVX-puro plasmid (Clontech) by XhoI and NotI.

Co-immunoprecipitation and western blots

Flp-in 293 cells were transfected with indicated plasmids. After 36 h cultivation, cells were collected and suspended in 1 mL lysis buffer (10 mM Hepes, pH 7.6, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, protease inhibitor from Roche). After 30 mins incubation on ice, cell lysate was centrifuged at 13000 rpm for 15 mins. The up-layer supernatants were incubated with 2 μg anti-GFP monoclonal antibody and 30 μL of protein G Sepharose beads (GE Healthcare Bio-Sciences) at 4°C overnight with rotation. After incubation, the protein beads were washed three times using 0.4 mL of lysis buffer.

For Western blot analysis, samples were boiled in SDS loading buffer at 95°C for 5-10 min, and applied to electrophoresis with 10-12% SDS-polyacrylamide gels, followed by transferring to PVDF membranes. For block and antibody dilution, 5% non-fat milk in PBST was used. After primary antibody incubation at 4°C overnight and secondary antibody incubation at room temperature for 1 h, the membranes were washed in 0.1% PBST three times, respectively. Chemiluminescent HRP substrate was purchased from Millipore (Cat#: WBKLS0500; Burlington, MA, USA). Images were taken in the BIO-RAD (Hercules, CA, USA) Universal Hood II machine with corresponding ImageLab software.

Protein purification
FMRP protein was expressed and purified as previously described with small modifications. Briefly, Rosetta 2(DE3) pLysS E. coli cells harboring pET21a–FMRP (provided by Mihaela-Rita Mihailescu), which encodes ISO1 fused with a C-terminal 6x histidine tag, were cultured in LB medium with addition of 200 μg/mL ampicillin and 15 μg/mL chloramphenicol. The cells were grown to an OD of 0.8 at 37°C and FMRP protein was induced by adding 1 mM IPTG for 12 h at 25°C. 5 g overexpressed cell mass was lysed in 30 mL lysis buffer (20 mM HEPES [pH 7.5], 500 mM NaCl, 5 mM BME, 0.05 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM PMSF and a mixture of protease inhibitors) and sonicated. The lysed sample was centrifuged for 30 min at 40,000 g. The supernatant with 10 mM imidazole was added to Ni-NTA beads (Qiagen) prewashed with equilibration buffer (same as lysis buffer but without PMSF or protease inhibitors), and then allowed for protein binding with rotation for 3 h. The beads were then washed with equilibration buffer supplemented with gradually increased concentrations of imidazole (10 mM, 20 mM and 50 mM). The protein was eluted with 10 mL buffer containing 300 mM imidazole. The protein was concentrated with PEG 2000 to 2 mL and then dialyzed by equilibration buffer with decreasing imidazole (200 mM, 100 mM, and 0 mM). The purified protein was stored in -80°C.

**In vitro pull-down**

The same amount of purified FMRP and TRDMT1(6) (0.3μg) were mixed in 500 μL lysis buffer (10 mM Heps, pH 7.6, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, protease inhibitor from Roche). The mixture was incubated with 2 μg anti-FMRP antibody and 30 μL of protein G Sepharose beads (GE Healthcare Bio-Sciences) at 4°C overnight with rotation. After incubation, the protein beads were washed three times using 0.4 mL of lysis buffer. The beads were resuspended in 20μL SDS loading buffer and boiled at 95°C for 5-10 min, followed by Western blot analysis.

**Dot blot assay**

Total poly(A)+ mRNA from U2OS-TRE was purified with a Dynabeads™ mRNA DIRECT™ Purification Kit (Cat#: 61011, ThermoFisher Scientific, Waltham, MA, USA). The same amount of mRNA from different samples was diluted to the same concentration with 10 mM Tris-HCl from the kit. The mRNA solutions were loaded on a positive-charged Nylon66 membrane (Biodyne B transfer membrane, 0.45 μm, 60209), and linked by a UV Stratalinker 2400 (Stratagene; La Jolla, CA, USA) at 1,200 μJ twice with 254 nm wavelength. Then, the membrane was washed in 0.02% PBST for 10 min. Primary antibody was diluted 1:100 in 5% non-fat milk in 0.02% PBST, and then washed three times. The membrane was stained by 0.1% methylene blue (Cat#: M9140-25G, Sigma-Aldrich; St. Louis, MO, USA) in 0.5 M NH₄OAc.

