Supplemental Figure Legends

Supplemental Figure S1. A, Location and marker expression of Lgr6$^+$ cells in human lung. Coexpression of Lgr6 and E-Cadherin in cells near vasculature and small bronchioles. **B,** E-Cad/Lgr6$^+$ expressing cells in human lung tissue (yellow arrow). **C,** Co-expression of Lgr6 and Lgr5 in human lung. **D,** Lgr6$^+$ cells located near vascular tissue (CD-31). **E,** Lgr6$^+$ cells located near small bronchioles (Clara cells in green) but do not express CC-10.

Supplemental Figure S2. A, Flow cytometry dot plot showing Lin- cells stained for Lgr6 and c-Kit. **B**, High magnification microscope image showing immunofluorescence staining of human lung tissue for Lgr6 (red) and c-Kit (green). **C**, Immunofluorescence image of human lung tissue for Lgr6 (red) and Integrin-α6 (green). **D**, Co-expression of Lgr5 (green) in some CC-10 (red), Clara cells. **E**, Colony assay of E-Cad/Lgr6$^+$ cells from lung samples from four independent patients. **E**, HLSCs with a CC-10-EGFP reporter (green) cultured on laminin differentiate into AQ5 (red) expressing AT1 elongated cells.

Supplemental Figure S3. A, Expression of the lung markers SP-C and CC-10, together with Lgr6 and E-Cadherin in clonally derived aggregates of HLSCs. HLSCs in culture (after passage 50), differentially express (**B**) lung specific, (**C**) stem, (**D**) signalling, and (**E**) integrin markers.

Supplemental Figure S4. A, Single EGFP$^+$ HLSCs were injected in human lung explants cultured and bleomycin treated *ex vivo*. **B**, Injection of HLSCs expressing a PGK-EGFP reporter in *ex vivo* cultured lungs, at day 1, 2, 5 or 7. Cells migrated and seeded into different areas. **C**, Cells integrate within the alveoli structures and differentiate. SP-C (red) positive

AT2 endogenous and injected (green) cells interact. **D**, Bleomycin lung injury mouse model. Tail vein injected HLSCs (green arrowheads) exit the small vessels in mouse lungs and migrate to different locations (red and white arrowheads). **E**, The engrafted HLSCs (green) integrated into the mouse lung structures and differentiated into AT1 (AQ5), AT2 (SP-C) or Clara (CC-10) cells (doble positive cells in yellow). A representative experiment of 3 to 5 independent replicates is shown. **D**, The human origin of the engrafted cells (green) was confirmed using a specific anti-human mithocondrial antibody (red).

Supplemental Figure S5. A, Differentiation of HLSCs (green cells injected in human lung explants treated with bleomycin *ex vivo*, into AT1 (AQ5), AT2 (SP-C) and Clara (CC-10) cells (in red). **B**, H&E staining of human lung samples untreated or treated with bleomycin showing the fibrosis induced by the drug.

Supplemental Figure S6. Bleomycin-induced lung injury mouse model to assess regeneration potential of HLSCs. **A**, HLSCs (green) engrafted in the lung differentiate into Clara cell like cells (CC-10 positive) when located near small bronchioles. **B**, HLSCs (green) engaged in the alveoli differentiate into AT2 cells (SP-C positive).

Supplemental Figure S7. A, Kidneys showing the burden at 4 or 8 weeks after injection of 1, 10 or 10^4 cells under the kidney capsule. **B**, HLSCs expressing a PGK-EGFP reporter under the kidney capsule 2 weeks after injection of 10^4 cells. **C**, $EGFP^+$ cells (green) in the grafts do not express the CD-73 (endothelial, hematopoietic) or vimentin (mesenchymal) markers (in red). **D**, Kidney grafts of HLSCs produced an epithelium (KG) that resembles lung tissue (LT). **E**, HLSCs engrafted in the kidneys formed alveolar (A) and bronchiolar-like (SB)

structures and differentiated into AT1, AT2 or Clara cell-like cells. **F**, Differentiation of HLSCs (green) into elongated AQ5 (red) expressing AT1 cells, engrafted in the kidney capsule.

