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SUPPLEMENTAL METHODS and DATA

Expression Of Mutant *Sftpc* In Murine Alveolar Epithelia Drives Spontaneous Lung Fibrosis

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SUPPLEMENTARY METHODS

Sftpc^{I73T} Targeting Vector and Recombineering strategy for generation of SP-C^{I73T} founder line

The *Sftpc^{I73T}* targeting vector was commercially produced by Gene Bridges, GmbH (Heidelberg, Germany) using a BAC-encoded fragment of interest from a murine C57BL/6 strain. Synthesis of a genomic DNA fragment carrying a hemagglutinin tag (HA) tag in Exon1 (the NH_2 terminus), an Ile for Thr substitution in Exon 3a, and the *FRT-PGK-gb2-Neo/km-FRT* cassette in Intron 1 was performed. *FRT-PGK-gb2-Neo/km-FRT* (herein termed "*PGK-Neo"* cassette) encodes for *gb2* driven kanamycin resistance in *E. coli* and mouse phosphoglucokinase promoter (PGK) driven expression of neomycin resistance in mammalian cells and is flanked by short flippase recognition target (FRT) sites The modified DNA fragment carrying the point mutation was then inserted into the *Sftpc* locus of the corresponding Zeocin/*Sftpc* BAC by one Red/ET step (triple recombination) and the resultant BAC encoded product subcloned into a minimal pMV vector. The functional regions (FRT, point mutation, restriction sites, and resistance cassette) were verified by sequencing.

ES cell electroporation of this targeting vector, clone selection, and microinjection of targeted ES clones into blastocysts of Balb/c mice were performed by University of Pennsylvania Transgenic Core Facility. F_0 offspring with a high degree of coat chimerism were then crossed with C57/Bl6 6J mice and germ line transmission of the recombineered allele screened by color, PCR, and Southern blot hybridization.

PCR based genotyping of mice

DNA was extracted from tail snips obtained from pre-weaned mouse pups and then used as template for detection of the following alleles by polymerase chain reaction (PCR):

Sftpc$^{Wild\text{-}type}$, Sftpc^{I73T}, and SP-C$^{I73T\text{-}Neo}$ - Multiplex PCR was done using a common reverse primer 3' downstream of the PGK-Neo insertion site, with alternate forward priming sites located upstream of or within the PGK-Neo cassette. Amplification was performed using Platinum Taq (Invitrogen), with the following primer sequences:

SP-C Fwd: TCACCCCTGTCCTCTCTGTC
PGK Fwd: TGGATGTGGAATGTGTGCGA
SP-C Rev: CCCAACTACATGGTGGTGCTA

The thermal cycling conditions were: 95°C for 3 minutes; 37 cycles of 95°C (30sec), 64°C (30 sec), 72°C (60 sec). For *Sftpc$^{Wld\text{-}type}$* this results in amplification of a 433 BP product. For *Sftpc^{I73T}* alleles post-excision, 113 base pairs (including the FRT and HA sites) are added, resulting in a 446 bp band. For alleles pre-excision (*SP-C$^{I73T\text{-}Neo}$*), an alternate forward priming site within the PGK promoter permits preferential amplification of a 271 bp band.

R26Flp-O ER - Multiplex PCR was done using a common forward primer and two reverse primers (as described by Jackson Laboratories, Inc.). Amplification was performed using Platinum Taq (Invitrogen), with the following primer sequences:

Common Forward (oIMR8545): AAAGTCGCTCTGAGTTGTTAT
I73T Mutant Reverse (10507): TTATGTAACGCGGAACTCCA
Wild type Reverse (oIMR8546): GGAGCGGGAGAAATGGATATG

The thermal cycling conditions used were: 95°C for 3 minutes; 10 cycles of 95°C (30sec) 65 - 0.5 °C/cycle (30sec), 68°C (60sec); 28 cycles of 95°C(30 sec), 60°C (30sec) 72°C (60 sec). These conditions result in amplification of a 603 bp band (Flp-O negative) and a 309 bp band (Flp-O positive).

Efficiency of Neomycin Cassette Excision

The efficiency of removal of the intronic neomycin cassette from *Sftpc* alleles of I^{ER}-SP-C^{I73T}Flp$^{+/-}$ mice was measured by q-PCR on DNA isolated from AT2 cells obtained 7 days after tamoxifen administration. Primers were designed using Primer Express 3.0.1 software (Applied Biosystems) to amplify an 86 base-pair DNA sequence flanking the first FRT minimal sequence, with a reverse primer located inside the excised portion:

I73TNeo Forward: TCACTATAGGGCTCGAGGAAGTTC
I73TNeo Reverse: TCATGATCTGTGTGTTGGTTTTTG

Relative quantification of intact SP-C$^{I73T\text{-}Neo}$ alleles was performed using a protocol previously described by Mancini et al with fast SYBR Green Master Mix (Applied Biosystems). Rpp30 served as a housekeeping control:

Rpp30 Forward: TCCAGTGTGCAAGAAAGCTAAATG
Rpp30 Reverse: GGCAGTGCGTGGAGACTCA

Standard curves and melting point curves were constructed to validate assay specificity and efficiency.

Measurement of pulmonary function

Invasive measurement of static lung compliance and airways resistance was performed as part of a terminal harvest. Mice were anesthetized with intraperitoneal pentobarbital, tracheas cannulated with a 20-gauge metal stub adapter, and placed on a small-animal ventilator (flexiVent; SCIREQ, Inc. Toronto Canada) at 150 breaths per min and a tidal volume of 10 mL/kg of body weight. Static lung compliance (using a 2-s breath pause) and airway resistance were determined with the manufacturer's software. Arterial hemoglobin oxygen saturations were measured in anesthetized, ventilated mice using a rodent pulse oximeter (PhysioSuite with MouseSTAT Pulse Oximeter (Kent Scientific, Torrington, CT).

MicroCT imaging

Chest CT images of spontaneously breathing mice were acquired in the SARRP core facility in the Department of Radiation Oncology at the Perelman School of Medicine of the University of Pennsylvania. Mice immobilized with 2.5% isoflurane anesthesia with medical air as the carrier gas were placed on a customized platform in the ventral recumbent position in a facemask allowing gas scavenging. The platform was placed on the stage of the SARRP (Xstrahl Life Sciences, Suwanee GA) and a Cone-Beam CT (CBCT) image was initiated with the X-ray tube operating at 65 kV, 0.5 mA with aluminum filtration. The images were reconstructed with Xstrahl's MuriSlice Software. Image stacks (233 total images) were processed using ITK-SNAP software (v 3.4.0)(www.itksnap.org)(1).

Measurement of autophagy flux in vitro

Autophagy flux was measured following established consensus guidelines as we previously published (2;3). Purified AT2 cells were cultured in HITES media with 10% FCS for 48 hours, on 1:10 on Matrigel® (Corning Life Sciences, Corning NY) coated culture wells. Culture media was changed to HITES media without serum and Torin (#4247, Tocris Bioscience, Bio-Techne Corp., Minneapolis, MN), bafilomycin A1 ($^{\#}$B1793 Sigma-Aldrich, St Louis, MO), or DMSO added culture wells for 4 hours. Cells collected in sample-loading buffer were analyzed by western for expression of LC3 and p62/SQSTM1.

In Vitro cell line transfection

For in vitro expression studies, human A549 epithelial cells originally obtained through American Type Culture Collection (ATTC) were grown to 85-90% confluency on glass coverslips and transiently transfected with SFTPC plasmids (2-5 μg / dish) using $CaPO_4$ (4;5). Forty-eight hours post-transfection coverslips were fixed in 4% PFA and either immunostained with anti-HA or anti-proSP-C, or left unstained (for EGFP) and examined by confocal microscopy.

Transmission electron microscopy

Preparation of lung tissue and acquisition of transmission electron microscopy (TEM) images of lung sections was performed in the Electron Microscopy Resource Laboratory in the Perelman School of Medicine (University of Pennsylvania) based on the method of Hayat that includes post-fixation in 2.0% osmium tetroxide with 1.5% potassium ferricyanide as we published (6). Cut thin sections (60-80 nm) were stained in situ on copper grids with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software.

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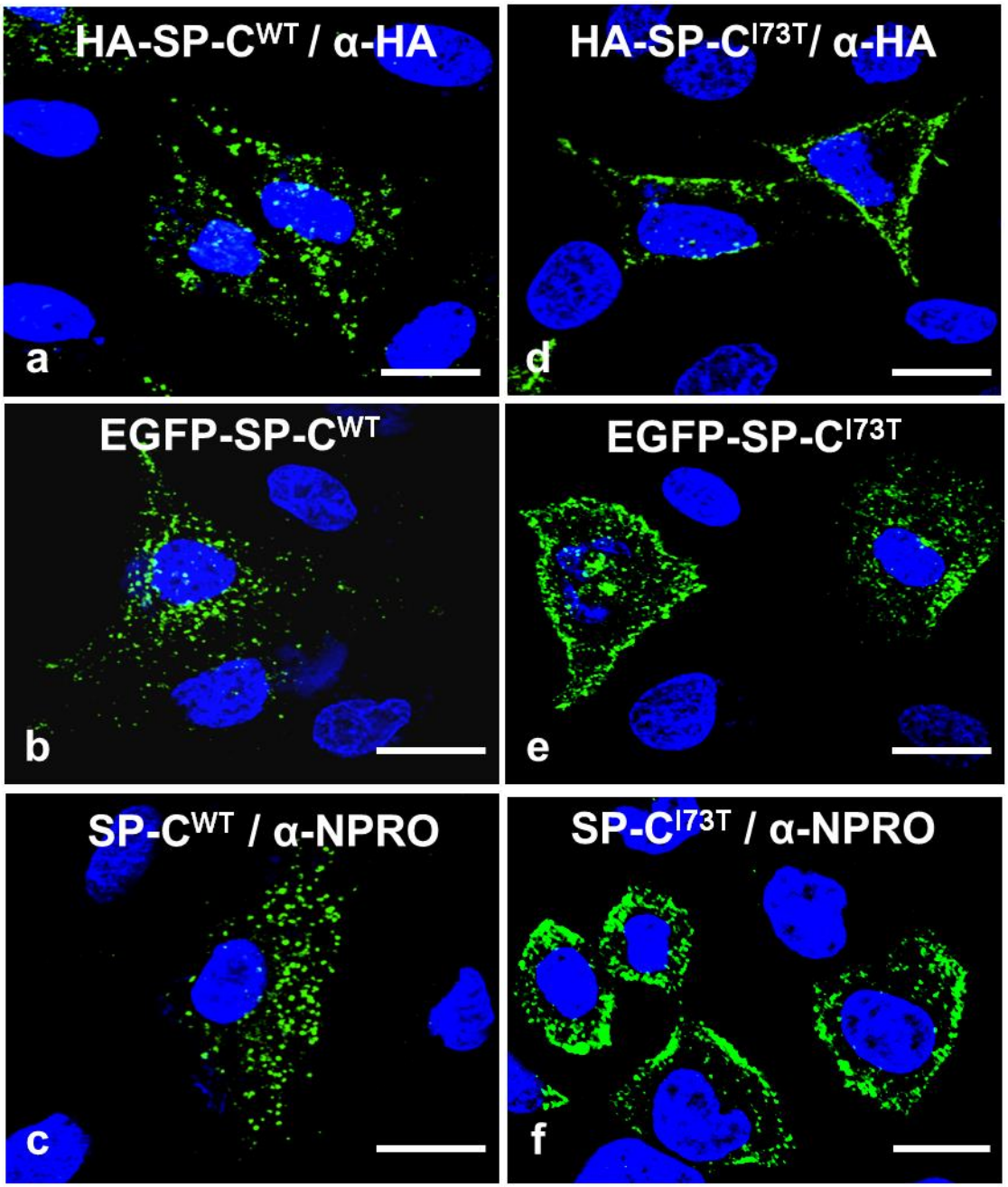
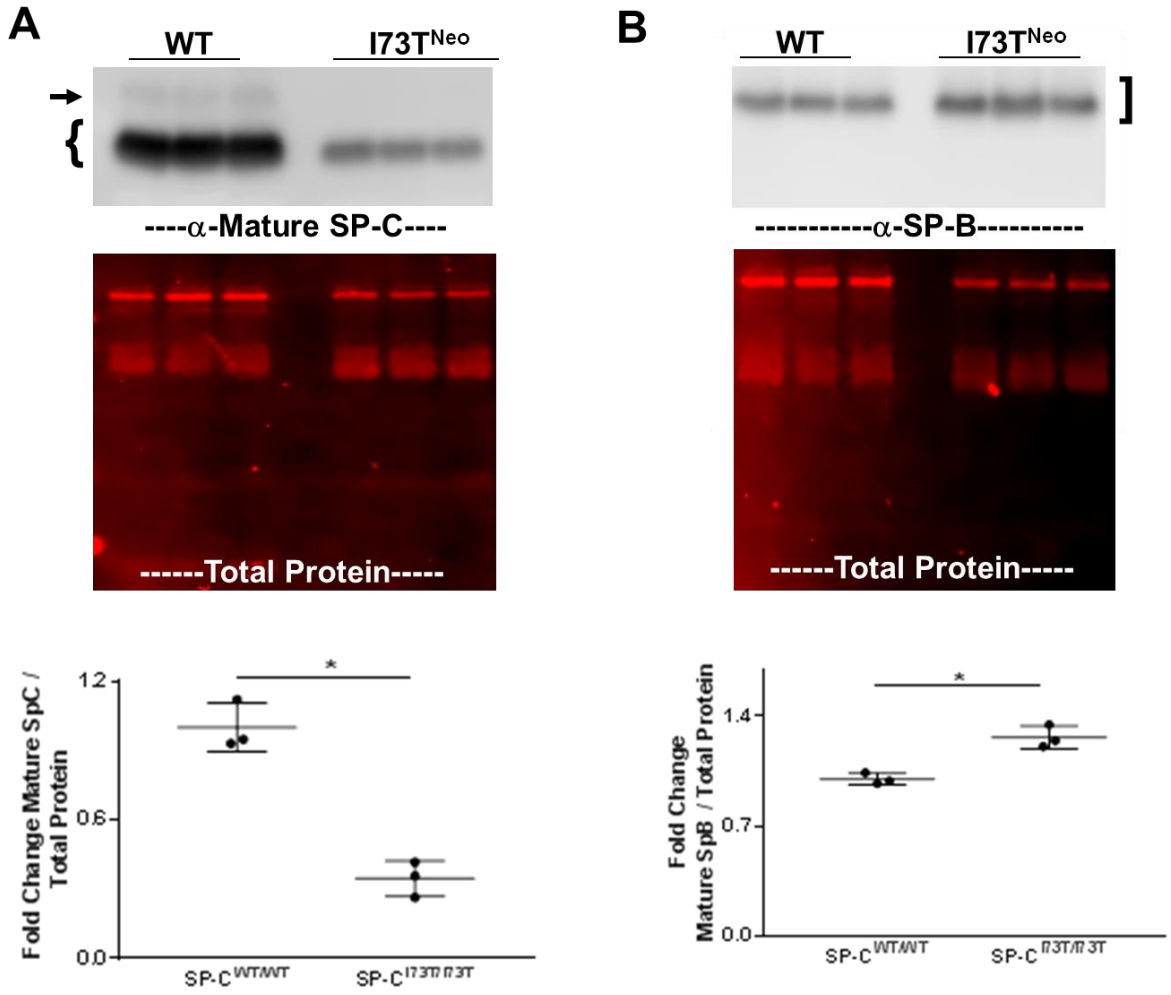
SUPPLEMENTAL FIGURES



