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Supplementary Materials for

Sentinel *p16^{INK4a}*+ cells in the basement membrane form a reparative niche in the lung

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MATERIAL AND METHODS

Generation of INKBRITE mouse model

Three H2B-GFP cassettes were cloned in tandem into one cassette with each separated by a 2A self-cleaving peptide (3X 2A-H2B-GFP). The 3X-2A-H2B-GFP fusion cassette was then inserted into bacterial artificial chromosome (BAC) containing the *Cdkn2a* locus (BAC clone RP24-322D20 from the RPCI-24 C57BL/6J male mus musculus BAC library http://bacpac.chori.org/library.php). The 3X 2A-H2B-GFP cassette was inserted in frame with the $p16^{INK4A}$ reading frame in exon 2 at the identical insertion site as the 3MR model as previously described *(5)*. The insertion site prevents the production of a full length $p16^{INK4a}$ transcripts and also produces a premature stop codon in the p19ARF reading frame within the shared exon 2 of the two transcripts, so no mature transcripts of p16 or p19 are produced by the BAC. Furthermore, the insertion frame of the cassette would produce a frame shift mutation in the p19ARF reading frame without translation of the H2B-GFP protein, thus only $p16^{INK4A}$ expression is reported. A neo cassette 3' to the 3X 2A-H2B-GFP cassette used for selection was removed before the BAC injection. Pronuclear injection of the BAC was performed in eggs from C57BL/6 strain donors and implanted to establish founders. One single founder sequenced to determine a single integration site in chromosome 18 was used for all subsequent experiments.

Human Lung Tissue

Donor lungs rejected for transplantation were received from Donor Network West. Lungs were rejected for various reasons, including a mismatch in sex, race, size, age, geography

between the donor and available recipients, or other elements of the donor history or clinical course. Our studies utilized donors without prior history of lung diseases. Peripheral regions of the lungs were obtained for cellular extraction.

Animal Studies

All animals were housed and treated in accordance with the Institutional Animal Care and Use Committee (IACUC) protocol approved at the University of California, San Francisco Laboratory Animal Resource Center (LARC). Generation and genotyping of the *Gli1*$^{cre/ERT2}$, *R26R*^{EYFP}, *R26R*^{tdTomato}, *Scgb1a1*^{creERT2}, Dermo1^{cre}, and NOD-scid mice (NGS^{TM}) mouse lines were performed as previously described by The Jackson Laboratory. The *Ereg* null mouse line was purchased from Mutant Mouse Resource and Research Centers (MMRRC, #0161510-UNC). The *p16*$^{flox/flox}$ allele as previously described was a gift from Ned Sharpless. The *p16*^{creERT} mouse line was previously described (*15*). 8-12 week old littermate mice were gender balanced and randomly assigned to experimental groups. A minimum of three mice was used in each experimental group. For lineage tracing studies, tamoxifen was dissolved in corn oil and administered intraperitoneally (IP) at 200 mg/kg body weight per day for 3-5 consecutive days. Animals were injured with naphthalene by administering naphthalene dissolved in corn oil IP at 150-300 mg/kg one time. Lung tissue was collected at 7, 14, or 28 days post injury. Lipopolysaccharide, LPS, (Sigma, cat# L2880-25MG) was delivered intranasally once at 5mg/kg body weight in 50µl of sterile 1X PBS and tissue was collected 3 days post injury. Controls received 50µl of sterile 1X PBS and considered 0 days post injury. Dasatinib (5mg/kg, VWR, Cat#101758-296) and Quercetin (25mg/kg, Selleck Chemical, cat#50-136-2357) were administered orally daily for

2 weeks (total of 10 doses). Both compounds were dissolved in 4 % DMSO, 30% PEG300 (Sigma, Cat#91462-1KG), 5% Tween80 (Sigma, Cat#RES3063T-A103X), 61% dH_20. EdU (Santa Cruz Biotechnology, Cat# sc-284628) administration was a single or weekly intraperitoneal (IP) injections at 50mg/kg concentration in 1% DMSO and 1X PBS. Il1b (Biolegend, Cat# 575102) was delivered intranasal at 1µg per mouse in 50ul 1X PBS. BrdU (Sigma, Cat# 50-188-2663) administration was performed *ad libitum* at a concentration of 0.5 mg/ml with 1% sucrose in dH_2O. BrdU water was refreshed twice a week for the duration of each experimental period.

Intratracheal Cell Transfer

In brief, recipient NGS™ mice received a single dose of Bleomycin (UCSF Pharmacy Cat#10189569) at 1.5U/kg 10 days prior to intranasal delivery of fibroblasts. INKBRITE mouse fibroblasts were harvest then pulsed with Cell Trace™ Far Red (CTFR, Thermo Fisher, cat# C34564) at 1µM for 1 million cells per 1 ml of 1XPBS for 20 minutes at 37ºC. Cells were rinsed with DMEM-F12 with 10%FBS and 1%PS and transplanted (3.5million cells/mouse) into the lungs of NGS™ mice *via* intranasal inhalation. Lungs from NGS™ mice were collected 4 days later for FACS and histological analysis. Cryosections for histology were stained with DAPI and visualized using Zeiss Lumar V12 microscope.

Hyperoxia treatment

INKBRITE litters were randomized at birth and assigned to either room air (normoxia) or hyperoxia. Nursing mothers and their pups were transferred into the hyperoxia chamber (BioSpherix) on P4 and maintained in hyperoxia until harvest on P8 for FACS analysis. The

chamber was attached to a medical oxygen source controlled by a ProOx single gas controller (BioSpherix) which was set to maintain 75% oxygen. Mice remained under typical 7a-7p light cycling, and chambers were checked daily to monitor temperature, humidity, and gas controller function.

Histology and Immunofluorescence

Detailed description on harvesting the lung, cryo/paraffin embedding of tissues, sectioning, and immunohistochemistry methods are previously described *(20)*. Primary antibodies and dilution used for histology include: anti-GFP 1:250 (chicken, Aves Labs, #1020, and goat, Abcam, #6673), anti-SCGB1A1 1:500 (goat, Santa Cruz, #9772), anti-beta IV Tubulin 1:200 (mouse, Abcam, #11315), anti-laminin 1:200 (rabbit, Sigma, #9932), anti-PDGFRα (rabbit, Cell Signaling, #3174), anti-BrdU 1:200 (rat, Abcam, #6326), anti-Collagen 1 1:200 (rabbit, Abcam Cat#21286), anti-F4/80 1:100 (rat, ebioscience, Cat#14-4801-82), anti-E-cadherin 1:200 (rabbit, Cell Signaling Cat#24E10). For cell culture staining, primary antibodies and dilutions are: anti-yH2AX 1:250 (rabbit, Cell Signaling, Cat#9718T), Ki67 1:100 (rabbit, Abcam, Cat#16667), anti-p65 1:500 (mouse, Santa Cruz Biotechnology, Cat#sc-8008), anti-phospho Erk(1/2), (rabbit, Cell Signaling cat#4695S). Sections were imaged for quantification on a Zeiss Lumar V12 microscope. At least three samples per genotype/condition were used, and at least 5-10 randomly selected sections were chosen for each sample. Cell counts for SGCGB1A1+, Brdu+, GFP+, tdTomato+, F4/80, p-Erk, and DAPI cells were performed with Fiji using the “Cell Counter” plug-in. For quantification of airway length and Collagen area, the freehand line feature coupled with “Measure” was utilized. Images were blinded to experimental condition for quantification.

Cleared Thick Slice Imaging

Mouse lung was extracted as above and fixed in 4% PFA overnight at 4ºC. Tissue was washed with changes of 1X PBS for 2 hours and cut into 200 µm sections on a vibratome. Sections were blocked for at least 1 hour in 0.3% Triton X, 5% FBS, 0.5% BSA in PBS with azide at 4ºC. Sections were then incubated in primary antibodies in PBS with 0.15% Triton X, 7.5% FBS, and 0.75% BSA for 1-3 days at 4ºC. Sections were washed with 0.15% Triton X and incubated in secondary antibodies overnight at 4ºC. After several washes with 0.15% Triton X, DAPI was added in PBS for 30 minutes and sections were rinsed again. Sections were cleared using RIMS (40 g Histodenz, 30 mL PBS, 5 µL Tween 20, 50 µL 10% azide) for 30-60 min and mounted. Images were taken on a Nikon A1R multi-photon confocal microscope. Surfaces were created for each marker of interest to generate images.