Supplemental Figure S8. A, $EGFP^+$ cells isolated from the kidney grafts contained different populations expressing SP-C, CC-10 and/or AQ5 lung markers. **B**, SP-C (AT2) or CC-10 (Clara) lung markers (green) co-expressed with the human-nuclei specific marker (red) in the engrafted cells in the kidneys.

Supplemental Figure S9. A, p38α knockdown in HLSCs using siRNAs directed to different segments of p38α mRNA. **B,** MAPKAPK-2 protein blots with different siRNAs against (MK2) mRNA. **C,** Lung specific markers mRNA levels induced by Fibronectin or Laminin in WT (black) or SH3 (grey) cells. **D,** Integrins mRNA expression induced by Fibronectin or Laminin in WT (black) or SH3 (grey) cells. **E,** Changes in lung specific and integrins protein levels in response to extracellular matrix proteins. **F,** Protein blots showing rescued levels of Integrins-α6 and β1 in WT, SH3, SH3+si-miR, and siMK2 HLSCs. Results are mean ± SEM.

Supplemental Figure S10. Expression of (**A**) lung specific or (**B**) integrin markers in WT or SH3 HLSCs cultured in fibronectin or laminin. **C,** Flow cytometry of WT (EGFP) or SH3 (H2B-Cherry) HLSCs. **C,** Levels of pri-miR-17-92 in clonal HLSCs, and freshly sorted E-Cad^+/$Lgr6^+$ or E-Cad^+/$Lgr6^-$ cells.

Supplementary Experimental Procedures

Isolation and culture of human lung progenitor cells. Human lung tissue was obtained from patients undergoing lung resection at Papworth Hospital, UK. Normal lung was used for cell isolation and histological evaluation. After pleura were separated bluntly, lung specimens were finely minced and resuspended in collagenase (0.5 – 3 mg/ml, Whorthington)/dispase (1 mg/ml, Invitrogen) containing DMEM (Invitrogen) and incubated for 30 – 45 min at 37°C in a shaking incubator. The suspension was spun for 5 min at 1200 rpm and the supernatant removed. The pellet was resuspended in fresh DMEM containing 0.1 mg/ml DNase (optional) and incubated for further 5 – 10 min. The suspension was washed with PBS, filtered through cell strainers (100 μm, 70 μm, BD) and treated with red blood cell lysis buffer (Roche Applied Science). Following further filtration (40 μm mesh) and centrifugation (5 min at 1200 rpm), the isolated cells were cultured in RH-B (Stem Cell Science) medium containing 2% FCS, with additional insulin (5 μg/ml, Pepro Tech), EGF (10 ng/ml, Pepro Tech) and FGF2 (20 ng/ml, Pepro Tech) for two days. Cells were then sorted in a flow cytometer and put in culture or used for in vivo assays. Cells in culture were maintained in serum-free medium containing growth factors (37°C in a 7% humidified CO_2 incubator).

Lentiviral vector preparation. The lentiviral vector pSINPGKEGFP was used to generate pSINhCC10EGFP, containing the human CC10 promoter. Lentiviral particles were produced by co-transfecting 293T cells with pSINPGKEGFP or pSINhCC10EGFP, pCMVΔ8.9 and pMD.G (encoding VSV-G), as previously described (Capowski et al., 2007; Naldini et al., 1996).

Four siRNAs (Thermo scientific) were tested to knockdown p38α:

si1- GGAAUUCAAUGAUGUGUAU; si2: UCUCCGAGGUCUAAAGUAU;

si3: GUAAUCUAGCUGUGAAUGA; si4: GUCCAUCAUUCAUGCGAAA.

si2 and si3 were selected to generate short-hairpins to stably knockdown p38α.

Oligoduplexes (ShA CCGGT-GTCCATCATTCATGCGAAA-TTCAAGAGA-TTTCGCATGAATGATGGACTG-TTTTTT-G, ShB AATTC-AAAAAA-CAGTCCATCATTCATGCGAAA-TCTCTTGAA-TTTCGCATGAATGATGGAC-A) were cloned into the PLKO.1-TRC (Sigma) lentiviral vector to generate a p38α knockdown construct (SH3). Infectious virus was added to cells in the presence of 8mg/ml polybrene (hexadimethrine bromide, Sigma) and incubated for 6 hours.

