Increased Susceptibility to Skin Carcinogenesis in TREX2 Knockout Mice

David Parra, Joan Manils, Bárbara Castellana, Arnau Viña-Vilaseca, Eva Morán-Salvador, Nuria Vázquez-Villoldo, Gemma Tarancón, Miquel Borràs, Sara Sancho, Carmen Benito, Sagrario Ortega, and Concepció Soler

Departament de Patologia i Terapèutica Experimental, Facultat de Medicina, Campus de Bellvitge, Universitat de Barcelona, L’Hospitalet de Llobregat; Unitat de Toxicologia Experimental, Parc Científic de Barcelona; and Protecció Radiològica, Universitat de Barcelona, Barcelona, Spain; Promed, Fribourg, Switzerland; and Spanish National Cancer Research Center, Madrid, Spain

Abstract
TREX2 is a proofreading 3′-5′ exonuclease that can be involved in genome maintenance; however, its biological role remains undefined. To better understand the function and physiologic relevance of TREX2, we generated mice deficient in TREX2 by targeted disruption of its unique coding exon. The knockout mice are viable and do not show relevant differences in growth, survival, lymphocyte development, or spontaneous tumor incidence compared with their wild-type counterparts over a period of up to 2 years. Also, we did not observe chromosomal instability or defects in cell proliferation and cell cycle upon loss of TREX2. We have observed that TREX2 expression is not ubiquitous, being expressed preferentially in tissues with stratified squamous epithelia, such as the skin or esophagus, and specifically in keratinocytes. Interestingly, TREX2-null mice are more susceptible to skin carcinogenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA) compared with wild-type mice. This phenotype correlates with a reduction of DMBA-induced apoptosis in both the epidermis and keratinocytes of TREX2-null mice. Altogether, our results suggest a tumor suppressor role for TREX2 in skin carcinogenesis through which it contributes to keratinocyte apoptosis under conditions of genotoxic stress.

Introduction
3′-5′ Exonucleases remove nucleotides from DNA 3′ termini in multiple processes of DNA metabolism, ranging from DNA synthesis to DNA degradation, and thereby can play a pivotal role in maintaining genome stability and preventing cancer, aging, immunologic abnormalities, and inherited diseases. The 3′-5′ exonuclease activity has been found in multidomain proteins, such as DNA polymerses (Polδ, Polγ, and Polκ), base excision repair apurinic/apyrimidinic endonucleases (APE1 and APE2), double strand break DNA repair proteins (MRE11), DNA repair nucleases (EXO1 and NMM23-H1), helicases (WRN, Dna2), cell cycle checkpoint proteins (RAD9 and RAD1), and the transcription factor p53. In addition, this activity has been described in the single-domain exonucleases TREX1 and TREX2 (1–5). Despite the numerous 3′-5′ exonucleases present in the cell with putative overlapping activities, in vivo functional studies have revealed specific and nonredundant biological roles for many of these proteins (1, 6–11). Among these, the biological function of TREX2 is still largely unknown.

TREX2, which was identified in a database search by means of its homology with TREX1, displays a robust nonprocessive 3′-5′ exonuclease activity that can proofread the work of DNA polymerases and process 3′ ends during DNA replication and repair processes in vitro (12–14). In this regard, it has been reported that TREX2 may interact with Polδ and increase its fidelity when deoxynucleotide triphosphates pools are imbalanced (15). TREX2 biochemical and structural properties are similar to TREX1, although they are not identical. The two proteins share a dimeric structure and can process ssDNA and dsDNA substrates in vitro with almost identical Km values. However, several features related to enzyme kinetics, structural domains, and subcellular distribution distinguish TREX2 from TREX1. TREX2 present a 10-fold lower affinity for DNA substrates in vitro compared with TREX1. In contrast with TREX1, TREX2 lacks a COOH-terminal domain that can mediate protein-protein interactions (12, 16–19). TREX2 is localized in both the cytoplasm and nucleus (20), whereas TREX1 is found in the endoplasmic reticulum, and is mobilized to the nucleus during granyme A–mediated cell death (21) or after DNA damage (22). Loss of TREX1 function does not trigger an increase in cancer development (11), but rather leads to cell-intrinsic immune activation by endogenous nondegraded ssDNA, resulting in the development of autoimmunity (23–26). Indeed, TREX1 has been shown to be involved in the degradation of ssDNA generated from the processing of aberrant replication intermediates (22) and endogenous retroelements (26). Otherwise, TREX2 seems to be important for chromosomal stability. As such, TREX2 deletion in embryonic stem (ES) causes high levels of Robertsonian Translocations (27), but this effect is independent of its exonuclease activity and DNA binding domains (28). The biochemical activities and the mechanisms by which TREX2 prevents chromosomal instability and the consequences of TREX2 deficiency in the organism remain unknown.