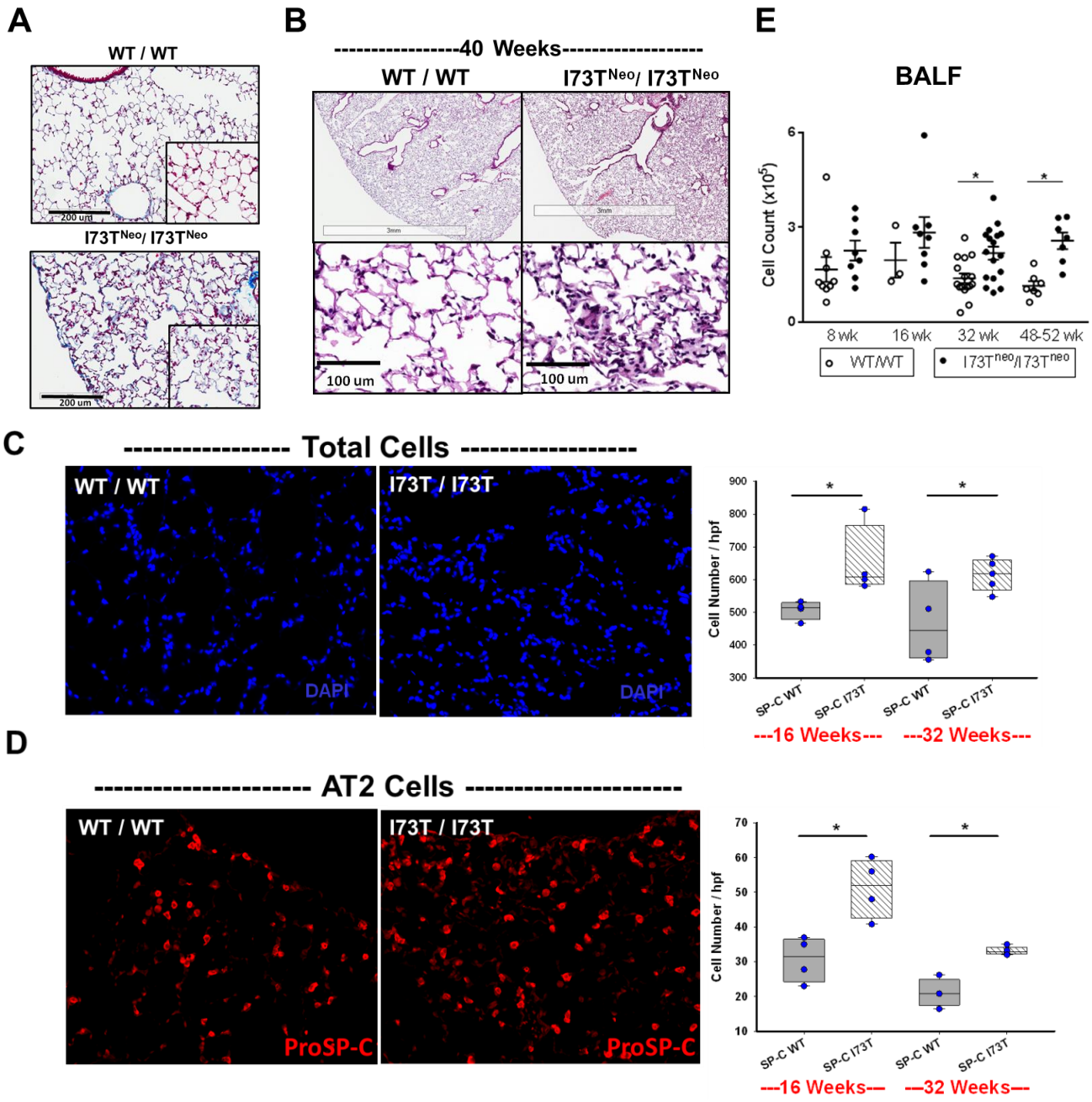
Supplemental Figure1. Expression patterns of epitope tagged and untagged SP-C isoforms

Plasmids containing cDNA for human (**a**) HA-SPC^{WT}, (**b**) EGFP/SP-C^{WT}, (**c**) untagged SP-C^{WT} , (**d**) HA-SPC^{I73T} , (**e**) EGFP/SP-C^{I73T} , or (**f**) untagged SP-C^{I73T} were each introduced into A549 cells by CaPO4 precipitation. 48 hours after transfection, cells were fixed. HA-tagged constructs (**a and d**) were stained with primary polyclonal anti-HA (eBioscience) and secondary Alexafluor488-conjugated goat anti-rabbit IgG. Cells expressing untagged proSP-C isoforms (**c and f**) were stained with anti-NPRO-SP-C and secondary Alexafluor 488 conjugated goat anti-rabbit IgG. Confocal images of EGFP or Alexafluor488 fluorescence were acquired using a 488nm laser line package of an Olympus Fluoview confocal system attached to an Olympus IX81 microscope (X60 oil objective). Bar = 10uM.

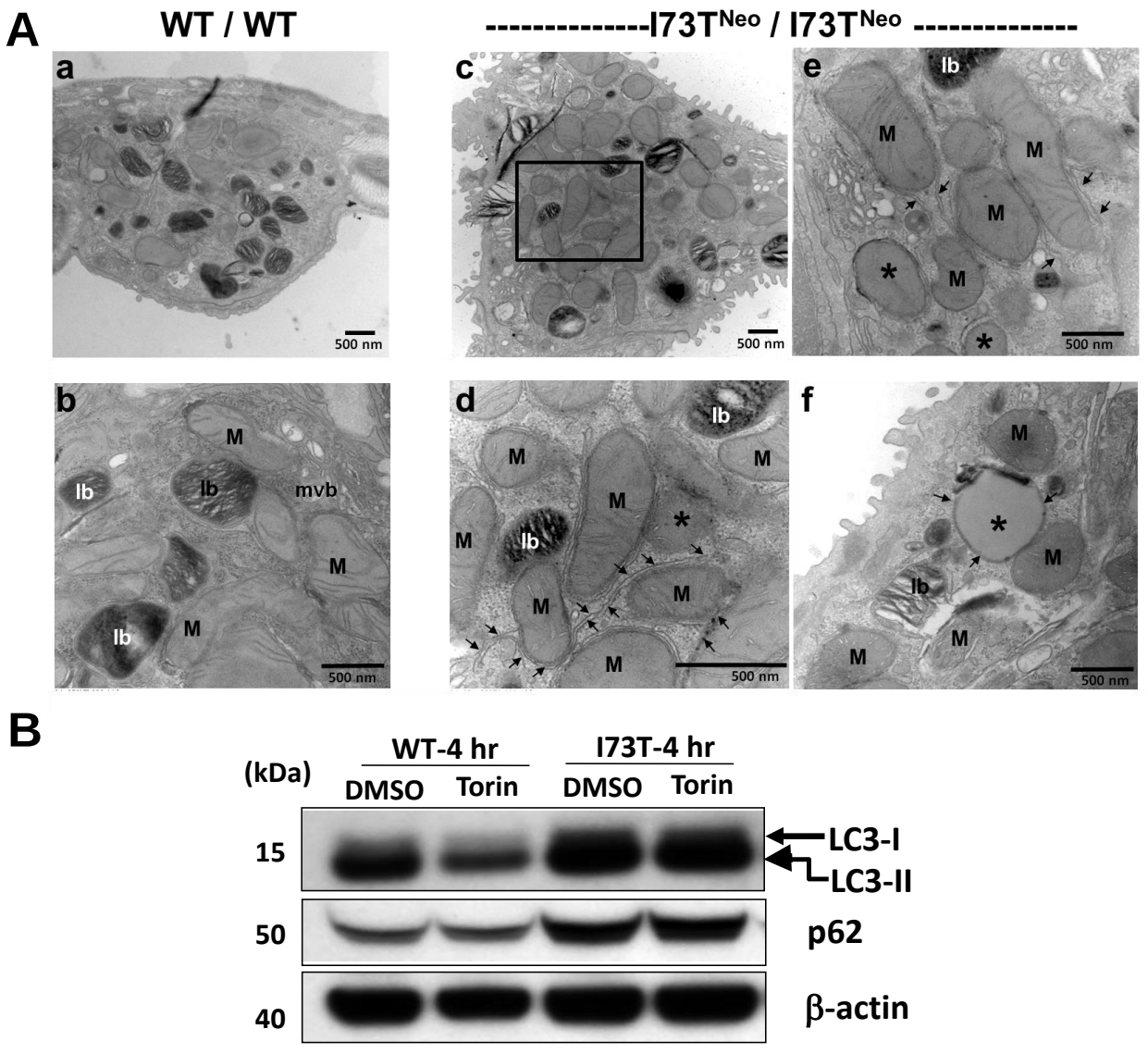


Supplemental Figure 2. Mature SP-C and SP-B content of lung surfactant from SP-C$^{I73T-Neo}$ mice

Western Blotting for mature SP-C and mature SP-B protein in lung surfactant fractions prepared from BALF of SP-C$^{I73T/I73T}$ and SP-C$^{WT/WT}$ (control) at 8 weeks of age. Each lane contained 20 μg total protein. SDS/PAGE was performed using pre-cast Tris-Bis gels and membranes containing transferred protein were first scanned for total protein content (as loading control) and then probed with anti-mature SP-C or anti-SP-B antisera. The 4 kD mature SP-C band (**{**) and non-reducible dimeric form (**arrow)** and 8 kD SP-B band (**]**) were visualized by enhanced chemoluminescence and captured and quantitated using a LiCor imaging station. Dot-plot and Mean ± SEM of relative SP-C or SP-B content (normalized to total protein) are expressed as fold change (fraction) of SP-C$^{WT/WT}$ group. * $p < 0.05$ by unpaired two-tailed student's t-test.