**Microscopy and activation of KR**

The Olympus FV1000 confocal microscopy system (Cat#: F10PRDMYR-1, Olympus; Waltham, MA, USA) and FV1000 software were used for acquisition of images. Cells were cultured in 35-mm glass-bottom dishes (P35GC-1.5-14-C, MatTek; Ashland, MA, USA) before observation. Activation of KR in bulky cells was completed by exposing them to a 15-W Sylvania (Wilmington, MA, USA) cool white fluorescent bulb for 25 min in a UVP stage. The intensity was measured by ImageJ 1.50i software. P values were calculated by the Student’s t test.

**Immunoassays and m5C staining**

Cells for immunofluorescence observation were fixed in 4% PFA (19943 1 LT, Affymetrix/ThermoFisher Scientific) for 15 min at room temperature and further treated with 0.2% Triton X-100 for 8 min. They were then blocked by 5% BSA (A-7030, Sigma-Aldrich) for 1 h at room temperature. Primary antibodies were diluted in 5% BSA and incubated with cells overnight at 4°C. The samples were then washed three times with 0.05% PBST, and the cells were incubated with secondary antibodies for 1 h at room temperature followed by three washes with 0.05% PBST. Incubation with (1:1,000 dilution) DAPI for 10 min at room temperature was optional. Antibodies used in this study are summarized in Supplementary Table 1.

For m5C staining using the heat method, cells were fixed and permeabilized in a 35-mm glass-bottom dish, incubated...
in buffer (10 mM Tris-HCl, 2 mM EDTA, pH 9), and steamed on a 95°C heating block for 20 min to expose the antigen. The dish was cooled, washed three times by PBS and blocked using 5% BSA in 0.1% PBST for 1 h at room temperature. The primary and secondary antibodies were diluted in the same buffer (5% BSA in 0.1% PBST) and followed the standard IF protocol. The S9.6 staining was done using the same heat method.

**CRISPR-Cas9-based KO cell line generation**

Oligonucleotides were designed for FMRP KO in the human genome that targets the following sequences: upstream 5’-TTAGCTAACCACCAACAGCA-3’, and downstream 5’- TACATTTGCCGTAAGTCTTC -3’ synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). They were inserted into a PX330 plasmid and co-transfected with pEGFP-C3 into U2OS-TRE or Flp-in 293 cells. 48 h after transfection, single GFP positive cells were separated in 96-well dishes to obtain monoclonal colonies. The successfully KO cells were screened by Western blot with FMRP antibody.

**In vitro RNA-protein pull down assay**

3’-biotin-labeled ssRNA (5’-UGACUAAUCGAAGUUGAUACACGACGUUA-3’) and complementary ssDNA (5’-TCGTCTGTTCTCCGTGTGTGTC-3’) were synthesized from IDT. For m5C ssRNA, all five cytidines in the ssRNA sequence were substituted with m5C-modified cytidines. The ssRNA with or without m5C modification was annealed with ssDNA in a 1:1 ratio. GFP-FMRP protein in cell lysate was pulled down with the annealed DNA:RNA hybrid using the Pierce magnetic RNA-Protein pull-down kit (Cat#: 20164, ThermoFisher Scientific) following the manufacturer’s instructions. The flow-through and final elution were analyzed by Western blot.

**Demethylation assay**

For demethylation with FMRP pulldown fraction from cell lysate, Flp-in 293 FMRP KO cells transfected with Flag-FMRP or mutant plasmid were collected and cell lysates were immunoprecipitated with FMRP antibody. FMRP pulldown fraction were incubated with m5C RNA:DNA hybrid in 50 μl buffer containing 283 μM of (NH₄)₂Fe(SO₄)₂·6H₂O, 300 μM of α-KG, 2 mM of L-ascorbic acid, 50 μg ml-1 of BSA, and 50 mM of HEPES buffer (pH 7.0). The reaction was incubated for 3 h at room temperature and then analyzed by dot blot.

