BRCA1 controls homologous recombination at Tus/Ter–stalled mammalian replication forks

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Replication fork stalling can promote genomic instability, predisposing to cancer and other diseases3–9. Stalled replication forks may be processed by sister chromatid recombination (SCR), generating error-free or error-prone homologous recombination (HR) outcomes8–10. In mammalian cells, a long-standing hypothesis proposes that the major hereditary breast/ovarian cancer predisposition gene products, BRCA1 and BRCA2, control HR/SCR at stalled replication forks3. Although BRCA1 and BRCA2 affect replication fork processing10–12, direct evidence that BRCA gene products regulate homologous recombination at stalled chromosomal replication forks is lacking, due to a dearth of tools for studying this process. Here we report that the Escherichia coli Tus/Ter complex13–16 can be engineered to induce site-specific replication fork stalling and chromosomal HR/SCR in mouse cells. Tus/Ter–induced homologous recombination entails processing of bidirectionally arrested forks. We find that the Brca1 carboxy (C)-terminal tandem BRCT repeat and regions of Brca1 encoded by exon 11—two Brca1 elements implicated in tumour suppression—control Tus/Ter–induced homologous recombination. Inactivation of either Brca1 or Brca2 increases the absolute frequency of ‘long-tract’ gene conversions at Tus/Ter-stalled forks, an outcome not observed in response to a site-specific endonuclease-mediated chromosomal double-strand break. Therefore, homologous recombination at stalled forks is regulated differently from homologous recombination at double-strand breaks arising independently of a replication fork. We propose that aberrant long-tract homologous recombination at stalled replication forks contributes to genomic instability and breast/ovarian cancer predisposition in BRCA mutant cells.

Tus binds the 23 base pair (bp) Ter site to induce polar replication fork arrest in E. coli3–16. To determine whether Tus/Ter can arrest mammalian replisomes, we introduced six Ter sites into a plasmid containing the Epstein–Barr virus nuclear antigen 1 (EBNA1)-binding origin of replication (p6×TerOri, Fig. 1a). EBNA1 recruits mammalian replication factors, mediating predominantly unidirectional plasmid replication, due to a replication block at EBNA1-bound FR (family of repeats)17. In p6×TerOri, the major clockwise fork approaches the ‘non-permissive’ (fork-stalling) face of Tus/Ter (Fig. 1a). We used two-dimensional DNA gel electrophoresis with Southern blotting18 to visualize replication through 6×Ter. Transfection of 293E cells, which express EBNA1 (ref. 19), with p6×TerOri and control empty vector revealed plasmid replication intermediates (arc A, Fig. 1b). Co-transfection of p6×TerOri and myc-tagged Tus revealed site-specific stalling of the clockwise fork (spot B, Fig. 1b, c and Extended Data Fig. 1). TusH144A, a Ter-binding-impaired mutant18, induced minimal fork stalling. Reversal of 6×Ter to the ‘permissive’ orientation (6×REVTer, Fig. 1b) also supported Tus-dependent stalling of the clockwise fork, albeit less efficiently than non-permissive 6×Ter (Fig. 1b, c). The FR/EBNA1 replication block is incomplete19. A weaker Tus/Ter-dependent double-Y spot (C, Fig. 1b and Extended Data Fig. 1) reflects bidirectional fork arrest at 6×Ter. We estimate the FR/EBNA1 and Tus/6×Ter replication block efficiencies as ~70% (Extended

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Data Fig. 2). Thus, Tus/Ter mediates bidirectional site-specific arrest of mammalian replication forks.

To determine whether Tus/Ter induces HR/SCR at a defined chromosomal locus in mammalian cells, we placed 6×Ter in a homologous recombination reporter of short- and long-tract gene conversion (termed STGC and LTGC, respectively) between sister chromatids. Duplication of a red fluorescent protein (RFP) cassette distinguishes LTGC (length ≥ 1252 bp; GFP + RFP + ) from STGC (length < 1252 bp; GFP + RFP + ; Fig. 2a). 6×Ter abuts an I-SceI site, interrupting an enhanced green fluorescent protein gene (6×Ter-I-SceI-GFP, Fig. 2a). Recombination of the staked left-hand fork (Fig. 2a) with the 5′-truncated GFP copy (Tr-GFP) of the sister chromatid generates wild-type GFP. If chromosomal fork arrest was bidirectional, this could produce a two-ended break, generating predominantly STGCs (Fig. 2a and Extended Data Fig. 3a). In contrast, unidirectional fork arrest with one-ended breaks would favour LTGC, and any STGCs arising from one-ended breaks would necessarily be terminated by non-canonical mechanisms (Extended Data Fig. 3b).

