Trex2 Enables Spontaneous Sister Chromatid Exchanges Without Facilitating DNA Double-Strand Break Repair

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ABSTRACT Trex2 is a 3′ → 5′ exonuclease that removes 3′-mismatched sequences in a biochemical assay; however, its biological function remains unclear. To address biology we previously generated *trex2*^{null} mouse embryonic stem (ES) cells and expressed in these cells wild-type human *TREX2* cDNA (*Trex2*^{hTX2}) or cDNA with a single-amino-acid change in the catalytic domain (*Trex2*^{H188A}) or in the DNA-binding domain (*Trex2*^{R167A}). We found the *trex2*^{null} and *Trex2*^{H188A} cells exhibited spontaneous broken chromosomes and *trex2*^{null} cells exhibited spontaneous chromosomal rearrangements. We also found ectopically expressed human *TREX2* was active at the 3′ ends of I-SceI-induced chromosomal double-strand breaks (DSBs). Therefore, we hypothesized Trex2 participates in DNA DSB repair by modifying 3′ ends. This may be especially important for ends with damaged nucleotides. Here we present data that are unexpected and prompt a new model. We found *Trex2*-altered cells (null, H188A, and R167A) were not hypersensitive to camptothecin, a type-1 topoisomerase inhibitor that induces DSBs at replication forks. In addition, *Trex2*-altered cells were not hypersensitive to γ-radiation, an agent that causes DSBs throughout the cell cycle. This observation held true even in cells compromised for one of the two major DSB repair pathways: homology-directed repair (HDR) or nonhomologous end joining (NHEJ). *Trex2* deletion also enhanced repair of an I-SceI-induced DSB by both HDR and NHEJ without affecting pathway choice. Interestingly, however, *trex2*^{null} cells exhibited reduced spontaneous sister chromatid exchanges (SCEs) but this was not due to a defect in HDR-mediated crossing over. Therefore, reduced spontaneous SCE could be a manifestation of the same defect that caused spontaneous broken chromosomes and spontaneous chromosomal rearrangements. These unexpected data suggest Trex2 does not enable DSB repair and prompt a new model that posits Trex2 suppresses the formation of broken chromosomes.

DNA double-strand breaks (DSBs) are cytotoxic lesions generated by exogenous and endogenous agents (Friedberg *et al.* 1995). Breaks may also occur spontaneously at collapsed replication forks (Petermann and Helleday 2010). There are two major pathways that repair DNA DSBs: homology-directed repair (HDR) and nonhomologous end joining (NHEJ). Even

though much is known about these processes, they are not fully understood. Both HDR and NHEJ are frequently used in mammalian cells (Jasin 2000) and disruption of either leads to gross chromosomal rearrangements that may cause cancer (Hoeijmakers 2001) or aging-like phenotypes (Vijg and Dolle 2002; Hasty *et al.* 2003; Lombard *et al.* 2005). HDR repairs general DSBs by annealing the broken strand to a homologous template usually provided by the sister chromatid during DNA replication (West 2003; San Filippo *et al.* 2008). Importantly, the generation of 3′ overhangs is required before strand annealing can take place. These ends may need modification if damaged nucleotides are present. NHEJ repairs general DSBs by joining the ends together without the use of a homologous template (Lieber *et al.* 2004) and like HDR, the ends may need modification if

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damaged nucleotides are present (Roberts *et al.* 2010). Thus a protein with nonprocessive 3' → 5' exonuclease activity could be useful for HDR and NHEJ to remove damaged nucleotides from 3' ends.

Trex2 is a nonprocessive 3' → 5' exonuclease that removes 3' mismatches in biochemical assays (Mazur and Perrino 2001). It is present from worms to mammals but is not found in lower life forms like bacteria and yeast. Trex2 functions as a homodimer and has a DNA-binding domain along with the catalytic domain. Single-amino-acid mutations can selectively disrupt these functions, including H188A that completely ablates exonuclease activity but also impairs DNA binding by ~60% and R167A that impairs DNA binding by ~85% without diminishing exonuclease activity (Chen *et al.* 2007b). Therefore, these activities are separable, at least in part.

To understand biological function, we generated *trex2^{null}* mouse embryonic stem (ES) cells (Chen *et al.* 2007a). We introduced into these *trex2^{null}* cells wild-type human *TREX2* cDNA or human *TREX2* cDNA with a single-amino-acid change in the catalytic domain (*Trex2^{H188A}*) or DNA-binding domain (*Trex2^{R167A}*); these cells were made by knock-in such that the human cDNA is expressed by the mouse *Trex2* promoter *in situ* (Dumitrache *et al.* 2009). The *trex2^{null}* and *Trex2^{H188A}* cells, but not the *Trex2^{hTX2}* or *Trex2^{R167A}* cells, exhibited high levels of spontaneous broken chromosomes. Broken chromosomes were especially apparent in the *Trex2^{H188A}* cells, suggesting a dominant activity. These breaks were often located in or adjacent to the pericentromere, a highly repetitive region that is composed of 6–8 Mb of tandem major satellite repeats (MSRs) that undergoes dynamic regulation (Guenatri *et al.* 2004). This observation suggests Trex2 maintains highly repetitive regions. In addition, the *trex2^{null}* cells exhibited spontaneous chromosomal rearrangements (*Trex2^{H188A}* and *Trex2^{R167A}* cells were not analyzed for rearrangements) (Chen *et al.* 2007a). Therefore, Trex2 suppresses spontaneous broken chromosomes and spontaneous chromosomal rearrangements.