The demethylation assay with purified FMRP protein was performed with 5mC Tet1 Oxidation Kit (Wisegene, IL, USA) according to the manufacturer’s protocols. Single stranded RNA containing m5C or m5C RNA:DNA hybrid were used as substrates. The reaction was incubated at 37°C for 2 h and the nucleic acid substrates were extracted by phenol-chloroform method. The purified substrates were subsequently digested with nuclease P1 at 37°C for 2 h and then with alkaline phosphatase at 37°C for 2 h. The resulting ribonucleoside mixture was then subjected to LC-MS analysis at Harvard Center for Mass Spectrometry.

**ChIP-PCR**

U2OS TRE cells cultured in a 10 cm dish were transfected with TA-KR and Flag-tagged plasmids for 36 h, light irradiated for 30 min in PBS, allowed to recover for 1 h, and fixed with formaldehyde for 10 min with agitation. The ChIP assay was performed using the ChIP-IT Express Enzymatic kit (Active Motif). Briefly, after fixation, the cells were lysed using a Dounce homogenizer. The nuclei were collected and digested with enzymatic shearing cocktail for 8 min at 37°C. The sheared chromatin was collected and added to Flag beads in ChIP reaction buffer. After incubating at 4°C overnight, the beads were washed three times and eluted. The eluted chromatin was reverse-crosslinked and deproteinized before the endpoint PCR analysis. A region of the TRE locus was amplified by PCR using primers: Forward: 5’- CCACATGAAGCAGCAGACGAC-3’, Reverse: 5’- CTGGGTGCTCAGGTAGTGGT-3’.

**DR-GFP HR assay and flow cytometry**

DR-GFP U2OS stable cells were used for HR assay. Cells were transfected with the pCMV-I-SceI plasmid. Two days after transfection, the cells were collected for flow cytometry analysis. The normal cell population was gated in P1 by SSC-A and FSC-A. The HR rate was then calculated from the population of GFP-positive cells.
CRISPR-based LMNA-HR reporter assay

For the assay, a DSB 28 nucleotides upstream to the translational start site of LMNA was created by CRISPR/Cas9, while pUC19-LMNA-mClover contains mClover cDNA flanked by 5’ homology and 3’ homology arms of LMNA, which are homologous to sequences upstream and downstream to the break site generated by CRISPR/Cas9 at the LMNA genomic locus. pUC19-LMNA-mClover lacks a mammalian promoter to drive mClover expression and thus does not express mClover fluorescent protein. U2OS cells were seeded in a 6-well plate and transfected the next day. 2 μg pX459-mCherry-sgLMNA (expressing Cas9, mCherry and a guide RNA targeting LMNA locus), and 2 μg pUC19-LMNA-mClover were transfected using FuGene 6 (Promega). Three days after transfection, cells were analyzed by FACS to assess the number of mClover-positive cells in a mCherry-positive population as described in a previous study (9).

Cell survival assay

Approximately 400 U2OS cells were seeded in each 6-cm dish and cultured as described above. They were treated with IR 10-12 h after seeding. After 7-10 days, colonies were fixed and stained with 0.3% crystal violet in methanol, and the number of colonies was counted manually.

RSI calculations

Clinical information and RNA seq gene expression in breast cancer downloaded from the cancer genome atlas (TCGA). RSI was calculated using the previously published rank-based linear regression algorithm (10). Higher RSI indicates possible radioresistance compared to lower RSI. RSI = −.0098009 * AR + 0.0128283 * cJun+ 0.0254552 * STAT1 – 0.0017589 * PKC – 0.0038171 * RelA + 0.1070213 * cABL – 0.0002509 * SUMO1 – 0.0092431 * PAK2 – 0.0204469 * HDAC – 0.0441683 * IRF1. As previously described, the median value of FMRP and BRCA2 TPM was defined as the cutoff value to classify patients into high expression and low expression groups.

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