We targeted the 6 × Ter/HR reporter as a single copy to the ROSA26 locus of mouse embryonic stem (ES) cell line 11CO/47T (Brca1fl/BRCT). Brca1fl/BRCT encodes a C-terminal truncated protein; the BRCT-encoding elements of Brca1fl can be conditionally deleted (generating Brca1Δ9). Indeed, Tus, but not TusH144A, induced HR within 6×Ter/HR Brca1fl/BRCT cells, the major HR product being STGC (Fig. 2b). Tus failed to induce HR in Brca1fl/BRCT cells containing a ROSA26-targeted HR reporter lacking the Ter array (Fig. 2b). Thus, Tus-Ter-induced chromosomal HR requires cognate Tus–Ter binding. The ratio LTGC/total HR, a measure of the probability that HR resolves as LTGC, was ~7% in three independent Tus-transfected clones (Fig. 2c and Extended Data Fig. 4a). Three additional independent clones of Brca1fl/BRCT ES cells, each containing a single-copy randomly integrated chromosomal 6×Ter/HR reporter, behaved similarly (Extended Data Fig. 4b). The predominance of STGC and the consistent results at different loci suggested that Tus/Ter-induced HR entails bidirectional fork arrest (Extended Data Fig. 3a). We resolved this definitively by Southern blot analysis of Tus/Ter-induced STGCs. Unidirectional fork arrest/breakage (Extended Data Fig. 3b) could produce a one-ended break, generating STGC products of variable size. In contrast, bidirectional fork arrest (Fig. 2a and Extended Data Fig. 3a) could produce a two-ended break, with STGC termination by annealing. This would generate STGC products of fixed size, resembling the parental reporter but lacking the 6×Ter array or I-SceI site (Fig. 2a). Indeed, 44/44 Tus/Ter-induced STGCs in 6×Ter/HR reporter Brca1fl/BRCT cells revealed this latter structure (Fig. 2d). As expected, I-SceI-induced HR behaved similarly (Fig. 2d). A second arrested fork (right-hand fork, Fig. 2a) must provide the homologous second end during Tus/Ter-induced STGC. Therefore, Tus/Ter-induced STGC is the product of bidirectional replication fork arrest. Overall, I-SceI-induced HR in Brca1fl/BRCT 6×Ter/HR reporter cells was ~20% of that in isogenic ROSA26-targeted Brca1fl/BRCT HR reporter cells, which lack a 6×Ter array (data not shown). The reasons for this difference are unclear.

To investigate further the non-polar behaviour of Tus/Ter in mammalian HR, we studied the Tus mutant F140A that binds duplex Ter with higher affinity than Tus but is defective for the Ter C-6 base-flipping ‘lock’ mechanism that contributes to polar fork arrest in E. coli. TusF140A induced higher levels of HR than Tus in 6×Ter/HR Brca1fl/BRCT cells (Extended Data Fig. 4c, d), showing that the C-6 ‘lock’ is dispensable for Tus/Ter-induced HR in mammalian cells. This might be explained by the different polarities of the E. coli DnaB and vertebrate MCM replicative helicases. Ter C-6 is located on the leading strand of the fork approaching the non-permissive end of Ter. Unlike DnaB, which translocates along the lagging strand, the MCM helicase translocates along the leading strand and might occlude Ter C-6 within its barrel, thereby denying Tus access to the C-6 lock mechanism. To determine the minimal number of Tus/Ter complexes needed for HR induction, we generated reporters containing 3, 2 or 1 Ter sites (Extended Data Fig. 4e). We targeted each, in parallel, as a single copy to the ROSA26 locus of Brca1fl/BRCT ES cells and found that a minimum of 3 Ter sites is required for robust Tus-induced HR (Extended Data Fig. 4f). We inverted the 6×Ter array orientation to generate a 6×REVTer/HR reporter. When targeted as a single copy to the ROSA26 locus of Brca1fl/BRCT ES cells, this reporter supported Tus-induced HR as robustly as the 6×Ter/HR reporter (Extended Data Fig. 4f). Our findings do not exclude a polar component to Tus/Ter-induced fork stalling on a mammalian chromosome but this polarity, if present, is relative and not absolute.

Interstrand DNA crosslink repair of plasmids replicating in Xenopus laevis egg extracts entails endonucleolytic attack of bidirectionally stalled forks. Interestingly, Tus/Ter-induced HR was suppressed by depletion of the endonuclease scaffold Sxl4/FancP23,24 to a greater extent than I-SceI-induced HR (Extended Data Fig. 5), indicating that Sxl4 contributes specifically to Tus/Ter-induced HR. However, it is not clear whether Sxl4 mediates endonucleolytic attack of stalled forks during
Tus/Ter-induced HR. Work in Schizosaccharomyces pombe suggests that alternative mechanisms, such as template switching, could mediate HR at stalled mammalian forks.

To determine whether BRCA1 regulates HR at stalled replication forks, we transduced 6×Ter/HR Brca1<sup>ΔBRCT</sup> cells with adeno-Cre and screened for Brca1 loss (Fig. 3a). The resulting Brca1<sup>ΔBRCT</sup> cells are viable hypomorphic with growth characteristics similar to Brca1<sup>ΔBRCT</sup> cells<sup>11</sup>. We studied Tus-induced HR in three independent Cre-treated clones of each genotype (Fig. 3). Surprisingly, Tus-induced STGC in 6×Ter/HR Brca1<sup>ΔBRCT</sup> cells showed no reduction compared to Brca1<sup>ΔBRCT</sup> cells, but LTGC was elevated twofold (Fig. 3b). Correspondingly, the probability of engaging LTGC during Tus/Ter-induced HR was doubled to ~15% (Fig. 3b). Consistent with our recent findings, I-SceI-induced HR in 6×Ter/HR Brca1<sup>ΔBRCT</sup> cells was diminished and biased in favour of LTGC (Fig. 3b)<sup>11</sup>. Southern blot analysis of Tus/TER-induced STGC and LTGC products in 6×Ter/HR Brca1<sup>ΔBRCT</sup> cells revealed patterns similar to Brca1<sup>ΔBRCT</sup> cells (Extended Data Fig. 6). However, in Brca1<sup>ΔBRCT</sup> cells, 6/41 (15%) Tus/TER-induced STGC and 3/15 (20%) LTGC clones retained an additional copy of the parental reporter (Extended Data Fig. 6). This was not separable by cloning, suggesting that it was retained by non-disjunction. A total of 4/41 (9.8%) I-SceI-induced STGC Brca1<sup>ΔBRCT</sup> clones revealed non-disjunction; thus, non-disjunction is not specific to Tus/TER-induced HR. The fact that the donor sister was unaltered during LTGC excludes crossing-over as a cause of the LTGC outcome in these clones<sup>23,26</sup>.

