HHS Public Access

Author manuscript

Nature. Author manuscript; available in PMC 2014 March 26.

Published in final edited form as:

Nature. 2013 September 26; 501(7468): 569–572. doi:10.1038/nature12500.

Two Replication Fork Maintenance Pathways Fuse Inverted Repeats to Rearrange Chromosomes

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Abstract

Replication fork (RF) maintenance pathways preserve chromosomes, but their faulty application at nonallelic repeats could generate rearrangements causing cancer, genomic disorders and speciation[1-3]. Potential causal mechanisms are homologous recombination (HR) and error-free postreplication repair (EF-PRR). HR repairs damage induced DNA double strand breaks (DSBs) and single-ended DSBs within replication. To facilitate HR, the recombinase RAD51 and mediator BRCA2 form a filament on the 3' DNA strand at a break to enable annealing to the complementary sister chromatid[4] while the RecQ helicase, BLM (Bloom syndrome mutated) suppresses crossing over to prevent recombination[5]. HR also stabilizes[6,7] and restarts[8,9] RFs without a DSB[10,11]. EF-PRR bypasses DNA incongruities that impede replication by ubiquitinating PCNA (proliferating cell nuclear antigen) using the RAD6/RAD18 and UBC13/MMS2/RAD5 ubiquitin ligase complexes[12]. Some components are common to both HR and EF-PRR like RAD51 and RAD18[13,14]. Here we delineate two pathways that spontaneously fuse inverted repeats to generate unstable chromosomal rearrangements in wild type mouse embryonic stem (ES) cells. Gamma-radiation induced a BLM-regulated pathway that selectively fused identical, but not mismatched repeats. By contrast, UV light induced a RAD18-dependent pathway that efficiently fused mismatched repeats. Furthermore, TREX2 (a 3'→5' exonuclease) suppressed identical repeat fusion but enhanced mismatched repeat fusion, clearly separating these

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Author contribution

LH, TMK, PRY, CM, LCD and PH designed experiments and interpreted results. LH, TMK, MYS, SAK, CLH, DHK and PH performed experiments. ST provided the *rad18*$^{-/-}$ and IB10 ES cells. PH wrote the paper with comments from the other authors.

The authors declare no competing financial interests.

pathways. TREX2 associated with UBC13 and enhanced PCNA ubiquitination in response to UV light, consistent with it being a novel member of EF-PRR. RAD18 and TREX2 also suppressed RF stalling in response to nucleotide depletion. Interestingly, RF stalling induced fusion for identical and mismatched repeats implicating faulty replication as a causal mechanism for both pathways.

The identical and mismatched repeat reporters (IRR & MRR, Fig. 1a, b) were designed to investigate pathways that rearrange chromosomes through repeat fusion. Both reporters contain a 313bp major satellite repeat (MSR) at each junction of an inversion in *miniHPRT*. These repeats are indirect so repeat fusion restores *miniHPRT* to enable survival in HAT selection media by a potential mechanism shown in figure 1c. The only difference between these reporters is the MRR's 3' repeat contains seven mismatches with the longest contiguous homology being 67 bases. The IRR and MRR were stably transfected into wild type AB2.2 and IB10 ES cells. About the same number of HAT-resistant colonies spontaneously grew for both reporters (Fig. 1d, $p>0.85$, student T-test) indicating spontaneous repeat fusion occurred in wild type cells.

The fused 5' repeat for the MRR was sequenced to determine the switch location (Fig. 1e, Extend data Fig. 1). Strand exchange in fission yeast predominately occurred at the palindrome center after RFs were induced to stall, an event called a U turn[9]. We found six of 14 switches exhibited this U-turn at the base of a putative hairpin (all green), while two occurred at the apex (all orange) and six occurred in the stem (green-orange). Thus, strand exchange occurred at multiple locations.

It is possible the switched strand replicated to the telomere forming a dipericentric (Fig. 1c). Two-color fluorescence in situ hybridization (FISH) was performed on clones with the IRR and MRR using a pericentromeric and telomeric probe. Dipericentrics and chromosomes with extra pericentromeres and telomeres (EPTs)[15] were observed for cells with both reporters (Extended data Fig. 2a, Extended data Tables 1, 2). EPTs appeared unstable since the pericentromere number and location varied between metaphase spreads from the same clone implicating secondary events consistent with breakage-fusion-bridge cycles[16]. Spectral karyotyping (SKY) on three MRR clones showed multiple fusion points confirming rearrangement complexity (Extended data Table 3). Duplications of chromosome 1 (Fig. 1f1) and translocations between chromosomes 14 and 11 (Fig. 1f2) or 14 and 13 were frequently observed from the same clone and even in the same metaphase spread implicating a role in genome topology[17]. Two-color FISH was performed on a single clone (clone 18 from Extended data Tables 2 and 3) with the MRR probe and either chromosome 1 or 14. This analysis revealed unstable structures since the MRR could be found at either chromosomes 1 or 14 (Extended data Fig. 2b) implicating faulty DNA synthesis[18]. Furthermore, the MRR pattern changed from a discrete dot to multiple dots interspersed with chromosomal sequences similar to segmental duplications described during evolution[19]. Thus, both reporters caused unstable and complex rearrangements, yet the causal pathways are not known.

Complex genomic rearrangements could manifest from faulty chromosome maintenance. Therefore, we tested if γ-radiation or UV light enhanced repeat fusion for wild type AB2.2

cells with the IRR or MRR. Exposure to 4 Gy γ-radiation induced repeat fusion for the IRR (Fig. 2a left, p=0.017, student T test) but not the MRR (Fig. 2a right, p=0.16) while exposure to 20 J/m^2 UV light had the opposite effect on the IRR (Fig. 2b left, p=0.35) and MRR (Fig. 2b right, p=0.006). This contrast suggests different pathways fused identical and mismatched repeats.