In this context, we aimed to assess the function and relevance of TREX2 in vivo. To that end, we generated and characterized the TREX2 knockout mice, Trex2−/−. Furthermore, to evaluate putative tissue-specific functions, we analyzed TREX2 tissue expression. TREX2 knockout mice seem viable and fertile and do not show a significant increase in spontaneous tumor incidence. Interestingly, TREX2 deficiency increases susceptibility to carcinogen-induced skin tumorigenesis that correlates with impaired apoptosis. These findings suggest a tumor suppressor role for TREX2 under...
Figure 1. Targeted disruption of the Trex2 gene in mice. A, targeting strategy. Schematic representation of germ line Trex2 locus, targeting vector, mutated conditional (Trex2\textsuperscript{lox\textast}}), and knockout (Trex2\textsuperscript{*}) locus. The mouse Trex2 locus contains two exons (gray boxes). The full protein coding sequence is included in exon 2. LoxP sites (filled triangles) were placed flanking exon 2. The pgk-neo\textsuperscript{r} cassette and pgk-tk cassette, flanked by frt sites (open boxes), used for selection of homologous recombinant ES clones are indicated. Flp, Flp recombinase; Cre, Cre recombinase. RV (EcoRV), RI (EcoRI). B, Southern blot (SB) and PCR analysis of recombinant ES clones and mice carrying the indicated alleles. The origin of the Southern blot probes is indicated in A. Sizes of the diagnostic DNA fragments are indicated in A and B. C, RT-PCR analysis of Trex2 mRNA expression in skin from wt, Trex2\textsuperscript{lox\textast}}, and Trex2\textsuperscript{lox/lox} mice. D, Western blot (WB) analysis of skin extracts and immunoprecipitates from wt, Trex2\textsuperscript{*}, and Trex2\textsuperscript{lox/lox} mouse with antibodies to mouse TREX2. Preimmune (PI) serum was used as a negative control for immunoprecipitation (IP). Bands corresponding to the 236 amino acid mouse TREX2 protein and to heavy immunoglobulin chain (Ig\textsubscript{H}) are indicated.
conditions of genotoxic stress in keratinocytes, cells where we have found that this exonuclease is predominantly expressed.

Materials and Methods

Generation of Trex2−/− mice and genotyping. To conditionally disrupt the mouse Trex2 gene, a targeting strategy was designed to knock in two loxP sites flanking exon 2 via homologous recombination in ES cells. The targeting construct was constructed by subcloning genomic DNA fragments encompassing exon 2 and the 5′ and 3′ flanking fragments into the loxP conditional vector pDELBOY-3×, as schematically shown in Fig. 1A. Genomic fragments corresponding to exon 2 (1 kb) and the 5′ (2.9 kb) and 3′ (2.2 kb) arms were amplified using genomic DNA extracted from mouse R1 ES cells by PCR using the Expand High Fidelity PCR system (Roche). The targeting vector was electroporated into mouse R1 ES cells and recombinant clones were selected in the presence of G418 and ganciclovir. Genomic DNA from resistant clones were digested with EcoRI, and tested for correct targeting events via Southern blotting using both 5′ (probe a) and 3′ (probe b) external probes. Targeted ES clones (Trex2loxneo) are hemizygous because the Trex2 gene is located on the X chromosome and R1 ES cells are XY. Three ES clones, in which correct homologous recombination had occurred, were aggregated with eight-cell-stage CD-1 embryos. Male chimeras transmitted the targeted allele.
(Trex2loxneo) to their offspring. Female Trex2+/loxneo mice were crossed with transgenic Flp (pCAG-flpe/) male mice to delete the neomycin resistance gene. Then, female Trex2+/loxneo mice were bred with transgenic Cre (CMV-Cre, ubiquitous expression) male mice to delete exon 2 and simultaneously segregate the Flp transgene. The resulting Trex2−/−, Trex2+/− females and Trex2−/− mice were subsequently intercrossed to eliminate the Cre transgene. Crosses between these mice generated TREX2 knockout mice, Trex2−/−. Excisions were verified via Southern blotting and PCR analysis of genomic DNA from tail biopsies of the mice (Fig. 1B). Targeting vector construction, primers, and PCR conditions are described in Supplementary Materials and Methods.