It is possible that Trex2 participates in repairing DSBs by either HDR or NHEJ. Trex2's potential role in HDR and NHEJ is especially attractive since it is a nonprocessive 3' → 5' exonuclease that would be useful for modifying 3' ends with damaged nucleotides. To support this possibility ectopically expressed human *TREX2* removed nucleotides from the 3' ends of DNA DSBs generated by *I-SceI*, a site-specific endonuclease (Bennardo *et al.* 2009). Typically two to five nucleotides were removed such that the *I-SceI* site was destroyed. Presumably these ends did not have damaged nucleotides, presenting the possibility that Trex2 activity can modify 3' DNA ends even without damage. However, these cells ectopically expressed human *TREX2* at levels 10 times higher than endogenous mouse *Trex2* as measured by mRNA. Therefore, we do not know whether these endogenous levels of mouse *Trex2* are sufficient to modify *I-SceI*-induced 3' ends. We also do not know whether Trex2 participates in the repair of DSBs induced by

chemical agents or ionizing radiation. Thus, it is possible that Trex2-defective cells exhibit elevated levels of broken chromosomes and chromosomal rearrangements due to a defect in DSB repair.

Here, we test the possibility that Trex2 participates in the repair of DNA DSBs by either HDR or NHEJ. We report that Trex2-altered cells did not exhibit hypersensitivity to agents that generate DSBs (camptothecin or γ -radiation) even in cells compromised for either HDR or NHEJ. *Trex2* deletion enhanced the repair of an *I-SceI*-induced DSB by both HDR and NHEJ without affecting pathway choice. In addition, *Trex2* deletion did not diminish the formation of sister chromatid exchanges (SCEs) in cells elevated for HDR-induced crossing over. Instead, *Trex2* deletion diminished spontaneous SCEs, suggesting a defect in a strand-exchange pathway that is independent of HDR and DSB repair. Our data suggest Trex2 does not participate in DSB repair by either HDR or NHEJ; therefore, Trex2 may instead suppress DSB formation.

Materials and Methods

Tissue culture conditions

ES cells were maintained in M15 [high-glucose DMEM supplemented with 15% fetal bovine serum, 100 μ M β -mercaptoethanol, 2 mM L-Glutamine, 3 mg/ml penicillin, 5 mg/ml streptomycin, 1000 units/ml ESGRO (LIF)] and seeded on 0.1% gelatin-coated plastic plates in a 37° incubator at atmospheric O₂.

Substrains and gene targeting

We performed an epistatic analysis for this study and used genetically modified cells derived from multiple investigators. These cells are called AB2.2, IB10, TC1, and J1. All cells were derived from the 129 mouse strain; however, 129 mice rapidly undergo genetic changes so cells derived from different substrains should not be considered isogenic (Simpson *et al.* 1997; Threadgill *et al.* 1997). Therefore, we mutated *Trex2* in control and mutant cells of each parental cell to make a valid comparison. All these cells are XY; therefore, the epistatic analysis was facilitated by *Trex2*'s location on the X chromosome.

For our previously published studies we used AB2.2 cells. We made a null by deleting a single exon that contains all coding sequence (*trex2^{null}* and *trex2^{null-2}*) (Chen *et al.* 2007a; Dumitrache *et al.* 2009). In addition, by knock-in we introduced the wild-type human short isoform of *TREX2* (*Trex2^{hTX2}*) and two variants with a single-amino-acid change adjacent to the *Trex2* promoter (Chen *et al.* 2007b; Dumitrache *et al.* 2009). One variant is mutated in the catalytic domain (*Trex2^{H188A}*) while the other variant is mutated in the DNA-binding domain (*Trex2^{R167A}*). IB10 is the genetic background used for immunostaining. These cells were used because they are better than AB2.2 cells at forming monolayers on plastic chamber slides. TC1 cells were used to generate the *h2ax^{-/-}* cells (Bassing *et al.* 2002)

and J1 cells were used to generate the *ku70*^{-/-} cells (Gu *et al.* 1997). AB2.2 cells were used to generate the *blm*^{tm3Brd/tm4Brd} cells (Luo *et al.* 2000); for simplicity these cells are referred to as *blm*^{-/-} cells for the remainder of the text.

Gene targeting was very similar for each ES cell type except different selection cassettes were used. The *Hprt* minigene was used for AB2.2 cells since they are *Hprt* negative. However, the other strains are *Hprt* positive. For IB10 cells we mutated the *Hprt* gene (below) and therefore used the same targeting vector as described for AB2.2 cells (Chen *et al.* 2007a). However, *Hprt* was not mutated for TC1 and J1 cells; therefore, we modified the *Trex2* targeting vector by using a different selection cassette, *puΔtk* (Figure 1A and below). We also used a different selection cassette to delete *Trex2* in *blm*^{-/-} cells, puromycin phosphotransferase (Figure 1B and below). Even though these different selection cassettes were used, the mutation is the same, deletion of the single exon that contains all *Trex2* coding sequences.