We tested if HR proteins fused identical repeats since HR corrects damage caused by γ-radiation but not UV light[4]. We tested BLM-defective ES cells (*blm$^{tm3Brd/tm4Brd}$*, simply called *blm$^{-/-}$*)[20] since BLM regulates HR through Holliday junction dissolution[5]. Repeat fusion was significantly higher in *blm$^{-/-}$* cells as compared to AB2.2 cells for the IRR (Fig. 2c, compare 1 & 2, p<0.0001), but not the MRR (Fig. 2c, compare 6 & 7, p=0.47). Next we tested *blm$^{-/-}$* cells haploinsufficient for RAD51 or BRCA2 since BRCA2 enables RAD51 filament formation on DNA single stands to mediate strand annealing and Holliday junction formation. We found *blm$^{-/-}$ Rad51$^{+/\Delta ex2\text{-}4}$* cells (Extended data Fig. 3) and *blm$^{-/-}$ brca2$^{+/\Delta ex27\text{-}n}$* cells (Extended data Fig. 4a) exhibited reduced repeat fusion (Fig. 2c, compare 2 to 3 & 4, p<0.0001). Deleting the remaining *Brca2* exon 27 copy (Extended data Fig. 4b) further reduced repeat fusion (Fig. 2c, compare 4 & 5, p=0.049). Thus, BLM suppressed RAD51/BRCA2-mediated identical repeat fusion consistent with an HR-based pathway (these data do not address RAD51/BRCA2's potential role in mismatch repeat fusion).

We tested if EF-PRR fused mismatched repeats since UV light, but not γ-radiation, induced PCNA ubiquitination in mammalian cells[21]. IB10 ES cells deleted for RAD18[22] were analyzed. These cells exhibited modestly lower levels of repeat fusion for the IRR as compared to IB10 control cells (Fig. 2d, compare 1 & 2, p=0.06). This reduction could reflect RAD18's nonessential participation in HR[14]. By contrast RAD18-deletion significantly lowered fusion of mismatched repeats (Fig. 2d, compare 3 & 4, p=0.0005). The reduction of mismatched repeat fusion is greater than identical repeat fusion (p<0.0001) demonstrating RAD18's role in fusing mismatched repeats is more prominent than identical repeats. These results are consistent with EF-PRR fusing mismatched repeats. Yet, RAD18 is an E3 ubiquitin ligase so it could have broad function; therefore, mutations in other genes in the poorly understood EF-PRR pathway should be observed.

TREX2 could be a novel member of EF-PRR. Previously, we analyzed *trex2^{null}* cells and cells that expressed wild type human *TREX2* (TREX2^{WT}) and human TREX2 mutated in the catalytic domain (TREX2^{H188A}) and DNA binding domain (TREX2^{R167A}, ~85 reduction in DNA binding)[23,24]. We found TREX2 deletion elevated levels of spontaneous isochromatid breaks and chromosomal rearrangements[24,25]. TREX2^{WT} rescued the null phenotype while TREX2^{H188A} exacerbated this phenotype suggesting a dominant effect[24]. These observations suggested defective DSB repair. However, *trex2^{null}* cells exhibited increased DSB repair and normal BLM-regulated sister chromatid exchanges (SCEs)[26]. Therefore, we hypothesized TREX2 did not repair DSBs but instead suppressed DSB formation through an unknown pathway, possibly EF-PRR. In support, *trex2^{null}* cells displayed reduced levels of spontaneous SCEs[26,27].

TREX2-altered cells were tested for fusion of identical and mismatched repeats. *trex2*^{null} and TREX2^{H188A} expressing cells exhibited elevated levels of identical repeat fusion as compared to control cells (AB2.2 and *Trex2*^{hTX2} cells) (Fig. 2e, compare 1 & 3 to 2 & 4 $p<0.05$) corroborating our previous observations that HR is elevated in *trex2*^{null} cells and that an HR-based pathway fuses identical repeats. A similar anti-recombination effect on identical repeats was seen for the 3' exonucleases Exo1 and ExoVII in *E. coli* suggesting 3' exonuclease activity inhibits these fusions[28]. We also found *trex2*^{null} and TREX2^{H188A} expressing cells exhibited very low levels of mismatch repeat fusion as compared to AB2.2, *Trex2*^{hTX2} and *Trex2*^{R167A} cells (Fig. 2f, compare 1, 3 & 4 to 2 & 5 $p<0.0006$). Furthermore, TREX2 mediated UV light-induced fusion of mismatched repeats (Fig. 2b right panel, $p=0.003$). These data clearly separate the pathways that mediate identical and mismatch repeat fusion and demonstrate sequence identity determined pathway choice. These data also demonstrate the importance of TREX2's catalytic activity in mediating repeat fusion. Exonuclease activity would predictably remove intermediate 3' mismatches or flaps that could occur at the DNA incongruity or during strand exchange and strand displacement. Furthermore, these data are consistent with TREX2 being part of the EF-PRR machinery.

Three experiments were performed to test if TREX2 is a member of EF-PRR. First, TREX2 located to the nascent replication strand after UV light exposure (Extended data Fig. 5a); thus, it was at the right place at the right time. Second, TREX2 associated with UBC13, but not MMS2, by GST pull down (Extended data Fig. 5b); UBC13/MMS2 is the E2 heterodimer that polyubiquitinates PCNA[12,21]. In addition, TREX2 associated with UBC13 after ectopic expression in HeLa cells that was enhanced by UV light (Extended data Fig. 5c); thus, it associated with the PCNA ubiquitination machinery. Third, we tested the impact TREX2 and RAD18 had on PCNA ubiquitination. As a control we found UV light, but not γ-radiation, enhanced PCNA ubiquitination as previously seen in human cells[21] (Extended data Fig. 6a). TREX2 and RAD18 were needed for efficient PCNA ubiquitination after exposure to UV light (Extended data Fig. 6b-d). In addition, cells deleted for both RAD18 and TREX2 (Extended data Fig. 7) showed no further reduction in PCNA ubiquitination suggesting they are epistatic (Extended data Fig. 6b-d). These observations are consistent with TREX2 being part of the EF-PRR machinery and implicate RAD18 and TREX2 in RF maintenance.