We asked whether Brca1 domains additional to the BRCT repeat regulate Tus/Ter-induced HR. Indeed, short interfering (siRNA)-mediated Brca1 depletion suppressed STGC but increased LTGC in both Brca1<sup>ΔBRCT</sup> and Brca1<sup>ΔBRCT</sup> cells (Extended Data Fig. 7). In Brca1-depleted Brca1<sup>ΔBRCT</sup> cells, ~40% of all HR products were LTGCs. More than half of the Brca1 polypeptide is encoded by exon 11, which is a target of inactivating germline mutations in hereditary breast/ovarian cancer; exon 11 is also alternatively spliced, generating an in-frame nuclear Aexon11 gene product that retains an N-terminal RING domain and C-terminal BRCT functions<sup>11</sup>. To test whether Brca1 exon 11 regulates Tus/Ter-induced HR, we targeted a single copy of the 6×Ter/HR reporter to the ROSA26 locus of mouse Brca1<sup>ΔExon11</sup> ES cells. The Brca1<sup>ΔExon11</sup> allele lacks exon 11; exon 11 of Brca1<sup>Δ</sup> can be conditionally deleted to generate Brca1<sup>ΔΔ</sup> (Fig. 4a).<sup>38</sup> Note that Brca1<sup>Δ</sup> and Brca1<sup>ΔΔ</sup> denote distinct Brca1 alleles in the two Brca1 conditional systems described here. Following adenovirus Cre treatment, we retrieved 6×Ter/HR Brca1<sup>ΔExon11</sup> and 6×Ter/HR Brca1<sup>ΔΔExon11</sup> clones. Each of three independent 6×Ter/HR Brca1<sup>ΔΔExon11</sup> clones reduced Tus/TER-induced STGC but increased absolute frequencies of LTGC in comparison with three independent 6×Ter/HR Brca1<sup>ΔExon11</sup> clones (Fig. 4b). Deletion of Brca1 exon 11 increased the probability of engaging Tus/TER-induced LTGC ~4-fold to ~30% (Fig. 4b). In contrast, the absolute frequency of I-SceI-induced LTGC was reduced in Brca1<sup>ΔExon11</sup> cells and ~20% of HR products were I-SceI-induced LTGC.

Figure 3 | The Brca1 tandem BRCT repeat regulates Tus/Ter-induced homologous recombination. a, Brca1 gene in Brca1<sup>ΔExon11</sup> ES cells. Brca1<sup>ΔExon11</sup> encodes a truncated protein. Cre converts Brca1<sup>Δ</sup> to the exon 22–24-deleted Brca1<sup>Δ</sup> allele. Grey boxes, Brca1 exons; black triangles, loxp sites; pA, polyadenylation signal; SA, splice acceptor; neo, neomycin resistance gene; pgk, phosphoglycerate kinase promoter. b, Tus- and I-SceI-induced HR in Brca1<sup>Δ</sup> and Brca1<sup>ΔBRCT</sup> 6×TER-HR cells (three independent clones each). Mean of triplicate samples, n = 4. Error bars represent s.e.m. Student’s t-test Brca1<sup>Δ</sup> versus Brca1<sup>ΔBRCT</sup> in all six panels t < 0.05. c, Upper panel, endogenous Brca1 immunoblot in Brca1<sup>Δ</sup> and Brca1<sup>ΔBRCT</sup> ES cells. Asterisk indicates a background band. Lower panel, β-actin loading control. d, Quantitative polymerase chain reaction with reverse transcription (qRT–PCR) for Brca1 mRNA. Exon 22–23 is deleted in Brca1<sup>ΔBRCT</sup> cells.

Figure 4 | Brca1 Exon11 regulates Tus/Ter-induced homologous recombination. a, Brca1 gene in Brca1<sup>ΔExon11</sup> ES cells. The Brca1<sup>ΔExon11</sup> encodes the Aexon11 protein. Cre converts Brca1<sup>Δ</sup> to exon11-deleted Brca1<sup>ΔΔ</sup> allele. Symbols as in Fig. 3. PCR primers a, b and d shown. b, Tus- and I-SceI-induced HR in Brca1<sup>ΔExon11</sup> and Brca1<sup>ΔΔExon11</sup> 6×TER-HR cells (three independent clones each). Mean of triplicate samples, n = 4. Error bars represent s.e.m. Student’s t-test Brca1<sup>ΔExon11</sup> versus Brca1<sup>ΔΔExon11</sup> in all 6 panels P < 0.005. c, Upper panel, endogenous Brca1 immunoblot in Brca1<sup>ΔExon11</sup> and Brca1<sup>ΔΔExon11</sup> ES cells. The asterisk denotes a background band. The lower panel denotes a β-actin loading control. d, PCR genotyping of Brca1<sup>ΔExon11</sup> and Brca1<sup>ΔΔExon11</sup> clones from panel b. P, untargeted Brca1<sup>ΔExon11</sup> ES cell, Empty (no DNA) control. Brca1<sup>Δ</sup> product, 531 bp; Brca1<sup>ΔΔ</sup> product, 621 bp.
LTGCs (Fig. 4b). Thus, Brca1 exon 11 contributes to Tus/Ter-induced HR both quantitatively and qualitatively.