Trex2 was deleted in IB10 control cells using the same targeting vector and targeting strategy as previously described (Chen *et al.* 2007a). This targeting vector has a floxed *miniHPRT* cassette (Holcomb *et al.* 2007); therefore, the *Hprt* gene was first mutated in control IB10 ES cells. We used a replacement targeting vector that is similar to the one previously described (Thomas *et al.* 1992). However, a floxed *puΔtk* cassette was used for selection instead of neomycin phosphotransferase (not shown). The *puΔtk* cassette is a positive/negative selection cassette that fuses puromycin N-acetyltransferase (*pu*) to a truncated version of herpes simplex virus type 1 thymidine kinase (*Δtk*) (Chen and Bradley 2000). Cells expressing *puΔtk* are resistant to puromycin and sensitive to 1-(-2-deoxy-2-fluoro-1-β-D-arabino-furanosyl)-5-iodoracil (FIAU). RE mutant *loxPs* (Araki *et al.* 1997) are located on each side of *puΔtk* so it can be removed upon Cre expression. The 5' end of RE mutant *loxP* (TACCGTTCGTATAATGTATGCTATACGAAGTTAT) differs from that of wild-type *loxP* (ATAACTTCGTATAATGTATGCTATACGAAGTTAT); the difference is in boldface type. Targeted clones were selected in tissue culture with 3–9 μg/ml puromycin for 5 days and then 10 μM 6-thioguanine + 3 μg/ml puromycin for another 5 days, using previously described conditions (Hasty *et al.* 1991).

Trex2 was targeted with a conditional vector in cells mutated for *H2ax* (Bassing *et al.* 2002) and *Ku70* (Gu *et al.* 1997). This targeting vector introduces cDNA coding for the human short isoform of *TREX2* (Chen *et al.* 2007b), similar to a previously described knock-in vector (Chen *et al.* 2007a) except *puΔtk* was used instead of *miniHPRT* for selection since these cells are *Hprt* positive (Figure 1A). *SfiI* sites flank this selection cassette to allow sticky directional cloning as previously described (Holcomb *et al.* 2007).

Trex2 was targeted with a deletion vector in cells mutated for *Blm* (Luo *et al.* 2000). This deletion vector is the same as the one previously described (Chen *et al.* 2007a) except a puromycin acetyltransferase selection cassette was

used for selection instead of *miniHPRT* (Figure 1B). *SfiI* sites also flank this selection cassette as previously described (Holcomb *et al.* 2007).

The floxed *puΔtk* in *Hprt* and *Trex2* were deleted using a Cre-recombinase expression vector (pPGKcrepA) that was transiently transfected into ES cells using previously described conditions (Holcomb *et al.* 2007; Kim *et al.* 2008). PCR was performed on FIAU-resistant clones to verify *puΔtk* deletion (Figure 1A). Primers for *Trex2* deletion are Tx2Cre2 (5'-CCAAAGGCCTCATGAGATGG-3') and Tx2CreRev3 (5'-AAGGCATGGACTAGCTCTCTGC-3'). The PCR conditions are as follows: 1 cycle of 98° for 5 min; then 35 cycles of 98° for 1 min, 62° for 1 min, and 72° for 30 sec; and then 1 cycle of 72° for 10 min.

Dose response to camptothecin and γ-radiation

The camptothecin and γ-radiation dose response was performed as described (Marple *et al.* 2004).

Immunostaining

IB10 cells were used as opposed to AB2.2 cells for immunostaining because they are better at forming monolayers soon after seeding when cultured on plastic chamber slides (AB2.2 cells proliferate on top of each other even immediately after seeding). IB10 ES cells were plated at 5 × 10⁴ cells per well in a four-well chamber slide and cultured at atmospheric pressure for 1 day as described for murine fibroblasts (Parrinello *et al.* 2003). Cells were washed in PBS, fixed with 2% formaldehyde in PBS for 10 min at room temperature, washed three times with PBS, permeabilized with 0.5% Triton X-100 for 10 min, washed three times with PBS, blocked for 30 min, and incubated overnight at 4° with primary antibody. After washing three times with PBS, cells were incubated with secondary antibody for 45 min at room temperature, washed three times with PBS, and stained with DAPI (1 μg/ml). The cells were photographed using a Zeiss Axioplan2 with a 60× objective and a Nikon Digital Camera DXM1200. The primary antibody for 53BP1 was a rabbit polyclonal from Bethyl (BL182) and the secondary antibody was a goat-anti-rabbit conjugated to Alexa fluor 488 from Molecular Probes.

Three-color FISH to measure DSBs

ES cells were seeded onto plastic tissue culture plates pretreated with 0.1% gelatin for 1 hr. The next day cells were exposed to 100 nM camptothecin for 5 hr and 540 nM colcemid for 4 hr and then trypsinized. The remainder of the procedure has been described (Chen *et al.* 2007a).

Sister chromatid exchange assay

ES cells (1 × 10⁶) were seeded onto a 10-cm plate pretreated with 0.1% gelatin for 1 hr. The next day BrdU was added (10 μM final concentration) and cells were incubated for 24 hr. Then the media were removed and new media added, cells were incubated for an additional 12 hr, and then 540 nM colcemid was added for 4 hr. Cells were then