Potential mechanisms for repeat fusion are faulty DNA repair and faulty DNA replication[2]. Repeat fusion could manifest from faulty DNA repair since γ-radiation and UV light increased fusion. However, the odds that damage actually occurred in or near the reporter sequences is small (even after exposure to agent); thus, the agents could cause a compensatory increase in repair pathways. RAD51/BRCA2/BLM are involved in both DSB repair and RF maintenance[6,7,10,11,15,29] so either are possible while direct evidence that RAD18 and TREX2 maintain RFs is lacking in mammalian cells. Therefore, *rad18*$^{-/-}$ and *trex2*^{null} cells were exposed to a brief pulse of low concentration HU (0.5 mM 90 min.) that depletes nucleotides to stall RFs without causing DSBs[6,7,10,29]. We found *rad18*$^{-/-}$ and *trex2*^{null} exhibited elevated levels of stalled RFs compared to control cells (Fig. 3a, $p<0.0001$) similar to depletion of the RAD5 ortholog, HLTF[30]. We further tested faulty replication as causal for repeat fusion by exposing cells with the IRR or MRR to this mild

HU concentration (Fig. 3b). This exposure increased repeat fusion for the IRR (p=0.00025, student t-test) and MRR (p=0.0037). Our observations suggest a BLM-regulated pathway consistent with HR fused identical repeats while a RAD18/TREX2-dependent pathway consistent with EF-PRR fused mismatched repeats during replicative stress. These pathways are good candidates for causing complex rearrangements found in cancer and genomic disorders in people and chromosomal variation that leads to species diversification.

METHODS

Construction of the IRR and MRR

The IRR and MRR contain a puromycin phosphotransferase (puro) selection cassette and an *HPRT* minigene[31] (*miniHPRT*). Puro was positioned 5' to *miniHPRT* and used to select for stable transfectants. *MiniHPRT* contains a phosphoglycerate kinase 1 (PGK) promoter[32], exons 1 and 2, intron and exons 3-8 with polyadenylation sequences. The 3' half of *miniHPRT* was inverted from intronic Xba1. Major satellite repeats (MSRs)[33] were positioned at inversion junctions in an indirect orientation. The same MSR sequence (below) is located at both junctions for the IRR (Fig. 1a, green arrow) and at the 5' junction for the MRR (Fig. 1b, green arrow) while a divergent MSR (seven mismatches) is located at the 3' end for the MRR (Fig. 1b, orange arrow). These mismatches are the only difference between the reporters.

MSR sequence, mismatched nucleotides underlined (Fig. 1a, b, green arrow):
5'TGGAATATGGCGAGAAAACTGAAAATCATGGAAAATGAGAAATACACACTTC
AGGACGTGAAATATGGCGAGGAAAACTGAAAAAGGTGGAAAATTTAGAAATGT
CCACTGTAGGACGTGGAATATGGCAAGAAAACTGTAAATCATGGAAAATGAGA
AACATCCACTTGACGACTTGAAAAATGACAAAATCACTAAAAAACATGAAAAA
TGAGAAATGCACACTGAAGGACCTGGAATATGGCTAGAAAACTGAAAATCACG
GAAAATGAGAAATACAAACCTTAGGACTTGAAATATGGCGAGGAAAACT3'

MSR sequence, mismatched nucleotides are underlined (Fig. 1b, orange arrow)
5'TGGAATATGGCGAGAAAACTGAAAATCATGGAAAATGAGAAATACACACTTT
AGGACGTGAAATATGGCGAGGAAAACTGAAAAAGGTGGAAAATTTAGAAATGT
CCACTTTAGGACGTGGAATATGGCAAGAAAACTGAAAATCATGGAAAATGAGA
AACATCCACTTGACGACTTCAAAAATGACGAAATCACTAAAAAACGTGAAAAA
TGAGAAATGCACACTGAAGGACCTGGAATATGGCGAGAAAACTGAAAATCACG
GAAAATGAGAAATACAAACCTTAGGACTTGAAATATGGCGAGGAAAACTG3'

PCR amplification of repeat fusion

PCR amplify fusions with primers 5' (HPRT4) and 3' (HPRT recom Rev) to Xba1. Sequence PCR products with the same primers.

HPRT4: 5'TCTCAAGCACTGGCCTATGC 3'

HPRT recom Rev: 5' AGACAGAATGCTATGCAACC 3'

Conditions: 1 cycle at 98°C for 10 min., 35 cycles: 98°C for 1 min., 62°C for 1 min., 72°C for 20 sec.

Tissue culture for mouse ES cells

Maintain ES cells in M15 [high glucose DMEM with 15% fetal bovine serum, 100 μM β-mercaptoethanol, 2 mM L-Glutamine, 3 mg/ml penicillin, 5 mg/ml streptomycin, 1000 U/ml ESGRO (LIF)] on plastic plates precoated with gelatin (0.1%, ~1 hour) and seeded with 2.5 × 10^6 primary murine embryonic fibroblasts (MEFs, mutated for *Hprt* and resistant to puromycin, exposed to 30 Gy γ-irradiation) and incubate at atmospheric O_2, 5% CO_2, 37°C. ES cells were also cultured on gelatinized plates without feeders.

Repeat fusion assay

Repeat fusion is seen in cells transfected with the IRR or MRR (Figs. 1d, 2, 3b). Transfect ES cells (5 × 10^6, 800 μl PBS) with 5 μg of uncut IRR or MRR by electroporation (Bio-Rad Gene Pulsar at 230 V, 500 μF). Seed cells onto 3-6 3.5 cm plates with mitotically inactivated MEFs. Each well is a replicate because they remain separate. Add puromycin (3 μg/ml) next day. About 100-200 puromycin resistant colonies grow for each well. Seven days later, pool puromycin resistant colonies for each well and passaged onto a 3.5 cm plate precoated with gelatin. Three days later passage cells onto a 10 cm plate precoated with gelatin. See below for agent expose cells. For unexposed cells, next day seed 1 × 10^6 cells onto a gelatin coated 10 cm plastic plate in M15 supplemented with 1 × HAT (1 mM sodium hypoxanthine, 4 μM aminopterin, and 160 μM thymidine). Count HAT-resistant colonies 10 days later. To control for seeding efficiencies, seed 2000 cells for each replicate onto a gelatin coated 3.5 cm plastic plate and culture in M15 without selection. Determine percentage of HAT resistant colonies by dividing the number of HAT resistant colonies by the number of cells electroporated multiplied by the seeding efficiency.

