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

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Running title: Trex2 does not repair DSBs
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 trex2null mouse embryonic stem (ES) cells and expressed in these cells wild type human TREC2 cDNA (Trex2hTX2) or cDNA with a single amino acid change in the catalytic domain (Trex2H188A) or in the DNA binding domain (Trex2R167A). We found the trex2null and Trex2H188A cells exhibited spontaneous broken chromosomes and trex2null cells exhibited spontaneous chromosomal rearrangements. We also found ectopically expressed human TREC2 was active at the 3’ ends of I-SceI-induced chromosomal DSBs. Therefore, we hypothesized Trex2 participates in DNA double-strand break (DSB) repair by modifying 3’ ends. This may be especially important for ends with damaged nucleotides. Here we present data that is unexpected and prompts 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 impacting pathway choice. Interestingly however, trex2null 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 prompts a new model that posits Trex2 suppresses the formation of broken chromosomes.
DNA 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: HDR and 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 (HASTY et al. 2003; LOMBARD et al. 2005; VIJG and DOLLE 2002). HDR repairs general DSBs by annealing the broken strand to a homologous template usually provided by the sister chromatid during DNA replication (SAN FILIPPO et al. 2008; WEST 2003). 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 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, 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 about 60% and R167A that impairs DNA binding by about 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 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 (*Trex2H188A*) or DNA binding domain (*Trex2R167A*); these cells were made by knockin such that the human cDNA is expressed by the mouse Trex2 promoter in situ (DUMITRACHE et al. 2009). The *trex2*null and *Trex2H188A* cells, but not the *Trex2H188A* or *Trex2R167A* cells, exhibited high levels of spontaneous broken chromosomes. Broken chromosomes were especially apparent in the *Trex2H188A* cells suggesting a dominant activity. These breaks were often located in or adjacent to the pericentromere, a highly repetitive region that is composed of 6-8Mb 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 (*Trex2H188A* and *Trex2R167A* 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 2-5 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 10X higher than endogenous mouse Trex2 as measured by mRNA. Therefore, we do not know if
these endogenous levels of mouse Trex2 are sufficient to modify I-SceI-induced 3’ ends. We also do not know if 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 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, 100µM β-mercaptoethanol, 2mM L-Glutamine, 3mg/ml penicillin, 5mg/ml streptomycin, 1000U/ml ESGRO (LIF)] and seeded on 0.1% gelatin coated plastic plates in a 37°C 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 in order 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*<sup>null</sup> and *trex2*<sup>null-2</sup>) (CHEN et al. 2007a; 
DUMITRACHE et al. 2009). In addition, by knockin we introduced the wild type human short 
isoform of *TREX2* (*Trex2<sub>hTX2</sub>*) 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<sub>H188A</sub>*<sup>null</sup>) while the other variant is mutated in the DNA binding domain 
(*Trex2<sub>R167A</sub>*<sup>null</sup>). 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*<sup>/−</sup> cells (BASSING et al. 2002) and J1 cells were used to 
generate the *ku70*<sup>/−</sup> cells (GU et al. 1997). AB2.2 cells were used to generate the *blm*<sup><sub>tm3Brd/tm4Brd</sub></sup> 
cells (LUO et al. 2000); for simplicity these cells are referred to as *blm*<sup>/−</sup> 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* (Fig. 1A and below). We also used a 
different selection cassette to delete *Trex2* in *blm*<sup>/−</sup> cells, puromycin phosphotransferase (Fig. 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 FIAU [1-(2-deoxy-2-fluoro-1-β-D-arabino-furanosyl)-5-iodouracil]. RE mutant _lox_ Ps (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 _lox_ P (TACCGTTTCGTATAATGTATGCTATACGAAGTTAT) differs from that of wild type _lox_ P (ATAAUCTTCGTATAATGTATGCTATACGAAGTTAT), the difference is in bold. Targeted clones were selected in tissue culture with 3-9μg/ml puromycin for 5 days and then 10μM 6-thioguanine + 3μg/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 knockin vector (CHEN et al. 2007a) except _puΔtk_ was used instead of miniHPRT for selection since these cells
are *Hprt* positive (Fig. 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 mini*HPRT* (Fig. 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 (Fig. 1A). Primers for *Trex2* deletion are Tx2Cre2 (5’-CCAAAGGCCTCATGAGATGG-3’) and Tx2CreRev3 (5’-AAGGCATGGACTAGCTCTCTGC-3’). The PCR conditions are: 1 cycle of 98°C for 5min then 35 cycles of 98°C for 1min, 62°C for 1min, 72°C for 30 sec then 1 cycle of 72°C for 10min.