A commercial (Applied Biosystems) siRNA was used to knockdown the pri-miR-17-92: sense- GGAGAGCUCAAUCUGCACAtt.

Luciferase Assays. To test miR-17-92 promoter activity and transcription factors 3'-UTR processing we have used a pGL3 reporter vector containing the firefly luciferase. As a control of the transfection efficiency we used a vector expressing the Renilla luciferase.
The 0.5kb and 0.7kb fragments of the miR-17-92 promoter were obtained by PCR using primers:

MIR17HG-Pr-NheI-4363Forward(0.5kb):
5'-GGTGCTAGCGATGTTAAAGTCACTGGATTTTAC

MIR17HG-Pr-NheI-4121Forward(0.7kb):
5'-GGTGCTAGCGGTTCTTTCCGAGCTATGCTATTT

MIR17HG-Pr-XhoI-4869Reverse:
5'-AATCTCGAGATGAAGGGCGGCGGCTCCCGCCTCAACG

PCR products were cloned in the promoterless pGL3 basic vector after NheI/XhoI restriction digestion.

The 3'-UTRs of the C/EBPα, GATA6 and TTF1 genes were obtained by PCR using primers:

C/EBPa 3'UTR XbaI Forward: CTTGTCTAGAGAGCCAGGACTAGGAGAT

C/EBPa 3'UTR XbaI Reverse: GATTTCTAGAGAGAACCAAGCCGTCCTTCCC

TTF1 3'UTR XbaI Forward: GACGTCTAGACCGGTGGATCATCTGAGGTC

TTF1 3'UTR XbaI Reverse: CTCGTCTAGAGAGTAGAACTCTGGCCATTG

GATA6 3'UTR XbaI Forward: ATCATCTAGAGCCCTGGCCCTGGCCTGAGC

GATA6 3'UTR XbaI Reverse: CTACTCTAGACAGGGAGCCACAGTTAAAGG

PCR products were cloned in the pGL3-Vector control after XbaI digestion.

Mutant 3'-UTRs were created introducing a mutation in the miR-92a target sequence of the C/EBPα and GATA 6 3'-UTRs using the appropriate oligos :

C/EBPα 3'UTR C992G Forward: CAGCAGTGCCTTGTGGAATGTGAATGTGCGC

C/EBPα 3'UTR C992G Reverse: GCGCACATTCACATTCCACAAGGCACTGCTG

GATA6 3'UTR C657G Forward:

TGTATACAATAATTTTTTTAAAAAGTGGAATTTGCGTTGCAGCAATCAGTGT

GATA6 3'UTR C657G Reverse:

ACACTGATTGCTGCAACGCAAATTCCACTTTTTAAAAAAATTATTGTATACA

Flow cytometry. Single cell FACS was performed (MoFlo, Dako) following incubation with anti-Lgr6 (Biolegend), anti-E-cadherin (Biolegend), anti-Integrinα6 (CD49f, Biolegend), anti-αSMA (Sigma), anti-CD31 (Biolegend), anti-CD34 (Biolegend), Human-nuclei specific (Millipore) and anti-CD45 (Biolegend) antibodies. An aliquot was fixed, permeabilized and labeled to confirm them as SP-C^+/$CC10^+$ (Dako CyAn flow cytometer).

***In vitro* assessment of differentiation potential.** Cells were grown to low confluence on 10 μg/ml fibronectin (Millipore) or laminin (in PBS, Sigma) coated tissue culture dishes (24- or 6- well plates). All differentiation cultures were maintained for 10 – 15 days, medium (containing 2 - 5% FCS) being renewed every 72 h.

Immunofluorescent staining. Cytospun (800g, 3 min) cultured cells were fixed with 4% PFA (paraformaldehyde) for 15 – 20 min at RT. Samples were blocked and permeabilized with 0.1% Triton-X/4% goat serum/PBS for 60 min at RT. Cells were incubated with primary antibodies overnight at 4°C and then washed 3 times with PBS at room temperature (5 min per wash). Secondary antibody (Alexa Fluor 488 and/or Alexa Fluor 594 secondary antibodies, Invitrogen) incubation took place for 1 hour at RT. Cells were visualized following DAPI (4',6-Diamidino-2-phenylindole) counterstaining.