For cells exposed to agent [γ-radiation or UV light or hydroxyurea (HU)] the protocol is the same for the transfection, selection in puromycin and expansion of puromycin resistant cells (see above). After expansion, expose cells to either 4 Gy γ-radiation (^{137}Cs at a rate of 0.125 Gy/sec., Mark1 gamma radiation source from Shepard and Associates) or 20 J/m^2 UV light (a duel wavelength UV transilluminator from Alpha Innotech Corp. at a rate of 1 J/m^2 per second) or HU (0.5 mM 90 min.). For γ-radiation and UV light, expose cells directly on the plate after removing media. Then add 10 mls of pre-warmed (37°C) fresh media and incubated for 48 hours. Then seed 1 × 10^6 cells onto a gelatin coated 10 cm plastic plate in M15 supplemented with 1 × HAT. Count HAT-resistant colonies 10 days later. To control for seeding efficiencies and survival fraction, seed 2000 cells for each replicate onto a gelatin coated 3.5 cm plastic plate and culture in M15 without selection. Survival fraction is ~10%, 0.6% or 100% after exposure to γ-radiation (4 Gy), UV (20 J/m^2) or HU (0.5 mM 90 min.), respectively.

Two-color FISH with the pericentromeric and telomeric probes

Perform two-color FISH (Extended data Fig. 2a) on HAT resistant colonies expanded with the IRR or MRR. Seed cells in HAT selection media on plastic plates precoated with gelatin. Next day add fresh media (without HAT). Treat cells with colcemid (540 nM, 4 hours) then trypsinize. Slide preparation: Spin cells (1000 rpm for 10 min.), wash twice in PBS (pH 7.4) and resuspend pellet in 300 μl 75 mM KCl, dropwise, flicking tube. Incubate in a 37°C water bath (15 min.). Add dropwise 300 μl methanol/acetic acid (2:1 fixative) while flicking

tube, spin 3000 rpm, 30 sec. Wash cells in 300 μl 2:1 fixative, dropwise, flicking tube, spin @ 3000 rpm, 30 sec; repeat wash. Hybridization: Place slides in methanol over night, then incubate in 70% formamide at 70°C, place slides in 30% formamide at 37°C in dark with 500 μl/slide of 0.25 mg/ml pericentromeric (CY-3 5' TGGAATATGGCGAGAAAACTGAAAATCATGGAAAATGAGA 3') and telomeric [6-FAM 5' $(CCCTAA)_7$ 3'] probes for 15 min., wash in PBS, 10 dips, coverslip in DAPI.

Spectral Karyotyping (SKY)

Perform SKY (Fig. 1f) as described[34] with commercial SKY paint probes from Applied Imaging (Applied Spectral Imaging Inc. Carlsbad CA.). Define rearrangements with nomenclature rules from the International Committee on Standard Genetic Nomenclature for Mice[35].

Two-color FISH with the MRR and chromosome 1 or 14 paint

Perform two-color FISH (Extended data Fig. 2b) with custom made chromosome paint probes specific for murine autosomes 1 and 14 labeled with the Spectrum Green (Dyomics, Jena Germany) using a standard DOP-PCR protocol (http://atlasgeneticsoncology.org/Deep/ComparCancerCytogID20011.html). Label MRR with Spectrum Orange dUTP (Dyomics, Jena Germany) by nick translation and hybridize to chromosomal preparations derived from clone 18 (Extended data Table 3). After overnight hybridization (37°C), wash slides and counterstain with DAPI and image random fields with an inverted Zeiss Axiovert 200 using fine focusing oil immersion lens (x60, NA 1.35). Equip microscope with a Camera Hall 100 and Applied Spectral Imaging software.

Generation of mouse *Rad51* targeting vector

Construct mouse *Rad51* targeting vector (Extended data Fig. 3) as described[36]. Amplify left (5') and right (3') homologous arms with high-fidelity PCR using genomic DNA extracted from AB2.2 ES cells and iProof DNA polymerase (Bio-Rad Laboratories) in 25 μl containing 5 μL of 5X iProof HF buffer, 0.5 μL of 10 mmol/L deoxynucleotide triphosphates, 0.75 μL of 4 μmol/l forward and reverse primers (below), 100 ng of genomic DNA, and 0.25 μl of iProof DNA polymerase.

Left arm primers:

Rad51KiLA for: 5'-CACACTCGAGTCCCCTCTACGCTGAGAAGCCGGAGAAAG-3'

Rad51KiLA rev: 5'-CACAGCGGCCGCAGGCCACTAAGGCCAGAACTGCAGCTGGCCCTCCCTATCCAC-3'.

Right arm primers:

Rad51KiRA for: 5'-CACAGCGGCCGCAGGCCTGCGTGGCCGGATTATAGGAATGTCAGCTTCTCATAGAC-3'

Rad5KiRA rev: 5'-
CACAGTCGACGGTACTGGTTAGTTCATAATGTTGTTCCA-3'.

PCR conditions for both arms: 1 cycle: 98°C for 5 min. 35 cycles: 98°C for 1 min., 64.7°C-70.2°C gradient for 1 min., 72°C for 1 min. and 30 sec. 1 cycle: 72°C for 10 min.

After amplifying arms, digest left arm (3.9kb) with SalI and NotI and clone into a plasmid backbone, pKO, cut with XhoI and NotI. Then, digest right arm (3.0 kb) with XhoI and NotI and clone into the same backbone digested with SalI and NotI to delete *Rad51* exons 2-4. Then, clone floxed SAβgeo-*miniHPRT* (Extended data Fig. 3a) into unique SfiI sites as described[36].

Transfect targeting vector (5 μg, cut with Pac1) into *blm*$^{-/-}$ ES cells (5 × 10^6 cells in 800 μl PBS) by electroporation (Gene Pulser Cuvettes with a 0.4 cm electrode gap at 230 V, 500 μF with a Gene Pulser Apparatus from Bio-Rad). After electroporation, seed cells onto two 10 cm plates with mitotically inactive MEFs. Next day, add M15 medium containing 1 × HAT (0.1 mM hypoxanthine, 0.0004 mM aminopterin, and 0.016 mM thymidine). Pick HAT resistant colonies 7 days later onto a 96-well plate and maintain in HAT selection. Replica plate to freeze one plate and use the other to isolate genomic DNA[37]. Screen for targeted clones with PCR (Extended data Fig. 3b).