Lung digestion and Fluorescence Activated Cell Sorting (FACS)

For mouse, whole lung was dissected from adult animals and tracheally perfused with a digestion cocktail of Collagenase Type I (225 U/mL), Dispase (15 U/mL) and Dnase I (50 U/mL) and removed from the chest. The lung was further diced with a razor blade and incubated in digestion cocktail for 45 mins at 37ºC with continuous shaking. The mixture was then washed with sorting buffer (2% FBS and 1% Penicillin-Streptomycin in DMEM). The mixture was passed through a 70 µm cell strainer and resuspended in red blood cell (RBC) lysis buffer, then passed through a 40 µm cell strainer. Cell suspensions were incubated with the appropriate conjugated antibodies in sorting buffer for 30 min at 4ºC and washed with sorting buffer. Doublets and dead cells were excluded based on forward and

side scatter and DRAQ7 (Cat#7406S; Cell Signaling Technologies) or DAPI fluorescence, respectively. Immune and endothelial cells were excluded using anti-CD45 (PECy7; Cat#25-0451-081; Invitrogen; used 1:200 or BV421; Cat#563890, BD) and anti-CD31 (APC/Fire750; Cat#102528; BioLegend; used 1:200 or BV421; Cat#48-0311-82, Fisher), respectively. Epithelial cells were also excluded using anti-CD326/Epcam (BV421; Cat#563214; BD; used 1:200), and finally, PDGFRα+ cells were included (APC; Cat#17-1401-81; Thermo; used 1:200). After selection for PDGFRα+ cells, the GFP- and GFP+ fibroblasts were further separated and collected into sorting buffer. Immune (CD45-biotin, Biolgened, Cat#103104), epithelial (CD326-biotin, Biolegend, Cat#118204) and endothelial (CD31-Biotin, Biolegend, Cat#102404) cells are removed with EasyStep™ mouse streptavidin RapidShperes™ (StemCell, Cat#19860A), when applicable. For fibroblasts separation FACS panel we used the same antibodies for lineage exclusion of immune, epithelial, endothelial cells, additionally excluding erythroid lineage Ter119+ (Biotin, Invitrogen, Cat#MA5-17819, used at 1:200) and endothelial CD146+ (biotin, Biolegend, Cat#134716) cells. To separate the adventitial mesenchyme with used live lineage negative (CD45-ter119-Epcam-CD146-CD31-) Sca1+ (PE-Cy7, Abcam, Cat#ab93537) marker. The peribranchial mesenchymal population was separated by lineage-Sca1-CD9+ (PE, Sigma, Cat#SAB4700570-100UG) and lastly the alveolar mesenchyme was separated as the lieaneage-Sca1-CD9- PDGFRa+ population. To isolate monocytes, we used the following panel of antibodies: CD45 (Invitrogen, Cat#25-0451-82), CD11b (Biolegend, Cat#101241), Ly6c (Invitrogen, Cat#12-5932-80), and Ly6g (Biolegend, Cat#127624). We used the following antibodies for our INKBRITE FACS immune panel: SinglecF (BD, Cat#740956), CD11b (Biolegend, Cat# 101241), CD11c (Biolegend, Cat# 117318), CD3 (Biolegend, Cat#

100347), CD19 (BD, cat# 11554), CD45 (BD, Cat# 564279), MHCII (BD, Cat#748845), CD64 (BD, Cat# 558539), Ly6G (Biolegend, Cat#127624), and Ly6c (Invitrogen, Cat# 12-5932-80). For *Scgb1a1*^{creERT2}*:R*^{tdt} epithelial cells, a live/dead stain was done first then tdTomato+ cells were sorted and collected into sorting buffer. Analysis was performed using FlowJo software.

For human, we followed established protocol extensively described our prior report (*20*). In brief, human tissues was digested in sterile HBSS buffer containing 15 U/ml Dispase II (Thermo fisher, Cat#17105041), 225 U/mL Collagenase Type I (Thermo Fisher, Cat#17100017), 100 U/mL Dnase I (Sigma, Cat#DN25), and 1% penicillin/streptomycin for about 2 hours. Single cell suspension was depleted of red blood cells, blocked with human Fc block reagent, and depleted of immune (CD45-biotin, Biolegend, Cat#304014), macrophages (CD11b-Biotin, BD, Cat#557754), and endothelial cells (CD31-biotin, Biolegend, Cat#303120) using EasyStep™ streptavidin RapidShperes™ (StemCell, Cat#50001). Club cell progenitors were collected by taking live lineage (CD45-CD11b-CD31-) negative HT2-280- (Terrace Biotech, Cat# TB-27AHT2-280), CD66c+ (BD, Cat#742689) epithelial cells as previously described (*25*).

Click-iT Plus EdU

Click-iT Plus EdU pacific Blue Kit (Cat# C10636, Thermo Fisher) was used for EdU FACS experiments. In brief, lung cells were harvest as describe above and stain with primary antibodies to mark each lineage. After primary antibody incubation cells were fixed and

permeabilized using Click-iT fixative and permeabilization and wash reagent. To detect Click-iT^{Tm} EdU, the cells were incubated in Plus Reaction cocktail for 30 minutes at room temperature protected from light. Analysis was performed using FlowJo software. EdU uptake index was calculated by taking the %EdU+ / %EdU- populations for each representative cell type.

Single Cell Sequencing and Analysis

Single cell suspension of the lung was prepped and sorted as described above. Cells were loaded onto a single lane per sample as 1000cells/µl into the Chromium^{TM} Controller to produce gel bead-in emulsions (GEMs). GEMs underwent reverse transcription for RNA barcoding and cDNA amplification, with the library prepped using the Chromium Single Cell 3' Reagent Version 3 kit. Each sample was sequenced in 1 lane of the HiSeq2500 (Illumina) in Rapid Run Mode. To build transcript profiles of individual cells the CellRanger v3.0.3 software with default settings was used for de-multiplexing, aligning reads with STAR software to mouse genome GRCm38, and counting unique molecular identifiers (UMIs). We used the Seurat R package along with a gene-barcode matrix provided by CellRanger for downstream analysis. In total, we filtered the data in 2 different steps. We first filtered the dataset by only accepting cells that expressed a minimum of 200 genes and genes that were expressed in at least 3 cells. The UMI were log-normalized and we to the identified genes with high expression and those are variable we used the mean variance relationship method. Our second filtered was set to accept cells with less than 6500 unique gene counts and up to 15% mitochondrial gene counts. Using regress out function, we mitigate the effect of mitochondrial gene counts. Next, we used principle component analysis (PCA) to identify components that can be found within our dataset for

unsupervised clustering. We used the JackStrawPlot function in the Seurat function to create Scree plots were and compare p-value (significance) for each PC. We selected 10 different PCA's for clustering of the 4600 cells. Clustering results were visualize using the Uniform Manifold Approximation and Projection (UMAP) algorithm in the Seurat package. 4 clusters were identified in p16+ cells. The standard workflow was carried out using ScaleData, RunPCA, RunUMAP, FindNeighbors and FindClusters functions to cluster cells. For individual gene visualization on all clusters, we used the FeaturePlot function. For interactome analysis we used the vignette NicheNet package from Seurat.

Bulk RNA Sequencing and Analysis

For bulk RNAseq sequencing was done using Sanger/Illumina 1.9, and there were an average of 45 million reads per sample with a total of 3 biological replicates per condition. Quality control of reads was conducted by using FastQC (Babraham Bioinformatics). Ligation adaptors were removed using the Cutadapt and Sickle. Sequencing reads were aligned using HISAT and assembled with Stringtie software to the reference genome *Mus musculus*, UCSC version mm10. All gene counts of the biological replicates were concatenated while running DEseq2 for differential gene expression (DGE). Upstream regulators were generated with Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). To identify previously annotated SASP, we crossed referenced our gene list with previously identified SASP factors (*22*). To assess the enrichment for SASP factors in $p16^{INK4a}$+ fibroblasts, we calculated the statistical significance of the overlap between all the annotated SASP factors (gene set 1, Table S1) and total number of SASP factors upregulated in $p16^{INK4a}$+ fibroblasts (gene set 2, Table S1) utilizing the webtool on nemate.org, which calculates the

representation factor along with the p value of overlap as a measure of the significance of enrichment utilizing hypergeometric probability testing.

Cell Culture

Freshly isolated PDGFRα+ cells from *INBRITE* lungs (GFP- or GFP+) were cultured on gelatin-treated tissue culture plates in DMEM-F12 with 10% FBS and 1% Penicillin-Streptomycin (PS). Media was refreshed every other day and primary lung mesenchymal cells were maintained for no more than three passages. For cell size assessment, the cells were fixed on the plate and stained with DAPI and F-actin with phalloidin-555 (Thermo, #34055) for 1 hour at room temperature. Phalloidin was used at (125 U/ml) concentration. Cell size was measured by using the freehand line feature to outline each cell from 40X magnification images and Measure to get area. All cells in GFP-, GFPlow, GFPhigh, or GFP+ fibroblasts wells were assayed and averaged and compared for statistical analysis.

