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Short communication

TREX2 exonuclease defective cells exhibit double-strand breaks and chromosomal fragments but not Robertsonian translocations

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ABSTRACT

TREX2 is a 3′ → 5′ exonuclease that binds to DNA and removes 3′ mismatched nucleotides. By an *in vitro* structure function analysis, we found a single amino acid change (H188A) completely ablates exonuclease activity and impairs DNA binding by about 60% while another change (R167A) impairs DNA binding by about 85% without impacting exonuclease activity. For a biological analysis, we generated *trex2*^{null} cells by deleting the entire *Trex2* coding sequences in mouse embryonic stem (ES) cells. We found Trex2 deletion caused high levels of Robertsonian translocations (RbTs) showing Trex2 is important for chromosomal maintenance. Here we evaluate the exonuclease and DNA binding domains by expressing in *trex2*^{null} cells coding sequences for wild type human *TREX2* (*Trex2*^{hTX2}) or human *TREX2* with the H188A change (*Trex2*^{H188A}) or the R167A change (*Trex2*^{R167A}). These cDNAs are positioned adjacent to the mouse *Trex2* promoter by Cre-mediated knock-in. By observing metaphase spreads, we found *Trex2*^{H188A} cells exhibited high levels of double-strand breaks (DSBs) and chromosomal fragments. Therefore, TREX2 may suppress spontaneous DSBs or exonuclease defective TREX2 may induce them in a dominate-negative manner. We also found *Trex2*^{hTX2}, *hTrex2*^{H188A} and *hTrex2*^{R167A} cells did not exhibit RbTs. Thus, neither the exonuclease nor DNA binding domains suppress RbTs suggesting TREX2 possesses additional biochemical activities.
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1. Introduction

The mammalian three prime repair exonuclease (TREX) proteins, TREX1 and TREX2 are homologous to the proofreading exonuclease in bacterial DNA polymerases important for postreplication repair [1–4]. *In vitro*, both function as homodimers, bind to DNA and effectively remove 3′ mismatched sequences via their 3′ → 5′ exonuclease activity [5]. To better understand biological function, *Trex1* was mutated in mice; however, surprisingly *trex1*$^{-/-}$ mice did not show genomic instability as would be expected for defective postreplication repair but instead died from cardiomyopathy caused by inflammatory myocarditis [6]. This unexpected phenotype suggested that TREX1 is not directly involved in DNA repair despite its sequence homology to exonucleases and its exonuclease activity. To help explain this surprising phenotype, TREX1 was later shown to process cytosolic DNA that may arise from endogenous retroelements or aberrant replication intermediates [7,8]. Failure to do so leads to the accumulation of cytosolic DNA that ultimately induces a pathological autoimmune response designed to defend against viruses or a chromic DNA damage checkpoint. To support these findings, TREX1 mutations cause a variety of autoimmune disorders in humans [9,10]. In addition, TREX1 is apart of the Granzyme A mediated cell death pathway since it binds to the SET complex and degrades nuclear DNA in concert with the endonuclease NM23-H1 [11]. Thus, TREX1 performs several functions, but does not appear to be important for DNA repair while much less is known about TREX2 biological function.

To better understand TREX2 biological function, we generated *trex2*^{null} mouse ES cells by deleting all the known *Trex2* coding sequences via gene targeting [12]. We found *trex2*^{null} cells exhibited high levels of Robertsonian translocations (RbTs). RbTs are chromosome rearrangements involving centric fusion of two acrocentric chromosomes to form a single metacentric chromosome that results from deletion of the p arms from both chromosomes [13,14]. RbTs may influence speciation [15,16] and may increase cancer risk [17], spontaneous abortions [18] and male infertility [19]. Thus, Trex2 maintains chromosomal integrity but we do not know if the exonuclease and DNA binding activities are important for suppressing RbTs.

TREX2 works as a homodimer to bind to DNA and remove 3′ mismatched sequences. Single amino acid mutations were made in TREX2 to separate DNA binding from exonuclease activity [20–22]. The H188A alteration completely ablates exonuclease activity but also impairs DNA binding by about 60% while the R167A change impairs DNA binding by about 85% without diminishing exonuclease activity [21]. Therefore, these functions may be genetically

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separated, at least in part, yet the biological significance of these activities is not known.

