Supplemental Information

Safety of Whole-Body Abrogation of the TRF1 Shelterin Protein in Wild-Type and Cancer-Prone Mouse Models

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Supplementary Figure 2

The graph shows the intestine telomere fluorescence (A.U.F.) for different genotypes: Ink4Arf−/−, p53−/−, and WT. The bars represent the mean ± standard error of the mean (n=4-6 per group). The following comparisons are indicated:

- *p < 0.05
- ns = no significant difference

The genotypes are distinguished by the following symbols:

- Black bars: Trf1+/+
- Gray bars: Trf1lox/lox

The number of observations (n) for each group is indicated above each bar.
Supplementary Figure 3

A

- **Trf1^+/+**
- **Trf1^lox/lox**

**Ink4Arf^−/−**, **p53^−/−**, **WT**

% of brain Sox2 positive cells

- **ns**
- **n=3**
- **n=4**
- **n=4**
Supplementary Figure 4

A

Lymphoma  HS  Sarcoma

Trf1<sup>++</sup>  

Trf1<sup>lox/lox</sup>  

Not found

B

Lymphoma (thymus)  Sarcoma  Skin carcinoma

Trf1<sup>++</sup>  

Trf1<sup>lox/lox</sup>  

Not found
Supplementary figure legends

Supplementary Figure 1. PCR analysis of Trf1 deletion upon tamoxifen treatment. Related to Figure 1. (A) Analysis of Trf1 excision by PCR in Ink4Arf-deficient mice. (B) Analysis of Trf1 excision by PCR in p53-deficient mice. (C) Analysis of Trf1 excision by PCR in wild-type background.

Supplementary Figure 2. Telomere length analysis upon Trf1 deletion in the different genetic backgrounds. Related to Figure 1. (A) Telomere Q-FISH analysis in the intestine of the indicated genotypes. Data are represented as mean ± SEM. n represents number of mice. Statistical analysis: unpaired t-test.

Supplementary Figure 3. Trf1 deletion does not decrease brain stem cell markers. Related to Figure 6. (A) Representative images (up) and quantification (down) of Sox2-positive cells in the brain after Trf1 deletion in the indicated backgrounds. Scale bar 20 μM. Data are represented as mean ± SEM. n represents number of mice. Statistical analysis: unpaired t-test.

Supplementary Figure 4. Tumor histology in InkArf-deficient and p53-deficient mice. Related to Figure 7. (A) Representative images of the different tumor types by H&E at the human end-point. Scale bar 100 μM. (B) Representative images of the different tumor types by H&E at the human end-point. Scale bar 20 μM.
Transparent Methods

Mice

Trf1\textsuperscript{lox/lox} mice (Martínez et al., 2009) were crossed with a mouse strain carrying ubiquitously expressed, tamoxifen-activated recombinase, hUBC-CreERT2 (Ruzankina et al., 2007) to generate Trf1\textsuperscript{lox/lox} or Trf1\textsuperscript{+/+}, hUBC-CreERT2 mice. These mice were further crossed with Ink4Arf\textsuperscript{-/-} (Serrano et al., 1996) and p53\textsuperscript{-/-} (Jacks et al., 1994) mouse lines to generate Trf1\textsuperscript{+/+} or Trf1\textsuperscript{lox/lox}, hUBC-CreERT2, Ink4Arf\textsuperscript{-/-} and Trf1\textsuperscript{+/+} or Trf1\textsuperscript{lox/lox}, hUBC-CreERT2, p53\textsuperscript{-/-} mice. These mice were fed ad libitum with tamoxifen containing diet for long-term, starting at 6 weeks of age for p53-deficient background and 10-weeks of age for Ink4Arf-deficient and wild type backgrounds. p53-deficient mice started tamoxifen treatment earlier due to the shorter lifespan of this mice. All mice were maintained at the Spanish National Cancer Centre under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). All animal experiments were approved by the Ethical Committee (CEIyBA) from the Spanish National Cancer Centre and performed in accordance with the guidelines stated in the International Guiding Principles for Biomedical Research Involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS).

Immunofluorescence analyses in tissue sections

For immunofluorescence analyses, tissues were fixed in 10% buffered formalin (Sigma) and embedded in paraffin. After desparaffination and citrate antigen retrieval,
sections were permeabilized with 0.5% Triton in PBS and blocked with 1%BSA and 10% Australian FBS (GENYCELL) in PBS.

Rat polyclonal anti-TRF1 antibody (homemade) was applied overnight in antibody diluents with background reducing agents (Invitrogen). Anti-rat Alexa 555 secondary antibody (Life Technologies, S.A) was incubated 1 hr at room temperature also in antibody diluents with background reducing agents (Invitrogen).

Immunofluorescence images were obtained using a confocal ultraspectral microscope (Leica TCS-SP5). Quantifications were performed with Definiens software.