LPS *in vitro* stimulation

Freshly sorted GFP-, GFPlow, GFPhigh or GFP+ fibroblasts (CD45-Epcam-CD31-PDGFRα+cells were plate and use within 3 passages. LPS (Sigma, Cat# L2880-25MG) was used at 10μg/ml in media (DMEM-F12, 10%FBS, 1%P/s) for 1 hour for nuclear p65 detection. To collect samples for qPCR analysis we treated cells at the same concentration for 6 hours and collected in 500μl trizol. For stimulation washout experiment media was refreshed every 2 days. BAY11-7082 (Sigma, Cat#B5556-10MG) was used at a concentration of 500nM for 6 hours.

Monocyte fibroblast co-culture

Freshly isolated monocytes (live CD45+CD11b+Ly6g-Ly6c+) from wildtype mice were placed directly onto already plated INKBRITE fibroblasts (passage 1) in DMEM-F12 10%FBS, 1%PS with recombinant murine M-CSF (10ng/ml, Peprotech, Cat#315-02-10UG). After 24 hours co-culture GFPhigh, low, neg fibroblasts were collected for RNA work with FACS.

Cell Trace Far Red Assay

Fibroblasts were harvested and allowed 72-hour period to settle on a culture dishes. We detached, counted, and pulsed cells in suspension (1 million cells / ml of 1X PBS) with Cell Trace^{TM} Far Red (Thermo Fisher, Cat# C34564) at 1μM for 20 minutes at 37°C. Stained cells were rinsed twice with media for 5 and 15 minutes at 37°C 5% CO_2 with centrifugation between each rinse, 550g for 5 minutes. After last centrifugation, cells were counted and all conditions were seeding at 50% confluency in normal media (DMEM-F12, 10%FBS, 1%PS). Serum starve controls were placed in DMEM-F12 with only 1%PS.

Lentivirus infection

Mesenchymal cells were seeded and infected the following day with dual-lentivirus (containing the TRE-p16/rTTA construct, $p16^{INK4a}$ shRNA, or control shRNA). Dual-lentivirus was used at a concentration of $2X10^3$ PFU per 1 ml of DMEM-F12 with 10% FBS with helper virus and polybrene at 5μg/ml. On day 2 cells were washed with 1X PBS 4 times and placed on regular media (DMEM-F12, 10%FBS, 1% PS). Doxycycline (1μg/ml) treatment began 72 to 96 hours later.

Adenovirus Cre recombinase infection

Mouse $p16^{flox/flox}$ lung fibroblasts were isolated and plated. The settled fibroblasts were infected with Adenovirus-cre recombinase (UI Viral Vector Core, Cat#Ad5CMVCre) at MOI of 1250:1 and polybrene at 5µg/ml in normal media for 72 hours.

Cytospin

Freshly sorted cells were rinsed in 1X PBS for 5 minutes spun down at 550g for 5 minutes then fixed with 4% PFA for 15 minutes at room temperature. After fixation cells were rinse with 1X PBS, centrifuged down to get rid of supernatant, and then resuspended in 50µl of fresh 1X PBS. Cells were spun onto superfrost slides at 340g for 5 minutes. Stain for F-actin and DAPI was performed as described above. For SABgal stain we rinsed fixed cells with 1mM $MgCl_2$ at room temperature followed by X-gal stain (1mg/ml X-gal 0.12 mM $K_3Fe[CN]_6$ and $K_4Fe[CN]_6$ in 1X PBS at pH 6.0) overnight at 37°C without CO_2.

3D organoid assay

For mouse, *INKBRITE* adult lungs were FACS sorted for CD45-Epcam-CD31-PDGFRa+GFP-, GFPlow or GFPhigh, GFP+ mesenchymal cells and epithelial cells were sorted from *Scgb1a1*^{creERT2}*:R*^{tdt} tamoxifen induced animals. Scgb1a1+ epithelial cells and GFP-, GFPlow, GFPhigh, or GFP+ mesenchymal cells were co-cultured ($5x10^3$ epithelial cells: $3x10^4$ mesenchymal cells/well) in a modified MTEC media diluted 1:1 in growth factor reduced matrigel. Modified MTEC culture media is comprised of small airway basal media (SABM) with selected components from SAGM bullet kit (Lonza) including Insulin,

Transferrin, Bovine Pituitary Extract, Retinoic Acid, and human Epidermal Growth Factor. 0.1 µg/mL cholera toxin, 5% FBS, and 1% Penicillin-Streptomycin were also added. Cell suspension-matrigel mixture was placed in a transwell and incubated in growth media with 1 µM ROCK inhibitor in a 24 well plate for 48 hours, after which the media was replenished every other day (lacking ROCK inhibitor). Each experimental condition was performed in triplicates. BAY11-7082 (50nM, Sigma, Cat#B5556-10MG) was added to the media after 48 hours and replenished in every media change. Colonies were assayed after 12-14 days. Each transwell was imaged using EVOS M5000 at 1.25X magnification and quantified on ImageJ blind to experimental condition.

For human, Cell Trace^{Tm} pulsed mesenchyme was separated by FACS into proliferating (p16low) and non-proliferating (p16hi) populations. Mesenchyme was co-cultured with human club cell progenitor cells with same ratios as described above and kept in cultured 14 days with media replenished every 48hours. Each transwell was imaged using EVOS M5000 at 1.25X magnification and quantified on ImageJ blind to experimental condition.

Quantitative RT-PCR

Total RNA was isolated from epithelial cells isolated from organoid assays or cultured primary lung fibroblasts using the PicoPure RNA Isolation Kit (Thermo Fisher, Cat#KIT0204) or the RNeasy Kit (Qiagen, Cat#74034), following the manufacturers' protocols. RNA from mouse lung tissue was obtained by removing the entire left lobe, homogenizing in trizol, and extracting using the E.Z.N.A Total RNA Kit (Omega, Cat#R6834-01) following manufacturer instructions. cDNA was synthesized from total RNA

using the SuperScript Strand Synthesis System (Invitrogen, Cat#11904018). Quantitative PCR was performed using the SYBR Green system. Primers are listed in Table S1. Relative gene expression levels after qRT-PCR were defined using the ΔΔCt method and normalizing to *Gapdh*, except in the case of the BAY inhibitor experiments that used *Rpl19* for normalization. We used a minimum of three biological replicates for each genotype/condition. One-tailed t tests were used to perform statistical analysis of fold changes between genotypes/conditions when one comparison is made between two samples, and two-way analysis of variance (ANOVA) was used to test statistical significance across conditions and genotypes when more than one comparison is made for more than two samples.

Gene primers	**Forward**	**Reverse**
Mouse		
Gapdh	ggcccctcctgttattatgggggt	ccccagcaaggacactgagcaaga
p16^{INK4a}	tcctcgcagttcgaatctg	aactctttcggtcgtacccc
Scgb1a1	ggatgccagataaccagactct	atgaagatcgccatcacaatcac
Ereg	ccgaggataactgtaccgcc	ctcacatcgcagaccagtgt
Il6	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
Cdkn1a	taaggacgtcccactttgcc	cgtctccgtgacgaagtcaa
p19^{ARF}	tggtcactgtgaggattcagc	ttgcccatcatcatcacctgg
Lmnb1	gggaagtttattcgcttgaaga	atctcccagcctcccatt
Col1a1	ccaagaagacatccctgaagtca	tgcacgtcatcgcacaca
Rpl19	atgtatcacagcctgtacctg	ttcttggtctcttcctccttg
Ccl8	tctacgcagtgcttctttgcc	aaggggatcttcagctttagta
Tnfsf10	ggaagacctcagaaagtggcag	tttccgagaggactcccaggat
Tnfaip3	ggtgaccctgaaggacagtg	tcaaacctaccccggtctct
Mmp3	ctctggaacctgagacatcacc	aggagtcctgagagatttgcgc
Tnfa	ctgaacttcggggtgatcgg	ggcttgtcactcgaattttgaga

Human		
EREG	cttatcacagtcgtcggttccac	gccattcagacttgcggcaact
CCL8	tatccagaggctggagagctac	tggattccctgacccatctctc
TNFSF10		
IL6	agacagccatctacctcttcag	ttctgccagtgcctctttgctg
MMP3	cactcacagacctgactcggtt	aagcaggatcacagttggctgg

shRNA target	**Sequence**
p16^{INK4a}	ACTGAATCTCCGCGAGGAAAGCTCGAGCTTTC CTCGCGGAGATTCAGT

Immunofluoescence Image Quantification

Sections were imaged for quantification on a Zeiss Lumar V12 microscope. At least three samples per genotype/condition were used, and at least 5-10 randomly selected sections were chosen for each sample. Cell counts for SGCGB1A1+, Brdu+, GFP+, RFP+, pERK, and DAPI cells were performed with Fiji using the “Cell Counter” plug-in. Immunofluorescence staining of cultured cells were done in triplicate wells with at least 5 images per well. For quantification of airway length, the freehand line feature couple with Measure. Cell size was measured by using the freehand line feature to outline each cell from 40X magnification images and Measure to get area. All cells per GFP-, GFPlow, GFPhigh or GFP+, fibroblasts were averaged and compared for statistical analysis.