**Dose response to camptothecin and γ-radiation.** The camptothecin and γ-radiation doses 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 X 10⁴ cells per well in a 4-well chamber slide and cultured at atmospheric pressure for 1day as described for murine fibroblasts (PARRINELLO et al. 2003). Cells were washed in PBS, fixed with 2% formaldehyde in PBS for 10min at room temperature, washed three times with PBS, permeabilized with 0.5% Triton X-100 for 10min, washed three times with PBS, blocked for 30min and incubated overnight at 4°C with primary antibody. After
washing 3 times with PBS, cells were incubated with secondary antibody for 45min at room temperature, washed three times with PBS and stained with DAPI (1µg/ml). The cells were photographed using a Zeiss Axioplan2 with a 60X 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 1hr. The next day cells were exposed to 100nM camptothecin for 5hrs then 540nM colcemid for 4hrs and then trypsinized. The remainder of the procedure has been described (CHEN et al. 2007a).

**Sister chromatid exchange assay.** ES cells (1 X 10⁶) were seed onto a 10cm plate pretreated with 0.1% gelatin for 1hr. The next day BrdU was added (10µM final concentration) and cells were incubated for 24hrs. Then the media was removed and new media added, cells were incubate for an additional 8hrs then 540nM colcemid was added for 4hrs. Cells were then 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 solution of 0.5% Tw20, 10µl FITC conjugated anti-BrdU antibody (Becton Dickinson) and 0.25mg/mL major satellite repeat (CY-3 5’ TGG AAT ATG GCG AGA AAA CTG AAA ATC ATG GAA AAT GAG A 3’) for 10min, at 37ºC, in dark. The slides were washed with PBS (pH 7) for 30sec, treated with DAPI (Vectashield) coverslip and analyzed under microscope (Axioplan).

**I-Scei assays.** Integration of DR-GFP and EJ5-GFP into the Pim1 locus of AB2.2 control and trex2null 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/relation 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 (Fig. 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 _trex2null_ and _Trex2H188A_ cells for camptothecin-induced chromosomal breaks by looking at metaphase spreads using three-color FISH (fluorescence in situ hybridization). 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 _trex2null_ and _Trex2H188A_ cells exhibited increased levels of spontaneous broken chromosomes as compared to control cells. These breaks were almost exclusively in the pericentromere. In addition, the _Trex2H188A_ cells showed higher levels of spontaneous broken chromosomes than the _trex2null_ cells suggesting a dominant negative-like effect (DUMITRACHE et
al. 2009). We looked at the level of broken chromosomes in cells after 5hrs exposure to camptothecin at 50nM, 100nM or 200nM followed by 4hrs exposure to colcemid. For control and \textit{trem2\textsuperscript{null}} cells we found a progressive increase in chromosomal breaks with dose, yet deleting Trex2 did not increase the number of camptothecin-induced breaks (Fig. 2B, C, see figure legend for statistics). Camptothecin exposure did not significantly increase the number of chromosomal breaks for \textit{Trex2\textsuperscript{H188A}} cells beyond the high level of spontaneous broken chromosomes (Fig. 2B, 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 \textit{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 in maintaining highly repetitive regions since the pericentromere is composed of major satellite repeats (Guenatri \textit{et al.} 2004).