Figure 1 *Trex2* knockouts. (A1) Targeting *Trex2* with the conditional vector in *h2ax*^{-/-} and *ku70*^{-/-} cells. The targeting vector contains floxed *puΔtk* (Chen and Bradley 2000) and the human short TREX2 isoform (Chen *et al.* 2007b). After gene targeting the mouse *Trex2* coding sequences (red) were replaced with the human short TREX2 isoform (green). (A2) Screen for targeted clones. PCR amplification with TX2LR55 and hTX2Rev is shown. (A3) Cre-mediated deletion of human TREX2 and *puΔtk*. (A4) Screen for *Trex2*-deleted clones by PCR amplification with Cre1 and Cre2. (A5) Verification of *Trex2* deletion by RT-PCR with mTx2For and mTx2Rev primers shown in A1. Detection of Rad51 mRNA was used as a loading control (Holcomb *et al.* 2007). WT, wild-type control. (B1) Replacing *Trex2* in *blm*^{-/-} cells with puromycin acetyltransferase expressed by the phosphoglycerate kinase (PGK) promoter. (B2) Screen for targeted clones. PCR amplification with TX2LR55 and HATrev is shown. (B3) Verification of targeted clones with RT-PCR using mTx2For and mTx2Rev primers shown in A1. Detection of Rad51 mRNA was used as a loading control.

trypsinized and prepared for slides using the same procedure for three-color FISH as previously described (Chen *et al.* 2007a). Once slides were prepared, they were immersed into a 40ul solution of 0.5% Tw20, 10 μ l FITC-conjugated anti-BrdU antibody (Becton Dickinson), and 0.25 mg/ml major satellite repeat (CY-3 5'-TGG AAT ATG GCG AGA AAA CTG AAA ATC ATG GAA AAT GAG A-3') for 10 min, at 37°, in the dark. The slides were washed with PBS (pH 7) for 30 sec, treated with a DAPI (Vectashield), and analyzed under a microscope (Axioplan).

I-SceI assays

Integration of DR-GFP and EJ5-GFP into the *Pim1* locus of AB2.2 control and *trex2*^{null} cells was performed as described (Bennardo *et al.* 2009). Transfection of the I-SceI expression plasmid was performed as described (Bennardo *et al.* 2009). Repair junctions were analyzed as described (Bennardo and Stark 2010).

Results and Discussion

Trex2-deleted cells are not hypersensitive to camptothecin

To test the possibility that Trex2 is important for repairing agent-induced DSBs at DNA replication forks, we compared control cells to *Trex2*-altered cells for sensitivity to camptothecin, a type 1 topoisomerase inhibitor. Camptothecin blocks rejoining of the cleavage/religation reaction and causes the accumulation of covalent reaction intermediates that generate DSBs after collision with a replication fork in S phase (Liu *et al.* 2000). We found all *Trex2*-altered cells exhibit the same level of sensitivity to camptothecin as control cells (Figure 2A). These data indicate that Trex2 is dispensable for chromosome break repair since mouse ES cells defective for HDR are hypersensitive to camptothecin (Marple *et al.* 2006). In addition, the survival fraction curve suggests

the dominant negative-like effects of the H188A mutation are not due to interference with DSB repair pathways.

We further investigated *trex2*^{null} and *Trex2*^{H188A} cells for camptothecin-induced chromosomal breaks by looking at metaphase spreads using three-color fluorescence *in situ* hybridization (FISH). Metaphase spreads from these cells were stained with a telomeric probe (green), a MSR probe in the pericentromere (red), and DAPI (blue) (Guenatri *et al.* 2004). Previously we showed *trex2*^{null} and *Trex2*^{H188A} cells exhibited increased levels of spontaneous broken chromosomes compared to control cells. These breaks were almost exclusively in the pericentromere. In addition, the *Trex2*^{H188A} cells showed higher levels of spontaneous broken chromosomes than the *trex2*^{null} cells, suggesting a dominant negative-like effect (Dumitrache *et al.* 2009). We looked at the level of broken chromosomes in cells after 5 hr exposure to camptothecin at 50 nM, 100 nM, or 200 nM followed by 4 hr exposure to colcemid. For control and *trex2*^{null} cells we found a progressive increase in chromosomal breaks with dose, yet deleting Trex2 did not increase the number of camptothecin-induced breaks (Figure 2, B and C; see legend for statistics). Camptothecin exposure did not significantly increase the number of chromosomal breaks for *Trex2*^{H188A} cells beyond the high level of spontaneous broken chromosomes (Figure 2, B and C). These data show Trex2 does not suppress camptothecin-induced broken chromosomes and suggest it is unessential for DSB repair.

The breaks were almost exclusively in the pericentromere for all genotypes at all CPT doses; thus, it is possible that mouse ES cells are susceptible to broken pericentromeres. However, our recent publication shows FancB-deleted mouse ES cells exhibited elevated levels of broken chromosomes in the long arm and the crosslinking agent mitomycin exacerbated this phenotype (Kim *et al.* 2011). Therefore, the pericentromere seems to be vulnerable to Trex2 deletion and type-1 topoisomerase inhibition. This phenotype suggests Trex2 and type-1 topoisomerase play an important role

Figure 2 Camptothecin (CPT)-induced broken chromosomes. (A) *Trex2*-altered cells exhibited the same dose response to camptothecin as control cells (AB2.2). Four clones of *Trex2^{hTX2}*, *Trex2^{H188A}*, and *Trex2^{R167A}* were compared to the parental clones of *trex2^{null}* and *trex2^{null-2}* (Dumitrache *et al.* 2009) and control cells. These experiments were done three times. The four clones from each experiment were averaged and then the three experiments were averaged. Standard deviation is shown. SF, survival fraction. (B) The percentage of metaphase spreads (MPS) with microscopically visible spontaneous and camptothecin-induced broken chromosomes. AB2.2, control cells. (C) Summary of results. Shown is the number of MPS with zero broken (br) chromosomes, one broken chromosome, two broken chromosomes, or three broken chromosomes. No metaphase spread had more than three broken chromosomes. Statistics (Student's *t*-test) refer to lanes in B for comparisons: 1 and 2, $P = 0.0001$; 1 and 3, $P < 0.0001$; 1 and 4, $P = 0.0003$; 2 and 3, $P = 0.0141$; 2 and 4, $P = 0.026$; 3 and 4, $P = 0.398$; 5 and 6, $P = 0.061$; 5 and 7, $P = 0.0003$; 5 and 8, $P = 0.0008$; 6 and 7, $P = 0.033$; 6 and 8, $P = 0.013$; 7 and 8, $P = 0.391$; 9 and 10, $P = 0.708$; 9 and 11, $P = 0.321$; 9 and 12, $P = 0.481$; 10 and 11, $P =$