To determine whether BRCA2/Rad51 regulates Tus/Ter-induced HR, we used siRNA to deplete Brca2 or Rad51 during HR induction. Depletion of Brca2 suppressed Tus/Ter-induced STGC but elevated LTGC frequencies in both Brca1ΔBrCT and Brca1ΔBRCT cells (Extended Data Fig. 8). In Brca1ΔBrCT and Brca1ΔBRCT cells depleted of Brca2, ~30% and ~50% respectively of all Tus/Ter-induced HR products were LTGCs, whereas the equivalent probabilities for Rad51-depleted cells were ~40% and ~70% (Extended Data Fig. 9). Thus, suppression of LTGC at stalled forks is a shared function of BRCA1, BRCA2 and Rad51. Inhibition of 53BP1 partially reversed defective I-SceI-induced HR in Brca1ΔBrCT cells, as expected,21 but did not affect Tus/Ter-induced HR in either Brca1ΔBrCT or Brca1ΔBRCT cells (Extended Data Fig. 10). This suggests that BRCA1’s functions in Tus/Ter-induced and SceI-induced HR are, in part, distinct. LTGC at stalled forks may include pathological responses analogous to break-induced replication in yeast3,8,29,30. Our work identifies loss of BRCA1/BRCA2/Rad51-dependent suppression of LTGC at stalled replication forks as a potential contributor to breast/ovarian cancer predisposition.

METHODS SUMMARY

A description of the molecular biology methods, cell culture and recombination assays can be found in the full Methods.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 13 July 2013; accepted 31 March 2014.

Published online 28 April 2014.

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METHODS

Molecular biology, siRNAs and antibodies. The vector for mammalian expression of myc epitope-tagged, nuclear localized, codon-optimized wild-type Tus (pCMVβ myc-NLS–Tus), vectors p6×TerOrí and p6×REVTerOrí and the Ter HR reporters were constructed by conventional cloning methods using a previously described RFP-SCR reporter. Ter-containing plasmids were cultivated in JJC33 (Tus +) strains of E. coli. siRNA SMARTpools were purchased from Dharmacon. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.1% sodium deoxycholate, 1% NP-40 containing protease and phosphatase inhibitors PMSF and Roche complete protease inhibitor tablet). Extracted protein was resolved by 4–12% Bis-Tris SDS–PAGE (Invitrogen) and analysed by immunoblotting using the following antibodies; Brca1 (a gift of the Baer laboratory, 1:100), beta-tubulin (Abcam ab6046, 1:4000), beta-actin (Abcam ab8226, 1:10000), Myc (Abcam ab9106, 1:10000), hRad51 (aliquot B32, 1:500), and HA (Santa Cruz sc-805, 1:200).

Cell lines and cell culture. Mouse embryonic stem (ES) cells were grown in ES medium on either MEF feeders or gelatinized plates as described previously. 31–33. A total of 10 μg of the 6× Ter HR reporter ROSA26 targeting plasmid was linearized by KpnI digest and introduced by electroporation to generate three independent experiments (not number of replicates). For each experiment, 106 293E (ATCC CRL-10852) cells were plated per 15-cm dish 24 h prior to transfection. Cells were transfected with 4.5 μg pC Iori plasmids and 1 μg of control empty vector or pcDNA3–myc-NLS–Tus in antibiotic-free media using Lipofectamine2000 reagent, and media changed 24 h after transfection. Then 40 h after transfection, plates were rinsed with 1× PBS and cells washed off the plate with ice-cold PBS, washed again with ice-cold PBS and Hirt extracted as described below. Purified DNA was restriction digested 8–16 h and run on a 14 × 16 cm 0.4% agarose gel, and bands were visualized under UV light after 160 V for 7.5 h in the cold room at 4 °C. Hirt EP PCR extraction from 293 cells. The plasmid was extracted as described. 35. Briefly, PBS-washed 293HEK or 293C cells were lysed in 2.25 mL 0.6% sodium dodecyl sulphate 33 mM Tris-HCl, 6 mM EDTA, 66 μg/mL RNase followed by digestion with 0.5 μg proteinase K for 90 min at 37 °C. Samples were subject to brief, 2×, base extraction with 0.75 mL 0.1 M NaOH and proteins precipitated by addition of 1 mL 4.2 M guHCl, 0.9 M potassium acetate pH 4.8. Cell debris was pelleted at 39,000g and supernatant loaded onto a Qiagen Miniprep spin column (Qiagen Sciences, Maryland). Columns were washed with 0.5 mL Qiagen Buffer PE (5 M guHCl, 30% ethanol, adding 10 mL Tris-HCl pH 6.6) and 0.75 mL Qiagen Buffer PE (10 M Tris-HCl pH 7.5, 80% ethanol) and plasmid DNA eluted using two volumes of 40 μl Qiagen EB buffer.

Southern blotting. Southern blotting of genomic DNA was performed using GFP cDNA or ROSA26 5′ probes as described previously. 34,35. For all experiments, including mouse ES cells containing a randomly integrated reporter not at ROSA26, clones containing only one intact copy of the reporter were used. Genomic DNA was extracted from confluent ES cells on 6-well plates (5 × 105 to 10 × 105 cells) using a Puregene DNA Isolation Kit (Genta Systems). Episomal plasmid DNA was extracted by Hirt extraction described above and Southern blotting performed using random labelled probe produced from the KpnI/HindIII restriction fragment of p6×TerOrí.