To evaluate DNA lesions, we measured the number of nuclear foci containing 53BP1 (p53 binding protein 1) 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 \textit{et al.} 2004). For this experiment, we deleted \textit{Trex2} in IB10 cells since immunostaining is easier in these cells as compared to AB2.2 cells (see Materials and Methods), the parental cells described in our previous publications. We found control and \textit{trem2\textsuperscript{null}} cells exhibited the same level of spontaneous and camptothecin-induced 53BP1 foci (Fig. 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. Gamma-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 (Fig. 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 (Fig. 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. 2010; XIE et al. 2004). 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 γ-radiation (Fig. 4B). To look at classical NHEJ-
defective cells, we deleted *Trex2* in control J1 cells and J1 cells mutated for *Ku70* (Gü *et al.* 1997) (Fig. 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 (Couedel *et al.* 2004; Pierce *et al.* 2001). However, deleting *Trex2* did not further sensitize these cells to γ-radiation (Fig. 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 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. Based on these results, it is possible that endogenous *Trex2* levels are sufficient to modify the I-SceI-cut 3’ ends. Therefore, we tested if 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 if endogenous *Trex2* levels affect the repair of an I-SceI-induced DSB in a substrate specific for either HDR (DR-GFP, Fig. 5A) or NHEJ between distal ends of two tandem DSBs (EJ5-GFP, Fig. 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 (Bennardo *et al.* 2008; Stark *et al.* 2004). In addition, DR-GFP does
not detect SSA since the iGFP donor fragment lacks essential 3’ elements of the GFP expression cassette. The generation of GFP+ cells with EJ5-GFP can involve either 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; Bennardo et al. 2009). We found the frequency of HDR and NHEJ was increased in trex2null cells relative to control (Fig. 4C; Student T test p<0.001) supporting the possibility these DSB repair pathways are elevated in trex2null cells. We also found the relative HDR/NHEJ ratio was similar between trex2null (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 trex2null cells.

Elevation in HDR and NHEJ in trex2null 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 trex2null 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 (Fig. 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; Bennardo et al. 2009). Even though formation of I-SceI- NHEJ products are 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 (Fig. 5B, right), can be independent of both Ku70 and Xrcc4, and promoted by ectopic expression of
Trex2 (Bennardo et al. 2008; Bennardo et al. 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 trex2null cells: 45+/−7% I-SceI restoration for control, 43+/−5% I-SceI restoration for trex2null (Fig. 5D). Therefore, Trex2-deletion did not impact 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 then sequenced individual clones. From this analysis, we found no significant difference between control and trex2null 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, 12/23 for trex2null). 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 trex2null 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 versus NHEJ, and the types of NHEJ products). To explain the increase in DSB repair, we suggest DSB repair is elevated in trex2null 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-mediated repair of DSBs induce SCE (Dong and Fasullo 2003; Dronkert et al. 2000; Sonoda et al. 1999; Takata et al. 2000; Takata et al. 2001). Therefore, we observed spontaneous SCEs by FISH. We found trex2null cells exhibited reduced levels of spontaneous SCEs (Fig. 6A, Yates-Corrected Chi-Square Test: p<0.0001). Spontaneous SCEs may result from HDR-dependent crossing over or perhaps an HDR-independent pathway.

In order to determine if these SCEs were HDR-dependent, we deleted Trex2 in blm+/− cells (Fig. 1B, 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; Liberi et al. 2005; Raynard et al. 2006; Wu and Hickson 2003). Additionally, the role of Blm 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 (Fig. 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 trex2null 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 TREX2H188A
further increased the level of spontaneous broken chromosomes as 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 if this endogenous level was sufficient to impact 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 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\textsuperscript{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 \textit{et al.} 2007b)
and since reduced TREX2/Trex2 levels inhibited proliferation in HeLa cells (CHEN \textit{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 suggests this exonuclease mutant interferes with proper replication fork maintenance. 3) Trex2 may enable postreplication repair since it was reported to associate with Polδ (Shevlev 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 (Moteギ et al. 2006). These observations support the possibility that Trex2 suppresses the formation of DSBs by maintaining replication forks perhaps in association with polδ or perhaps by enabling a strand exchange mechanism that is HDR independent. In the absence of Trex2 it is possible that replication forks stall, then collapse to form a DSB that is then a substrate for chromosomal rearrangements.

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We are grateful to Ms. Charnae Williams for technical support and Drs. Fred Alt (Ku70) and Allan Bradley (B1m) for mutant cells and their control cells. We are also thankful to Dr. Allan Bradley for puΔtk. The work was supported by the National Institutes of Health [UO1 ES11044, 1 RO1 CA123203-01A1 to PH, RO1 CA120954 to JMS, and T32 CA86800-03 to LCD].
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MARPLE, T., H. LI and P. HASTY, 2004 A genotoxic screen: rapid analysis of cellular dose-response to a wide range of agents that either damage DNA or alter genome maintenance pathways. Mutat Res 554: 253-266.


