Supporting Information

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SI Methods

Mice. Mice were housed at the Johns Hopkins University School of Medicine campus, and procedures were approved by its Institutional Animal Care and Use Committee. Mice with *Terf2*$^{Fl/Fl}$ (1), *Trp53*$^{-/-}$ (2), *Trp53*$^{Fl/Fl}$ (3), and *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$ (4) genotypes (referred to as "*Trf2*$^{Fl/Fl}$," "*p53*$^{-/-}$," "*p53*$^{Fl/Fl}$," and "Rosa-*mTmG*," respectively) were purchased from the Jackson Laboratory. *mTR*$^{-/-}$, *Sftpc-Cre*, and *Sftpc-CreER* mice were generated as previously described (5–7). All mouse strains were on the C57BL/6J background except those involving the Rosa-mTmG reporter line, which was on a mixed B6/129 background.

Tamoxifen (5 mg) (Sigma) was administered intraperitoneally (i.p.) on days 1 and 3. In one experiment, tamoxifen was mixed with the diet for 21 d (TD.130856; Harlan Laboratories). For experiments involving *Sftpc-CreER* mice, sex-matched 8- to 12-wk-old mice were studied. Otherwise, the ages of mice are noted in figure legends. Proliferation studies in adult mice were carried out by implanting a 14-d miniosmotic pump (Alzet) containing 10 mg/mL EdU, a nucleotide analog, dissolved in sterile saline as described (8). For 72-h experiments, 100 μg EdU was delivered i.p. daily.

AEC2 Cell Isolation and Alveolosphere Assay. Single-cell suspensions of minced lungs were prepared as described with a few modifications (9). Briefly, mice were euthanized by isoflurane inhalation or Euthasol (pentobarbital sodium and phenytoin sodium; Virbac) followed by transection of the inferior vena cava. The lungs were perfused with 5 mL PBS. Lungs were inflated with 1 mL digest solution containing 450 U/mL collagenase type I (Gibco), 4 U/mL elastase (Worthington Biochemical Corporation,), 5 U/mL dispase (BD Biosciences), and 0.33 U/mL DNaseI (Roche) in DMEM/F12 or PBS (Gibco). After inflation, the trachea was ligated, and the lungs were removed and placed in 3 mL of digest solution. Lung tissue was minced and incubated at 37 °C for 30 min with frequent agitation. The cell suspension was passed through a 70-μM filter, red cell lysed, and suspended in DMEM/F12 with 2% FBS. Cells were sorted on a Becton Dickinson FACSAria IIu or FACS Vantage SE. Analyses were performed using FlowJo software (TreeStar Inc.).

For alveolosphere assays using non–lineage-labeled AEC2s, we stained single-cell suspensions and gated based on the following criteria: positive for EpCAM and negative for CD31/CD45/podoplanin/Sca1/CD24/PI. For isolation of non–lineage-labeled stromal cells, we gated positive for Pdgfrα and negative for EpCAM/CD31/CD45. For antibody staining, cells were placed in staining solution (1× PBS with 2% BSA and 2% FBS). They were blocked in staining solution with TruStain fcX (BioLegend). Antibody were obtained as follows: EpCAM PE-Cy7, CD31 biotin, and CD45 biotin from eBioscience. PE/Cy5 streptavidin, podoplanin PE, Sca-1 488, CD24 BV421, and PDGFRα/CD140a APC were purchased from BioLegend.

Alveolosphere assays were performed as described (2). Briefly, AEC2 cells were plated in MTEC/Plus and were mixed with growth factor-reduced Matrigel (BD Biosciences). Then 5×10^3 AEC2s and 1×10^5 PDGFRα-GFP^{hi} cells were plated into a 24-well Transwell insert (Falcon), and 500 μL MTEC/Plus was placed in the lower chamber. PDGFRα-GFP^{hi} cells (1×10^5) were plated alone to measure contaminating epithelial cells from stromal donors. Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor (10 μM; Y0503; Sigma-Aldrich) was included in the medium for the first 2 d of culture, which was at 37 °C in 5% CO_2/air (2). For alveolosphere assays of lineage-labeled cells, GFP$^+$ cells were flow sorted and mixed with lineage-labeled stromal cells as described (2).

For immunohistochemistry of alveolosphere colonies, the Matrigel disk was removed, dehydrated through ethanol, and transitioned into paraffin as described (10). All quantification is based on triplicate values for each mouse at each time point. All alveolosphere assays were harvested and analyzed blinded to mouse genotypes.