Mice were maintained in a pathogen-free environment. All animal procedures were approved by the Animal Care and Use Committee of Spanish National Cancer Research Center and the Ethics Committee of the University of Barcelona.

RNA analysis. For reverse transcription-PCR (RT-PCR) analysis of TREX2 mRNA expression, total RNA from tissues and cells of Trex2−/−, Trex2+/−, and Trex2+/+ mice was isolated using the Nucleospin RNA extraction kit (Macherey-Nagel). Total RNA from human tissues was obtained from Spanish National Cancer Research Center and the Ethics Committee of the University of Barcelona. Reverse transcription was done with Transcriptor reverse transcriptase using oligo dT, according to the manufacturer’s instructions (Roche). For a quantitative analysis, after cDNA was synthesized, real-time PCR was performed using a LightCycler machine with LightCycler FastStart DNA Master (Roche). Values were normalized to hypoxanthine phosphoribosyltransferase (HPRT1) mRNA expression. Primer sequences, gene accession number, length of PCR amplicons, and PCR conditions are shown in Supplementary Table S2.

Immunoprecipitation, Western blot, and antibodies. Cells and tissues from mice were homogenized in ice-cold lysis buffer [1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl] together with protease inhibitors [10 µg/mL aprotinin, 10 µg/mL leupeptin, 86 µg/mL iodoaceticamide, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. For immunoprecipitation, 1 mg of protein was incubated with 10 µL of rabbit polyclonal antibodies to mouse TREX2 coupled to protein A-Sepharose beads for 3 h. Immunocomplexes were washed four times with buffer containing 20 mmol/L HEPES (pH 7.5), 0.1% Nonidet P-40, 10% glycerol, and 150 mmol/L NaCl. Samples were boiled in Laemmli buffer and separated on 10% SDS-PAGE. They were then transferred to polyvinylidene difluoride membranes (Hybond-ECL; GE Healthcare). Membranes were blocked for 1 h at room temperature in 5% nonfat milk in TBST buffer (150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4) and 0.05% Tween 20), incubated with diluted antibody (anti-TREX2, 1:10,000) and followed by horseradish peroxidase–conjugated protein A/G (Bio-Rad) and detected with enhanced chemiluminescence (ECL; GE Healthcare). Antibodies against mouse TREX2 were generated using an MBP-fusion mouse TREX2 protein for rabbit immunization. Antibodies were raised and affinity purified by standard procedures. This antibody does not cross-react with TREX1. The specificity of this antibody was unequivocally validated by the absence of immunodetection of TREX2 protein by immunoprecipitation, Western blot and immunofluorescence in samples from Trex2−/− mice compared with samples from Trex2+/+ mice (Figs. 1D and 2C).

Exonuclease assay. Cells and tissues from mice were homogenized in an ice-cold lysis buffer containing 1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 1 mmol/L DTT, 100 mmol/L NaCl, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mmol/L PMSF, and 1 mmol/L sodium orthovanadate. Lysates were then assayed for DNA 3’ exonuclease activity, as described elsewhere (11).

Flow cytometry analysis of B and T cells. Cells from the bone marrow, thymus, spleen, and blood of 2-month-old Trex2−/− and Trex2+/+ mice were stained with the following antibodies to B- and T-cell surface markers: FITC-anti-CD19, PE-anti-CD4, Cy5-anti-CD8, FITC-anti-B220, and PE-anti-CD19 (Becton Dickinson) following standard protocols. Analysis was performed with an Epics XL flow cytometer (Coulter Corporation).