Statistical Analysis

All statistical analyses were performed in GraphPad Prism 6.0. One-tailed t tests were used to perform statistical analysis of fold changes between genotypes/conditions when one comparison is made between two samples, and two-way analysis of variance (ANOVA)

was used to test statistical significance across conditions and genotypes when more than one comparison is made for more than two samples. Data in graphs are presented as mean ± SEM.

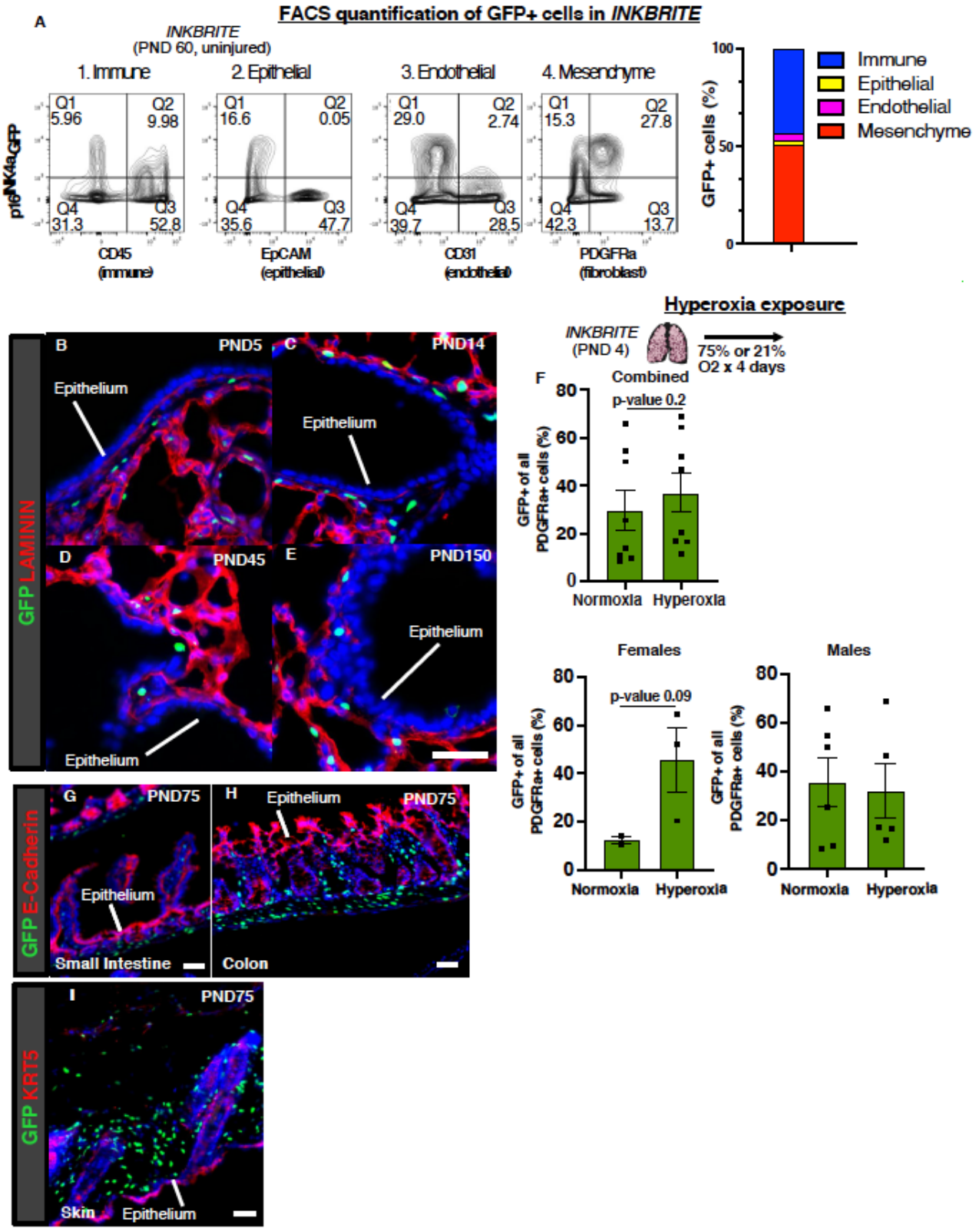


Fig. S1. Detection of *p16*^{INK4A}+ cells in the *INKBRITE* reporter.

(A) Lineage marker analysis of GFP+ cells on FACS from uninjured *INKBRITE* lungs and quantification of % GFP+ cells in each major lung lineage. (**B** to **E)** IHC of GFP overlaid with laminin during alveologenesis (PND5,14) and homeostasis (PND45,150) phases of postnatal lung maturation. (**F**) Bar graphs representing the percent of GFP+ fibroblasts (PDGFRα+) of PND4 pups either kept on normal or hyperoxic conditions (n=8 per condition). (**G** to **I**) IHC of GFP in skin, small intestine, and colon at PND75. AW=airway,PND = postnatal day. Scale bars 100um. Each point in graph represents one animal with mean ± s.e.m. All p values determined by one-tailed t-test.

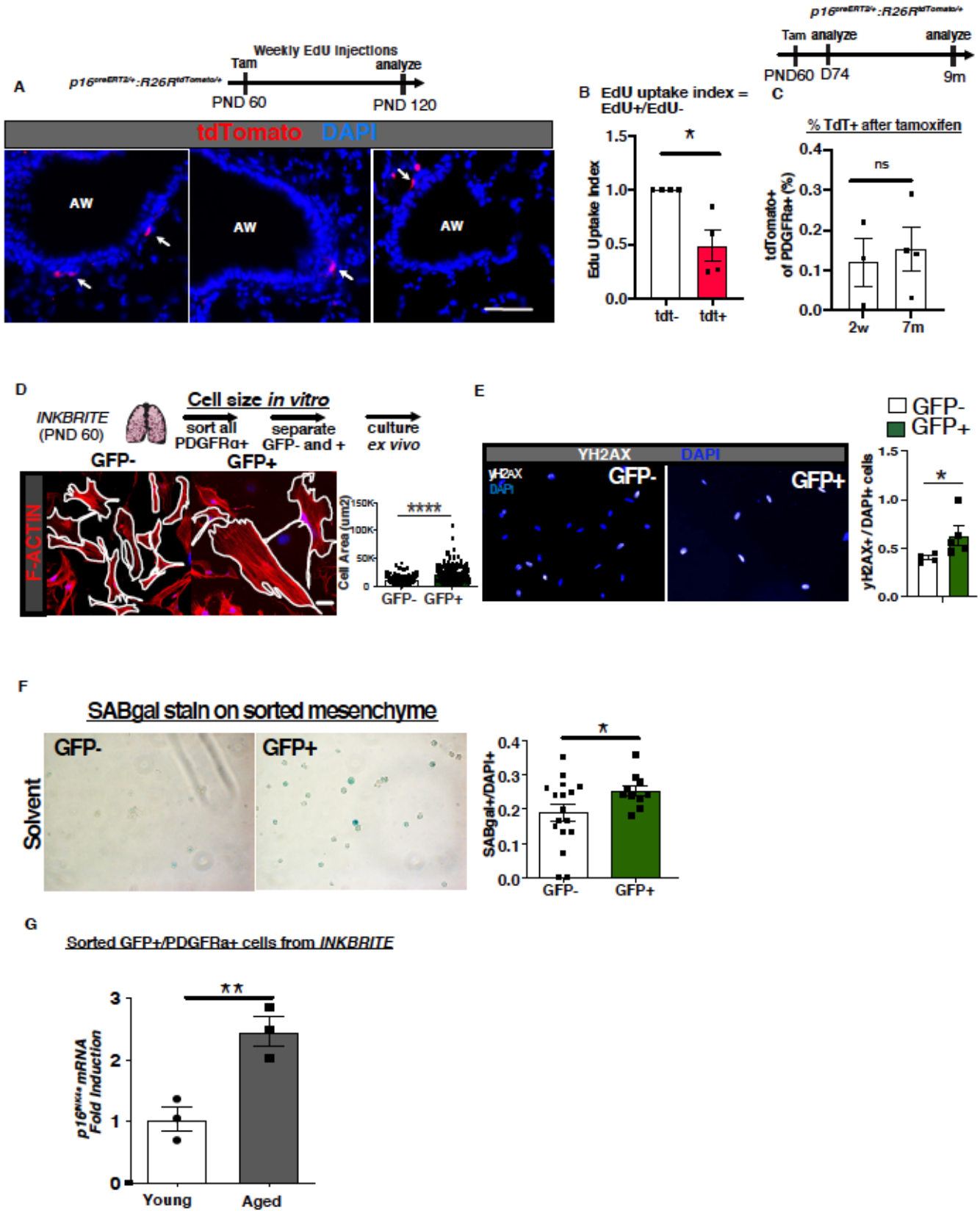


Fig. S2. Enrichment of senescent markers in *p16^{INK4A}*+ fibroblasts.