Immunohistochemistry and Immunofluorescence. Tissue sections were harvested from adult lungs and prepared using the detailed protocols previously outlined (8). Sorted cells were prepared for immunofluorescence using a Cytospin 3 centrifuge (Thermo Scientific) and were fixed in fresh 4% paraformaldehyde (PFA) for 10 min followed by permeabilization in 0.3% Triton X-100. Antibodies were obtained from the following sources: E-cadherin, γH2AX, p53^{Ser15}, and cleaved caspase-3 from Cell Signaling; pro-Spc from Chemicon; E-cadherin for whole-mount studies from Life Technologies; 53BP1 from Novus Biologicals; Club cell antigen from Santa Cruz Biotechnologies; CD3 from Abcam; GFP from Aves Labs; Mac-3 and p21 from BD Biosciences; and podoplanin (hamster) from Developmental Studies Hybridoma Bank (Clone 8.1.1). The ABCA3 antibody (guinea pig) was generously provided by Jeffrey Whitsett (University of Cincinnati, Cincinnati). Secondary antibodies conjugated to Alexa-488, Alexa-594, or Alexa-647 were purchased from Life Technologies. EdU staining was performed using a Click-iT imaging kit (Life Technologies). Telomere length on AEC2s was measured as previously described (8). Images were analyzed using the Telometer: Software for Telomere Counting as described previously at demarzolab.pathology.jhmi.edu/telometer/. Telomere dysfunction was assayed using 53BP1 immunostaining combined with telomere FISH as described (11). Images were obtained on a Zeiss Axioskop. For telomere dysfunction studies, a DeltaVision Elite system (GE Healthcare) controlling a Scientific CMOS camera (pco.edge 5.5) was used to analyze images at 0.2-μm z-sections which subsequently were deconvolved. Immunohistochemistry was performed using a Vectastain Elite ABC kit (Vector Laboratories). All microscopy imaging and analysis was performed blinded to genotype.

Pulmonary Function, Histology, and Bronchoalveolar Lavage. Pulmonary function and mean linear intercept studies were performed as previously described (8). Qualitative pathological examination was performed after slides were blinded, and inflammation severity was scored on a scale of 0 to 4 with 0 representing no inflammation and 4 representing very severe inflammation. Bronchoalveolar lavage fluid was collected by lavaging the lung twice with 0.75 mL of PBS. Samples were centrifuged to recover cells, pellets were resuspended in PBS-2%FBS, and viable cells were counted using a hemocytometer. Differential counts were performed on Cytospin preparations stained with a Hema 3 stain (Thermo Fisher). Histopathologic analysis of lung sections was performed by two of the authors (J.K.A. and R.M.T.) including a board-certified pathologist (R.M.T.). Parenchymal macrophages were quantified as described previously (8). To examine for fibrosis, we performed Masson trichrome staining and measured collagen mRNA levels on whole-lung lysates. To measure the extent of epithelial branching, we captured calibrated images and measured the number of independent epithelial branches identified by E-cadherin immunohistochemistry per lung area.

qRT-PCR. RNA was extracted from tissue in TRIzol (Life Technologies) followed by column purification using an RNeasy kit (Qiagen). qRT-PCR primer sequences for p21, p27, Hprt, and β2m were published previously (8). Additional primers were used as follows: Bax-forward: GCTGATGGCAACTTCAACTG, Bax-reverse: CACAAAGATGGTCACTGTCTG ; Ccng1-forward: CTCAGTTCTTTGGCTTTGACACG, Ccng1-reverse: ACATTCCTTTCCTCTTCAGTCGC; Col1a1-forward: GCCAAGAAGACATCCCTGAAG; Col1a1-reverse: TGTGGCAGATACAGATCAAGC; Col1a2-forward: CACCCCAGCGAAGAACTCATA, Col1a2-reverse: GCCACCATTGATAGTCTCTCC; Mdm2-forward: ATTGCCTGGATCAGGATTCAGTT, Mdm2-reverse: ACCTCATCATCCTCATCTGAGA, Il17c-forward: CTGGAAGCTGACACTCACG, Il17c-reverse: GGTAGCGGTTCTCATCTGTG; Mif-forward: TGCCCAGAACCGCAACTACAGTAA, Mif-reverse: TCGCTACCGGTGGATAAACACAGA.