H13F (in *miniHPRT*): 5'-
GTAAATGAAAAAATTCTCTTAAACCACAGCACTATTGAG-3'

SR3 (outside the right arm): 5'-
AGCCAGGTATAGTCTCAAAGGAATCTGCAATCC-3'.

PCR conditions: 1 cycle: 98°C for 5 min.; 35 cycles: 98°C for 1 min., 67°C for 1 min., 72°C for 1 min. 30 sec.; and 1 cycle: 72°C for 10 min.

Cre-mediated deletion of SAβgeo and 5' *miniHPRT*

Delete *SAβgeo* and 5' half of *miniHPRT* using Cre recombinase to generate *Rad51*$^{+/\Delta ex2\text{-}4}$ cells (Extended data Fig. 3c). Expand targeted ES cells in 1 × HAT to remove HPRT-negative cells that survive due to cross feeding. Removed HAT selection 2 days before transfection and cultured in 1 × HT (1 mM sodium hypoxanthine and 160 μM thymidine); electroporate 5 × 10^6 cells in 800 μL DPBS with 10 μg of pPGKcrepA using a Bio-Rad Gene Pulsar at 230 V, 500 μF. After electroporation, seed 200 μl onto a 10 cm feeder plate without selection for 2-4 days to allow time for *miniHPRT* removal and time for degradation of *HPRT* mRNA and protein. Then seed 4 × 10^4 cells onto a 10 cm feeder plate in 10 μM TG (6-thioguanine). Pick TG-resistant colonies 10 days later. Expand cells in 10 μM TG and replica plate. Freeze one plate and use the other to isolate genomic DNA[37]. Confirmed Cre-mediated deletion with PCR (1.4 kb fragment).

PCR primers:

RCF1 (in *RAD51* intron 1): 5'-GTGCTGAATCTCCTAGAACTG-3'

AS2 (in exon cluster 3-8 of *miniHPRT*): 5'- TGTCCCCTGTTGACTGGTCA-3'.

PCR conditions: 1 cycle: 98°C for 5 min., 35 cycles: 98°C for 1 min., 64°C for 1 min., 72°C for 30 sec. 1 cycle: 72°C for 10 min.

Targeting mouse *Brca2* exon 27

Replace the first copy of *Brca2* exon 27 with PGKneobpA[38] by cloning PGKneobpA into the Sfi1 sites of the *Brca2* exon 27 deletion targeting vector (Extended data Fig. 4a)[36]. Transfect as described for *Rad51*. Use PCR to detect targeted clones (Extended data Fig. 4a).

PCR primers:

NF (in neo): 5'AGCGCATCGCCTTCTATCGCCTTCTTGACG3').

Brca2 intron 27 reverse: 5'-CCCCGTCGACCGGAGAGCTAATGGCCTCTACTCCAACG-3' Conditions: 35 cycles of 98°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute and 30 seconds.

Replace the second copy of *Brca2* exon 27 with floxed *miniHPRT* (Extended data Fig. 4b)[36]. Use PCR to detect targeted clones (Extended data Fig. 4b).

PCR primers:

H13F: 5'-GTAAATGAAAAAATTCTCTTAAACCACAGCACTATTGAG-3'

B27R: 5'-CCCCGTCGACCGGAGAGCTAATGGCCTCTACTCCAACG-3' Conditions: 35 cycles of 98°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute and 30 seconds.

Removed the 5' half of *miniHPRT* by Cre-mediated recombination[36] to generate $Brca2^{\Delta ex27\text{-}h/\Delta ex27\text{-}n}$ cells. Use PCR to detect removal (Extended data Fig. 4b).

PCR primers:

Bi26: 5'-TCAATCAAGCAGTCCTCACC-3'

H3-8R: 5'-TGACCAGTCAACAGGGGACA-3'

Conditions: 35 cycles: 98°C for 1 minute, 65°C for 1 minute, 72°C for 45 seconds.

Co-IP of IdU and Myc-TREX2 after exposure to UV light

TREX2 associates with nascent strand DNA after UV exposure (Extended data Fig. 5a). Experiment performed as described[10] with minor modifications. Transfected HeLa cells with 5 μg Myc-TREX2 using FuGENE6 (Roche). Label cells with IdU (5 μM, 30 min), treat with 20 J/m^2 UV and recover with the indicated time. Crosslink cells in formaldehyde (1%, 15 min. 24°C). Remove cytoplasmic protein fraction by incubation in hypotonic buffer [10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, β-mercaptoethanol, PMSF, Protease Inhibitor (Roche) for 10min. on ice]. Resuspend pellets in nuclear exact buffer [20 mM Hepes, 20% Glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, β-mercaptoethanol, PMSF, Protease Inhibitor (Roche)]. Dilute nuclear exact protein (50 μg) solution with equal volume of IP dilution buffer [20 mM Hepes, 0.2 mM EDTA, 10% Glycerol, PMSF, Protease Inhibitor (Roche)] and pre-wash with Protein G Sepharose beads (10 μl, 1 hour). Remove

bead and immunoprecipitate supernatant by incubating with 1 mg of anti-BrdU (mouse anti-BrdU B44) at 4°C overnight. Incubate reaction solution with 20 μl Protein G Sepharose beads for 3 hour at 4°C and wash beads 4 times with IP wash buffer. Separate immunoprecipitated proteins with SDS/PAGE gel and blot with anti-Myc (BD Bioscience) antibody.

TREX2-UBC13 association

TREX2 associates with UBC13 by GST pull-down (Extended data Fig. 5b). Bind GST-MMS2, GST-UBC13, and GST-TREX2 fusion proteins (5 μg) to glutathione-Sepharose 4B (GE Healthcare) and incubate with [35S]-methionine-labeled TREX2 (4 μl, 1.5 hour, 23°C)[39]. Wash beads with NETN buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, pH 7.5, and 0.1% NP40) and subject to SDS-PAGE and phosphorimager analysis.