Table and Figure Legends

Table 1:

Bold, inserted sequence; Gray, microhomology; N/A, not applicable.

Figure 1. Trex2 knockouts. A1. Targeting Trex2 with the conditional vector in h2ax<sup>+/−</sup> and ku70<sup>+/−</sup> 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. 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 a loading control (HOLCOMB et al. 2007). Wild type control, WT. B1. Replacing Trex2 in blm<sup>−/−</sup> cells with puromycin acetyltransferase expressed by the phosphoglycerate kinase (PGK) promoter. B2. Screen for targeted clones. PCR amplification with TX2LR55 and HATrev. B3. Verification of targeted clones with RT-PCR using mTx2For and mTx2Rev primers shown in A1. Detection of Rad51 mRNA was used a loading control.

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<sup>h/tx2</sup>, Trex2<sup>H188A</sup> and Trex2<sup>R167A</sup> were compared to the parental clones of trex2<sup>null</sup> and trex2<sup>null−2</sup> cells (DUMITRACHE et al. 2009) and control cells. These experiments were done 3 times. The four
clones from each experiment were averaged and then the three experiments were averaged. Standard deviation is shown. Survival fraction, SF. B. The % of metaphase spreads (MPSs) with microscopically visible spontaneous and camptothecin-induced broken chromosomes. Control cells, AB2.2. C. Summary of results. Shown is the number of metaphase spreads (MPS) with 0 broken (br) chromosomes, 1 broken chromosome, 2 broken chromosomes or 3 broken chromosomes. No metaphase spread had more than 3 broken chromosomes. Statistics (student T test), refer to lanes in B for comparisons: 1&2 p=0.0001, 1&3 p<0.0001, 1&4 p=0.0003, 2&3 p=0.0141, 2&4 p=0.026, 3&4 p=0.398, 5&6 p=0.061, 5&7 p=0.0003, 5&8 p=0.0008, 6&7 p=0.033, 6&8 p=0.013, 7&8 p=0.391, 9&10 p=0.708, 9&11 p=0.321, 9&12 p=0.481, 10&11 p=0.517, 10&12 p=0.847, 11&12 p=0.608, 1&5 p=0.037, 1&9 p=0.0002, 2&6 p=0.872, 2&10 p=0.027, 3&7 p=0.511, 3&11 p=0.206, 4&8 p=0.546, 4&12 p=0.872, 5&9 p=0.0001, 6&10 p=0.037, 7&11 p=0.569, 8&12 p=0.411.

Figure 3. Camptothecin-induced 53BP1 foci. A. trex2null cells exhibit the same level of camptothecin-induced 53BP1 foci as control cells (IB10). B. Summary of results.

Figure 4. Exposure to γ-radiation. A. Trex2-altered cells exposed to γ-radiation. Four clones of Trex2hTX2, Trex2H188A and Trex2R167A were compared to the parental clones of trex2null and trex2null-2 cells (DUMITRACHE et al. 2009) and control cells. These experiments were done 3 times. And standard deviation is shown. Survival fraction, SF. B. trex2null h2ax−/− cells exposed to γ-radiation. Average of two h2ax−/− trex2null clones is shown. These experiments were done 3 times and standard deviation is shown. Control cells, TC1. C. trex2null ku70−/− cells exposed to γ-
radiation. Average of two \textit{t}re\textit{x}2\textit{null} \textit{k}u70\textsuperscript{−/−} clones is shown. These experiments were done 3 times and standard deviation is shown. Control cells, J1.