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Extended Data Figure 1 | Tus/Ter-induced replication fork stalling visualized by additional restriction digests. a, Phosphorimager quantification of spot B. One of five independent experiments that contributed to Fig. 1c. Four areas were quantified for each sample using ImageJ 1.48p software, as shown by cartoon. A, area containing a portion of replication fork arc A. B, area containing replication fork stall spot B (same shape/size as A). F, largest area of replication fork arc that is accessible to quantification in every sample. G, same shape as F, used to quantify background signal on membrane. Stall spot B intensity was calculated as: \( \frac{(B - A)}{(F - G)} \times 100\% \). Note, this value does not correspond to the probability of stalling at the Tus/Ter block, but is used to illustrate the relatively weaker arrest produced by 6xREV Ter.

b, Plasmid elements as in Fig. 1a. MluI/XmnI digested plasmid yields a linear fragment of 5.4 kb. Probe for Southern blotting indicated by the black bar.

c, Plasmid replication intermediates extracted from 293E cells transiently transfected with 6xTer-containing plasmids or no Ter control, co-transfected with empty vector (EV), TusH144A or Tus as shown. All samples are from one experiment. Plasmid DNA extracted from 293E cells was digested with XmnI and MluI and analysed by neutral/neutral two-dimensional gel-electrophoresis and Southern blotting. Replication intermediates as described in Fig. 1a.

d, Predicted replication intermediates generated by Tus/Ter-induced replication fork stalling with or without effective FR/EBNA1 replication fork block. Diagrams below plasmid maps show shape of the major Tus/Ter-dependent fork arrest species. Green dotted line shows predicted additional branch of double Y structure formed by stalling of anticlockwise fork at Tus/6xTer when FR/EBNA1 replication block fails. The length of the additional branch is shown in each diagram. Note that the relationship between spots B and C will vary according to the length of this additional branch.

e, Plasmid replication intermediates extracted from 293E cells transiently transfected with 6xTer-containing plasmids and co-transfected with empty vector (EV) or wild-type Tus as shown. Restriction digests of extracted plasmids as shown. All samples are from one experiment. Note: replication fork size and position of stall spot B in relation to replication arc A varies with restriction digest. For example, spot B in KpnI/MluI is close to the 2n linear position, since the Tus/Ter-stall site is only ~680 bp from the KpnI site. For the same reason, spots B and C are closely placed in the KpnI/MluI-digested sample. Note: the relatively weak spot C in the KpnI/MluI digest, which is consistent across multiple experiments, might reflect a proportionately large contribution of ssDNA (reflecting processed lagging strand DNA) to the ~680 bp lagging strand of the stalled anticlockwise fork.
Extended Data Figure 2 | Estimation of efficiencies of the FR/EBNA1 and Tus/6xTer replication fork barriers. a, Tus/Ter-mediated replication stall structures responsible for spots B and C. The relative abundance of the single stall spot B and the double Y stall spot C can be used to calculate the efficiency of the FR/EBNA1 replication fork barrier. b, Phosphorimager analysis of twelve independent Southern blot experiments (method described in Fig. 1b). Areas B, B', C and C' are the same shape and size within each experiment, but vary between experiments. B, stall spot B. B', background gel signal of same area as B. C, stall spot C. C', background gel signal of same area as C. Relative intensity of spot B/(B + C) estimates the stalling efficiency at FR/EBNA1 and is calculated as: (B/B')/(B + C - B') x 100%. The stalling efficiency at FR/EBNA1 is therefore 70 ± 2.2% (s.e.m.). Relative intensity of spot C is calculated as: (C/C')/(B + C - B') x 100%. c, Structure of p6xTer-2Ori plasmid. Stalled replication intermediates depict different combinations of FR/EBNA1 block/bypass and Tus/6xTer block/bypass. Spots B and B2 are defined as in the diagram. Spots C and C2 result from FR/EBNA1 bypass. Spot C2 requires successful arrest at both of the 6xTer arrays. Spot C results from bypass of one of the two 6xTer arrays. d, One of three independent experiments performed with p6xTer-2Ori. Methods as in Fig. 1b. Note presence of four stall spots in p6xTer-2Ori replicating in presence of Tus. Double Y stall spots C and C2 and background signal C' were quantified. Note that the shape and size of each area is identical within an individual experiment, but varies between experiments. By considering only double Y stall spots (that is, in which FR/EBNA1 bypass has occurred), the relative abundance of the double Y stall spots C and C2 are used to estimate the efficiency of the Tus/6xTer replication fork barrier. Let a = probability of the 6xTer array blocking the fork and b = probability of 6xTer bypass. Then a + b = 1. The probability of the two 6xTer arrays blocking each fork on one p6xTer-2Ori plasmid (generating spot C2) is a². The probability of one 6xTer array being blocked and the second array being bypassed (generating spot C) is 2ab. Relative densitometry of spots C and C2 (each with subtraction of background C') shows that spot C contributes 49.6% and C2 contributes 50.4% (s.e.m. 5.6%). Therefore 0.496a² = 0.504 × 2ab. Solving this, a = 0.67 Therefore the estimated efficiency of the Tus/6xTer replication fork block within the replicating plasmid is 67%. Note that the efficiency of the Tus/6xTer replication fork block within the chromosome is unknown.
Extended Data Figure 3 | Two-ended versus one-ended break repair models of Tus/Ter-induced homologous recombination. a, Bidirectional fork arrest would provide two DNA ends for sister chromatid recombination. Termination by annealing generates STGC products of a fixed size. Recombining GFP elements and HR reporter features other than Tus/Ter are not shown. Black strands represent parental DNA. Grey strands represent newly synthesized DNA. Arrowheads on DNA strands represent DNA synthesis. Blue/grey hexagons, Tus monomers. Red triangles, Ter sites. Green line, invading DNA strand. Green dotted line, nascent strand extension. b, Unidirectional fork arrest would provide only one DNA end for sister chromatid recombination. Following one-ended invasion of the neighbouring sister chromatid, any STGC products could not be terminated by annealing, as there is no homologous second end. Termination by non-canonical mechanisms would generate STGCs of unpredictable/variable size, as in ref. 21. DNA and protein elements labelled as in panel a. LTGC is not considered in this analysis, as the mechanisms of termination of the major LTGC products are not accessible from the current data. Each model invokes a hypothetical DSB intermediate. Tus/Ter-induced HR could be initiated by a template switching mechanism (that is, without the formation of an initiating DSB intermediate). However, the requirement for a homologous second end is not altered by consideration of a template switch model and this second end must be provided by the processing of a second arrested fork (the right-hand fork in panel a).
Extended Data Figure 4 | Tus/Ter-induced homologous recombination in Brca1fl/BRCT