TREX2 associates with UBC13 by Co-immunoprecipitation in HeLa cells (Extended data Fig. 5c). Transfect HeLa cells with 5 μg Myc-TREX2 and 5 μg HA-UBC13 plasmid (48 hour) using FuGENE6 (Roche), expose cells to 0 J/m^2 or 20 J/m^2 UV as described for the PCNA ubiquitination assay (below). Crosslink cells in formaldehyde (1%, 15 min., 24°C). Incubate in hypotonic buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, β-mercaptoethanol, PMSF, Protease Inhibitor (Roche) for 10 min. on ice to remove cytoplasmic protein fraction. Resuspend pellets in nuclear exact buffer [20 mM Hepes, 20% Glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, β-mercaptoethanol, PMSF, Protease Inhibitor (Roche)]. Dilute nuclear exact protein (50 μg) solution with equal volumes of IP dilution buffer [20 mM Hepes, 0.2 mM EDTA, 10% Glycerol, PMSF, Protease Inhibitor (Roche)] and incubate with 10 μl Protein G Sepharose beads (1 hour). Remove beads and immunoprecipitate supernatant by incubating with 2 μg anti-Myc (BD Bioscience) or anti-HA (Roche) antibody at 4°C overnight. Incubate reaction solution with 20 μl Protein G Sepharose beads for 3 hr. Wash beads 4 times with IP wash buffer. Separate immunoprecipitated proteins by SDS/PAGE gel and blot with anti-Myc or anti-HA antibody.

Detection of PCNA ubiquitination with chromatin-bound fraction

RAD18 and TREX2 participated in UV-induced PCNA ubiquitination (Extended data Fig. 6). Isolate chromatin-bound fraction as described[21,40] with modifications. Briefly, resuspend ~1.5×10^7 cells in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100 and protease inhibitor cocktail (Roche)], incubate and rotate 5 min. at 4°C then centrifuge (7000 rpm, 2 min., 4°C). Remove soluble fraction. Resuspended pellet in buffer then centrifuge (7000 rpm, 3min., 4°C). Extract chromatin-bound fraction, resuspend pellet in buffer B [20 mM Tris-Cl (pH 8.1), 2 mM EDTA (pH 8.0), 500 mM NaCl, 0.1% SDS, 1% Triton X-100 and protease inhibitor cocktail (Roche)], sonicate, treat with micrococcal nuclease (10 min., 37°C) and centrifuge (13000 rpm, 15 min., 4°C). Immunoprecipitate supernatant containing released chromatin-bound protein. Pre-incubate with protein G Sepharose beads (GE healthcare) (1-2hr., 4°C) to pre-cleaned protein and incubated with 1 μg of anti-PCNA antibody (PC10, Santa Cruz Biotechnology) overnight at 4°C. Precipitate anti-PCNA immune complexes with 30 μl protein G Sepharose beads for 3 hours at 4°C). Separate protein on 10% SDS/PAGE gel and transfer onto PVDF

membrane. Use monoclonal antibodies for Western blot: anti-Ub (P4D1, COVANCE; 1:1000-2000) or anti-PCNA (PC10, Santa Cruz Biotechnology; 1: 2000-2500). Used mouse True®Blot ULTRA (Anti-mouse Ig HRP, ROCKLAND; 1:1000-2500) to minimize IgG signal. Quantify band intensities with ImageJ software (http://rsb.info.nih.gov.ij).

Targeting *Trex2* in IB10 cells and *rad18*$^{-/-}$ cells

Electroporate *Trex2* targeting vector (5 μg of PacI-cut) (Extended data Fig. 7)[25] into IB10 cells and *rad18*$^{-/-}$ cells as described for *Rad51*.

Primers to detect Left arm integration:

TX2 LR55 (outside of Left arm, Table 2-1): 5'-TAT ATT TAG GAG ACA AAG TGG CCC TGC CAG AGC TG-3'

HATrev (in the *HPRT* minigene) 5'- CAT GCG CTT TAG CAG CCC CGC TGG GCA CTT GGC GC -3'

Conditions: 1 cycle: 98°C for 5 min. 35 cycles: 98°C for 1 min., 72°C for 1 min., 72°C for 2 min. 30 sec. 1 cycle: 72°C for 10 min.

Primers to detect Right arm integration:

HATfor (in the *HPRT* minigene) 5'-GTA AAT GAA AAA ATT CTC TTA AAC CAC AGC ACT ATT GAG-3'

TX2 RR33 (outside the Right arm). 5'-CCT GTT TCA CAA ATA TCA GGA CCT GAG TTT GTA TCC-3'

Conditions: 1 cycle: 98°C for 5 min. 35 cycles: 98°C for 1 min., 63.5°C for 1 min., 72°C for 2 min. 30 sec. 1 cycle: 72°C for 10 min.

Primers to confirm deletion of TREX2 open reading frame

mTX2For: 5'-AAAAGAATTCCCGCCACCATGTCTGAGCCACCCCGGGC-3'

mTX2Rev: 5'-AAAACTCGAGTCAGGCTTCGAGGCTTGGACC-3'

Conditions: 1 cycle: 98°C for 5 min. 35 cycles: of 98°C for 1 min., 65°C for 1 min., 72°C for 25 min. 1 cycle: 72°C for 10 min.

Microfiber analysis

RAD18 and TREX2 enabled replication fork restart (Fig. 3a). Perform DNA fiber analysis as described[10,15] with modifications. Pulse-label ES cells with IdU (25 μM, 20 min.), wash twice with medium, expose to HU (0.5 mM, 1.5 hour), wash twice with medium and pulse-label with CldU (250 μM, 20 min). Fix fibers in methanol and acetic acid (3:1) and air-dry. To denature fibers, treat slides with HCl (2.5 M, 75-80 min.) and wash twice with PBS then block 1 hour with 1% BSA (Bovine Serum Albumin) + 0.1% Tween 20. Incubate slides with primary antibodies against CldU (rat anti-BrdU BU1/75[ICR1], Abcam, 1:1000) and IdU (mouse anti-BrdU B44, 1:750) for 1.5 hours. Fix slides with 4% PFA and wash thrice with PBS. Apply AlexaFluor 555-conjugated goat anti-rat IgG (Molecular Probes, 1:500) and AlexaFluor 488-conjugated goat anti-mouse IgG (Molecular Probes, 1:500) to slides for 2

hours. Wash slides and mount in Fluroshield (sigma) and examine (Axioplan2, Zeiss fluorescent microscope).