Here we report the impact human wild type TREX2 and human TREX2 mutated in the exonuclease domain or DNA binding domain has on mouse ES cells. We find Trex2-deletion mildly increases the level of spontaneous chromosomal DSBs and fragments. This phenotype is rescued by expression of wild type human TREX2 [21] and TREX2 mutated in the DNA binding domain. However, expression of TREX2 mutated in the exonuclease domain resulted in a further increase in chromosomal breaks suggesting a dominant negative phenotype. Surprisingly, expression of wild type TREX2 and either mutant prohibited the formation of RbT's, suggesting that TREX2 possesses functions other than DNA binding and exonuclease activity that are important for genomic stability.

2. Materials and methods

2.1. Cell culture conditions

AB2.2 ES cells were maintained in M15 [high glucose DMEM supplemented with 15% fetal bovine serum, 100 μM β-mercaptoethanol, 2 mM glutamine, 3 mg/ml penicillin, 5 mg/ml streptomycin, 1000 U/ml ESGRO (LIF)] and grown on plates with 2.5×10^6 γ-irradiated murine embryonic fibroblasts (mitotically inactive feeders) seeded on 0.1% gelatin coated plastic at least the day before and grown in 5% CO_2 in a 37 °C incubator at atmospheric O_2.

2.2. Knock-in into ES cells

To integrate the human cDNA next to the mouse *Trex2* promoter, a Cre-mediated knock-in (CMKI) plasmid [23] with the short isoform of human cDNA [21] was transfected into *trex2*$^{null-2}$ cells that had been previously deleted for the 5′ half of the *HPRT* minigene [12]. 5×10^6 cells were co-electroporated with 20 μg of CMKI plasmid and 10 μg of a Cre-recombinase expression vector (pPGKcrepA) in a total of 800 μl Dulbecco's phosphate buffered saline (DPBS) using a Bio-Rad Gene Pulsar at 230 V, 500 μF. Then 200 μl of the electroporation was seeded onto a 10 cm feeder plate (primary embryonic fibroblasts mutated for *HPRT*). After 48 h of transfection, a final concentration of 1× HAT (1 mM sodium hypoxanthine, 4 μM aminopterin, and 160 μM thymidine) was added to the media. Eight to ten colonies were picked after 7–10 days of selection and expanded in HAT selection media to eliminate *HPRT* negative cells that survive by cross-feeding. These colonies were replica plated, and then one plate was frozen while the other plate was used to isolate DNA [24] for screening knock-in clones by genomic PCR.

2.3. Verification of knock-in

PCR verified knock-in by using Cre1 and hTX2Rev primers. PCR conditions: the forward primer (Cre1: 5′ CCATGAGTCCTCTTTAAAGTG 3′) and reverse primer (hTX2Rev: 5′ CTGCAGCGTCCGCACCACG 3′) were used under these conditions: one cycle of 98 °C 5 min followed by 35 cycles of 98 °C 1 min', 63.5 °C 1 min, 72 °C 1 min 40 s followed by one cycle of 72 °C 10 min).

RT-PCR also verified knock-in by using primers specific to human *TREX2* (hTX2For, hTX2Rev). PCR was performed on RNA with and without reverse transcriptase (+/−) to ensure there is no DNA contamination. RT-PCR conditions: The forward primer (hTX2For: 5′ AAA AGA ATT CCC GCC ACC ATG TCC GAG GCACCCCGGGC 3′) and reverse primer (hTX2Rev2: 5′ CTGCAGCGTCCGCACCACG 3′) were used under these conditions: one cycle of 98 °C 5 min followed by 35 cycles of 98C 1 min, 65 °C 1 min, 72 °C 25 s followed by one cycle of 72 °C 10 min).

2.4. Three-color fluorescence in situ hybridization (FISH)

Treat cells with 10 mg colcemid for 4 h then trypsinized cells. Slide preparation: spin cells (1000 rpm), 10′ wash cells 2× in PBS (all PBS washes are pH 7.4 unless otherwise noted). Resuspended pellet in 300 ml 75 mM KCl, dropwise, flicking tube. Incubate 37 °C water bath, 15′. Add 300 ml methanol/acetic acid (3:1 fixative), dropwise, flicking tube, spin 3000 rpm, 30″. Wash cells in 300 ml 3:1 fixative, dropwise, flicking tube, spin @ 3000 rpm, 30″; rpt wash. Hybridization: Place slides in 70 mM NaOH, 2′. Wash in PBS pH 8.5, 10 dips. Incubate 37°, 5′ in the dark, in 500 μl/slide of 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′) and telomeric [6-FAM 5′ $(CCCTAA)_7$ 3′] probes wash in PBS, 10 dips, coverslip in DAPI.