6 × Ter/HR cells conforms to an affinity/avidity model.

a, Primary data from Fig. 2c, showing directly measured frequencies of background HR, Tus-induced HR and I-SceI-induced HR in three independent Brca1fl/BRCT 6 × Ter/HR reporter clones. Cells were transfected with empty vector (EV, grey squares), myc-NLS-I-SceI (I-SceI, blue diamonds), or myc-NLS-Tus expression vectors (Tus, orange circles). Each point represents the mean of triplicate samples from three independent experiments (that is, n = 3). Error bars represent s.e.m. Student’s t-test of Tus versus EV: STGC P = 0.0001; LTGC P = 0.0001. Student’s t-test of I-SceI versus EV: STGC P < 0.0001; LTGC P < 0.0001. Student’s t-test of Tus versus I-SceI: STGC P < 0.0001; LTGC P = 0.0018; LTGC/Total HR P = 0.0186. b, Primary data comparing a single ROSA26-targeted Brca1fl/BRCT 6 × Ter/HR clone with three independently derived clones, each harbouring a single intact 6 × Ter/HR reporter randomly integrated at an unknown locus. Filled symbols, ROSA26-targeted clone (as in panel a). Open symbols, data from randomly integrated 6 × Ter/HR reporter clones. Each point represents the mean of six independent experiments, triplicate replicates for each experiment (that is, n = 6). Error bars represent s.e.m. Student’s t-test of pooled random integrants Tus versus EV: STGC P < 0.0001; LTGC P < 0.0001. Student’s t-test of pooled random integrants I-SceI versus EV: STGC P < 0.0001; LTGC P < 0.0001. Student’s t-test of pooled random integrants Tus versus I-SceI: STGC P < 0.0001; LTGC P = 0.3620; LTGC/total HR P = 0.00012. c, Primary data of STGC products observed in Brca1fl/BRCT 6 × Ter/HR cells transfected with empty vector (EV), wild-type Tus, DNA binding defective TusH144A, lock defective TusF140A or I-SceI. All expression vectors are codon-optimized for mammalian expression and encode N-terminal myc epitope and NLS sequences. Each column represents the mean of six independent experiments (that is, n = 6). Error bars represent s.e.m. Student’s t-test of Tus versus EV: P = 0.0002; Tus versus TusH144A: P = 0.0004; Tus versus TusF140A: P = 0.0042; Tus versus I-SceI: P = 0.0159; TusH144A versus EV: P = 0.4406; TusF140A versus EV: P < 0.0001; TusF140A versus TusH144A: P < 0.0001; TusF140A versus I-SceI: P = 0.0888. d, Myc-tagged protein abundance in transfected Brca1fl/BRCT 6 × Ter/HR cells. EV, empty vector. Other lanes as marked. Lower panel, β-tubulin loading control. e, Cartoons of the Ter/HR reporter constructs assayed in panel f. f, Frequencies of Tus-induced STGC in Brca1fl/BRCT cells carrying single copy ROSA26-targeted Ter/HR reporters shown in panel e. Left, HR in 6 × Ter, 3 × Ter, 2 × Ter and 1 × Ter HR reporters, as shown. Right, HR in three independently derived clones carrying single copy, ROSA26-targeted 6 × REVTer HR reporters. Each column represents the mean of three independent experiments (that is, n = 3). Error bars represent s.e.m. Student’s t-test of 6 × Ter versus 3 × Ter: P = 0.2604; 6 × Ter versus 3 × Ter: P = 0.5192; 6 × Ter versus 2 × Ter: P = 0.0547; 6 × Ter versus 2 × Ter: P = 0.0524; 6 × Ter versus 1 × Ter: P = 0.0507; 6 × Ter versus 1 × Ter: P = 0.8291; 3 × Ter versus 2 × Ter: P = 0.0650; 3 × Ter versus 2 × Ter: P = 0.0606; 3 × Ter versus 1 × Ter: P = 0.0576; 3 × Ter versus 1 × Ter: P = 0.0574; 3 × Ter versus 2 × Ter: P = 0.1832; 3 × Ter versus 2 × Ter: P = 0.1748; 3 × Ter versus 1 × Ter: P = 0.1677; 3 × Ter versus 1 × Ter: P = 0.1697. By one-way ANOVA (analysis of variance) test used to compare more than three sets of data, the trend in HR from 6 × to 1 ×, P = 0.0012.