Statistics

Student T test was used for statistics (two-sided without adjustments for multiple comparisons). The average was the center value. In all figures the s.e.m. is shown and the number of biological replicates are provided in the legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. Williams and S. Dodds for technical assistance and the Molecular Cytogenetic Core at Albert Einstein College of Medicine for help with the execution of SKY and two-color FISH. This work was supported by the National Institutes of Health (1 RO1 CA123203-01A1 to PH and CM, 2P01AG017242-12 to PH, P30CA013330 to CM) and with support from the CTRC (P30 CA054174).

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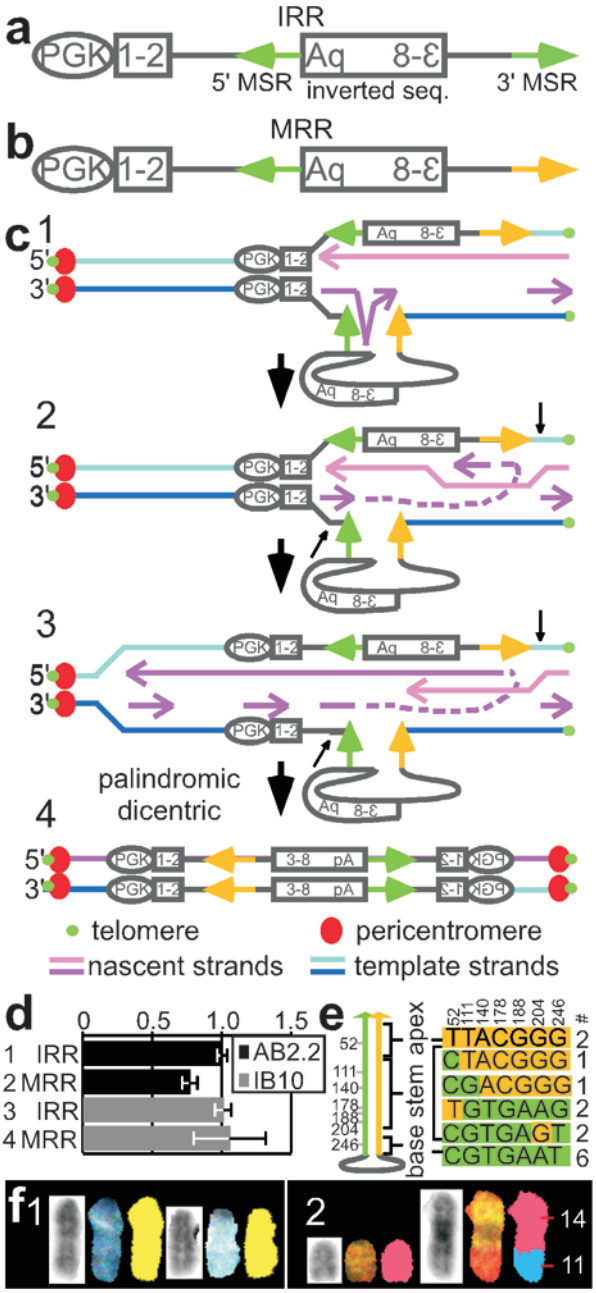

Figure 1. Inverted repeat fusion

(**a, b**) *MiniHPRT* reporters. Promoter (PGK) with intron that separates exons 1&2 from 3-8. Repeats at inversion junction. The IRR (**a**) and MRR (**b**) differ only in seven 3' repeat mismatches (green vs. orange arrow). **c,** Repeat fusion model. 1) Nascent lagging strand stalls at repeat hairpin and 2) switches to displace complementary template strand to 3) correct *miniHPRT* and 4) produce a dipericentric. **d,** Repeat fusion in AB2.2 and IB10 cells. Shown is the ratio of HAT resistant colonies compared to IRR. Percentages of HAT-resistant colonies for the IRR in AB2.2 and IB10 are 0.02% and 0.14%, respectively. Biological replicates for lanes 1-4: 19, 19, 18, 18. Standard error of the mean (SEM). **e,** Sequence of fused repeats for the MRR in AB2.2 cells (Extended data Fig. 1). **f,** SKY

analysis on clone 18 (Extended data Table 3). 1) Duplication of chromosome 1. 2) translocation of chromosomes 11 and 14.