**Immunohistochemistry analyses in tissue sections**

For immunohistochemistry analyses, tissues were fixed in 10% buffered formalin (Sigma) and embedded in paraffin. Immunohistochemistry was performed on de-paraffinated tissue sections processed with 10 mM sodium citrate (pH 6.5) cooked under pressure for 2 min. Slides were washed in water, then in Buffer TBS Tween20 0.5 %, blocked with peroxidase, washed with TBS Tween20 0.5 % again and blocked with fetal bovine serum followed by another wash.

Primary antibodies included those raised against: γH2AX Ser 139 (Millipore), Ki67 (Master diagnostica), Sox2 (C70B1, Cell signaling) and Nestin (RAT-401, Millipore). Slides were then incubated with secondary antibodies conjugated with peroxidase from DAKO.

Pictures were taken using Olympus AX70 microscope. The percentage of positive cells was identified by eye.

**Histological analyses in tissue sections**
Tissue samples were fixed overnight in 10% neutral buffered formalin, embedded in paraffin and sectioned 3 µm thick and dried. Slides were dewaxed and re-hydrated through a series of graded ethanol until water and were stained with hematoxilin-eosin (H-E). Histological observations and photomicrography were performed using an Olympus DP73 digital camera. Histopathologies were classified into “non-tumoral” lesions, “preneoplastic” lesions and tumor lesions.

“Non-tumoral” lesions included degenerative lesions (atrophy in both epidermis and hypodermis, dermal fibrosis, follicular atrophy), inflammatory lesions (dermatitis, hepatitis) and proliferative lesion (benign hyperplasias, hyperkeratosis).

“Pre-neoplastic” lesions were mainly proliferative and included epithelial dysplasia, malignant hyperplasia, nuclear atypia and cell depolarization.

Tumor lesions were characterized by cellular polymorphism, irregular stratification, both loss of polarity or basement membrane disruption of epithelial cells, nuclear hyperchromatism, nuclear atypia, enlarged nucleoli, increase number of mitotic figures and atypical mitotic figures. The most frequent tumors were lymphomas (in thymus or extending and infiltrating different organs, mainly the spleen, liver, and lymph nodes), histiocytic sarcomas (in liver, spleen and mesenteric lymph nodes), subcutaneous fibrosarcomas and angiosarcomas, vertebral osteosarcomas and squamous cell carcinomas (SCC) in skin.

**Telomere length analyses on tissue sections**

For quantitative telomere fluorescence *in situ* hybridization (Q-FISH) we deparaffinized paraffin-embedded sections and fixed them with 4% formaldehyde, followed by digestion with pepsine/HCl and a second fixation with 4% formaldehyde. Next, we dehydrated the sides with increasing concentrations of EtOH (70%, 90%,
100%) and incubated them with the telomeric probe for 3.5 min at 85ºC followed by
2h RT incubation in a wet chamber. After, the slides were extensively washed with
50% formamide and 0.08% TBS-Tween. Immunofluorescence images were obtained
using a confocal ultraspectral microscope (Leica TCS-SP5) and the analysis was
performed by Definiens software.

**Real-time qPCR**

Total RNA from frozen tissue was extracted with the RNeasy kit (QIAGEN)
following manufacturer’s instructions. The cDNA synthesis was performed using the
iSCRIPT cDNA synthesis kit (BIO-RAD) according to manufacturer’s protocols.
Quantitative real-time PCR was performed with the QuantStudio 6 Flex (Applied
Biosystems, Life Technologies) using Go-Taq qPCR master mix (Promega). All values
were obtained in triplicates.

Primers for mouse samples are listed below:

- TRF1-F 5′-GTCTCTGTGCGAGCCTTC-3′
- TRF1-R 5′-TCAATTGGTAAGCTGTAAGTCTGTG-3′
- TBP1-F 5′-ACCCTTCACCAATGACTCCTATG-3′
- TBP1-R 5′-TGACTGCAGCAATCGCTTG-3′

**PCR**

DNA from frozen tissue samples was extracted using
Phenol:Chloroform:Isoamyl:Alcohol (Sigma). Cre-mediated recombination by PCR
was determined using the following primers:

- F: 5′-ATAGTGATCAAATGTGGTCCTGG-3′
- R: 5′-GCTTGCCAATTTGGTTGG-3′
Quantification and statistical analysis

Survival data were analyzed by Kaplan Meier survival curves, and comparisons were performed by Log Rank test. Statistical analysis was performed using GraphPad Prism 5.03. Comparison of the percentage of mice in Figures 3, 4, 5, 7 and 8 was performed by Chi-Square test.

Immunofluorescence quantifications were performed with Definiens software and immunohistochemistry quantifications were performed by direct cell counting. Unpaired Student's t test was used to determine statistical significance. P values of less than 0.05 were considered significant. Statistical analysis was performed using Microsoft® Excel 2011.


References

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