(**A**) Schematic showing tamoxifen induction of $p16^{creERT2/+}$:$R26R^{tdtomato+}$ mice followed by weekly EdU injections. Images showing tdTomato+ cells adjacent to airways. (**B**) Quantification of EdU uptake index of tdTomato- and tdTomato+ cells after 2 months of weekly Edu injections (n=4 per condition). (**C**) Quantification of % tdTomato+/PDGFRα+ cells after 2 and 7 weeks post tamoxifen administration. (**D**) Images and quantification of cell size of PDGFRα+ cells grown in culture at passage 0 (3 wells n=13-14 images per cell type). (**E**) γH2AX staining and quantification of GFP+ and – fibroblasts in uninjured *INKBRITE* lungs (n= 5-7 images per condition).(**F**) SA-β-gal staining and quantification of GFP+ and – fibroblasts in uninjured *INKBRITE* lungs (n= 10-20 images per condition). (**G**) qPCR of $p16^{INK4A}$ expression in GFP+ mesenchymal cells in young (2 month) and aged (12 month) *INKBRITE* lungs. Scale bars 100um. Each point in graph represents one animal for *in vivo* studies or distinct image for *in vitro* studies with mean ± s.e.m.). All p values determined by one-tailed t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

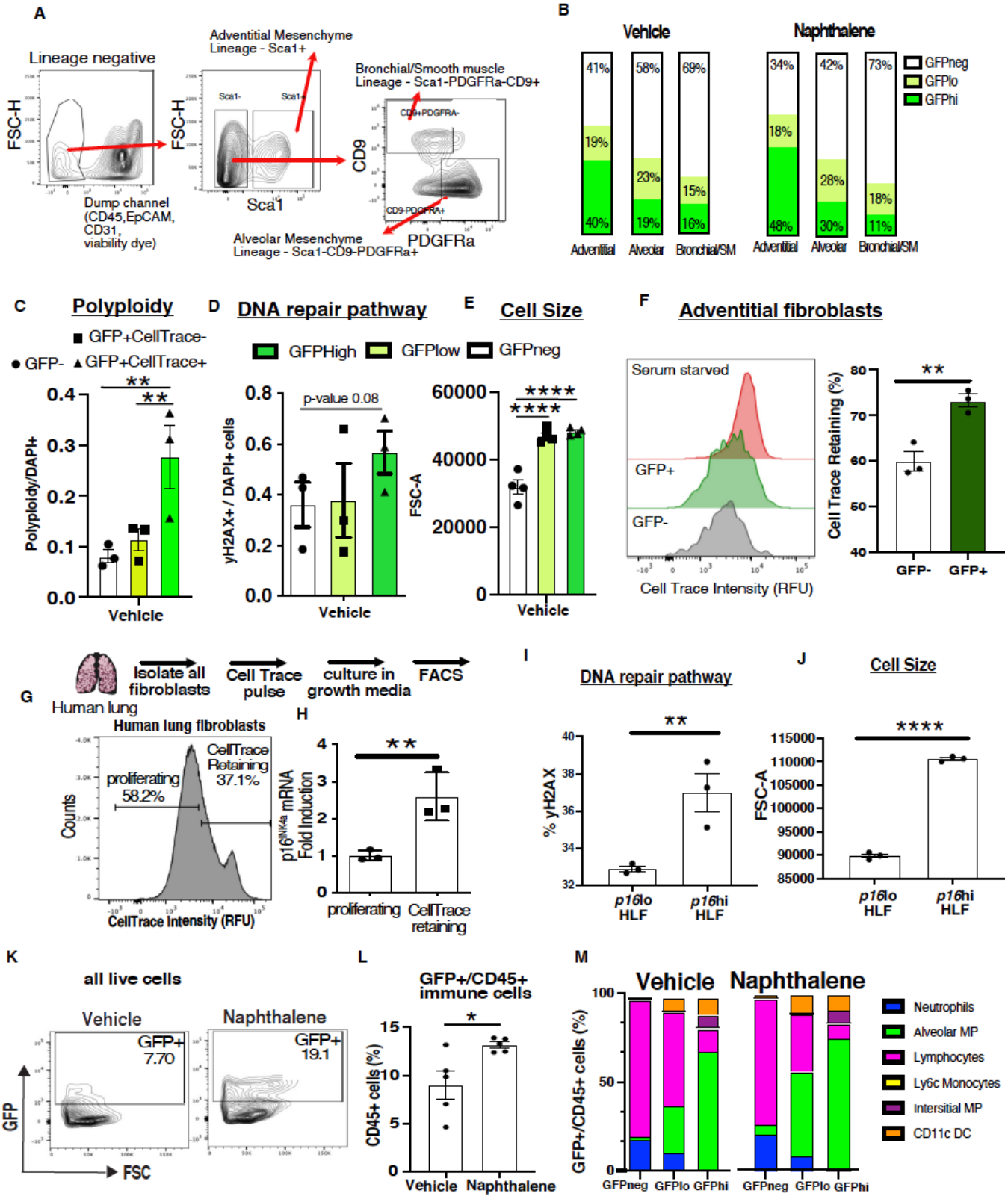
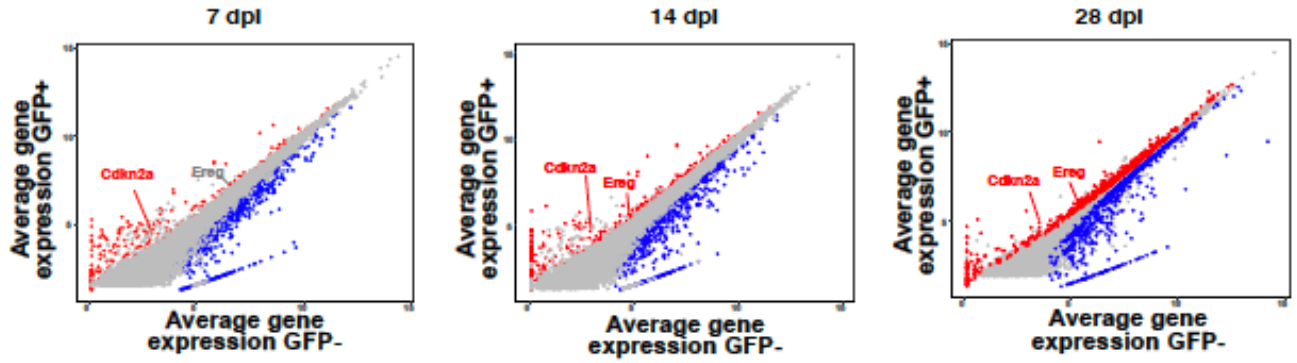


Fig. S3. Segregation of *p16*^{INK4A}-hi/lo/negative cells in the lung.

(**A**) FACS gating strategy for adventitial, peribronchial/smooth muscle, and alveolar fibroblast separation. (**B**) Distribution of GFPhi/lo/neg populations in the three main fibroblast subgroups of uninjured and naphthalene injured INKBRITE lungs. (**C** to **E**) Quantification of polyploidy, γH2AX, and cell size (FSC-A) of GFPhi/lo/neg fibroblasts from INKBRITE lungs. (**F**) Histogram showing CellTrace Far Red (CFTR) distribution of GFP- and GFP+ adventitial fibroblasts and the percentage of GFP- and GFP+ that are at cell cycle arrested (percentage of cells with serum-starved CellTrace intensity, n=3 per condition). (**G**) Human lung fibroblasts isolated from cadaveric donor (25 years old) for CTFR pulse and histogram showing CTFR distribution (Cell Trace Intensity). (**H**) qPCR of *p16*^{INK4a} expression in sorted proliferating and non-proliferating (Cell Trace retaining) human fibroblasts (n=3 per condition, 2 experiments). (**I** and **J**) Quantification of γH2AX, and cell size (FSC-A) of sorted *p16*hi or *p16*lo human lung fibroblasts**.** (**K**) FACS analysis of all live cells from the lungs of vehicle and naphthalene injured (14 dpi) *INKBRITE* animals. (**L**) quantification of the % GFP+ in all immune (CD45+) cells of vehicle and naphthalene injured (14 dpi) lungs (n=5 per condition, 2 experiments). (**M**) Immune cell distribution among GFPhi/lo/neg populations of vehicle and naphthalene injured (14 dpi) *INKBRITE* lungs. HLF = human lung fibroblasts. RFU = relative fluorescent units. Each point in graph represents one animal or one distinct image from *in vitro* study with mean ± s.e.m. All p values determined by one-tailed t-test or two-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