Mouse Experiments. All mouse experiments were performed according to UK Home Office Regulations. CD-1® nude mice (Charles River) were maintained under standard pathogen-free conditions.

Kidney capsule engraftments. 6 – 8 week old mice were anaesthetized with isofluorane (0.5 – 2%). Cells were disassociated with accutase to generate a single-cell suspension (1 x 10^6 cells in 20 μl PBS) and this suspension was injected under the kidney capsule. Mice were killed 2, 4, 6 and 8 weeks later and the kidneys harvested to examine *in vivo* differentiation of the injected cells. The engraftments were removed and prepared for immunofluorescent microscopy.

Bleomycin treatment of mice and lung progenitor cell transplantation. 6 – 8 week old mice were given one tail vein injection (tvi) of 5 U/kg of bleomycin in 100 μl PBS. Control animals were given an equivalent volume of PBS. Control groups received PBS and experimental groups received bleomycin. 48 hrs later, the experimental group received progenitor cells (tvi). Each group had 6 mice (repeated 3 times), which were analyzed 10 days post-bleomycin/progenitor cell injection. Lungs were fixed overnight with buffered neutral formalin 10% (VWR) or 4% PFA at room temperature. Tissues were then processed for paraffin embedding or for cryosectioning. Slides were stained in Mason's Trichrome stain (Fisher Scientific) and H&E (Dako), according to the manufacturer's instructions. Cryosections were analyzed for the presence of human lung progenitors by microscopy.

Total RNA Isolation and Quantitative RT–PCR. Total RNA was extracted using TRIzol (Invitrogen) and DNAse I (Promega) treated. 1 μg RNA was reverse transcribed per sample (Biorad), according to the manufacturer's instructions. Quantitative Real Time PCR (qPCR) was used to determine the expression levels of the different genes using human specific primer pairs (Eppendorf, Realplex^2). Reaction conditions for amplification were as follows: first step of 95ºC 20 seconds, then 40 cycles of three-step 95ºC 3 seconds, 60ºC 30 seconds and 68ºC 20 seconds with 2 μl of cDNA per reaction in 10 μl SYBR Green PCR Master Mix (Applied Biosystems). Specificity of PCR products was tested by dissociation curves. Threshold cycles of primer probes were normalized to a housekeeping gene (GAPDH or HPRT) and relative values calculated (Livak and Schmittgen, 2001).
We use 2µL of cDNA per 10µL of Taqman Fast Universal PCR Master Mix (2x). The cycles were: first step of 95ºC for 20 seconds, then 40 cycles of 2-step 95ºC for 1 second and 60ºC for 20 seconds (default setup in StepOne machine). VIC-labelled human GAPDH from Applied Biosystems was used as internal control. Pri-*miR-17-92* expression was quantified by

TaqMan RT-PCR and normalized to *GAPDH*-expression. The primers used for pri-*miR-17-92* are: sense: 5'-CAGTAAAGGTAAGGAGAGCTCAATCTG-3'; antisense: 5'-CATACAACCACTAAGCTAAAGAATAATCTGA-3',TaqMan-probe:6-FAM-TGGAAATAAGATC ATCATGCCCACTTGAGAC-TAMRA.

Immunoblot analysis. Proteins were extracted and analyzed as previously described (Ventura et al., 2007), to confirm that observed changes in mRNA expression were reflected in the amount of protein present.