Survival, growth, and spontaneous tumor development. Trex2−/− mice and their Trex2+/− littermates in the C57Bl/6/CD1/129 mixed genetic background were used in an untreated time course study, during which they were observed weekly for up to 2 y. Mice were monitored for body weight, fertility, survival, lymphocyte development, and spontaneous tumor growth, survival, and spontaneous tumor development.
development. Mice were euthanized when they seemed unhealthy, either because of visible lesions, morbidity, or significant weight loss. Autopsies were performed on these animals and in all of the mice when they reached the age of 24 mo. Mouse tissues were fixed in 10% neutral-buffered formalin solution and processed for paraffin embedding, cut into 5-μm sections, and mounted on slides. The sections were stained with H&E and examined by two pathologists who were blinded to the tissue grouping.

**Skin tumor induction experiments.** The strategy used for the carcinogenicity study followed well-established mouse models of DMBA and 7,12-dimethylbenz[a]anthracene (DMBA)+12-O-tetradecanoylphorbol-13-acetate (TPA)–induced tumors in the skin. For the DMBA-induced tumor experiments, DMBA (120 nmol) was applied twice weekly for 14 wk. For the DMBA+TPA tumor initiation/promotion experiments, mice were treated with a single dose of the carcinogen DMBA (120 nmol in 200 μL of acetone; Sigma) followed by applications of the tumor promoter TPA (20 nmol in 200 μL of acetone; Sigma) twice a week beginning 4 d after initiation, and for a duration of 12 wk. Treatments were applied to the shaved backs of 8-wk-old Trex2^−/−^ and Trex2^+/+^ mice. Mice of the C57BL/6/C01/129 mixed genetic background were backcrossed for six generations to indicated doses of the genotoxic agent. Ten days after treatment, the cultured on collagen I precoated plates (BD Biosciences), as described (30).

**Results**

Generation of Trex2^−/−^ mice. To create the Trex2^−/−^ mice, Trex2 gene was disrupted by deletion of exon 2, as detailed in Materials and Methods (Fig. 1A and B). Because exon 2 contains the full-length coding sequence of murine TREX2 (31), removal of this sequence completely abrogates TREX2 expression. As described below, we did find that TREX2 is predominantly expressed in the skin. Analysis of TREX2 mRNA (Fig. 1C) and protein expression (Fig. 1D) showed the presence of TREX2 expression in skin from wild-type (wt; Trex2^+/+^) and conditional gene–targeted mice (Trex2^lox/lox^), and its absence in gene-targeted mice (Trex2^−/−^), confirming the null mutation.

**Tissue- and cell-specific expression of TREX2.** TREX2 mRNA transcripts had been previously detected in various human tissues, suggesting ubiquitous expression (20, 31). However, quantitative expression was not assessed in those studies. Although the human and mouse TREX2 gene structure is highly homologue, mouse gene

|NOTE: Number of mice with the indicated tumors followed by the total number of mice (the corresponding percentage is given in parentheses). Some mice bore more than one tumor. Percentage of wt and Trex2^−/−^ mice bearing at least one tumor was 42% and 63%, respectively. There were no significant differences (Fisher's exact test) in tumor incidence between wt and Trex2^−/−^ mice. |  |