Extended Data Figure 5 | Slx4/FancP depletion suppresses Tus/Ter-induced HR. a, Frequencies of STGC in Brca1^fl/BRCT 6×Ter-HR cells co-transfected with Tus (orange) or I-SceI (blue) and with either control Luciferase siRNA (siLuc), Slx4 SMARTpool (siSlx4), Slx1 SMARTpool (siSlx1), Slx1 and Slx4 SMARTpools (siSlx1 siSlx4), Eme1 SMARTpool (siEme1), Eme1 and Slx4 SMARTpools (siEme1 siSlx4), Xpf SMARTpool (siXpf), Xpf and Slx4 SMARTpools (siXpf siSlx4). Each column represents the mean of triplicate samples from four independent experiments for each clone (that is, \( n = 4 \)). Error bars represent s.e.m. Tus-induced HR: Student’s \( t \)-test of siSlx4 versus siLuc: \( P = 0.0219; \) siSlx4 versus siSlx1: \( P = 0.0012; \) siSlx4 versus siSlx4 + Slx1: \( P = 0.5983; \) siSlx4 versus siEme1: \( P = 0.0171; \) siSlx4 versus siSlx4 + siEme1: \( P = 0.8721; \) siSlx4 versus siXpf: \( P = 0.0098; \) siSlx4 versus siSlx4 + siXpf: \( P = 0.4711; \) siSlx4 versus siLuc: \( P = 0.9332; \) siEme1 versus siLuc: \( P = 0.4631; \) siXpf versus siLuc: \( P = 0.7818; \) siSlx4 + siSlx1 versus siLuc: \( P = 0.0155; \) siSlx4 + siEme1 versus siLuc: \( P = 0.0215; \) siSlx4 + siXpf versus siLuc: \( P = 0.0365; \) I-SceI-induced HR: Student’s \( t \)-test of siSlx4 versus siLuc: \( P = 0.0907; \) siSlx4 versus siSlx1: \( P = 0.0195; \) siSlx4 versus siSlx4 + siSlx1: \( P = 0.4897; \) siSlx4 versus siEme1: \( P = 0.0568; \) siSlx4 versus siSlx4 + siEme1: \( P = 0.3411; \) siSlx4 versus siXpf: \( P = 0.0745; \) siSlx4 versus siSlx4 + siXpf: \( P = 0.2726; \) siSlx1 versus siLuc: \( P = 0.9198; \) siEme1 versus siLuc: \( P = 0.3349; \) siXpf versus siLuc: \( P = 0.9217; \) siSlx4 + siSlx1 versus siLuc: \( P = 0.1521; \) siSlx4 + siEme1 versus siLuc: \( P = 0.2864; \) siSlx4 + siXpf versus siLuc: \( P = 0.2063. \) b, qRT–PCR analysis of mRNA exon boundaries for Slx4, Slx1, Eme1 and Xpf mRNA in siRNA-SMARTpool-treated cells used in panel a.
Extended Data Figure 6 | Southern blot analysis of Tus/Ter- and I-SceI-induced HR products in Brca1<sup>DBRCC</sup> 6×Ter/HR cells. a, Structure of the 6×Ter/HR parental reporter, and major STGC or LTGC HR products (assuming two-ended breaks). Elements as shown in Fig. 2a. b, Southern blot analysis of Tus-induced and I-SceI induced HR products in Brca1<sup>DBRCC</sup> 6×Ter-HR cells. P, un-rearranged reporter; STGC and LTGC as shown. SN, STGC accompanied non-disjunction with retention of parental donor reporter; LN, LTGC accompanied non-disjunction with retention of parental donor reporter. B, BglII digest. BI, BglII + I-SceI digest. Membranes probed with full-length GFP cDNA. Panels underneath two SN events and one LN event show that re-cloning does not separate the two reporters, confirming that the cell contains two copies of the reporter (consistent with non-disjunction).
Extended Data Figure 7 | Brca1 contributes quantitatively and qualitatively to homologous recombination at stalled replication forks. a, Frequencies of Tus-induced and I-SceI-induced HR in Brca1 fl/BRCT and Brca1ΔBRCT 6×Ter/HR cells transiently co-transfected with Tus or I-SceI and with either control Luciferase siRNA (siLuc) or Brca1 SMARTpool (siBrca1). Each column represents the mean of triplicate samples for each independent clone from seven independent experiments (that is, n = 7). Error bars represent s.e.m. Tus-induced HR, Brca1 fl/BRCT cells, Student’s t-test siBrca1 versus siLuc: STGC: P = 0.0013; LTGC: P = 0.0206; LTGC/total HR: P = 0.0003; Brca1ΔBRCT cells, siBrca1 versus siLuc: STGC: P = 0.0016; LTGC: P = 0.4558; LTGC/total HR: P < 0.0001. I-SceI-induced HR, Brca1 fl/BRCT cells, Student’s t-test siBrca1 versus siLuc: STGC: P = 0.0001; LTGC: P = 0.0033; LTGC/total HR: P = 0.9214; Brca1ΔBRCT cells, siBrca1 versus siLuc: STGC: P = 0.0013; LTGC: P = 0.2348; LTGC/total HR: P = 0.0071. b, Brca1 protein levels and b-actin loading control in Brca1 fl/BRCT and Brca1ΔBRCT in siRNA-treated cells as shown. c, qRT–PCR analysis of Brca1 mRNA in siRNA-treated cells as shown.