in maintaining highly repetitive regions since the pericentromere is composed of major satellite repeats (Guenatri *et al.* 2004).

To evaluate DNA lesions, we measured the number of nuclear foci containing p53-binding protein 1 (53BP1) in ES cells. 53BP1 rapidly localizes to DNA DSBs and is a marker of unrepaired DNA damage and an important early component of the DNA damage response (Mochan *et al.* 2004). For this experiment, we deleted *Trex2* in IB10 cells since immunostaining is easier in these cells compared to AB2.2 cells (see *Materials and Methods*), the parental cells described in our previous publications. We found control and *trex2^{null}* cells exhibited the same level of spontaneous and camptothecin-induced 53BP1 foci (Figure 3), suggesting *Trex2* deletion does not alter the level of DSBs or the early DNA damage response to DSBs. This observation indicates *Trex2* deletion does not alter the early stages of the DNA damage response.

Trex2-altered cells are not hypersensitive to γ -radiation

Camptothecin causes DSBs associated with replication forks, so we tested the impact γ -radiation has on *Trex2*-altered cells since it causes DSBs throughout the cell cycle. γ -Radiation is a high-frequency electromagnetic wave that interacts directly with DNA or with surrounding molecules to make reactive species that cause DSBs (Friedberg *et al.* 1995). Cells defective for either HDR (Essers *et al.* 1997; Morimatsu *et al.* 1998) or classical NHEJ (Burma *et al.* 2006) are hypersensitive to γ -radiation. We found *Trex2*-altered cells exhibited the same level of sensitivity to γ -radiation as control cells, suggesting *Trex2* does not participate in repairing γ -radiation-induced DSBs (Figure 4A). Again, the survival fraction curve suggests the dominant negative-like effects of the H188A mutation are not due to interference with DSB repair pathways.

It is possible *Trex2* has an important, but nonessential role in DSB repair. Therefore, we deleted *Trex2* in ES cells defective for either HDR or NHEJ to look for an additive or synergistic phenotype. Such a phenotype may be revealed in DSB repair-compromised cells. To observe HDR-defective cells, *Trex2* was deleted in control TC1 cells and TC1 cells defective for *H2ax* (Figure 1A). *H2ax* is a histone phosphorylated in response to DSBs (γ -H2ax) (Bassing *et al.* 2002). γ -H2ax recognizes DNA DSBs (Pilch *et al.* 2003), recruits DNA repair proteins to DNA damage (Paull *et al.* 2000), and controls recombination between sister chromatids (Xie *et al.* 2004, 2010). Therefore, the *h2ax^{-/-}* cells are compromised for HDR and are therefore more likely dependent on NHEJ for repairing DSBs (Couedel *et al.* 2004). However, deleting *Trex2* did not further sensitize these cells to

0.517; 10 and 12, $P = 0.847$; 11 and 12, $P = 0.608$; 1 and 5, $P = 0.037$; 1 and 9, $P = 0.0002$; 2 and 6, $P = 0.872$; 2 and 10, $P = 0.027$; 3 and 7, $P = 0.511$; 3 and 11, $P = 0.206$; 4 and 8, $P = 0.546$; 4 and 12, $P = 0.872$; 5 and 9, $P < 0.0001$; 6 and 10, $P = 0.037$; 7 and 11, $P = 0.569$; and 8 and 12, $P = 0.411$.

Figure 3 Campthothecin-induced 53BP1 foci. (A) *trex2*^{null} cells exhibit the same level of campthothecin-induced 53BP1 foci as control cells (IB10). (B) Summary of results.

γ -radiation (Figure 4B). To look at classical NHEJ-defective cells, we deleted *Trex2* in control J1 cells and J1 cells mutated for *Ku70* (Gu *et al.* 1997) (Figure 1A). Ku70 forms a heterodimer with Ku80, called Ku that binds to DNA ends as an essential component of classical NHEJ (Burma *et al.* 2006). Therefore, *ku70*^{-/-} cells are more dependent on HDR (Pierce *et al.* 2001; Couedel *et al.* 2004). However, deleting *Trex2* did not further sensitize these cells to γ -radiation (Figure 4C). Thus, *Trex2* does not appear to be important for repairing general DSBs even in cells compromised for either HDR or classical NHEJ.