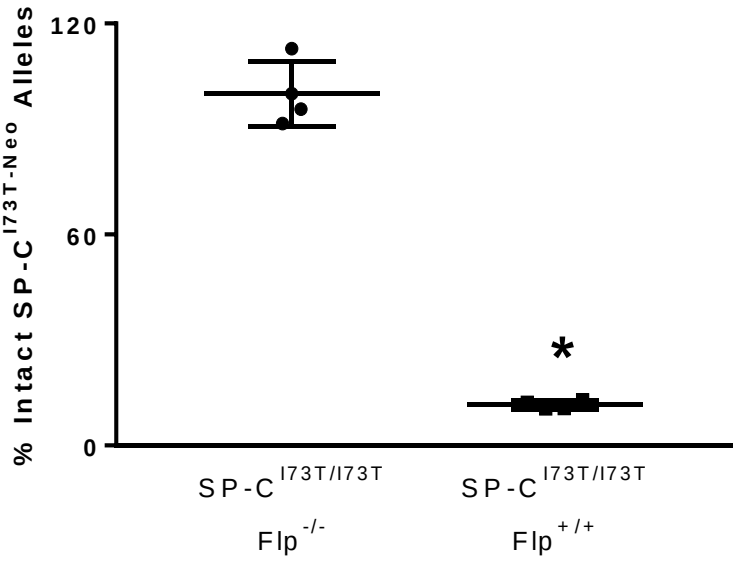
Supplemental Figure 3. Histological remodeling and BALF cellularity in SP-C$^{I73T-Neo}$ mice
(**A**) Masson's Trichrome staining of lung sections from 32 week old SP-C^{WT} (WT/WT) and homozygous SP-C$^{I73T-Neo}$ ("I73T / I73T") mice. Higher power view (insert) showing increased collagen deposition in the alveolar septae of SP-C$^{I73T-Neo}$ mice. (**B**) Low and high power views of H & E stained lung sections from 40 week WT/WT and I73T/I73T mice showing persistence of diffuse parenchymal lung remodeling characterized by septal thickening and cellular infiltrates. **(C)** Representative DAPI staining of lung sections from 32 week old WT/WT and I73T/I73T mice demonstrates increased numbers of nuclei in the alveolar septae of SP-C$^{I73T-Neo/I73T-Neo}$ mice. DAPI (+) cells were counted from 5 fields per section from 3-5 mice for each group. data expressed as number of DAPI (+) cells per field (Mean +/- SEM). * P<0.05 versus SP-C^{WT} using paired Student's t test. (**D**) Representative lung sections from 32 week old WT/WT and I73T/I73T mice stained for proSP-C. AT2 cells (defined as proSP-C staining cells) were counted from 5 fields per section per mouse for both strains and data expressed as number of SP-C cells per field (Mean +/- SEM). * P<0.05 versus SP-C^{WT} using paired Student's t test. (**E**) Dot-plot with Group Mean $\pm$ SEM shown of total BALF cells recovered from SP-C^{WT-WT} (control) and SP-C$^{I73T-Neo/I73T-Neo}$ mice 8, 16, 32, and 48-52 weeks of age. *p < 0.05 versus control group at individual time points by One Way ANOVA with post hoc Tukey test.;



Supplemental Figure 4. Defects in macroautophagy occur in AT2 cells of SP-C$^{I73T-Neo}$ mice

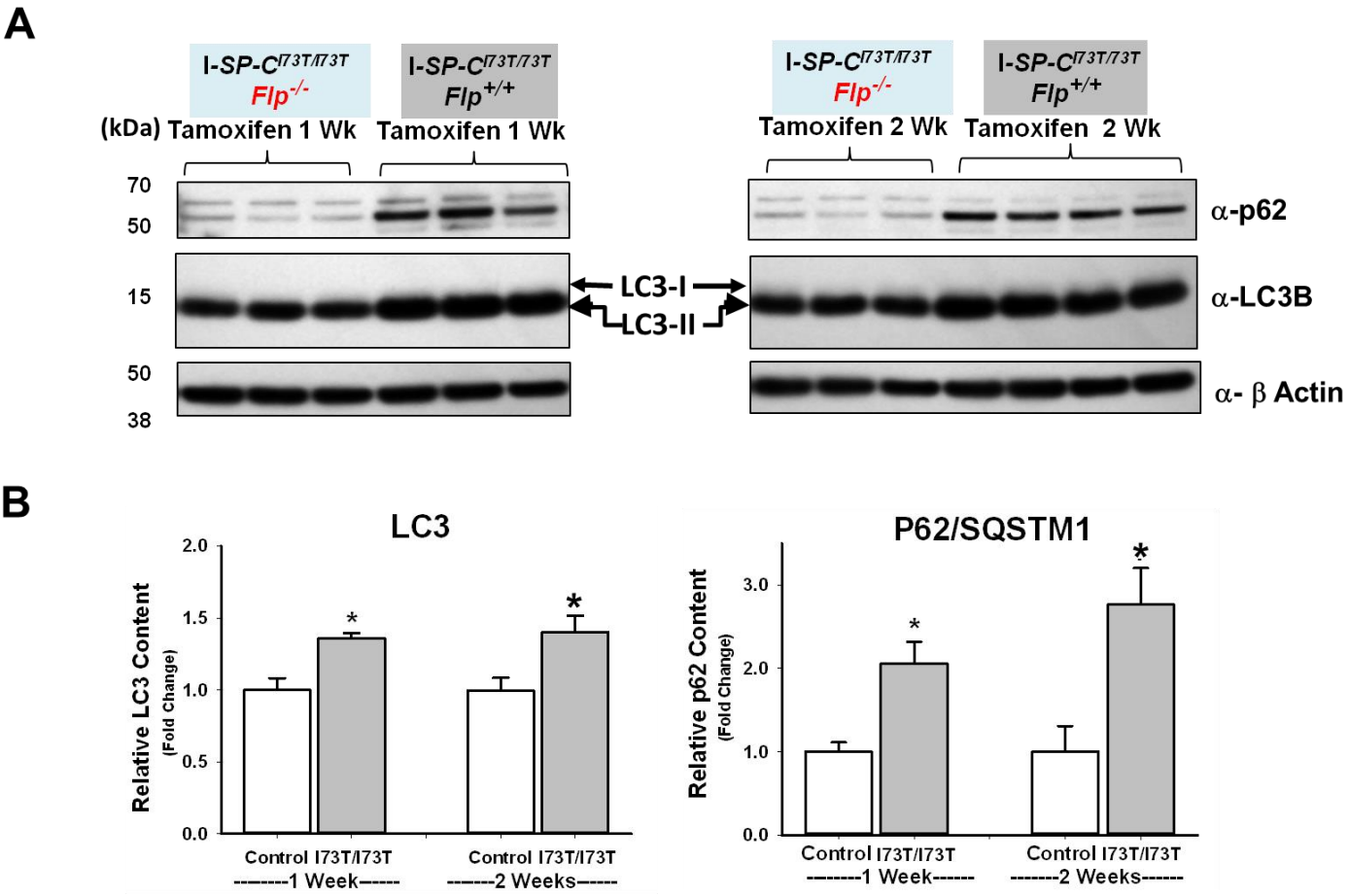
(A) Electron micrographs of AT2 cells (identified by the presence of lamellar bodies ("lb") in 29-32 week old C57/BL6 wild type ("WT/WT) and homozygous SP-C$^{I73T-Neo}$ mice ("I73T^{Neo}/ I73T^{Neo}). AT2 hypertrophy accompanied by ultrastructural changes including large autophagosomes identified by the presence of double membranes (arrows) containing mitochondria (M) as well as inclusion bodies (*) were observed in SP-C$^{I73T-Neo}$ mice. Bar = 500uM; mvb = multivesicular body.

(**B**) Autophagy flux assay using primary alveolar type 2 cells isolated from 32 week old wild type or homozygous SP-C$^{I73T-Neo/I73T-Neo}$ mice. Following culture for 24 hours, AT2 cells were treated for 4 hours with 5 uM Torin or vehicle (DMSO) for 4 hours. Lysates were subjected to SDS/PAGE and Western blotting for autophagy markers LC3 and SQSTM1/p62.



Supplemental Figure 5. Recombination efficiency of Sftpc$^{I73T-Neo}$ alleles after tamoxifen induction of I^{ER}-SP-C$^{I73T/I73T}$ Flp$^{+/+}$ mice.