Figure 5. I-Sce\textit{I}-specific DSBs. A. The HDR-specific substrate, DR-GFP. The I-Sce\textit{I} site interrupts the GFP sequence while the Bcg\textit{I} site is the wild type sequence at this location. After generation of an I-Sce\textit{I} DSB, the free ends invade the adjacent truncated GFP substrate that contains the wild type Bcg\textit{I} site. Nonreciprocal homologous recombination (gene conversion) restores GFP. B. The NHEJ-specific substrate, EJ5-GFP. There are two I-Sce\textit{I} sites separated by a puromycin phosphotransferase (puro) selection cassette. After generation of two I-Sce\textit{I}-induced DSBs distal end joining restores the GFP cassette. There are two potential outcomes. First, the I-Sce\textit{I} site is restored. This detects classical NHEJ (Ku70-dependent). Second, the I-Sce\textit{I} site is lost. This detects Alt-NHEJ (Ku70-independent). C. \textit{t}re\textit{x}2\textit{null} cells repaired I-Sce\textit{I}-induced DSBs more efficiently than control cells (AB2.2) for the DR-GFP (DR, \textit{p}<0.001, student T test) and EJ5-GFP (EJ, \textit{p}<0.0001) substrates. The mean of 6-8 experiments is shown. D. I-Sce\textit{I} digestion of PCR amplified DNA from GFP+ cells with the EJ5-GFP substrate. After I-Sce\textit{I} digestion, the amplified DNA is either uncut (U) or cut with I-Sce\textit{I} (S).

Figure 6. The average number of spontaneous SCEs per metaphase spread (MPS). (A) Spontaneous SCEs in control (AB2.2) and \textit{t}re\textit{x}2\textit{null} cells. \textit{T}re\textit{x}2-deletion reduced SCEs in control AB2.2 cells (Yates-Corrected Chi-Square Test, \textit{p}<0.0001).(B) Spontaneous SCEs in control (AB2.2), \textit{bl}m\textsuperscript{−/−} and \textit{t}re\textit{x}2\textit{null} \textit{b}lm\textsuperscript{−/−} cells. \textit{T}re\textit{x}2-deletion did not reduce SCEs but in \textit{bl}m\textsuperscript{−/−} cells (\textit{p}<0.11). Total numbers: AB2.2, 374 SCEs out of 1965 chromosomes (61 metaphase spreads); \textit{t}re\textit{x}2\textit{null}, 354 SCEs out of 4020 chromosomes (106 metaphase spreads); \textit{bl}m\textsuperscript{−/−}, 1348 SCEs out of
1900 chromosomes (50 metaphase spreads); \textit{trex2}^{null} \textit{blm}^{-/-}, 1348 SCEs out of 1894 chromosomes (50 metaphase spreads).
**A1**

hTREX2

PGK

puATk

---

**A2**

TX2 LR55

hTX2Rev

---

**A3**

Cre

Transfect Cre

select in FIAU

---

**A4**

Before Cre

h2ax<sup>-/-</sup>, ku70<sup>-/-</sup>

WT

---

**A5**

After Cre

h2ax<sup>-/-</sup>, ku70<sup>-/-</sup>

WT

**B1**

PGK

Puro

---

**B2**

PCR

AB2.2 blm<sup>-/-</sup>

---

**B3**

RT-PCR

blm<sup>-/-</sup> AB2.2

Trex2

Rad51

---
**A**

Graph showing the percentage survival (SF) of cells with different TREC2 variants against various concentrations of CPT.

**B**

Bar graph showing the percentage of chromosomes with a break for different TREC2 variants against various concentrations of CPT.

**C**

Table summarizing the number of MPS (microscopic chromosome structure) breaks for different TREC2 variants and concentrations of CPT.

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<th>2 br</th>
<th>3 br</th>
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<td>29</td>
<td>17</td>
<td>8</td>
<td>4</td>
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A

IB10

0 nM CPT  100 nM CPT

IB10

0 nM CPT  100 nM CPT

trex2null

0 nM CPT  100 nM CPT

B

<table>
<thead>
<tr>
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<th>nM CPT</th>
<th># cells observed</th>
<th># (%) cells ≥10 foci</th>
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<td>IB10</td>
<td>0</td>
<td>97</td>
<td>2 (2%)</td>
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<tr>
<td>trex2null</td>
<td>0</td>
<td>204</td>
<td>4 (2%)</td>
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<tr>
<td>IB10</td>
<td>100</td>
<td>278</td>
<td>137 (49%)</td>
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<tr>
<td>trex2null</td>
<td>100</td>
<td>217</td>
<td>111 (51%)</td>
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Table 1. I-SceI-resistant end joining junctions.

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<th>I-SceI site (CAPS)</th>
<th>microhomology (nt.)</th>
<th>AB2.2 # of clones</th>
<th>trex2null # of clones</th>
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<tr>
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