Microarray Analysis. Transcriptional profiling of purified AEC2 cells was performed using Mouse Exon 1.0 ST Arrays (Affymetrix) at the Johns Hopkins Microarray Facility. Total RNA (10 ng) was amplified using the Nugen Pico WTA System 2.0 (Nugen). Affymetrix CEL file data were extracted and normalized with Partek Genomics Suite software using the Robust Multichip Analysis algorithm. To ensure better coverage of all exons in the annotated transcriptome, all 641,138 extended-class probe sets were extracted (based on the Affymetrix Probeset annotation file) and were summarized into 115,838 transcripts.

ANOVA was run comparing transcript expression levels in control and mutant mice, and the 30,769 transcripts with gene-level annotation were selected for further analysis. The $\log_2$-fold change distribution of these genes then was evaluated to determine their SD up or down from the mean of 0 (i.e., no change). Genes with a change greater than 2 SD, corresponding to a linear fold change of approximately ± 1.37-fold, were considered to have significant differential expression. These 1,713 gene transcripts were uploaded to the Ingenuity Pathway Analysis platform to evaluate their functional relevance in canonical pathways (Table S1). *P* values for pathway studies were calculated using Fisher's exact test. Our minimum information about a microarray experiment (MIAME)-compliant microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (Series GSE56892).

Bleomycin Challenge. Mice were anesthetized with ketamine (90 mg/kg) and xylazine (18 mg/kg) (Sigma). The trachea was exposed, and mice were intubated with an 18-gauge cannula. Bleomycin (1.25 U/kg) was delivered, and mice were ventilated immediately to ensure even distribution. A single lot was used for all experiments. Body weight was collected every other day.

Embryo Studies. Embryos were dissected in PBS and fixed overnight in 4% PFA. For staining requiring frozen sections (lineage trace experiment), tissues were fixed for 3 h in 4% PFA, cryoprotected in sucrose, and embedded in optimum cutting temperature compound. EdU (300 μg) (Invitrogen) was injected i.p. 2 h before embryo harvest. For all timed matings, E0.5 was considered to be noon on the day vaginal plugs were observed. Whole-mount studies were performed as previously described (12). Briefly, lungs were dissected from E14.5 embryos and fixed for 3 h in fresh 4% PFA at 4 °C with gentle rocking. Lungs then were dehydrated by a series of methanol washes and were stored in 100% methanol at −20 °C until staining was performed. To quantify the morphogenesis defect, we imaged and counted the number of independent epithelial branches per embryonic lung area as identified by E-cadherin staining.

Immunoblot. Protein was isolated from homogenized lungs using a 26-gauge needle in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (Mini Complete and PhosSTOP; Roche). Protein was quantified using a bicinchoninic acid assay (Pierce), separated using SDS-gel electrophoresis, and transferred to PVDF membrane under reducing conditions (Invitrogen). Detection was performed using near-infrared fluorescent-conjugated secondary antibodies (LI-COR).

Statistics. We used GraphPad Prism v. 5.00 for Windows. Means were compared using Student's *t* test, and survival was analyzed using the Kaplan–Meier method and the log-rank test. *P* values <0.05 were considered significant, and all values shown are two-sided unless otherwise noted.