3. Results and discussion

Here we evaluate the TREX2 exonuclease and DNA binding domains by introducing human *TREX2* coding sequences adjacent to the mouse *Trex2* promoter in *trex2*^{null} cells. Previously we generated *trex2*^{null} AB2.2 ES cells (Fig. 1A) by replacing the entire known mouse *Trex2* coding sequence [12] with a floxed hypoxanthine phosphoribosyltransferase (*HPRT*) minigene [25], referred to as *miniHPRT*. *MiniHPRT* is selected for expression in HAT (hypoxanthine, aminopterin, thymidine) or for absence of expression in 6-TG (6-thioguanine) and is composed of a phosphoglycerol kinase (PGK) promoter with an intron that separates exons 1–2 from exons 3 to 8.

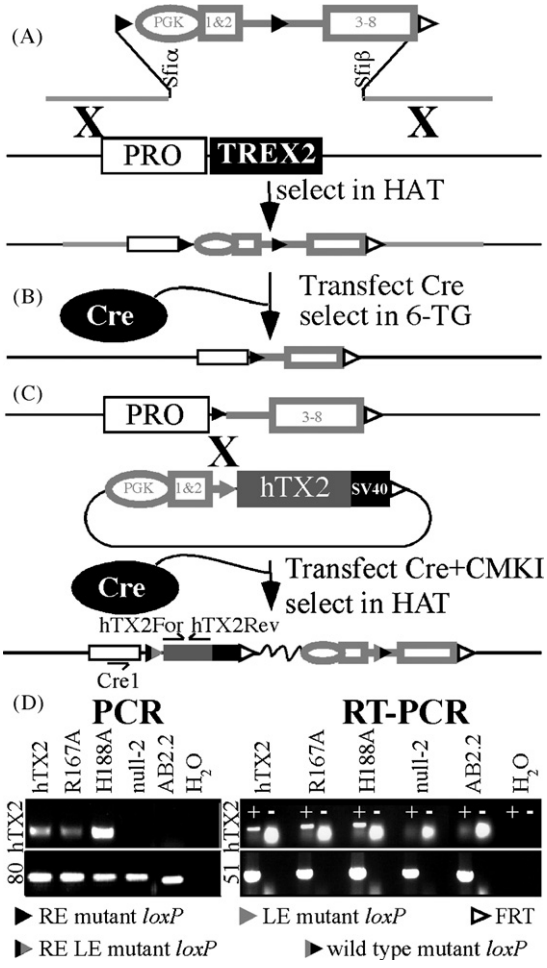

Fig. 1. Knock-in of human *TREX2* cDNA variants. (A) The *HPRT* minigene, expressed by the PGK promoter [25,29] is used for selection and contains exons 1 and 2 (box labeled 1&2), exons 3–8 + polyadenylation sequences (box labeled 3–8) separated by an intron (straight line). Select for minigene expression in HAT. A RE mutant *loxP* (black arrow head) [26] is 5′ to PGK and another RE mutant *loxP* is in the intron. An FRT (open arrow) is located 3′ to *miniHPRT*. Upon targeting the entire known mouse *TREX2* open reading frame (rectangle) is deleted as previously described [12]; this sequence corresponds to the human short isoform [21]. PRO, mouse *Trex2* promoter. (B) Removal of the 5′ half of *miniHPRT* by Cre recombination as previously described [12]. (C) Knock-in of the short isoform of human *TREX2* cDNA (hTX2). A Cre-mediated knock-in plasmid is cotransfected along with a Cre-expression plasmid. The knock-in vector contains the 5′ half of *miniHPRT*, a left element (LE) mutant loxP (Grey arrow head) and the cDNA with SV40 polyadenylation sequences as previously described [23]. Cells are selected in HAT for restoration of *miniHPRT*. The knock-in corrects *miniHPRT*, generates an RE LE mutant loxP (left, black grey arrow), a wild type loxP (right, grey black arrow) and places the cDNA adjacent to the mouse TREX2 promoter. (D) Verification of knock-in. Due to the stringent selection, all HAT resistant clones are positive for knock-in as verified by PCR (left) using Cre1 and hTX2Rev primers. Ku80 (80) was used as a loading control for PCR as previously described [12]. In addition, human *TREX2* expression is confirmed by RT-PCR (right) using primers specific to human *TREX2* (hTX2For, hTX2Rev). Note the primers are specific for human *TREX2* since mouse *Trex2* is not amplified in the AB2.2 control. PCR was performed on RNA with and without reverse transcriptase (+/−) to ensure there is no DNA contamination. *Rad51* (51) was used as loading control for RT-PCR as previously described [12].

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