<p>| Table 1. Incidence of common spontaneous tumors found in Trex2^−/−^ versus wt mice |</p>
<table>
<thead>
<tr>
<th>Tumor</th>
<th>M</th>
<th>F</th>
<th>Wt</th>
<th>M &amp; F</th>
<th>Trex2^−/−^</th>
<th>M</th>
<th>F</th>
<th>M &amp; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchioloalveolar adenoma</td>
<td>7/22 (32%)</td>
<td>2/16 (12.5%)</td>
<td>9/38 (24%)</td>
<td>8/22 (36%)</td>
<td>2/14 (14%)</td>
<td>10/36 (28%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchioloalveolar carcinoma</td>
<td>2/22 (9%)</td>
<td>0/16 (0%)</td>
<td>2/38 (5%)</td>
<td>4/22 (18%)</td>
<td>0/14 (0%)</td>
<td>4/36 (11%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepato cellular adenoma</td>
<td>3/22 (14%)</td>
<td>0/16 (0%)</td>
<td>3/38 (8%)</td>
<td>1/22 (5%)</td>
<td>0/14 (0%)</td>
<td>1/36 (3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hapatic hemangioma</td>
<td>2/22 (9%)</td>
<td>2/16 (12.5%)</td>
<td>4/38 (10.5%)</td>
<td>2/22 (9%)</td>
<td>7/14 (50%)</td>
<td>9/36 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splenic lymphoma</td>
<td>0/22 (0%)</td>
<td>3/16 (19%)</td>
<td>3/38 (8%)</td>
<td>2/22 (9%)</td>
<td>4/14 (28%)</td>
<td>6/36 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric node lymphoma</td>
<td>0/22 (0%)</td>
<td>2/16 (12.5%)</td>
<td>2/38 (5%)</td>
<td>1/22 (4.5%)</td>
<td>4/14 (28%)</td>
<td>5/36 (14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymic lymphoma</td>
<td>0/22 (0%)</td>
<td>4/16 (25%)</td>
<td>4/38 (10.5%)</td>
<td>4/22 (18%)</td>
<td>7/14 (50%)</td>
<td>11/36 (30%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any lymphoma</td>
<td>0/22 (0%)</td>
<td>6/16 (37.5%)</td>
<td>6/38 (16%)</td>
<td>4/22 (18%)</td>
<td>7/14 (50%)</td>
<td>11/36 (30%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
codes for a single 26-kDa isoform, whereas human gene can gives rise to two additional 30-kDa isoforms that contain an extra NH2-terminal region. The different human isoforms arise from mRNAs generated by alternative splicing, and by the use of alternative promoters. Analogous transcripts in human and mice code for the 236 amino acid proteins (26 kDa; short isoform). Other two human transcripts code for an extra 43 or 42 amino acids upstream the NH2 terminus of the 26 kDa isoform, and are translated into a protein of 279 and 278 amino acids, respectively (30 kDa; long isoform). Interestingly, our results from the quantitative RT-PCR analysis reveal a specific pattern of expression of TREX2 in both mice and human tissues. As seen in Fig. 2A, the unique mouse TREX2 mRNA transcript is highly expressed in the skin, tongue, esophagus, and forestomach. Compared with skin, 10-fold lower expression was detected in the bladder, prostate, and thyroid, and a 100-fold lower levels were found in the trachea, lung, glandular stomach, small intestine, pancreas, and uterus. Expression was more than a 1,000-fold lower or almost undetectable in the spleen, thymus, ganglia, kidney, colon, rectum, ovary, testis, seminal vesicle, adrenal gland, salivary gland, brain, liver, skeletal muscle, or adipose tissue. Similarly, in human tissues, we observed major expression in skin; moderate expression in the tongue and cervix, low expression in the kidney, spleen, stomach, and thyroid; and it was undetectable in liver. An analogous pattern of expression was observed for the two main human TREX2 transcripts, the predominant long isoform and the less abundant short isoform. Next, to evaluate protein expression, we performed immunoprecipitation followed by Western blot analyses. As shown in Fig. 2B, TREX2 protein is observed only in certain mouse tissues, such as the skin, tongue, esophagus, forestomach, or bladder, which express relatively high levels of mRNA for this gene. In these mouse tissues, we only detected a single TREX2 polypeptide with an apparent molecular weight of ~28 kDa, indicating the expression of a single protein isoform in mice. This protein corresponds to the 236 amino acid isoform, as confirmed by heterologous expression in fibroblasts of the mouse Trex2 cDNA coding for the 236 amino acid protein, which gives rise to an identical 28 kDa protein (data not shown). Collectively, mRNA and protein data indicate that TREX2 shows a tissue-specific expression.

To identify cell types expressing TREX2, we performed immunohistochemistry assays of major tissues expressing TREX2, such as the skin and esophagus (Fig. 2C). As a negative control for staining, we used corresponding knockout samples. We observed that TREX2 expression was confined to cells of the stratified squamous epithelia of these tissues. Because keratinocytes are the most abundant cells that integrate the stratified squamous epithelia, we next checked for TREX2 expression in primary keratinocyte cultures. Immunocytochemistry analyses illustrate that TREX2 is expressed in keratinocytes and localized mainly in the nucleus, showing a punctate distribution with many foci per cell (Fig. 2C). Quantitative mRNA analysis further confirmed that TREX2 is preferentially and highly expressed in keratinocytes. Thus, mouse TREX2 mRNA transcript levels were almost 105 times higher in keratinocytes compared with MEFs and ES cells, whereas the transcript was undetectable in macrophages.