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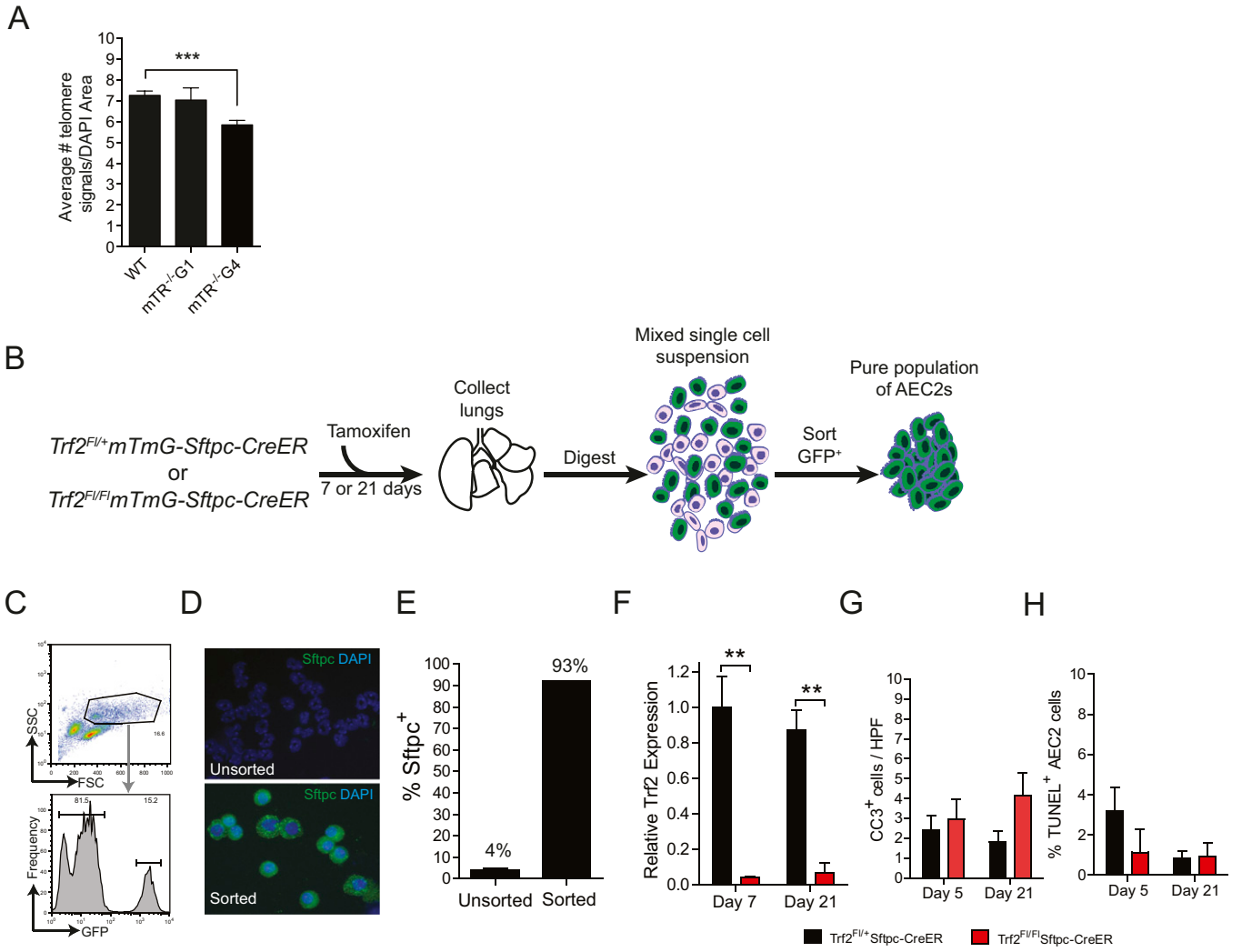

Fig. S1. Characterization of telomere length in AEC2 cells, validation of sorting methods, and apoptosis assays. (*A*) Telomere length in AEC2s from wild-type, $mTR^{-/-}$ G1, and $mTR^{-/-}$ G4 mice by quantitative FISH of $Abca3^{+}$ cells (10–20 nuclei per lung; $n = 6$ mice per group). (*B*) Schema for the experimental design for isolating lineage-labeled AEC2s from mouse lungs. (*C*) Representative histograms from flow plots showing morphology and GFP gates for cell sorting. FSC, forward scatter; SSC, side scatter. (*D*) Cytospin preparation of sorted cells stained with Sftpc antibody (green). Pre- and postsorting images show enrichment for AEC2s. (*E*) Quantification of the purity of the sorted cells in *C*. (*F–H*) Studies were performed on $Trf2^{Fl/+}$;mTmG-Sftpc-CreER (black bars) and $Trf2^{Fl/Fl}$;mTmG-Sftpc-CreER (red bars) mice. (*F*) Trf2 mRNA levels in purified AEC2s on days 7 and 21 after tamoxifen delivery and as measured by qRT-PCR. Levels were normalized to Hprt expression ($n = 3$ mice per group). (*G*) Number of $CC3^{+}$ cells by immunohistochemistry on days 1 and 3 after tamoxifen ($n = 3–5$ mice per group). HPF, high-powered field. (*H*) Frequency of apoptotic AEC2s was measured by the TUNEL assay combined with Sftpc immunofluorescence ($n = 3–5$ mice per group). Data are expressed as mean ± SEM. ns, nonsignificant, $^{**}P < 0.01$, $^{***}P < 0.01$, Student's *t* test.