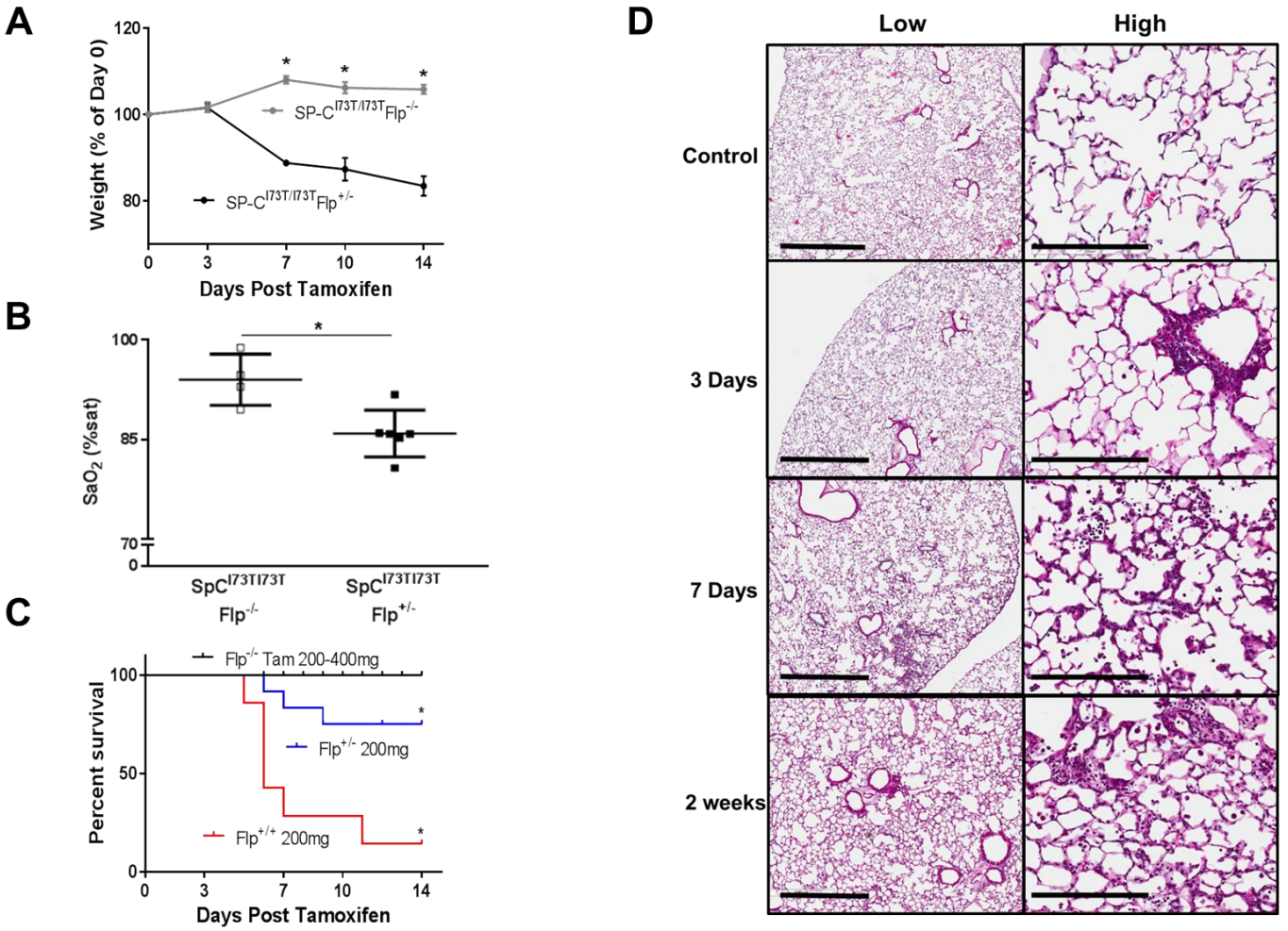
DNA was prepared from AT2 cells isolated from I^{ER}-SP-C$^{I73T/I73T}$ Flp$^{+/+}$ and I^{ER}-SP-C$^{I73T/I73T}$ Flp$^{--/-}$ controls (4 individual preparations) at 7 days post-treatment with 200 mg/kg intraperitoneal tamoxifen. qPCR was performed on AT2 DNA templates using primers designed to detect intact Neomycin cassettes within *Sftpc^{I73T}* alleles and conditions described in Supplemental Methods. dCT values obtained from controls represented unexcised alleles and were normalized to 100%. Shown is dot-plot with group means and S.E.M indicated for each cohort. $p < 0.05$ for I^{ER}-SP-C$^{I73T/I73T}$ Flp$^{+/+}$ versus unexcised controls by 2 tailed t-test. Excision efficiency was calculated as a percentage of intact alleles.



Supplemental Figure 6. Defects in macroautophagy occur as early as 1 week post iTAM induction.

(**A**) Lysates from freshly isolated AT2 cells from I^{ER}-SP-C$^{I73T/I73T}$ Flp$^{-/-}$ ("control") and I-SP-C$^{I73T/I73T}$Flp$^{+/+}$ mice at 1 and 2 weeks post tamoxifen induction were subjected to western blotting using antibodies against LC3B, p62/SQSTM1, and β–actin (loading control). Bands were visualized by enhanced chemluminescence and exposure to film. Arrows delineate position of 2 major LC3 isoforms. Each lane represents AT2 cells prepared from single mice and contains 30 μg total protein.

(B) Denistometric quantitation of bands detected on western blots reveals increases in total LC3 and early post-induction.consistent with a late block in autophagy. Data are expressed as fold change from time matched control group; * $p < 0.05$ by student's t-test. n= 3-4 per genotype and time point.



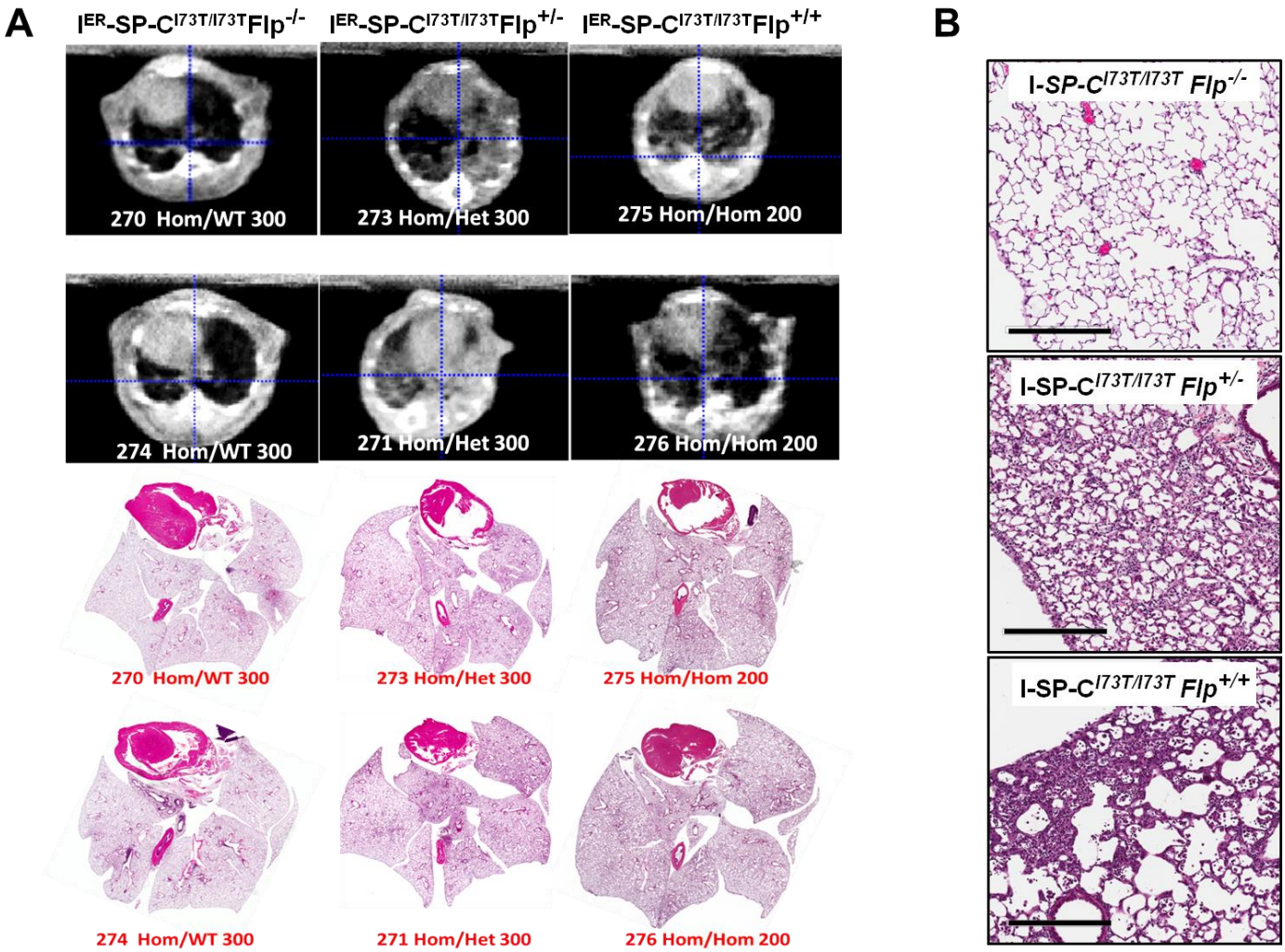
Supplemental Figure 7. Morbidity, mortality, and evolution of early lung injury in I^{ER}-SP-C^{I73T}Flp mice post tamoxifen induction.

(**A) Tamoxifen administration results in early time dependent weight loss.** Body weights for control (I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$)(N=6) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ (N=8) animals treated with 300 mg/kg intraperitoneal tamoxifen in oil were obtained at the indicated times. Measured weights were normalized to starting weight for each individual animal and expressed a percentage of Day 0 and group mean data (X +/- S.E.M) shown. *p < 0.05 vs I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control group at using unpaired two tailed t-test.

(B) I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ become hypoxemic following Tamoxifen administration. Dot-plot with Group Mean ± SEM shown of arterial hemoglobin saturation (SaO2) measured by pulse oximetry at day 7 following 250 mg/kg intraperitoneal tamoxifen in oil to I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mice. *p < 0.05 vs I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control group by unpaired two tailed t-test.

(**C**) **FLP-O allele dependent mortality.** Kaplan–Meier survival curves for 8-12 week old I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Flp$^{-/-}$) treated with 200-400 mg /kg IP Tamoxifen (N=27) or inducible I^{ER}-SP-C$^{I73T/I73T}$Flp mice either heterozygous(Flp$^{+/-}$) (N=12) or homozygous (Flp$^{+/+}$) (N=7) for the Flp-O allele all treated with 200 mg/kg tamoxifen. Death was defined as spontaneous death or need for euthanasia for body weight less than 75% starting weight on 2 consecutive days. * p < 0.05 versus I-SP-C$^{I73T/I73T}$ER$^{-/-}$ control group by Log Rank analysis.

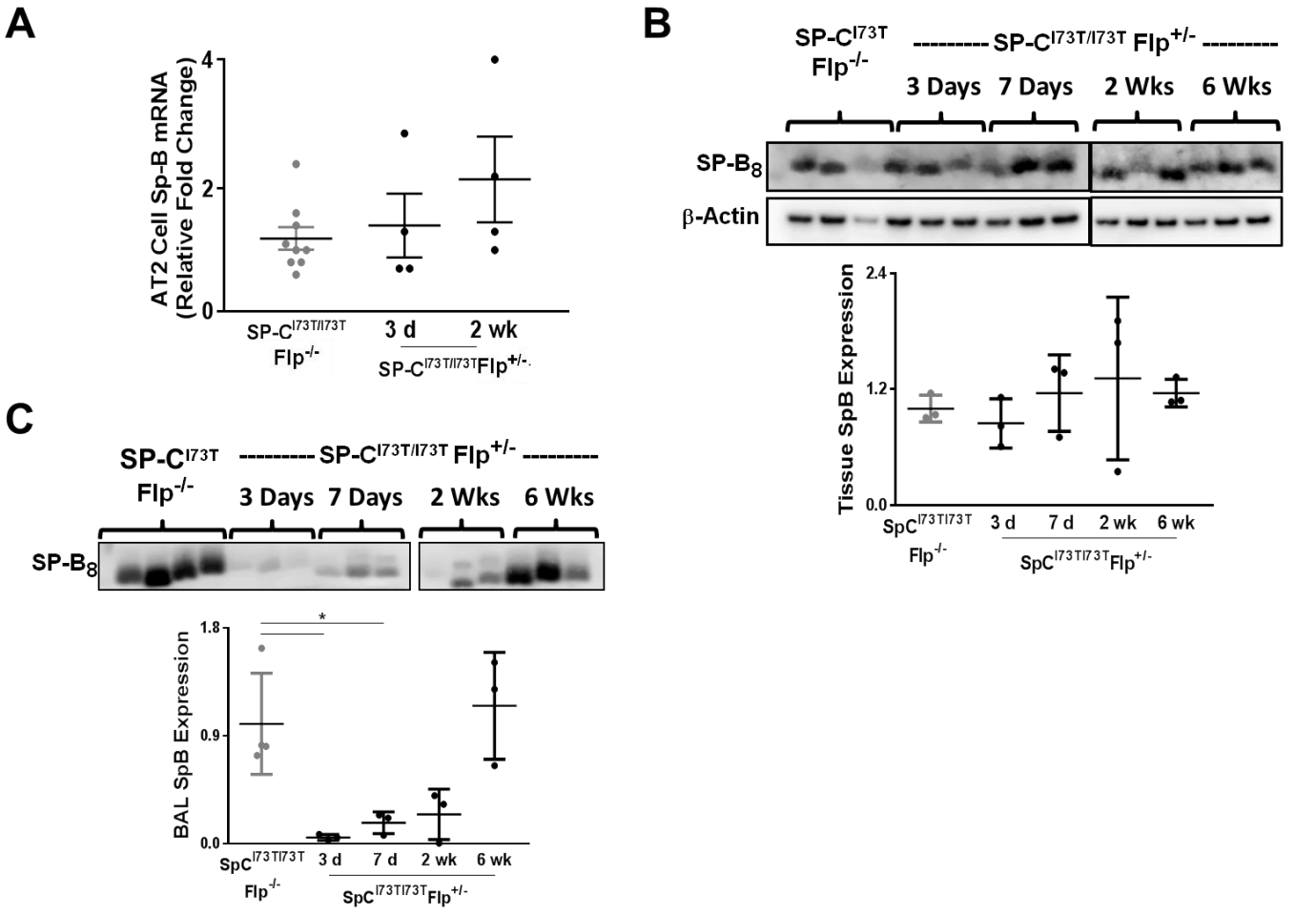
(**D**) **Histological evolution of lung injury following tamoxifen induction.** Low and high power views of representative H&E stained lung sections from I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mice treated with oil ("Control") or 3-14 days after treatment with a single 300 mg/kg dose of intraperitoneal tamoxifen. Low Power: Bar = 600 microns; High power: Bar = 200 microns.