Figure 4. DMBA-induced skin carcinogenesis in wt and Trex2−/− mice. Mice were untreated or treated with DMBA, or DMBA+TPA, as described in the Materials and Methods. A, average number of tumors per mouse in the skin of wt (n = 15) and Trex2−/− (n = 18) mice treated with DMBA. B, size distribution of skin tumors present in DMBA-treated wt and Trex2−/− mice at week 14. C, average number of tumors per mouse in the skin of wt (n = 25) and Trex2−/− (n = 25) mice following DMBA+TPA two-stage chemical carcinogenesis. D, tumor size distribution of skin tumors present in DMBA+TPA-treated wt and Trex2−/− mice at week 12. Tumor multiplicity data were statistically analyzed using the Mann-Whitney test (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Finally, to evaluate the contribution of TREX2 activity to the overall 3′-5′ exonuclease activity, we compared this activity in several tissues from Trex2−/− and Trex2+/+ mice (Fig. 2D). Consistent with the expression data, TREX2 does not contribute to the 3′-exonuclease activity of the dermis or liver, but does account for 50% of this activity in keratinocytes and the epidermis.

Altogether, the expression and activity data indicate that TREX2 is predominantly expressed in keratinocytes and, consequently, in tissues with stratified squamous epithelia.

Trex2 loss does not lead to tumorigenic phenotype or reduced survival. The TREX2 knockout mice were viable, born at the expected Mendelian frequency, fertile, and indistinguishable by average weight and growth from their wt littermates (Fig. 3A). Mice lacking TREX2 seemed healthy and no apparent anatomic or gross behavioral abnormalities were observed. No differences in life span (Fig. 3B) or spontaneous tumor development (Table 1) were seen between the two genotypes for up to 2 years. Compared with Trex2+/− mice, we did observe a slight increase in the percentage of Trex2−/− mice that developed splenic lymphomas; however, the differences were not statistically significant. Although TREX2 is highly expressed in the skin, its suppression does not lead to abnormalities in the skin or an increase in related diseases such as skin hyperpigmentation, alopecia, dryness and dermatitis, or spontaneous skin tumors. Both Trex2−/− and Trex2+/+ mice developed most common tumors in agreement with published rates in aging male and female C57BL/6/129 mice (32). Collectively, these results indicate that the loss of the Trex2 gene in mice does not compromise survival or trigger a clearly cancer-prone phenotype.

Chromosomal stability, proliferation, and immunologic development are not altered in cells from Trex2−/− mice. Another group has previously reported the reduced proliferation and presence of Robertsonian translocations, duplications, and deletions in two clones of Trex2-deficient ES cells (27). Surprisingly, we did not detect any chromosomal abnormalities in more than one hundred metaphases analyzed by SKY from MEFs and keratinocytes isolated from at least four knockout and wt Trex2 mice (Supplementary Fig. S1A). Furthermore, we did not find differences in the proliferation or cell cycle phases of several cell types, such as keratinocytes (Supplementary Fig. S1B and D) and MEFs (from primary passage 2 (P2) to 6 (P6); Supplementary Fig. S1C and D), obtained from Trex2−/− mice compared with Trex2+/+ cells.

Because defects in many genes involved in DNA repair and genome stability trigger impaired lymphocyte development (33), we analyzed B- and T-cell populations in the thymus, bone marrow, spleen, and blood from Trex2−/− mice and their wt littermates. Populations of lymphoid T cells bearing CD4, CD8, and T-cell receptor αβ (Fig. 3C) or lymphoid B cells expressing B220, CD19, or IgM (Fig. 3D) were not affected by loss of TREX2, revealing no defects in B- and T-cell development. Together, these findings indicate that TREX2 is dispensable for chromosomal stability and efficient cellular proliferation of several cell types.