***Trex2*-deleted cells were not defective for repairing I-SceI-induced DSBs by either HDR or NHEJ**

Previously we observed the impact ectopically expressed human *TREX2* had on the repair of I-SceI-induced DSBs, using multiple substrates in a variety of genetically altered mouse ES cells (Bennardo *et al.* 2009). The level of ectopically expressed human *TREX2* was 10-fold greater than that of endogenous mouse *Trex2* at the mRNA level. We found this high level of ectopically expressed *TREX2* reduced persistent I-SceI cutting through its nonprocessive exonuclease activity. Specifically, high levels of *TREX2* lead to I-SceI-resistant NHEJ products with modified 3' overhangs, which also reduced the frequency of NHEJ between distal ends of two tandem DSBs, but did not affect the frequency of HDR. On the basis of these results, it is possible that endogenous *Trex2* levels are sufficient to modify the I-SceI-cut 3' ends. Therefore, we tested whether endogenous levels of *Trex2*

had an impact on the frequency of HDR, the frequency of distal NHEJ, or end processing during NHEJ.

To begin with, we tested whether endogenous *Trex2* levels affect the repair of an I-SceI-induced DSB in a substrate specific for either HDR (DR-GFP, Figure 5A) or NHEJ between distal ends of two tandem DSBs (EJ5-GFP, Figure 5B); both substrates were targeted to *Pim1* (Bennardo *et al.* 2009). The generation of GFP⁺ cells with DR-GFP is specific for HDR, since it is facilitated by Rad51 (Stark *et al.* 2004; Bennardo *et al.* 2008). In addition, DR-GFP does not detect single-strand annealing since the iGFP donor fragment lacks essential 3' elements of the GFP expression cassette. The generation of GFP⁺ cells with EJ5-GFP can involve classical NHEJ factors (e.g., Ku70 and Xrcc4), which are important for I-SceI site restoration, but also can be generated independent of classical NHEJ when the I-SceI site is destroyed (Bennardo *et al.* 2008, 2009). We found the frequency of HDR and NHEJ was increased in *trex2*^{null} cells relative to control (Figure 4C; Student's *t*-test, *P* < 0.001), supporting the possibility these DSB repair pathways are elevated in *trex2*^{null} cells. We also found the relative HDR/NHEJ ratio was similar between *trex2*^{null} (1.17) and control (1.23) cells, suggesting that *Trex2* deletion increased DSB repair efficiency without affecting pathway choice. These observations support the possibility that HDR and NHEJ are elevated in *trex2*^{null} cells.

Elevation in HDR and NHEJ in *trex2*^{null} cells could be due to compensation for increased spontaneous DSBs or due to loss of *Trex2*-mediated processing of I-SceI overhangs prior to NHEJ. To address the latter possibility, we characterized distal NHEJ repair junctions from control and *trex2*^{null} cells. We sorted GFP⁺ cells from the EJ5-GFP reporter experiment and amplified the region flanking the I-SceI site. Since the two tandem I-SceI sites in EJ5-GFP are in the same orientation, one possible distal NHEJ repair product is restoration of the I-SceI site (Figure 5B, left). The generation of GFP⁺ cells with EJ5-GFP can involve classical NHEJ factors (e.g., Ku70 and Xrcc4), which are important for I-SceI site restoration (I-SceI⁺), but can also be generated independent of classical NHEJ when the I-SceI site is destroyed (I-SceI⁻) (Bennardo *et al.* 2008, 2009). Even though formation of I-SceI⁻ NHEJ products is independent of Ku70 and Xrcc4, we do not mean to imply that classical NHEJ factors never participate in these repair events in wild-type cells. Accordingly, loss of the I-SceI site (Figure 5B, right), can be independent of both Ku70 and Xrcc4 and promoted by ectopic expression of *Trex2* (Bennardo *et al.* 2008, 2009). Hence, we first examined the amplification products by I-SceI digestion analysis. From this experiment, we found that restoration of the I-SceI site was the same between control and *trex2*^{null} cells: 45 $\pm$ 7% I-SceI restoration for control and 43 $\pm$ 5% I-SceI restoration for *trex2*^{null} (Figure 5D). Therefore, *Trex2* deletion did not affect loss of the I-SceI site, which suggests that endogenous *Trex2* does not affect processing of 3' ends during NHEJ.

To further examine repair junctions, we isolated the I-SceI-resistant products for subcloning into a plasmid and

Figure 4 Exposure to γ -radiation. (A) *Trex2*-altered cells exposed to γ -radiation. Four clones of *Trex2*^{hTX2}, *Trex2*^{H188A}, and *Trex2*^{R167A} were compared to the parental clones of *trex2*^{null} and *trex2*^{null-2} cells (Dumitrache *et al.* 2009) and control cells. These experiments were done three times. Standard deviation is shown. SF, survival fraction. (B) *trex2*^{null} *h2ax*^{-/-} cells exposed to γ -radiation. Average of two *h2ax*^{-/-} *trex2*^{null} clones is shown. These experiments were done three times and standard deviation is shown. TC1, control cells. (C) *trex2*^{null} *ku70*^{-/-} cells exposed to γ -radiation. Average of two *trex2*^{null} *ku70*^{-/-} clones is shown. These experiments were done three times and standard deviation is shown. J1, control cells.

then sequenced individual clones. From this analysis, we found no significant difference between control and *trex2*^{null} in the sizes of deletions (Table 1). For example, in both cell lines, the majority of junctions showed 6–10 nucleotide deletions (14/23 for control and 12/23 for *trex2*^{null}). These experiments show that *Trex2* deletion did not affect either the efficiency of distal NHEJ or the nature of the repair products. Hence, these data suggest that endogenous *Trex2* levels are unlikely to modify the 3' ends of I-SceI-induced DSBs.