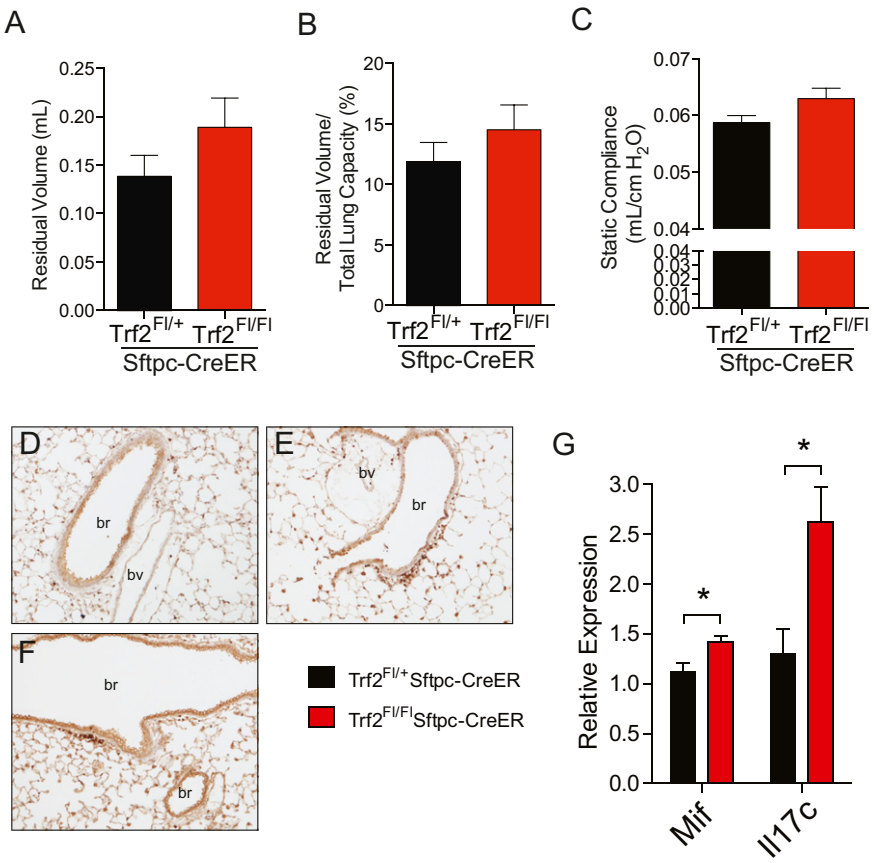

Fig. S2. Telomere dysfunction in AEC2s affects lung volume and mechanics and signals recruitment of inflammatory cells. (*A*–*C*) Residual volume (*A*), the percentage of the residual volume relative to the total lung capacity (*B*) and lung compliance (*C*) were measured on day 21 after tamoxifen. (*D*) CD3 immunohistochemistry in *Trf2*$^{Fl/+}$*;Sftpc-CreER* lungs shows no infiltrates in these controls in contrast to *Trf2*$^{Fl/Fl}$*;Sftpc*, which show patchy areas of T lymphocytes in peribronchiolar and perivascular distributions (*E* and *F*) (day 21 after tamoxifen). br, bronchiole distribution; bv, blood vessel distribution. (*G*) Relative mRNA expression of Il17 and Mif1 by qRT-PCR from sorted AEC2s 7 d after tamoxifen. *$P < 0.05$, Student's *t* test.