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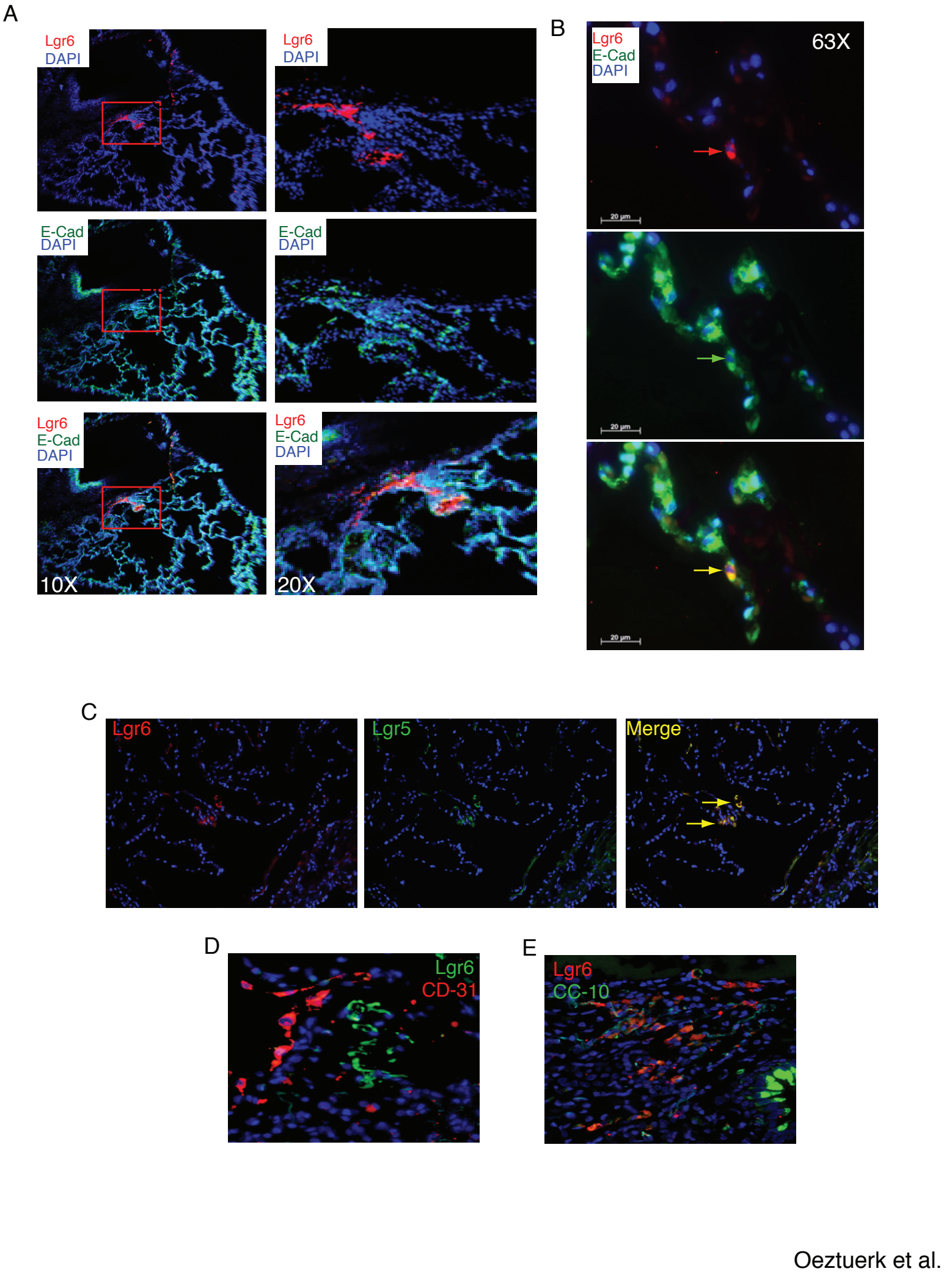
Suppl. Fig. S1