Why do *trex2*^{null} cells exhibit elevated levels of DSB repair? While the I-SceI reporter systems provide insight into the relative frequency of different repair outcomes, they do not assess the mechanism by which a genetic mutation affects each repair outcome equivalently. Accordingly, the main insight from these experiments indicates that *Trex2* deficiency does not affect the relative frequency of diverse

DSB repair outcomes (HR vs. NHEJ and the types of NHEJ products). To explain the increase in DSB repair, we suggest DSB repair is elevated in *trex2*^{null} cells as a general compensatory response to increased spontaneous chromosomal breaks (Dumitrache *et al.* 2009). In any case, these data do not support a role for endogenous *Trex2* in promoting DSB repair.

Trex2 facilitates spontaneous SCEs, but not SCEs induced by *Blm* deletion

SCE is the process by which a single chromatid breaks and then recombines with the intact sister chromatid during replication. During this process, regions of parental strands in the duplicated chromosomes are reciprocally exchanged (Wilson and Thompson 2007). HDR of DSBs induces SCE (Sonoda *et al.* 1999; Dronkert *et al.* 2000; Takata *et al.* 2000, 2001; Dong and Fasullo 2003). Therefore, we

Figure 5 I-SceI-specific DSBs. (A) The HDR-specific substrate, DR-GFP. The I-SceI site interrupts the GFP sequence while the *BcgI* site is the wild-type sequence at this location. After generation of an I-SceI DSB, the free ends invade the adjacent truncated GFP substrate that contains the wild-type *BcgI* site. Nonreciprocal homologous recombination (gene conversion) restores GFP. (B) The NHEJ-specific substrate, EJ5-GFP. There are two I-SceI sites separated by a puromycin phosphotransferase (*puro*) selection cassette. After generation of two I-SceI-induced DSBs, distal end joining restores the GFP cassette. There are two potential outcomes. First, the I-SceI site is restored. This detects classical NHEJ (Ku70 dependent). Second, the I-SceI site is lost. This detects Alt-NHEJ (Ku70 independent). (C) *trex2*^{null} cells repaired I-SceI-induced DSBs more efficiently than control cells (AB2.2) for the DR-GFP (DR, $P < 0.001$, Student's *t*-test) and EJ5-GFP (EJ, $P < 0.0001$) substrates. The mean of six to eight experiments is shown. (D) I-SceI digestion of PCR-amplified DNA from GFP⁺ cells with the EJ5-GFP substrate. After I-SceI digestion, the amplified DNA is either uncut (U) or cut with I-SceI (S).

Table 1 I-SceI resistant end joining junctions

	Microhomology (nt)	WT: no. of clones	Trex2 ^{null} : no. of clones
I-SceI site (CAPS) attcTAGGGATAACAGGGTAATggat			
Insertions/deletions			
Insertion + 1		1/23	1/23
TAGGG A ATAACAGGGTAAT + 1 insertion	0	1	
TAGGG ataac ATAACAGGGTAAT + 5 insertions	NA		1
Deletion, 1–5 nt		2/23	2/23
TAGGGAT...ACAGGGTAAT, 1-nt deletion	0		1
TAGGG...TAACAGGGTAAT, 1-nt deletion	0		1
TAGGGA...ACAGGGTAAT, 2-nt deletion	1	1	
TAGGG... <u>A</u> ACAGGGTAAT, 2-nt deletion	1	1	
Deletion, 6–10 nt		14/23	12/23
ttc... <u>TA</u> ACAGGGTAAT, 6-nt deletion	2		1
TAGGGA... <u>TAAT</u> GGAT, 8-nt deletion	3	3	
<u>TAGGG</u> ...TAAT, 9-nt deletion	4	9	9
agaa... <u>ATA</u> ACAGGGTAAT, 9-nt deletion	2	1	
TAGGGAT... <u>Atgg</u> , 9-nt deletion	1		1
aatt...ACAGGGTAAT, 9-nt deletion	0	1	
ttc...AGGGTAT, 10-nt deletion	1		1
Deletion, >10 nt		6/23	8/23
agaa...CAGGGTAAT, 12-nt deletion	2		1
ttcTA... <u>ggatcc</u> , 16-nt deletion	2	1	
agaa... <u>TAAT</u> , 17-nt deletion	1		1
ttgg...CAGGGTAAT, 19-nt deletion	2	1	
TAGGG... <u>tcat</u> ggctcg, 22-nt deletion, 4-nt insertion	0	1	
catc...AACAGGGTAAT, 24-nt deletion	1	1	
TAGGG...cgcc, 25-nt deletion	0		1
atca...GGGTAATGG, 27-nt deletion	2	1	
attt... <u>Tggat</u> , 30-nt deletion	2	1	
TAGGGA...atgg, 31-nt deletion	0		1
tcat...cacc, 41-nt deletion	2		1
tgct...GGTAAT, 48-nt deletion	2		1
tttt...CAGGGTAAT, 70-nt deletion	1		1
gcaa...AGGGTAAT, 79-nt deletion	2		1

Boldface type, inserted sequence; underlined, microhomology; NA, not applicable.

observed spontaneous SCEs by FISH. We found *trex2*^{null} cells exhibited reduced levels of spontaneous SCEs (Figure 6A, Yates-corrected chi-square test: $P < 0.0001$). Spontaneous SCEs may result from HDR-dependent crossing over or perhaps an HDR-independent pathway.