Supplemental Figure 8. Radiographic changes induced after Tamoxifen I^{ER}-SP-C$^{I73T/I73T}$Flp mice.

(**A)** Micro CT images on spontaneous breathing mice (**Top**) and corresponding H & E stained whole lung sections (muse number indicated) obtained immediately after imaging (**Bottom**) prepared from I^{ER}-SP-C$^{I73T/I73T}$ Flp$^{-/-}$ (*"Hom-WT"*), I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ (*"Hom-Het"*) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+=-}$ ("Hom-Hom") mice 2 weeks after induction with a single 300 mg/kg dose ("300") or 200 mg/kg (200") of intraperitoneal tamoxifen.

(**B**) Higher power representative H & E stained lung sections from each tamoxifen treated genotype as indicated. Bar = 300 microns.

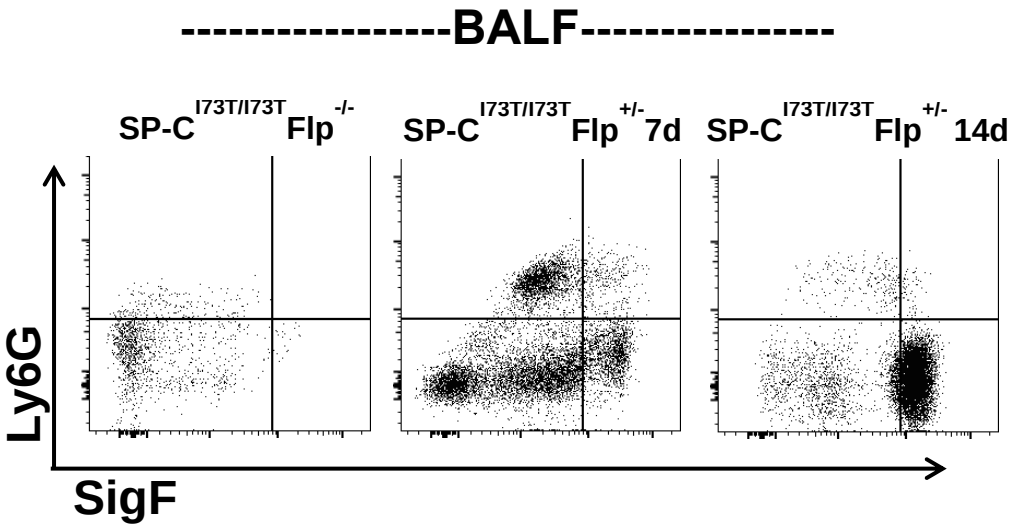


Supplemental Figure 9. Surfactant Protein B expression following induction of SP-C^{I73T}.

(A) *Sftpb* expression in AT2 cells. qRT-PCR was performed using total RNA from AT2 cells isolated from I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mice 3 days and 2 weeks following induction with 250 mg/kg intraperitoneal tamoxifen. Data were normalized for 18S expression and expressed as fold change from Control. p >0.05 versus Control by One way ANOVA, followed by Tukey post hoc t-test.

(B) Mature SP-B protein expression in lung tissue. Western Blotting for mature SP-B protein expression in lung homogenates from I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mice 3 days, 1 week, 2 week, and 6 weeks following 250 mg/kg intraperitoneal tamoxifen. Each lane contained 20 ug total protein. SDS/PAGE was performed using pre-cast Tris-Bis gels and membranes containing transferred protein were probed with anti-SP-B antisera (1:3000) and reprobed with anti β–actin as a loading control. The mature 8 kDa SP-B bands (upper) and 42 kDa β-actin bands (lower) visualized by ECL were captured and quantitated using LiCor imaging station. Dot-plot and Group Mean +/- SEM of relative tissue SP-B content normalized to β-actin are expressed as % SP-C$^{I73T/I73T}$ ER$^{-/-}$ control. For all time points p > 0.05 vs I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control group by One way ANOVA followed by Tukey post hoc t-test.

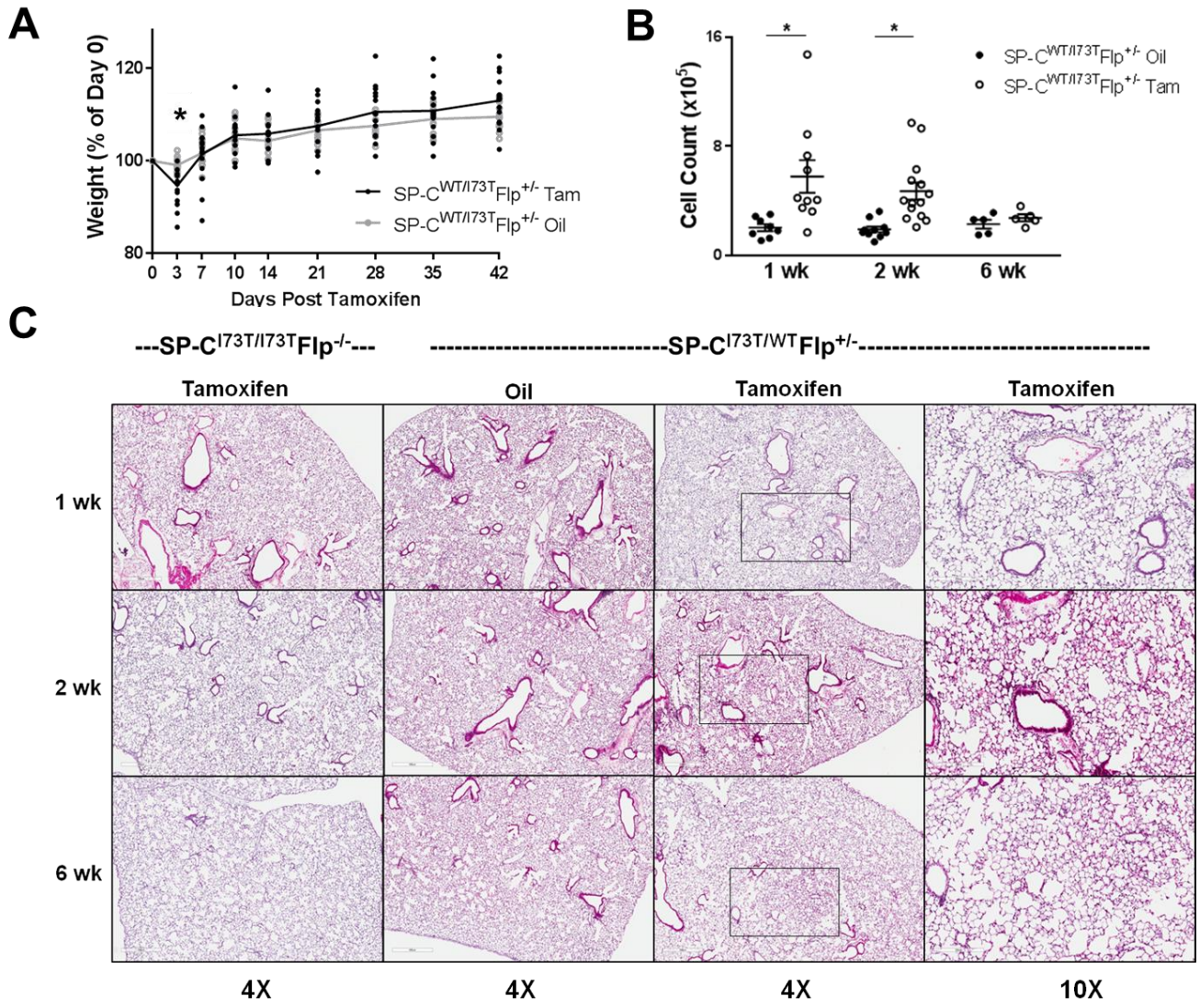
(**C**) **BALF SP-B levels are acutely decreased post-induction.** BALF (20 ul/lane) from I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mice 3 days, 1 week, 2 weeks, and 6 weeks following 250 mg/kg intraperitoneal tamoxifen was subjected to SDS/PAGE and Western blotting with anti-SP-B antisera (1:3000) as in (**B**) above. The secreted 8 kDa mature SP-B isoform visualized by chemiluminesence was detected and quantiated using a LiCor imaging station. * p < 0.05 versus I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control group by One Way ANOVA followed by Tukey post hoc t-test.



	% Neutrophils	% Eosinophils
SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control)	3.0 ± 0.8	2.2 ± 0.8
SP-C$^{I73T/I73T}$Flp$^{+/-}$ 7 days Post-Tamoxifen	26.6 ± 7.1*,**	14.7 ± 4.0*
SP-C$^{I73T/I73T}$Flp$^{+/-}$ 14 days Post-Tamoxifen	8.8 ± 1.5*	41.7 ± 8.8*,**

Supplemental Figure 10. Flow Cytometry of BALF for Neutrophils and Eosinophils

Representative flow cytometric analysis for identification of neutrophils and eosinophils in BALF from I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mice obtained 7 and 14 days after tamoxifen. Sorting strategy which was performed as detailed in *Methods*. Immune cells were identified by forward and side scatter followed by doublet discrimination of CD45^{+} viable cells. Relative amounts of neutrophils (SigF^{lo}Ly6G^{+}CD11b^{+}CD11c^{-}CD64^{-}) and eosinophils (SigF^{Hi}Ly6G^{-}CD11b^{+}CD11c^{-}CD64^{-}) identified were expressed as percentage of total CD45^{+} viable BALF cells. n = 5-7for each group. Multiple group comparisons within cell types were performed using One Way ANOVA with post-hoc Tukey testing. $^{*}p < 0.05$ versus I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control. $^{**}p < 0.05$ versus treated I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/+}$ (7 or 14 days)..