Increased susceptibility to DMBA-induced skin tumorigenesis in Trex2−/− mice. To further characterize TREX2 biological function, we tested whether TREX2 could play a relevant role in the DNA damage response to genotoxic stress. Specific expression of TREX2 in skin prompted us to examine DNA damage–induced skin...
Trex2 DNA fragmentation, were observed in DMBA-induced apoptosis, as measured by quantifying apoptotic Trex2 of induced by DNA damage, rather than in the promotion stage. Interestingly, the number of tumors was markedly increased in Trex2−/− mice compared with Trex2+/+ mice in both models (Fig. 4). As expected, in the DMBA model, the period of latency was longer and tumor multiplicity was lower than in the DMBA+TPA model. Tumor size, which can be correlated with tumor growth rate, was similar between the two genotypes in the DMBA+TPA group (Fig. 4D), and was slightly larger in Trex2−/− mice compared with Trex2+/+ in the DMBA group (Fig. 4B). Furthermore, in the DMBA-treated group, the average number of tumor-bearing mice was higher in the Trex2−/− mice than in the Trex2+/+ mice. By 7 weeks, ∼39% of the null mice had developed first tumors versus 6% of wt mice. By 9 weeks, 88% of null mice versus 60% of wt mice had developed tumors, and by 12 weeks, all mice had developed tumors. Therefore, Trex2−/− mice show an increased tumor susceptibility to DMBA-induced skin carcinogenesis compared with wt mice. This phenotype is independent of TPA-induced proliferation and tumor promotion, which suggests a suppressive role for TREX2 in the initiation phase of the tumors induced by DNA damage, rather than in the promotion stage.

**Reduced apoptosis in DMBA-treated skin and keratinocytes of Trex2−/− mice.** To determine the mechanisms by which the lack of TREX2 in the skin increases tumor development in response to genotoxic stress, we analyzed whether apoptosis was altered in the untreated, DMBA−, and DMBA+TPA-treated skin of Trex2−/− mice. Interestingly, an approximately 2- to 3-fold greater number of apoptotic cells was seen in the Trex2+/+ compared with the Trex2−/− epidermis (Fig. 5A and B). Next, we compared DMBA-induced apoptosis in keratinocytes from wt and knockout mice (Fig. 5C). In agreement with in vivo data, reduced levels of DMBA-induced apoptosis, as measured by quantifying apoptotic DNA fragmentation, were observed in Trex2−/− compared with wt keratinocytes. When caspases were inhibited by the pan-caspase inhibitor Z-VDAD-FMK, no differences in DNA damage–induced DNA fragmentation were observed between the two genotypes, indicating that TREX2 effects on apoptosis are dependent on prior caspase activation. Furthermore, data from survival colony assays indicated that Trex2−/− keratinocytes are more resistant than Trex2+/+ keratinocytes to DMBA-induced genotoxicity (Fig. 5D). Thus, increased susceptibility to DMBA-induced skin tumorigenesis in the Trex2−/− mice is associated with reduced carcinogen-induced keratinocyte apoptosis. Reduced apoptosis can contribute to survival of DNA-damaged keratinocytes and, therefore, to skin tumorigenesis.

**Discussion**

The present work shows that TREX2 is highly expressed in keratinocytes, and provides genetic evidence for a proapoptotic tumor suppressor role for this exonuclease in carcinogen-induced skin tumorigenesis. Cell requirements for 3′-5′ exonucleases can vary depending on homeostatic properties and environmental exposure to exogenous stimuli. Specific TREX2 expression in keratinocytes suggests that this exonuclease plays a relevant role in tissues containing these cells. In fact, keratinocytes are the first line of defense against exogenous genotoxic agents, such as radiations, and carcinogens. In this context, inactivation of the 3′-5′ exonucleolytic proofreading activity of Pol6 leads to a high incidence of skin tumors, indicating that epithelial cells are especially susceptible to polymerase error-induced cancers (6). The absence of a cancer-prone phenotype in the Trex2−/− mice indicates that the proofreading activity of TREX2 is not essential to maintain DNA replication fidelity or, alternatively, could be due to compensation by other proofreading exo nucleases. Interestingly, the increased susceptibility of the Trex2−/− mice to DNA damage–induced skin tumors suggests a relevant role of TREX2 under conditions of stress. In this regard, the Trex2 gene is similar to a few other tumor suppressor genes involved in genome maintenance, DNA repair, and apoptosis pathways, such as XPA, (34), XPC (35), CHK2 (36, 37), or caspase-activated DNase (38). Unlike p53 knockout mice, mice deficient in these genes do not develop tumors spontaneously or are not tumor-prone at early ages. However, these animals do show increased tumorigenic susceptibility when exposed to carcinogens.