A



B

SASP expression in GFP+ fibroblasts

0 dpi	7 dpi	14 dpi	28 dpi
CCL8	CCL8	CCL8	COL14A1
COL24A1	COL28A1	COL16A1	COL1A1
COL27A1	HGF	COL24A1	COL1A2
COL7A1	IL6ST	COL28A1	COL24A1
IL6	LAMA1	COL6A3	COL28A1
LAMC3	LAMC3	COL7A1	COL4A5
NRG1	TNFSF10	EREG	COL5A1
		LAMA2	COL5A2
		LAMB1	COL7A1
		LAMC3	EGFR
		MMP3	EREG
		TIMP1	IL6
			LAMA2
			LAMB1
			LAMC3
			NRG1
			PLAT

C

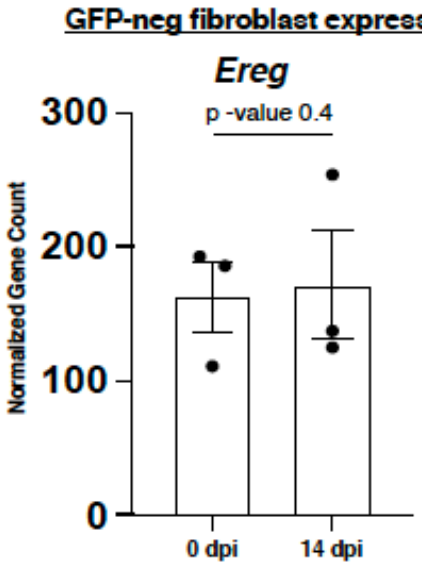


Fig. S4. Bulk RNAseq analysis of *p16^{INK4A}*+ fibroblasts in the lung. (**A**) Gene correlation plot showing expression of Cdkn2a and Ereg after injury. (**B**) Table of annotated SASP genes upregulated in p16^{INK4A}+ fibroblasts during homeostasis and injury. (**C**) Normalized gene count plot of *Ereg* expression in GFP-neg fibroblasts at 0 and 14 dpi (n=3 per condition). Dpi = days post injury. All p values determined by one-tailed t-test.

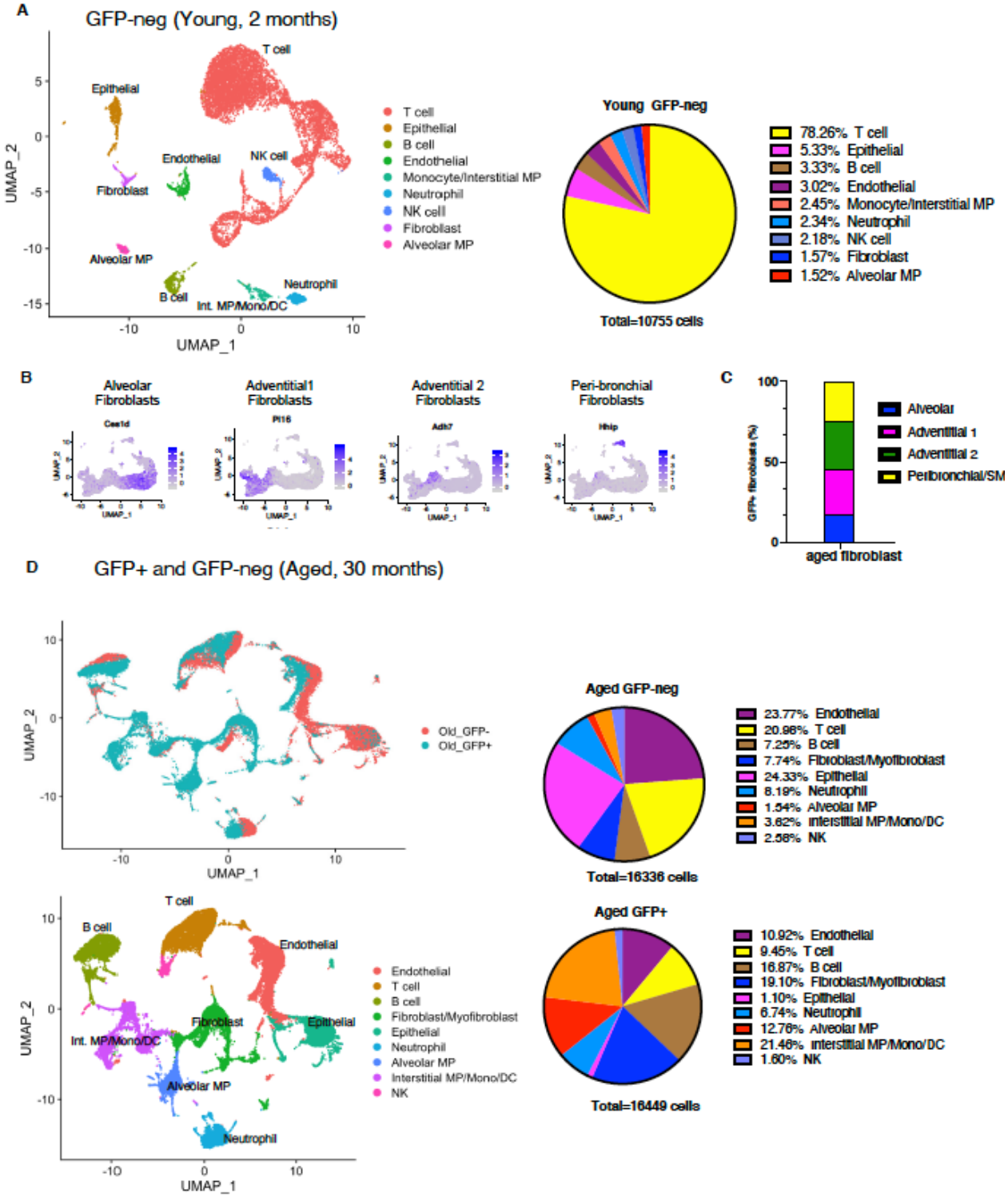


Fig. S5: Single cell RNA seq analysis of young and aged whole lung from INKBRITE mice. (**A**) Comparison between aged GFP-neg and aged GFP+ cells from INKBRITE

lungs (30 months). UMAP shows the merged Seurat objects (GFP- and GFP+) and distribution of cellular clusters. Pie charts show the proportion of different cellular lineages. (**B**) Expression of fibroblast subtype-specific genes in GFP+ fibroblasts isolated from INKBRITE lungs. (**C**) Distribution of GFP+ fibroblast subsets in aged INKBRITE lungs. (**D**) Comparison between aged GFP-neg and aged GFP+ cells from INKBRITE lungs (30 months). UMAP shows the merged Seurat objects (GFP- and GFP+) and distribution of cellular clusters. Pie charts show the proportion of different cellular lineages.