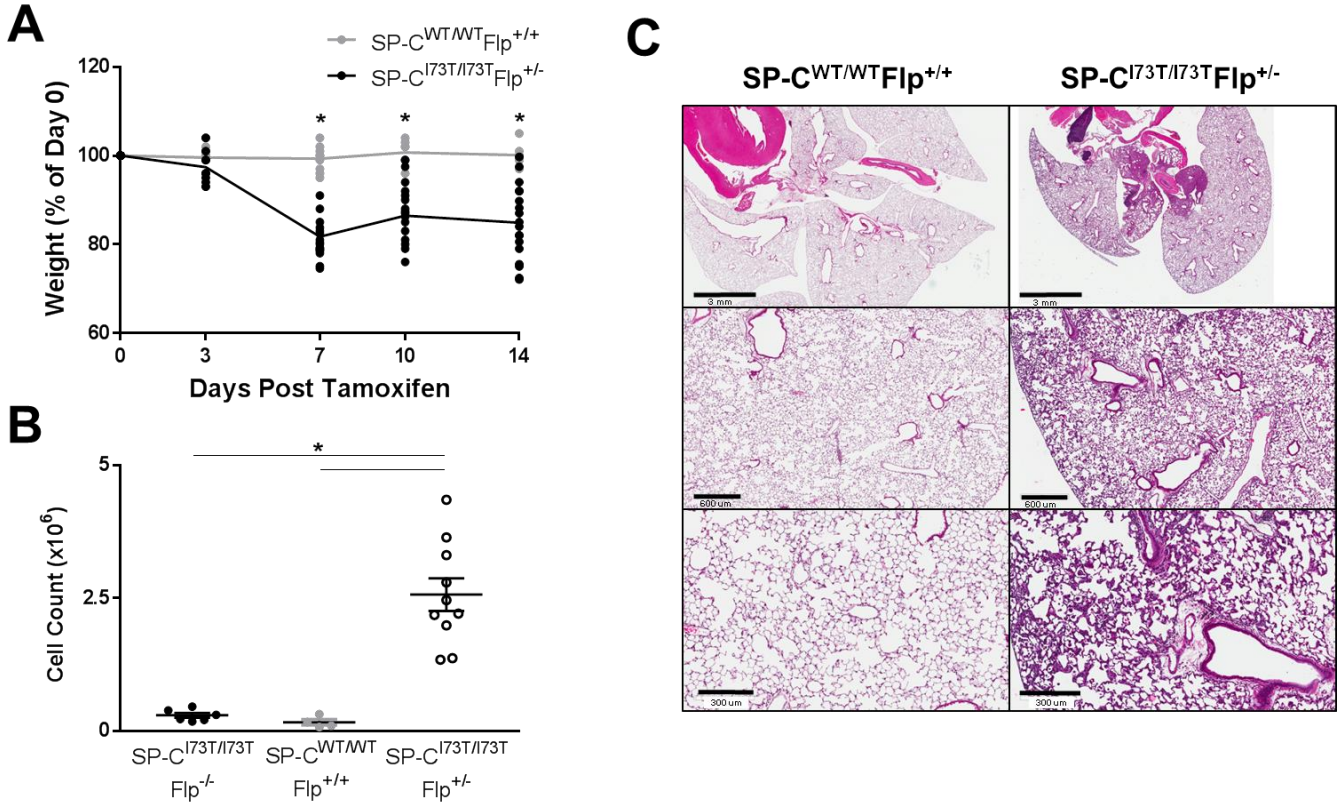


Supplemental Figure 11. Phenotype of heterozygous I^{ER}-SP-C$^{I73T/WT}$Flp$^{+/-}$ mice.
Double heterozygous I^{ER}-SP-C$^{I73T/WT}$Flp$^{+/-}$ mice received 400 mg/kg intraperitoneal tamoxifen (in 2 divided doses) or corn oil and followed for 14 days.

(**A**) Dot-plots for individual animal body weights measured at the indicated time points and expressed as percent (%) of starting weight. Line represents group mean data. * $p < 0.05$ versus oil at 3 days by two tailed t-test

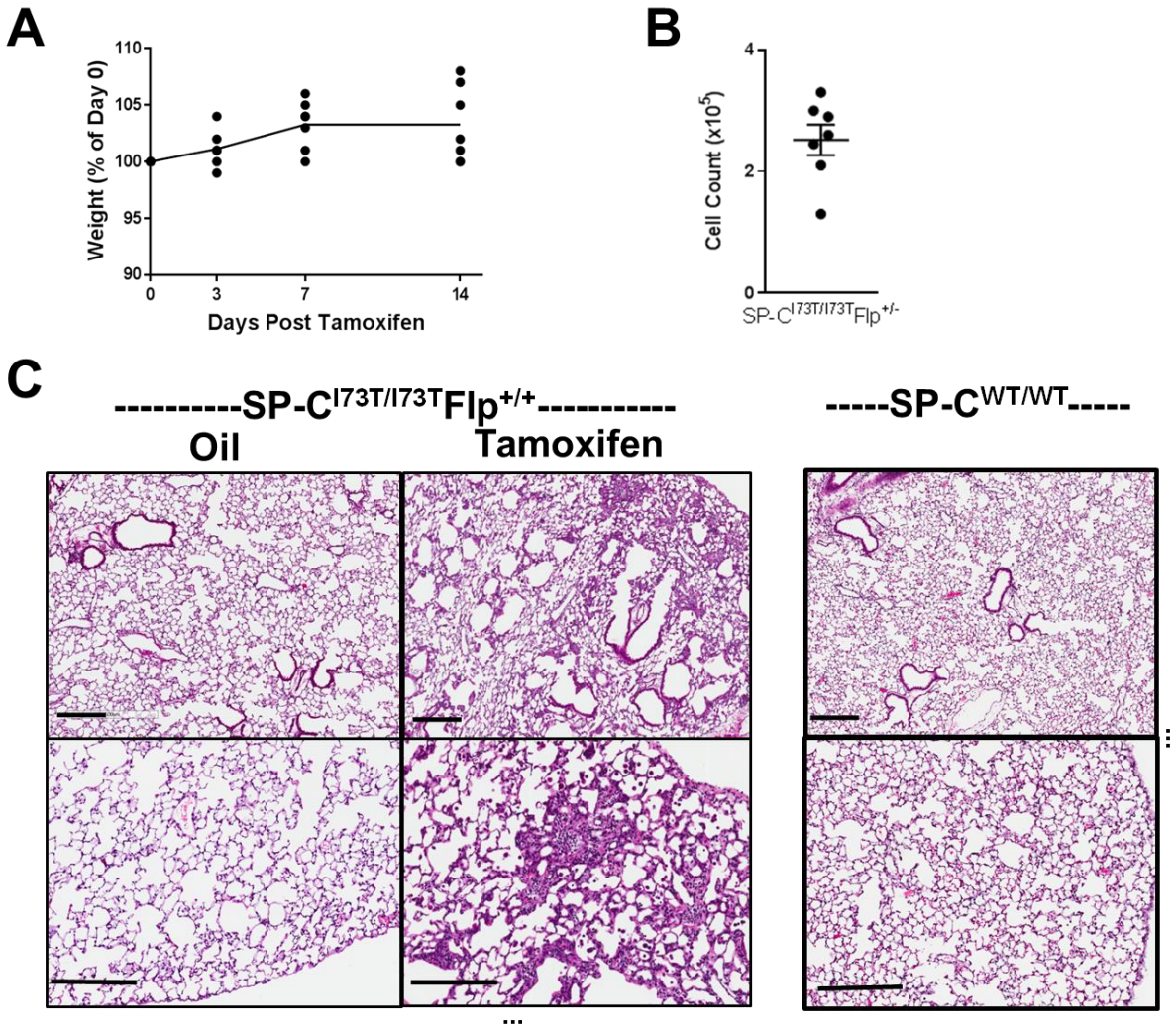
(**B**) Dot-plot with Group Mean $\pm$ SEM shown of total BALF cells recovered from I^{ER}-SP-C$^{I73T3/WT}$Flp$^{+/-}$ cohorts 1, 2, and 6 weeks after treatment with tamoxifen (Open circles) or oil (black circles) with tamoxifen. Multiple comparison between groups were made using an One Way ANOVA with post hoc Tukey test. *$p < 0.05$ for tamoxifen versus corresponding oil treated group.

(**C**) Representative low power (4X) micrographs from H&E stained lung sections from Tamoxifen and Oil treated I^{ER}-SP-C$^{I73T3/WT}$Flp$^{+/-}$ cohorts. The far right column represents enlargement of indicated box (10X). iTAM treated I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ section provided as an additional histological control (Left column).



Supplemental Figure 12. Induction of FlpO recombinase does not contribute to the early injury phenotype of I^{ER}-SP-C$^{I73T/I73T}$Flp mice.

Rosa26ERFlpO (= I^{ER}-SP-C$^{WT/WT}$Flp$^{+/+}$), I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ (as a positive inducible control), and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (as a non-inducible negative control) mice each received a single dose of 250 mg/kg intraperitoneal tamoxifen and were followed for 14 days. (**A**) Body weights measured at the indicated time points represented by dot-plots for each individual animal expressed as percent (%) of starting weight are shown. Line represents group mean data. * $p < 0.05$ vs I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ control group at each time point by unpaired two tailed t-test; (**B**) Total BALF cells recovered from I^{ER}-SP-C$^{WT/WT}$Flp$^{+/+}$, I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$, and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ cohorts 2 weeks after tamoxifen treatment. Data are represented with dot-plot with mean $\pm$ SEM shown. *$p < 0.05$ versus I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ by One Way ANOVA with post-hoc Tukey test; (**C**) Low, intermediate and high power micrographs of representative H&E stained lung sections showing normal lung histology in I^{ER}-SP-C$^{WT/WT}$ Flp$^{+/+}$ animals. Bar = 300 μm.



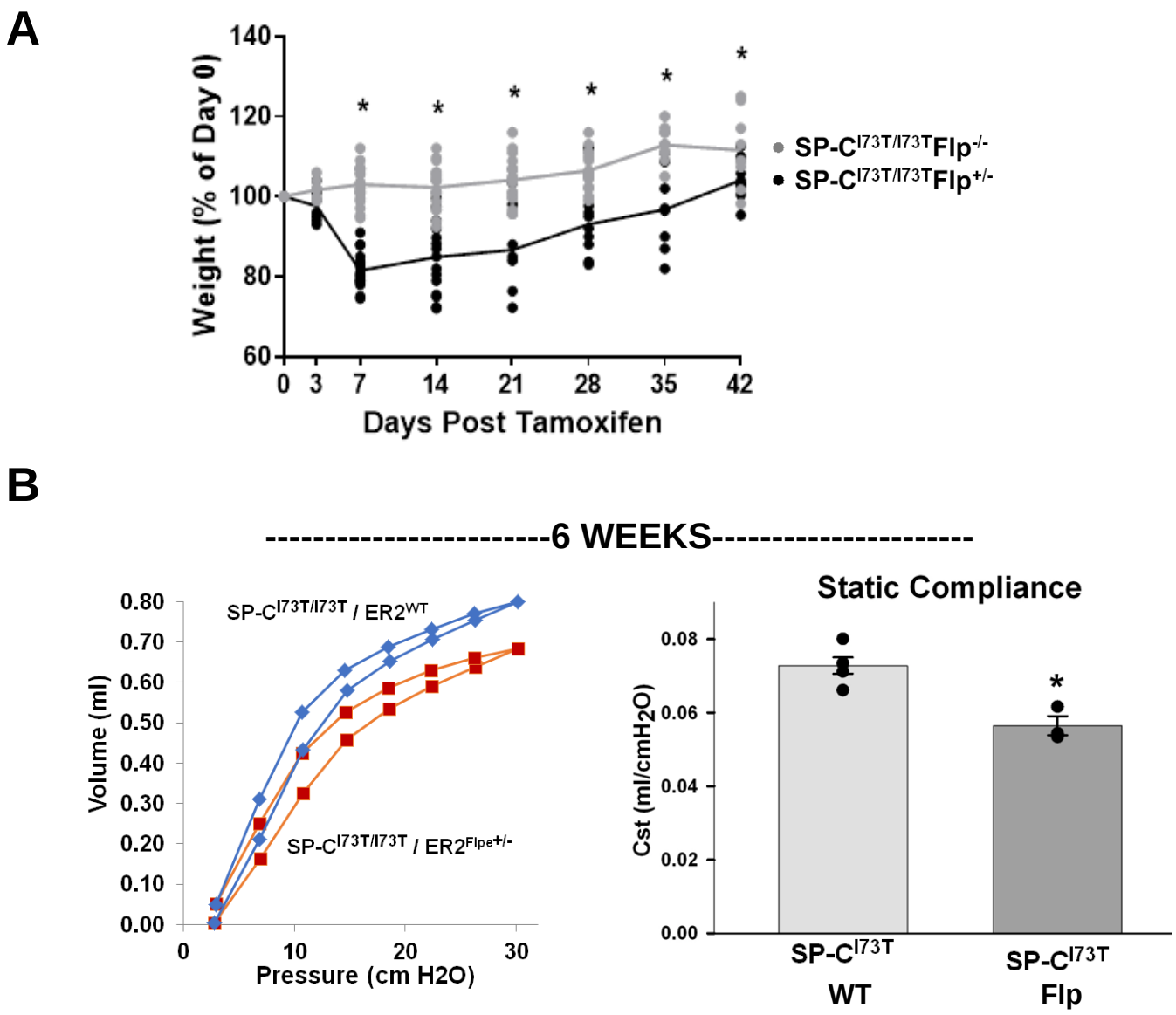
Supplemental Figure 13. Phenotype of Oil Treated I^{ER}-SP-C$^{I73T/I73T}$Flp mice.