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Extended Data Figure 8 | Brca2 contributes quantitatively and qualitatively to homologous recombination at stalled replication forks. **a**, Frequencies of Tus-induced and I-SceI-induced HR in *Brca1/hrCT* and *Brca1/D BRCT 6×Ter* HR cells transiently co-transfected with Tus, or I-SceI and with either control Luciferase siRNA (siLuc) or Brca2 SMARTpool (siBrca2). Each column represents the mean of triplicate samples for each independent clone from five independent experiments (that is, *n* = 5). Error bars represent s.e.m. Tus-induced HR, *Brca1/hrCT* cells, Student’s *t*-test siBrca2 versus siLuc: STGC: *P* = 0.0031; LTGC: *P* = 0.0007; LTGC/total HR: *P* = 0.0042; *Brca1/D BRCT* cells, siBrca2 versus siLuc: STGC: *P* = 0.0040; LTGC: *P* = 0.0013; LTGC/total HR: *P* = 0.0006. I-SceI-induced HR, *Brca1/hrCT* cells, Student’s *t*-test siBrca2 versus siLuc: STGC: *P* = 0.0028; LTGC: *P* = 0.0456; LTGC/total HR: *P* = 0.7945; *Brca1/D BRCT* cells, siBrca2 versus siLuc: STGC: *P* = 0.0010; LTGC: *P* = 0.2926; LTGC/total HR: *P* = 0.0316. **b**, qRT–PCR analysis of Brca2 mRNA in siRNA-treated cells as shown.

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Extended Data Figure 9 | Rad51 contributes quantitatively and qualitatively to homologous recombination at stalled replication forks.

**a**, Frequencies of Tus-induced and I-SceI-induced HR in Brca1fl/BRCT and Brca1D/BRCT Ter/HR cells transiently co-transfected with Tus, or I-SceI and with either control Luciferase siRNA (siLuc) or Rad51 SMARTpool (siRad51). Each column represents the mean of triplicate samples for each independent clone from seven independent experiments for Brca1fl/BRCT (that is, n = 7) and four independent experiments for Brca1D/BRCT cells (that is, n = 4). Error bars represent s.e.m. Tus-induced HR, Brca1fl/BRCT cells, Student’s t-test siRad51 versus siLuc: STGC: P < 0.0001; LTGC: P = 0.1578; LTGC/total HR: P = 0.0002; Brca1fl/BRCT cells, siRad51 versus siLuc: STGC: P = 0.0010; LTGC: P = 0.0676; LTGC/total HR: P < 0.0001. I-SceI-induced HR, Brca1fl/BRCT cells, Student’s t-test siRad51 versus siLuc: STGC: P = 0.0014; LTGC: P = 0.0002; LTGC/total HR: P = 0.6216; Brca1fl/BRCT cells, siRad51 versus siLuc: STGC: P = 0.0068; LTGC: P = 0.2064; LTGC/total HR: P = 0.0186.

**b**, Rad51 protein levels and β-tubulin loading control in Brca1fl/BRCT and Brca1D/BRCT siRNA-treated cells as shown.
Extended Data Figure 10 | Effect of 53BP1 inhibition on Tus/Ter-induced homologous recombination.  

**a**. Frequencies of Tus-induced and I-SceI-induced HR in Brca1<sup>fl/BRCT</sup> and Brca1<sup>Δ/BRCT</sup> 6×Ter/HR cells transiently cotransfected with Tus or I-SceI expression vectors and with either F53BP1 D1521R fragment (D1521R; non-chromatin-binding negative control for ‘dominant-negative’ 53BP1 fragment) or ‘dominant-negative’ F53BP1wt fragment (F53BP1wt). Each column represents the mean of triplicate samples for each independent clone from five independent experiments (that is, n = 5). Error bars represent s.e.m. Tus-induced HR, Brca1<sup>fl/BRCT</sup> cells, Student’s t-test D1521R versus F53BP1wt: STGC: P = 0.0442; LTGC: P = 0.5739; LTGC/total HR: P = 0.2250; Brca1<sup>Δ/BRCT</sup> cells, Student’s t-test D1521R versus F53BP1wt: STGC: P = 0.0086; LTGC: P = 0.6888; LTGC/total HR: P = 0.0328. Tus-induced LTGC/total HR, Brca1<sup>fl/BRCT</sup> versus Brca1<sup>Δ/BRCT</sup> cells, Student’s t-test F53BP1wt: 0.0064; Brca1<sup>fl/BRCT</sup> versus Brca1<sup>Δ/BRCT</sup> cells, Student’s t-test D1521R: 0.0014; I-SceI-induced LTGC/total HR, Brca1<sup>fl/BRCT</sup> versus Brca1<sup>Δ/BRCT</sup> cells, Student’s t-test F53BP1wt: 0.1556; Brca1<sup>fl/BRCT</sup> versus Brca1<sup>Δ/BRCT</sup> cells, Student’s t-test D1521R: 0.0208. 

**b.** Abundance of 53BP1 fragments, and β-tubulin (loading control) in treated Brca1<sup>fl/BRCT</sup> and Brca1<sup>Δ/BRCT</sup> 6×Ter/HR reporter ES cells in a. 

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