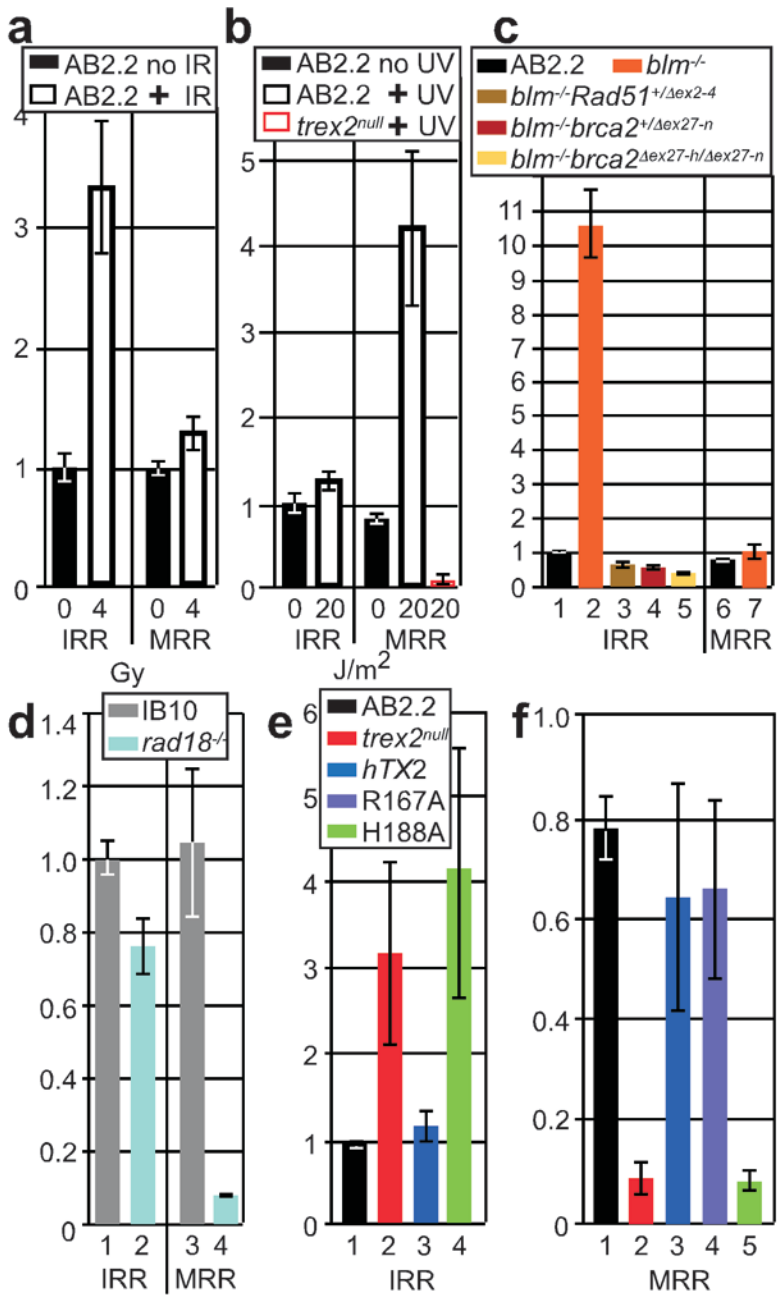

Figure 2. Two pathways enable repeat fusion that depend on sequence identity

Shown is the ratio of HAT resistant colonies transfected with IRR in control cells displayed in figure 1d. **a,** Gamma-radiation (4 Gy) increases fusion for the IRR (left) but not MRR (right). Survival fraction, ~10%. Biological replicates for lanes 1-4: 19, 11, 19, 11. SEM. b, UV (20 J/m^2) enables fusion for the MRR (right) but not IRR (left). Survival fraction, ~0.6%. Biological replicates for lanes 1-4: 19, 11, 19, 11. SEM. **c,** BLM suppressed repeat fusion for the IRR but not MRR. *blm*$^{-/-}$ cells deleted for one copy of *Rad51* exons 2-4 (*blm*$^{-/-}$ *Rad51*$^{+/\Delta ex2-4}$ cells), one copy of *Brca2* exon 27 (*blm*$^{-/-}$ *brca2*$^{+/\Delta ex27-n}$) or two copies of *Brca2* exon 27 (*blm*$^{-/-}$ *brca2*$^{\Delta ex27-h/\Delta ex27-n}$). Biological replicates for lanes 1-7: 19, 23, 12,

12, 12, 19, 23. SEM. **d,** RAD18 enabled fusion for the MRR more than IRR. Biological replicates: 18 for all lanes. SEM. (**e, f**) TREX2 suppressed fusion for the IRR (e) but enabled fusion for the MRR (f). Examined are *trex2*^{null} cells that express human wild type *TREX2* (*hTX2*) or human *TREX2* mutated in the DNA binding domain (R167A) or catalytic domain (H188A). Biological replicates for lanes e1-4: 19, 19, 20. 23 and for lanes f1-5: 19, 21, 21, 21, 23. SEM.

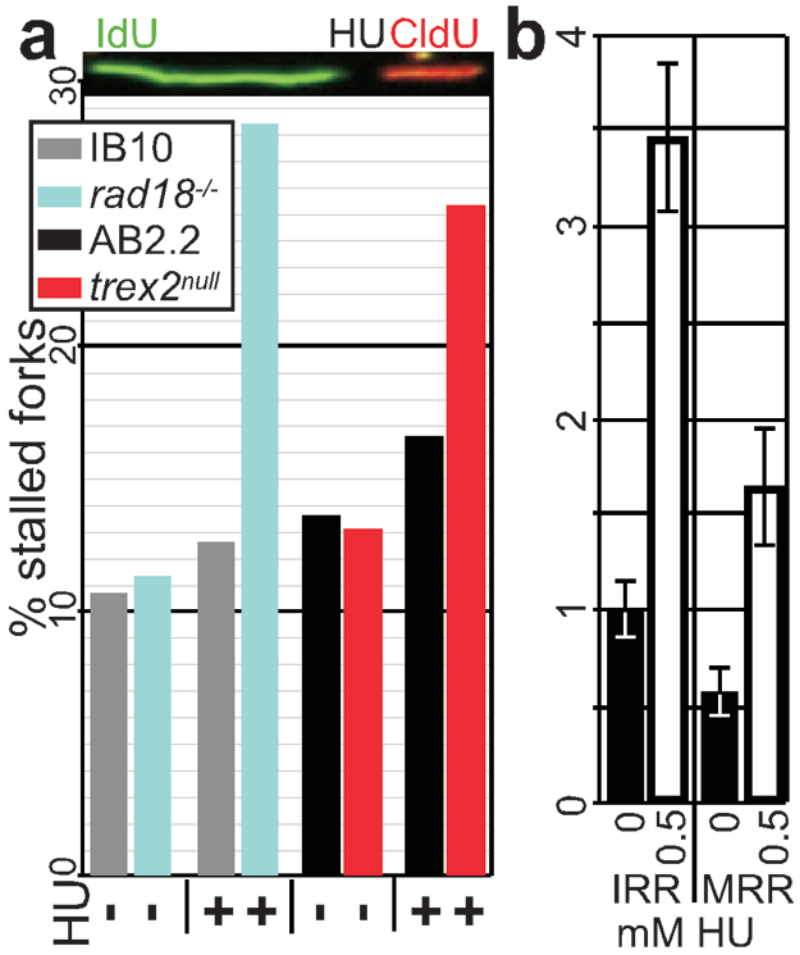

Figure 3. HU-induced nucleotide depletion

a, RAD18 and TREX2 maintain replication forks. The % of stalled replication forks after HU exposure. Experimental design: cells were cultured in IdU (20 min.) to label nascent strand and then exposed to HU (0.5 mM, 90 min.) to stall replication and then cultured in CldU (20 min.) to label restart. Fiber number observed without and with HU: IB10 (1943, 657), *rad18*$^{-/-}$ (1180, 1460), AB2.2 (452, 510), *trex2*^{null} (705, 448). **b,** The impact of HU (0.5 mM, 90 min.) on repeat fusion for the IRR (left) and MRR (right). The ratio of HAT resistant colonies as compared to AB2.2 cells transfected with the IRR (0.05%) is shown. Survival fraction is 100%. SEM. Biological replicates: 6 for all lanes.