Homozygous I^{ER}-SP-C$^{I73T3/I73T}$Flp$^{+/+}$ mice received corn oil or and followed for up to 28 days.

(**A**) Dot-plots for individual animal body weights for measured at the indicated time points and expressed as percent (%) of starting weight. Line represents group mean data.

(**B**) Dot-plot with Group Mean $\pm$ SEM shown of total BALF cells recovered 2 weeks after oil treatment.

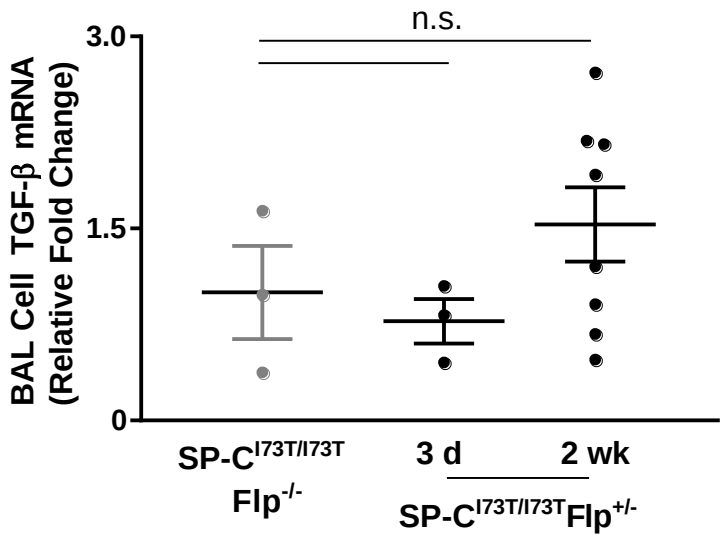
(**C**) Representative micrographs from H&E stained lung sections from Oil treated I^{ER}-SP-C$^{I73T3/WT}$Flp$^{+/-}$ cohort. As a reference representative micrographs from an I^{ER}-SP-C$^{I73T3/WT}$Flp$^{+/+}$ animal 28 days after 175 mg/kg tamoxifen and an untreated age matched wild-type C57/Bl6 mouse ("SP-C$^{WT/WT}$"). Bar = 300μm.



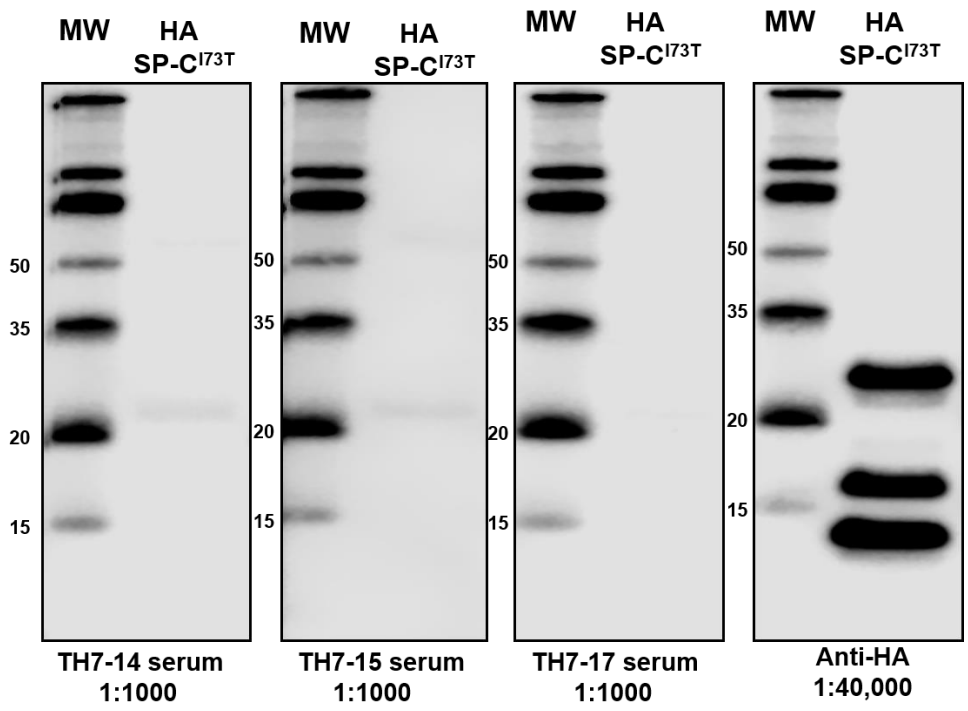
Supplemental Figure 14. Body weight changes and restrictive lung physiology develop post tamoxifen.

(**A**) Body weights for I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ animals treated with 300 mg/kg intraperitoneal tamoxifen were assessed out to 42 days. Dot-plots for each individual animal expressed as percent (%) of starting weight are shown. Line represents group mean data. * $p < 0.05$ vs I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control group at each time point by unpaired two-tailed t-test.

(**B**) I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ (Control) and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ (expressing 1 allele of the ER$_2$-Flp-O gene) were subjected to pulmonary function testing using a Flexivent 6 weeks after administration of 250 or 300 mg/kg tamoxifen. (**Left**) Pooled flow volume loops from affected mice (n=4 per group) are shown. (**Right**) Bar graph with mean ± SEM and superimposed dot-plot is shown. Calculated static compliance was reduced consistent with restrictive parenchymal lung disease. * $p < 0.05$ vs I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ by two tailed t-test.

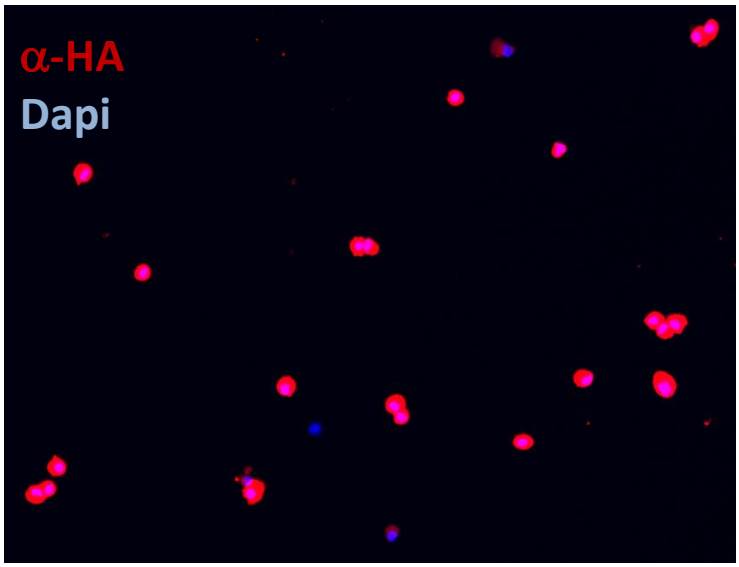


Supplemental Figure15. TGFβ$_1$ mRNA expression in BALF cells. RNA was extracted from unfractionated BALF cell populations harvested at 3 and 14 days post-tamoxifen. TGFβ$_1$ mRNA expression was determined by qRT-PCR using primers and conditions identical to Figure 7B. dCT values were normalized to 18S housekeeping gene. Dot-plot showing fold change I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ samples (black dots) versus non-inducible I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control (grey dots). n.s.= $p > 0.05$ versus I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ by One Way ANOVA.



Supplemental Figure 16. Tamoxifen induced I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/+}$ mice fail to develop anti-HA antibodies.

SDS/PAGE was performed on a standardized cell lysate (10 μg total protein / lane) prepared from freshly isolated AT2 cells expressing HA-proSP-C^{I73T} protein 2 weeks after intraperitoneal tamoxifen treatment of a I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ mouse ("HA SP-C^{I73T}"). Separated proteins were transferred to a single PVDF membrane and strips each containing an HA-SP-C^{I73T} lane and molecular weight markers (MW) were cut. Western blotting of each strip was performed using primary sera (dilution 1:1000) isolated from one of three individual double homozygous I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/+}$ mice six weeks after tamoxifen induction ("*TH 7-14, TH 7-15, and TH 7-17*") and secondary goat anti-mouse IgG. As a positive control, an identical antigen strip was immunoblotted with a primary commercial polyclonal anti-HA antiserum (abcam, Inc., Cambridge, MA) used at a dilution of 1:40,000. Using enhanced chemluminesence and scanning with a LiCOR imaging station, HA-proSP-C^{I73T} bands were detected with the commercial anti-HA antiserum but significant anti-HA titers were absent in all 3 experimental mice.



Mouse ID	HA+/Dapi+	Dapi+	% HA+
I73T Hypo 1	237	241	98.3
I73T Hypo 2	201	205	98.0
I73T Hypo 3	185	195	94.9
I73T Hypo 4	275	280	98.2
Avg			97.4
Std Dev			1.7

Supplemental Figure 17. Purity of isolated AT2 cell preparations.

Representative double label immunofluorescence staining for anti-HA (red) and Dapi (Blue) of coverslips coated in 10% Matrigel®containing AT2 cells isolated as detailed in *Methods* and cultured overnight to promote adherence. The number of Dapi+ and HA+/Dapi+ cells were manually counted from 4 preparations as shown and summarized at right.

SUPPLEMENTAL TABLES

Supplemental Table 1. Prenatal death in C^{Flp}-SP-C^{I73T} mice with excised Sftpc^{I73T} alleles.

Progeny at day e18.5 (Embryo)

SP-C$^{WT/I73T\text{-}Neo}$ /Flp$^{+/-}$ X SP-C$^{WT/I73T\text{-}Neo}$ / Flp$^{+/-}$

15 Litters (176 Pups)				Verified Progeny With Neo Excised Sftpc^{I73T} Alleles
***sftpc* Genotype**	**SP-C$^{WT/WT}$**	**SP-C$^{I73T/WT}$**	**SP-C$^{I73T/I73T}$**	
Expected	25%	50%	25%	
Observed (n)	40	92	44	**51/136**
Observed (%)	23%	52%	25%	**37.5%**
Average litter size		**7.8 +/- 0.33 (X +/- SEM)**		

Progeny at weaning (Post-Natal)

SP-C$^{WT/I73T\text{-}Neo}$ /Flp$^{+/-}$ X SP-C$^{WT/I73T\text{-}Neo}$ / Flp$^{+/-}$

19 Litters (69 Pups)				Verified Progeny With Neo Excised Sftpc^{I73T} Alleles
***sftpc* Genotype**	**SP-C$^{WT/WT}$**	**SP-C$^{I73T/WT}$**	**SP-C$^{I73T/I73T}$**	
Expected	25%	50%	25%	
Observed (n)	33	34	2	**0 / 36**
Observed (%)	48%	49%	3%	**0%**
Average litter size		**3.4 +/- 0.42 (X +/- SEM)**		

Breeding summary of attempts to generate viable C^{Flp}-SP-C^{I73T} progeny. Hypomorphic SP-C$^{I73T\text{-}Neo}$ mice were interbred with a FLPe deleter mouse line. The FLPe mouse has been shown to generate excision in both somatic and germline cells in a mosaic fashion. Incomplete somatic PGK-Neo cassette removal in all resulting F1 mice was detected by tail PCR (not shown). Crossing mosaic F1 mice resulted in progeny that included an admixture of genotypes including some fully Neo-excised mice heterozygous or homozygous for *Sftpc^{I73T}*. In day e18.5 embryonic lungs, *Sftpc^{I73T}* alleles were represented in Mendelian fashion with excised *Sftpc^{I73T}* alleles recovered only in embryonic. Among weaned cohorts, there was a significant reduction in litter size ($p < 0.05$ versus embryonic C^{Flp}-SP-C^{I73T} embryos), a complete absence of Neo-excised alleles, and a decrement in *Sftpc^{I73T}* prevalence relative to predicted Mendelian inheritance all consistent with a toxic gain of function imparted by the presence of excised *Sftpc^{I73T}* alleles.