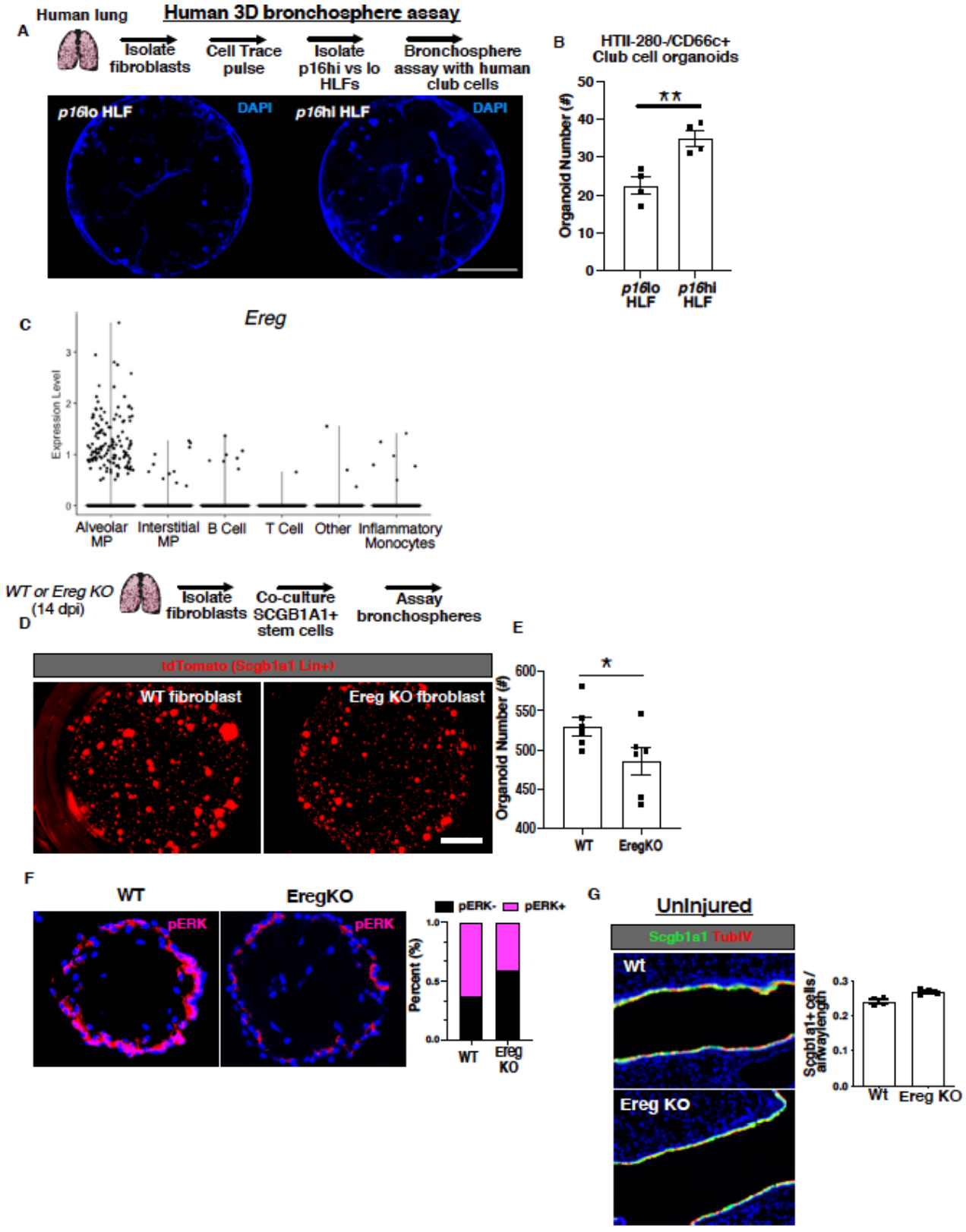


Fig. S6. *p16*^{INK4A}+ fibroblasts support epithelial regeneration.

(**A** and **B**) 3D bronchospheres generated using *p16*lo and *p16*hi human lung fibroblasts (43yrs, Male) co-cultured with HTII-280-/CD66c+ club cells (airway stem cells), imaged with DAPI counterstain and quantified for number of organoids per well. (**C)** Distribution of *Ereg* expression at the single cell level in different immune populations from 0 and 14 dpi INKBRITE lungs lungs. (**D** and **E**) Mouse 3D epithelial organoids co-cultured with mouse fibroblasts isolated from naphthalene injured (14 dpi) wildtype (WT) or Ereg knockout (KO) lungs with quantification of organoid number (n=6 for each condition). (**F**) Phospho-ERK (pERK) immunostaining in organoids and bar graph showing the percent of p-ERK+ bronchospheres cultured with WT or *Ereg*KO fibroblasts. (**G**) IHC and quantification of SCGB1A1+ club cells in the airways of *Ereg*KO vs. WT lungs during normal homeostasis (n=4 for each condition). Each point in graph represents one animal or one technical replicate from *in vitro* study with mean ± s.e.m. All p values determined by one-tailed t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

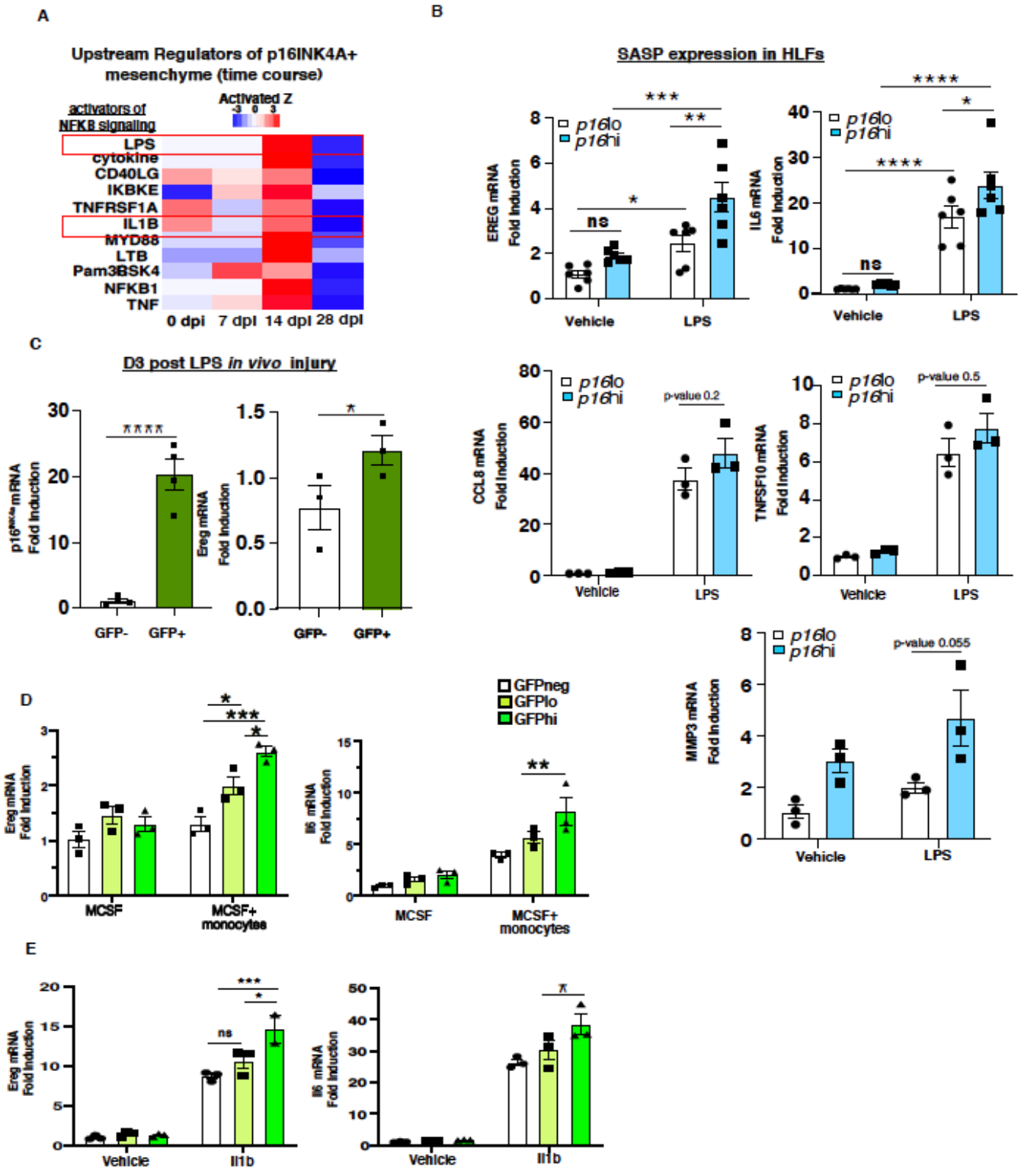


Fig. S7. *p16*^{INK4A}+ fibroblasts sense tissue inflammation.
(**A**) Heat map of Z scores of upstream regulators activating NF-κB signaling across time points in p16^{INK4A}+ fibrobasts. (**B**) SASP expression in *p16*lo and *p16*hi human lung fibroblasts (46yrs, Male; 78yrs, Female) after vehicle and 6 hour LPS stimulation (*EREG,*

IL6, CCl8, TNFSF10, MMP3; 2 experiments). (**C**) qPCR of *p16*^{INK4a} and *Ereg* expression in live-sorted GFP+ and GFP-neg fibroblasts from LPS injured (3 dpi) INKBRITE lungs (n=4 for each condition). (**D**) qPCR of *Ereg* and *IL6* in *p16*^{INK4A}hi/lo/neg fibroblasts directly co-cultured with or without monocytes (n=3 wells per conditions). (**E**) qPCR of *Ereg* and *IL6* in *p16*^{INK4A} hi/lo/neg fibroblasts treated with vehicle or recombinant Il1B for 6 hours (n=3 wells per conditions, 2 experiments). Each point in graph represents one animal or one technical replicate from *in vitro* study with mean ± s.e.m. All p values determined by one-tailed t-test or two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