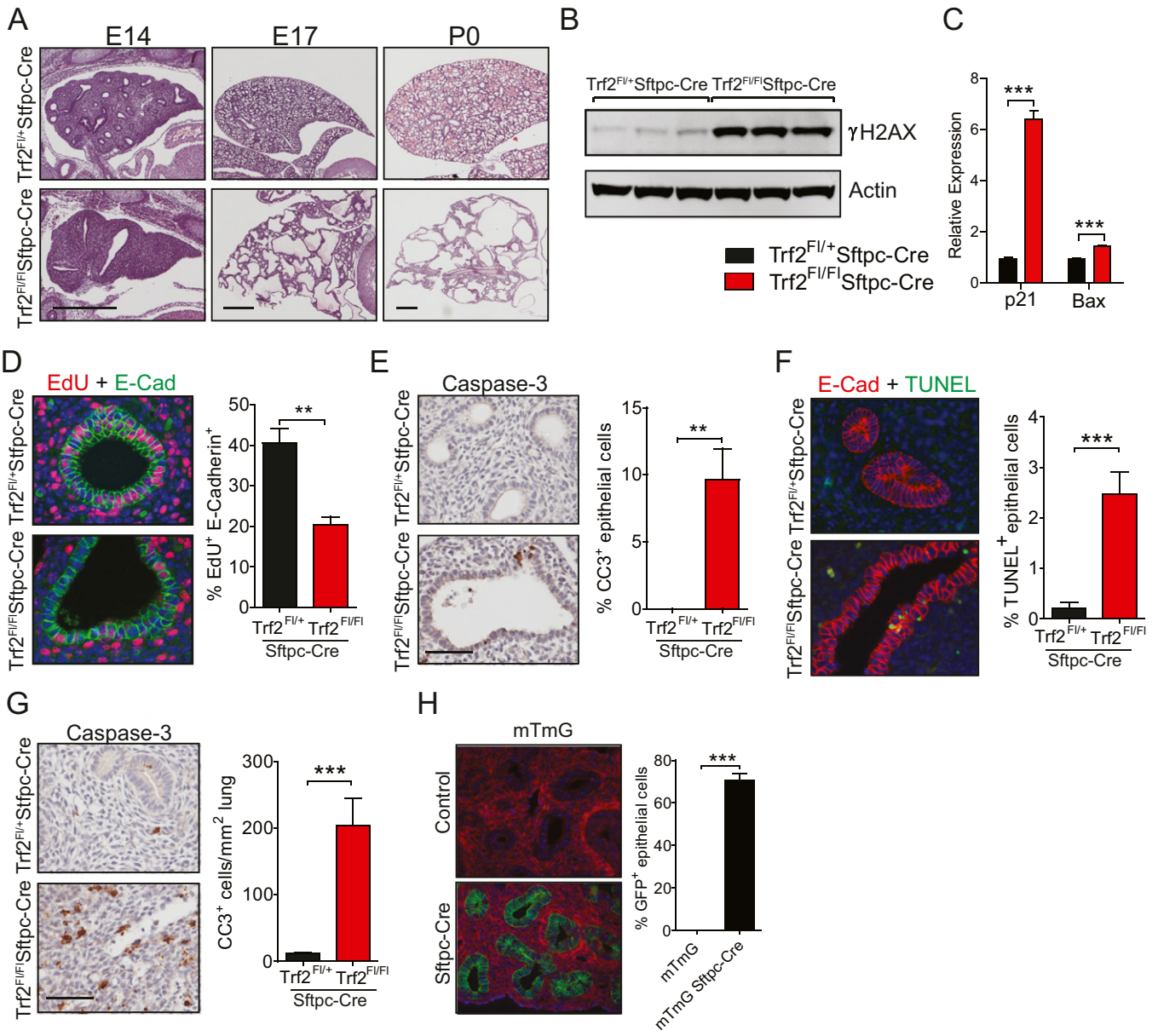

Fig. S3. Trf2 deletion in Sftpc$^+$ cells during lung development induces an epithelial-restricted DNA damage response. (*A*) H&E transverse lung sections from E14, E17, and postnatal day 0 (P0) lungs show that cyanotic newborn pups have a severe morphogenesis defect. (Scale bars: 500 μm.) (*B*) Western blot of whole-lung lysates. (*C*) mRNA levels measured by real-time PCR from whole-lung lysates. (*D*) Proliferation rate is quantified by EdU incorporation (red) in epithelial cells marked by E-cadherin (green) ($n = 5$ per group). (*E*) CC3 immunohistochemical staining on lung sections (*Left*) with quantification (*Right*) ($n = 5$ per group). (*F*) TUNEL staining (green) in epithelial cells (stained with E-cadherin; red) with quantification (*Right*) ($n = 5$ per group). (*G*) CC3 immunohistochemistry of mesenchymal cells (*Left*) and quantification (*Right*) ($n = 5$ per group). (*H*) mTmG-Sftpc-Cre mice were analyzed to test the specificity of Sftpc-Cre transgene expression. The fraction of lineage-labeled epithelial cells is quantified. Studies shown here were performed on E14.5 lungs, except in *A*. Data are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)