Previous studies in ES cells lacking Trex2 have shown that TREX2 is required for efficient proliferation and chromosomal stability (20, 27). Severe chromosomal instability is usually associated with lethality, growth retardation, premature aging, immunologic abnormalities, or cancer (33). However, we have not observed defects in cell proliferation or chromosomal abnormalities in cells from Trex2−/− mice. Also, Trex2−/− mice did not exhibit reduced survival, growth retardation, impaired lymphocyte development, or a cancer-prone phenotype. Furthermore, the fact that Trex2-null mice showed increased susceptibility to carcinogen-induced skin tumors and deficient apoptosis would be consistent with a lack of chromosomal instability in this mouse. Thus, the DNA-PK knockout mouse, which is predisposed to chromosomal instability, shows a decreased susceptibility to DMBA-induced tumorigenesis due to the preexisting DNA damage in these cells (39). The chromosomal instability observed in the two TREX2-deficient ES clones (27) could be a consequence of replication stress generated during ES growth and selection. Therefore, our data indicate that TREX2 suppression does not lead to a general chromosomal instability, at least in the absence of genotoxic stress.

The increased susceptibility to DMBA-induced skin carcinogenesis in the Trex2−/− mice could be a consequence of deficient DNA editing or 3′ end processing during nucleotide excision repair of DNA lesions generated by these genotoxins agents. However, unlike nucleotide excision repair–deficient cells (34, 35, 40), TREX2 deficiency in keratinocytes triggers a decrease in DNA damage–induced apoptosis and an increase in survival. Therefore, a putative role for TREX2 in nucleotide excision repair would probably not be the main mechanism underlying TREX2 function in carcinogen-induced skin tumorigenesis. Our data support a proapoptotic role for TREX2 in which it facilitates the removal of keratinocytes that have suffered DNA damage. Apart from p53 (5, 41), other 3′-5′ exonucleases, such as WRN (42), NM23-H1, and TREX1 (21), can display a proapoptotic role through multiple mechanisms. In this regard, TREX1 and NM23-H1 have been implicated in DNA degradation during cell death mediated by caspase-independent granzyme A in cells targeted by cytotoxic T cells (21). Similarly, TREX2 could facilitate caspase-dependent DNA degradation once 3′-hydroxyl DNA breaks are available, causing irreparable damage. In the absence of TREX2, a fraction of cells carrying mutagenic DNA damage could indeed survive. Analogous to TREX2-null mice, caspase-activated DNase knockout mice, in which apoptotic DNA fragmentation is disrupted, do not show an increase in spontaneous tumors, but are more susceptible to induced carcinogenesis (38).
Skin is exposed to diverse and multiple exogenous genotoxic agents, and a large number of genes are required to maintain genome stability (43). Interestingly, TREX2 expression is largely reduced in the mice lacking IKKα, which is required to maintain skin homeostasis and prevent skin cancer (44). Here, we found that loss of TREX2 does not lead to a tumor-prone phenotype, but rather increases susceptibility to induced skin carcinogenesis. Thus, TREX2 can serve as a proapoptotic tumor suppressor that may contribute to genome maintenance in tissues with stratified squamous epithelia under conditions of genotoxic stress. Further work to search for TREX2 expression and mutations in squamous cell carcinomas is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments


Grant support: Spanish Ministerio de Ciencia e Innovación grants BFU2006-06076 and CSD2006-00005 (C. Soler), and BIO2006-32133 (S. Ortega), the Fondo de Investigaciones Sanitarias grant PR21192 (C. Soler), and the University of Barcelona (C. Soler).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. D.J. Rossi (University of Helsinki) for providing the pDELBOY-3X vector, Dr. J.C. Gipouso and the Cytogetic Unit of the CNIO for SKY analyses, and C. Gómez and M. Riffo for ES clone aggregation and chimera crosses.

References

10. Chordury D, Berns DE. The exonuclease TREX1 is in the SET complex and acts in concert with NME3-1 to degrade DNA during granulocyte A-meditated cell death. Mol Cell 2006;23:133–42.
Increased Susceptibility to Skin Carcinogenesis in TREX2 Knockout Mice

David Parra, Joan Manils, Bàrbara Castellana, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-1208

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2009/07/30/0008-5472.CAN-09-1208.DC1

Cited articles
This article cites 43 articles, 23 of which you can access for free at: http://cancerres.aacrjournals.org/content/69/16/6676.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/69/16/6676.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.