To determine whether these SCEs were HDR dependent, we deleted *Trex2* in *blm*^{-/-} cells (Figure 1B and *Materials and Methods*) (Luo *et al.* 2000). *Blm* is a RecQ helicase that suppresses HDR-mediated SCEs by Holliday junction dissolution to inhibit crossing over (Ira *et al.* 2003; Wu and Hickson 2003; Liberi *et al.* 2005; Raynard *et al.* 2006). Additionally, the role of *Blm* in suppressing HDR-mediated SCEs was shown in mouse ES cells (Chu *et al.* 2010). *Trex2* deletion did not alter the level of spontaneous SCEs in *blm*^{-/-} cells (Figure 6B, $P = 0.11$). This observation suggests that *Trex2* does not participate in *Blm*-regulated SCEs but instead participates in a pathway that enables HDR-independent SCEs. Therefore, the SCEs present in the *trex2*^{null} cells are likely HDR dependent since we know HDR is responsible for at least some spontaneous SCEs and since HDR is elevated in these cells. This presents the possibility that two pathways

cause spontaneous SCEs, one that is dependent on HDR and another that is dependent on *Trex2*.

Conclusion

Previously we showed *Trex2* deletion caused increased levels of spontaneous chromosome breaks and rearrangements. In addition we found human TREX2^{H188A} further increased the level of spontaneous broken chromosomes compared to no expression or to expression of wild-type human TREX2. These phenotypes suggest defective DSB repair so we hypothesized *Trex2* participates in either HDR or NHEJ. This hypothesis is especially attractive since ectopic expression of human TREX2 modified the 3' ends of I-SceI-induced DSBs in mouse ES cells. TREX2's 3' → 5' exonuclease activity was essential for end modification. However, ectopic expression of human TREX2 was 10-fold greater than endogenous expression of mouse *Trex2*. Thus, we did not know whether this endogenous level was sufficient to affect DSB repair. Contrary to our hypothesis, our data suggest *Trex2* suppresses the formation of chromosomal breaks as opposed to repairing them. In fact, *Trex2* deletion significantly

Figure 6 The average number of spontaneous SCEs per metaphase spread (MPS). (A) Spontaneous SCEs in control (AB2.2) and *trex2^{null}* cells. *Trex2* deletion reduced SCEs in control AB2.2 cells (Yates-corrected chi-square test, $P < 0.0001$). (B) Spontaneous SCEs in control (AB2.2), *blm^{-/-}*, and *trex2^{null} blm^{-/-}* cells. *Trex2* deletion did not reduce SCEs in *blm^{-/-}* cells ($P = 0.11$). Total numbers: AB2.2, 374 SCEs from 1965 chromosomes (61 metaphase spreads); *trex2^{null}*, 354 SCEs from 4020 chromosomes (106 metaphase spreads); *blm^{-/-}*, 1348 SCEs from 1900 chromosomes (50 metaphase spreads); and *trex2^{null} blm^{-/-}*, 1348 SCEs from 1894 chromosomes (50 metaphase spreads).

increased the repair of I-SceI-induced DSBs by both HDR and NHEJ. Therefore, these pathways might be enhanced to compensate for the increased levels of spontaneous broken chromosomes.

We present a conceptual model that suggests *Trex2* suppresses the formation of spontaneous chromosomal breaks known to occur at collapsed replication forks (Petermann and Helleday 2010). Our model begins with a replication fork stalling as it encounters a defect. This defect may be the consequence of alternative structures found in repetitive DNA (like a hairpin) but could also be some form of damage. *Trex2* then enables replication fork progression to prevent collapse and break formation. In *Trex2*'s absence chromosomal breaks increase along with a compensatory upregulation of HDR and NHEJ. *TREX2^{H188A}* enhances break formation by interfering with another maintenance pathway. Thus, this model proposes *Trex2* suppresses the formation of chromosomal breaks as opposed to repairing them.

The following data support this conceptual model:

1. *Trex2* appears to be important for cell proliferation since *Trex2* was expressed during S-G2, but not mitosis (Chen *et al.* 2007b) and since reduced *TREX2/Trex2* levels inhibited proliferation in HeLa cells (Chen *et al.* 2007b) and ES cells (Chen *et al.* 2007a).
2. *Trex2* may play a specialized role in maintaining highly repetitive regions since the majority of spontaneous chromosomal breaks found in *Trex2*-altered cells occurred in the pericentromere, a region rich in major satellite repeats (Guenatri *et al.* 2004). This phenotype is especially obvious for cells expressing *TREX2^{H188A}* and sug-

gests this exonuclease mutant interferes with proper replication fork maintenance.

3. *Trex2* may enable postreplication repair since it was reported to associate with Pol δ (Shevelev *et al.* 2002). In addition we show *trex2^{null}* cells exhibit diminished spontaneous HDR-independent SCEs, suggesting *Trex2* participates in a strand exchange pathway that is independent of HDR. For example in prokaryotes, diminished spontaneous SCEs were indicative of a defect in newly synthesized DNA strands switching templates during replication (Goldfless *et al.* 2006). Furthermore, defects in error-free postreplication repair resulted in gross chromosomal rearrangements in yeast (Smith *et al.* 2004) and inactivation of HDR reduced the level of these rearrangements (Motegi *et al.* 2006).

These observations support the possibility that *Trex2* suppresses the formation of DSBs by maintaining replication forks perhaps in association with pol δ or perhaps by enabling a strand exchange mechanism that is HDR independent. In the absence of *Trex2* it is possible that replication forks stall and then collapse to form a DSB that is then a substrate for chromosomal rearrangements.

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