Supplemental Table 2: BALF cytokine levels following tamoxifen-induced *Sftpc^{I73T}* expression

	GM-CSF	IFNγ	IL-1β	IL-4	IL-10	IL-13	IL-17	MIP-1α	MIP-2	RANTES	TNF-α
SP-C^{I73TI73T}Flp$^{-/-}$ 7 d	2.7 ± 2.2	2.3 ± 0.3	1.0 ± 1.1	1.0 ± 0.0	2.0 ± 0.0	4.3 ± 4.3	1.0 ± 0.0	3.2 ± 0.0	0.0 ± 0.0	2.7 ± 0.3	2.0 ± 0.0
SP-C^{I73TI73T}Flp$^{+/-}$ 7 d	11.7 ± 1.9	2.1 ± 1.1	3.2 ± 0.0	2.3 ± 0.3	5.0 ± 1.5	12.8 ± 0.0	2.7 ± 0.9	41.7 ± 22.2	2.1 ± 1.1	3.0 ± 0.0	3.3 ± 0.3
SP-C^{I73TI73T}Flp$^{-/-}$ 2 wk	8.3 ± 1.3	2.3 ± 0.9	3.2 ± 0.0	1.0 ± 0.0	2.5 ± 0.3	12.8 ± 0.0	1.5 ± 0.3	3.2 ± 0.0	12.6 ± 10.5	3.0 ± 0.0	2.0 ± 0.0
SP-C^{I73TI73T}Flp$^{+/-}$ 2 wk	6.6 ± 1.5	2.1 ± 0.5	6.0 ± 1.6	**11.4 ± 2.9 ***	2.8 ± 0.2	12.8 ±0.0	2.4 ± 0.8	19.6 ± 12.8	23.3 ± 15.3	5.0 ± 0.8	50.8 ± 46.2
SP-C^{I73TI73T}Flp$^{-/-}$ 6 wk	8.3 ± 1.3	2.5 ± 0.5	3.1 ± 1.1	1.0 ± 0.0	3.8 ± 0.5	9.6 ± 3.2	1.3 ± 0.3	2.4 ± 0.8	19.8 ± 12.5	3.0 ± 0.0	2.8 ± 0.5
SP-C^{I73TI73T}Flp$^{+/-}$ 6 wk	3.0 ± 1.2	1.6 ± 0.5	2.1 ± 0.8	2.3 ± 1.1	2.9 ± 0.3	12.8 ± 0.0	1.1 ± 0.1	9.7 ± 7.1	16.6 ± 5.7	3.0 ± 0.0	2.4 ± 0.4

BALF isolated from control (I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$) and induced (I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$) mice 7, 14, and 42 days after 250-300 mg/kg tamoxifen treatment as indicated were analyzed by multiplex assay (MACS® beads) for expression of cytokines shown above as described in *Methods* and presented in Figures 9 and 10. n = 3-8 samples per group. Values at or below the limit of detection of the assay are highlighted in **gray boxes**. **For IL-4 only,** *$p < 0.05$ by Student's t-test versus I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control only at 2 weeks. All other values for each cytokine at each time point were $p > 0.05$ by unpaired one way t-test versus corresponding I^{ER}-SP-C$^{I73T/I73T}$Flp$^{-/-}$ control group.

Supplemental Table 3. Cytokines in Control SP-C$^{WT/WT}$Flp$^{+/+}$ Mice 2 weeks Following Tamoxifen

	CCL11 (pg/ml)	IL5 (pg/ml)	IL6 (pg/ml)	MCP-1 (pg/ml)	KC (pg/ml)
SP-C$^{WT/WT}$ Flp$^{+/+}$ 2wk	6.5 ± 1.6	1.5 ± 0.8	1.9 ± 1.1	2.0 ± 1.6	17.4 ± 5.1
SP-C$^{I73T/I73T}$ Flp$^{+/-}$ 2wk	164.5 ± 47.1*	452.7 ± 280.9*	4307.3 ± 1820.7*	284.9 ± 84.9*	107.3 ± 15.4*

BALF isolated from I^{ER}-SP-C$^{WT/WT}$Flp$^{+/+-}$ and I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ (Positive control) mice 2 weeks after 250-300 mg/kg intraperitoneal tamoxifen were analyzed by multiplex assay (MACS® beads) for expression of cytokines shown above as described in *Methods*. n = 3-8 samples per group. Values at or below the limit of detection of the assay are highlighted in **gray.** *p < 0.05 versus I^{ER}-SP-C$^{WT/WT}$Flp$^{+/+-}$ by unpaired two tailed t-test.

Supplemental Table 4. Endpoint Stratification By Sex Following iTAM Induction of SP-C^{I73T}

Time	Genotype	Cell Count (x10^5)		Protein Content (µg/ml)		Inspiratory Capacity (ml)		Static Lung Compliance (ml/cm$_{H2O}$)	
		Male	Female	Male	Female	Male	Female	Male	Female
3 d	CTL	0.21 ± 0.03	0.18 ± 0.02	411.1 ± 72.6	322.55 ± 61.89	-	-	-	-
	SP-C$^{I73T/I73T}$Flp$^{+/-}$	0.15 ± 0.02	0.25 ± 0.05	454.2 ± 52.6	679.7 ± 180.2	-	-	-	-
1 wk	CTL	0.24 ± 0.05	0.26 ± 0.07	507.9 ± 257.9	-	-	-	-	-
	SP-C$^{I73T/I73T}$Flp$^{+/-}$	1.06 ± 0.22	1.33 ± 0.55	2207.1 ± 722.6	2310.1 ± 560.2	-	-	-	-
2 wk	CTL	0.33 ± 0.03	0.26 ± 0.04	494.9 ± 42.2	514.8 ± 79.9	0.80 ± 0.06	0.75 ± 0.06	0.075 ± 0.006	0.067 ± 0.006
	SP-C$^{I73T/I73T}$Flp$^{+/-}$	3.02 ± 0.64	2.57 ± 0.37	2737.6 ± 1238.5	3863.2 ± 1055	0.46 ± 0.05	0.45 ± 0.09	0.027 ± 0.003	0.036 ± 0.016
4 wk	CTL	0.19 ± 0.02	0.29 ± 0.03	-	404.7 ± 85.3	0.76 ± 0.06	0.79 ± 0.08	0.067 ± 0.005	0.072 ± 0.008
	SP-C$^{I73T/I73T}$Flp$^{+/-}$	1.23 ± 0.54	1.03 ± 0.20	1278.3	2154.7 ± 1574.7	0.67 ± 0.17	0.47 ± 0.08	0.054 ± 0.022	0.033 ± 0.008
6 wk	CTL	0.24 ± 0.04	0.25 ± 0.02	496.8 ± 173.5	490.7	0.76 ± 0.04	0.75 ± 0.06	0.068 ± 0.004	0.069 ± 0.006
	SP-C$^{I73T/I73T}$Flp$^{+/-}$	0.62 ± 0.04	0.97 ± 0.18	978.6 ± 224.6	1027.2 ± 286.2	0.75	0.69 ± 0.03	0.061	0.058 ± 0.004

Subgroup analyses stratified by sex of inflammatory and physiological endpoints in inducible I^{ER}-SP-C$^{I73T/I73T}$Flp$^{+/-}$ and control I^{ER}-SP-C$^{I73T/I73}$Flp$^{-/-}$ mice 3, 7, 14, 28 and 42 days after 250-300 mg/kg intraperitoneal tamoxifen from Figure 3H, 4A, 5D and Supplemental Figure 14B. p > 0.05 for male versus female groups at all time points. ("-") indicates insufficient numbers after stratification or study not performed.

Supplemental Table 5 Antibodies

Histochemistry/Immunofluorescence				
Antibody	**Clonality**	**Dilution**	**Catalog Number**	**Manufacturer**
proSP-C	polyclonal	1:3000	22871	In-house
SP-B	polyclonal	1:5000	PT3	In-house
SP-D	polyclonal	1:20000	1754	In-house
HA	monoclonal	1:40000	#ab20084	Abcam
HA	polyclonal	1:40000	#AB24779	Abcam
HA	polyclonal	1:40000	#14-6756-81	eBiosciences
Mature SP-C	polyclonal	1:3000	#WRAB-76694	Seven Hills Bioreagents
LC3B	polyclonal	1:1000	#2775	Cell Signaling
SQSTM1/p62	polyclonal	1:1000	#5114	Cell Signaling
β-actin	monoclonal	1:10000	#A1978	Sigma Aldrich
Osteopontin	polyclonal	1:10000	#AB10910	Millipore
TTF-1	monoclonal	1:100	#18-0221	Invitrogen
Vimentin	monoclonal	1:100	#V2258	Sigma Aldrich
Aquaporin-5	monoclonal	1:100	#sc-9890	Santa Cruz
CD45	monoclonal	1:100	#sc-53665	Santa Cruz
Cleaved Caspase-3	polyclonal	1:10	#CP-229A	Biocare Maedical
Ki67	polyclonal	1:50	#ab16667	Abcam

Epithelial Cell Purity Analysis				
Antibody	**Fluorochrome**	**Clone**	**Catalog Number**	**Manufacturer**
EPCAM	PE	G8.8	12-5791-81	Thermo Fisher Scientific
CD45	FITC	30-F11	103108	Biolegend
Viability Dye	eFluo780	N/A	65-0865-14	eBiosciences
Flow Cytometry Analysis				
Antibody	**Fluorochrome**	**Clone**	**Catalog Number**	**Manufacturer**
CD16/32	-	FCR-4G8	MFCR00-4	eBiosciences
SiglecF	PE-CF594	E50-2440	562757	BD Biosciences
CD11b	eFluo450	M1/70	101205	eBiosciences
CD45	FITC	30-F11	103108	Biolegend
CD11c	BV705	HL3	561022	Biolegend
Ly6G	AF700	1A8	561236	Biolegend
CD64	APC	X54-5/7.1	139305	Biolegend
MHC-II	PE-Cy5	M5/114.15.2	107611	Biolegend
Ly6C	BV510	HK1.4	128033	Biolegend
Viability Dye	eFluo780	N/A	65-0865-14	eBiosciences
Immunofluorescence Secondary Antibodies				
Antibody	**Fluorochrome**	**Clone**	**Catalog Number**	**Manufacturer**
Goat anti-mouse IgG	Alexafluor594	Polyclonal-	115-585-146	Jackson Immuno Research
Goat anti-Rabbit IgG	AlexaFluor594	Polyclonal	111-585-144	Jackson Immuno Research
Goat anti-mouse IgG	AlexaFluor488	Polyclonal	115-545-062	Jackson Immuno Research
Goat anti-Rabbit IgG	AlexaFluor488	Polyclonal	111-545-144	Jackson Immuno Research

Supplemental Table 6. qPCR Primers

Taq Man PCR Primers		
Gene	**Catalog Number**	**Manufacturer**
Sftpc	#Mm00488144_m1	Applied Byosystems/Thermo fisher Scientific
Sftpb	#Mm00455678_m1	Applied Byosystems/Thermo fisher Scientific
***Ccl2* (MCP-1)**	#Mm00441242_m1	Applied Byosystems/Thermo fisher Scientific
***Ccl17* (TARC)**	#Mm01244826_g1	Applied Byosystems/Thermo fisher Scientific
***Cxcl1*(GroA/KC)**	#Mm04207460_m1	Applied Byosystems/Thermo fisher Scientific
Il6	#Mm00446190_m1	Applied Byosystems/Thermo fisher Scientific
Il5	#Mm00439646_m1	Applied Byosystems/Thermo fisher Scientific
***Cxcl11* (eotaxin)**	#Mm0041238_m1	Applied Byosystems/Thermo fisher Scientific
***tgfb1* (TGF-β1)**	#Mm01178820_m1	Applied Byosystems/Thermo fisher Scientific
Col3a1	#Mm01254476_m1	Applied Byosystems/Thermo fisher Scientific
18S	#Mm03928990_g1	Applied Byosystems/Thermo fisher Scientific