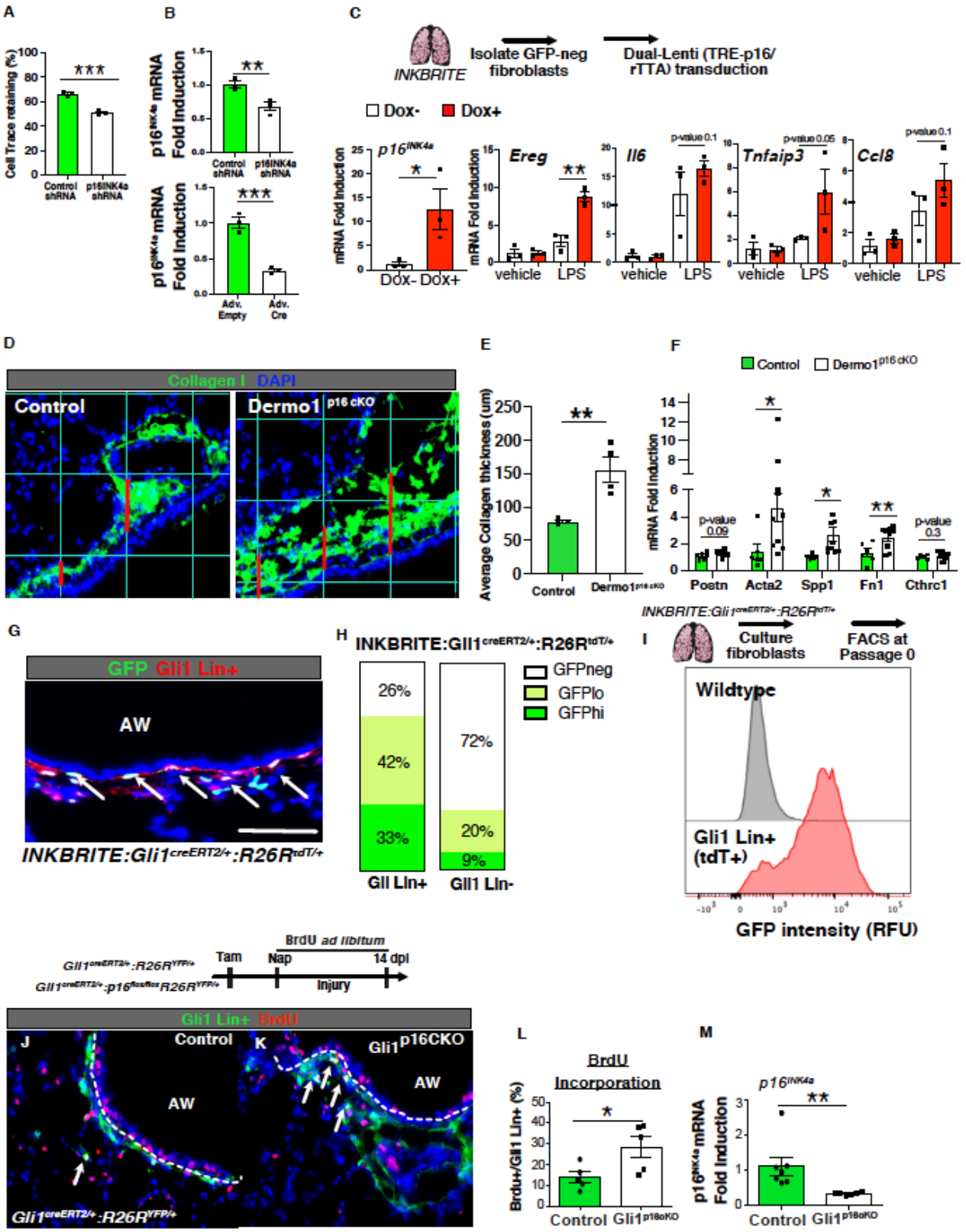


Fig. S8. Knockdown and overexpression of *p16INK4A* in lung fibroblasts. (**A**) CellTrace retention in GFP+ fibroblasts treated with control or *p16INK4a* shRNA (n=3 per conditions, 2

experiments). (**B**) qPCR of *p16*^{INK4a} expression in control and *p16*^{INK4a} shRNA treated GFP+ fibroblasts, and in *p16*$^{flox/flox}$ fibroblasts treated with adenovirus-cre recombinase or adenovirus-control. (**C**) qPCR of SASP genes in GFP-neg cells transduced with Lenti-TRE-p16/Lenti-rTTA/rTS +/- doxycycline to overexpress *p16*^{INK4a}, along with LPS stimulation (2 experiments). (**D** and **E**) Quantification of collagen thickness measurements under airways using stereologic grid in lungs of control and Dermo1^{p16cKO} mutants injured with naphthalene (n=6 Control, n=10 *Dermo1*^{p16CKO},2 experiments). (**F**) qPCR results of fibrotic makers (*Postn, Acta2, Spp1, Fn1, and Cthcr1*) in 14 dpi control or Dermo1$^{p16\ cKO}$ mutant lungs. (**G**) Airway image of *INKBRITE:Gli1*$^{creERT2/+}$*:R26R*$^{tdT/+}$ lungs. (**H**) FACS percentage of *Gli1* Lin+ and *Gli1* Lin- cells that are of GFPhi/lo/neg during homeostasis (n=4). (**I**) GFP intensity histogram of *INKBRITE:Gli1*$^{creERT2/+}$*:R26R*$^{tdT/+}$ fibroblasts at passage 0. (**J** to **L**) Images and quantification of BrdU incorporation in Gli1 Lin+ cells (white arrows) of control and *Gli1*^{p16CKO} lungs after naphthalene injury. (**M**) qPCR of *p16*^{INK4A} expression in whole lung RNA of control and *Gli1*^{p16CKO} lungs after naphthalene injury (n=7 Control, n=6 *Gli1*^{p16CKO}). AW = airway. RFU = relative fluorescent units. Each point in graph represents one animal or one technical replicate from *in vitro* study with mean ± s.e.m. All p values determined by one-tailed t-test or two-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

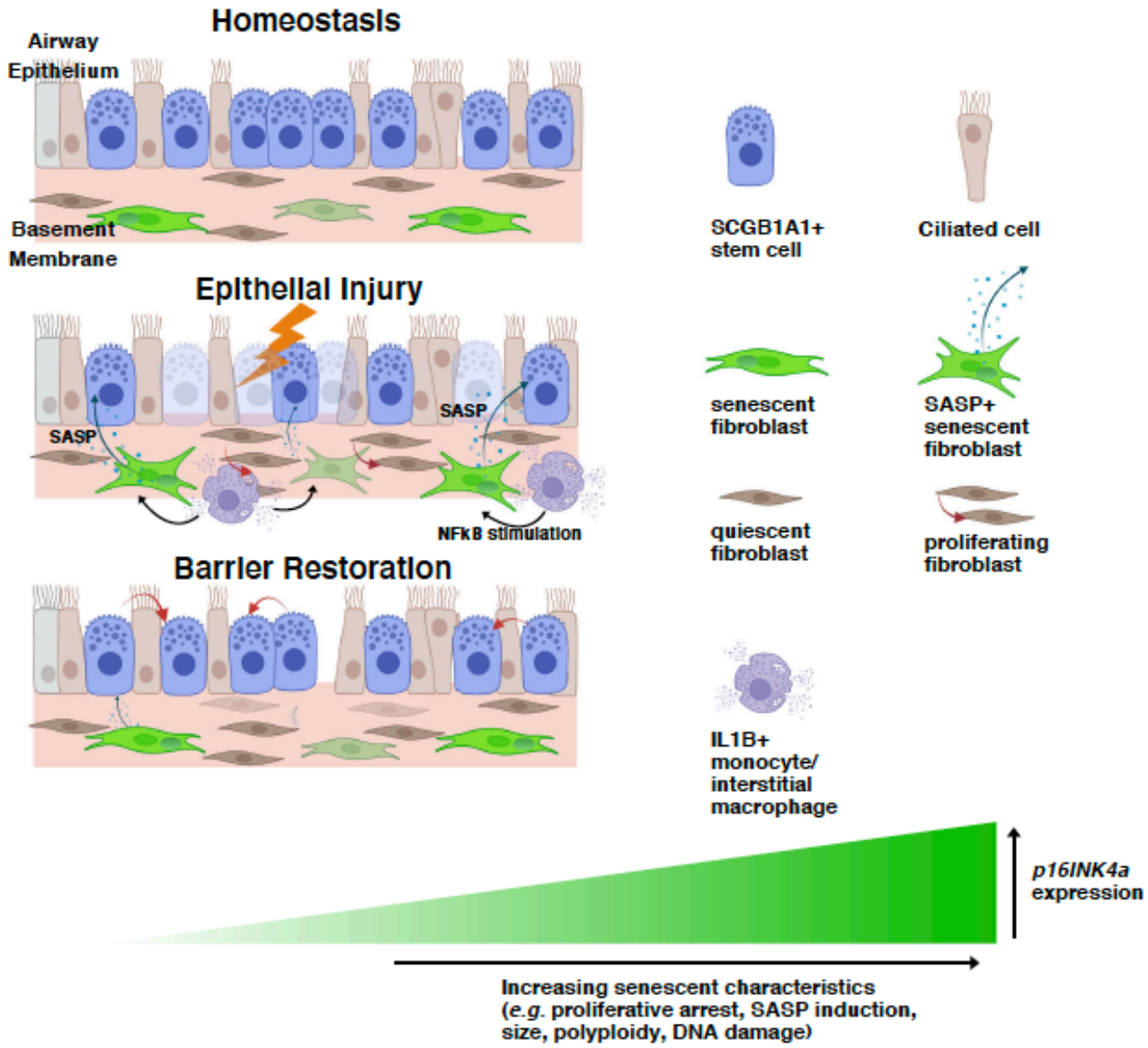


Fig. S9. Model of *p16*^{INK4A}+ fibroblasts' role as tissue-resident sentinels responding to inflammation to augment barrier repair.

Table S1. Bulk RNAseq (p16+ vs. p16- fibroblasts) DEGs and SASP factors