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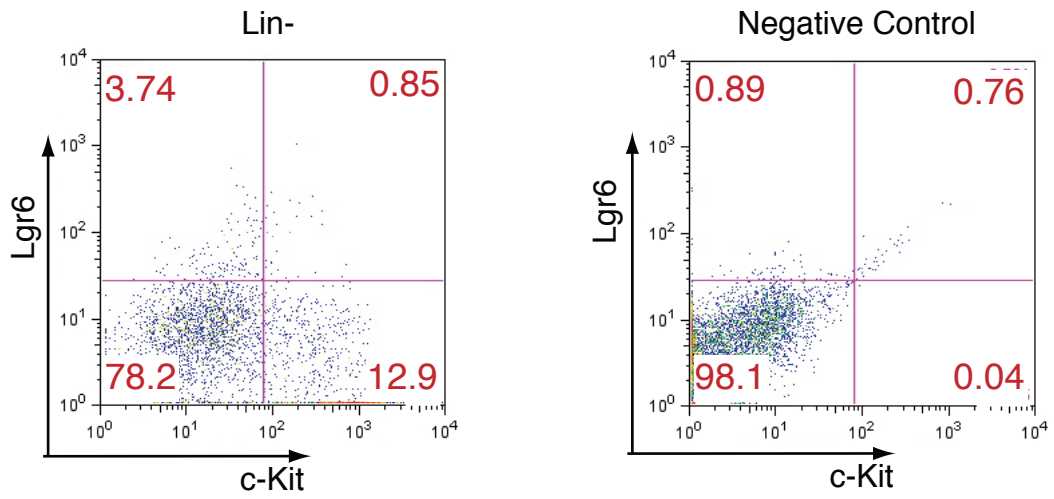
Suppl. Fig. S2

A



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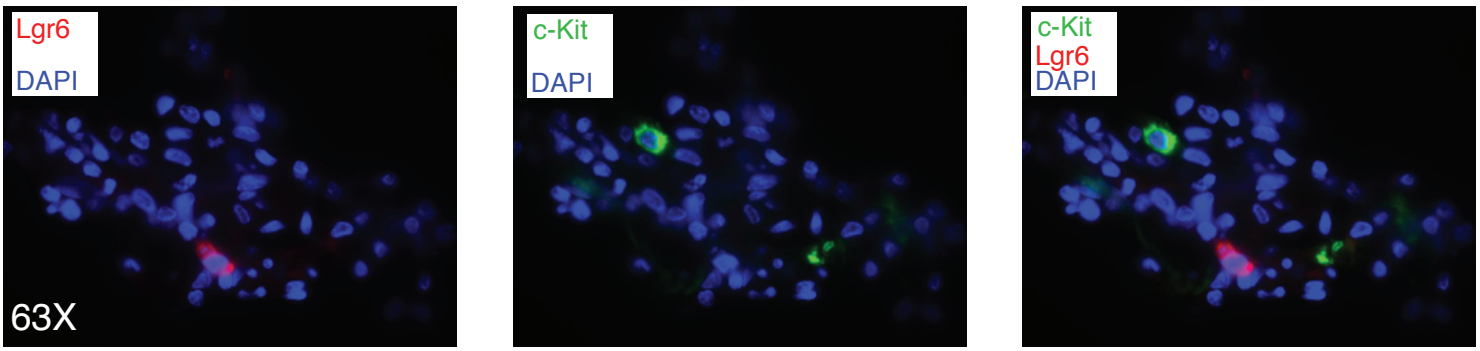

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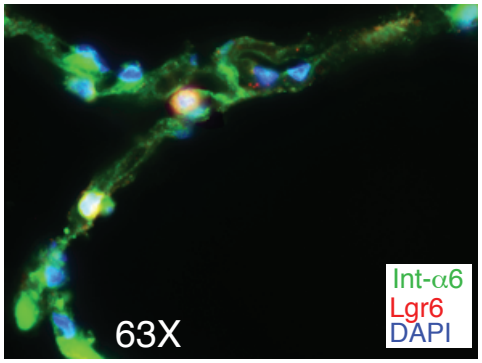

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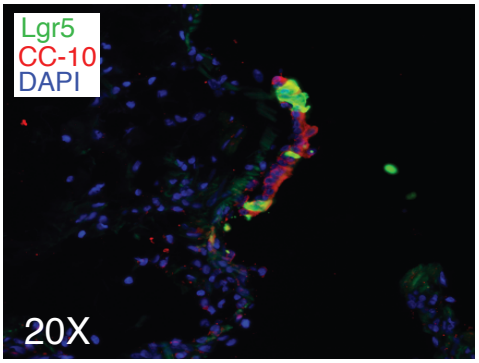
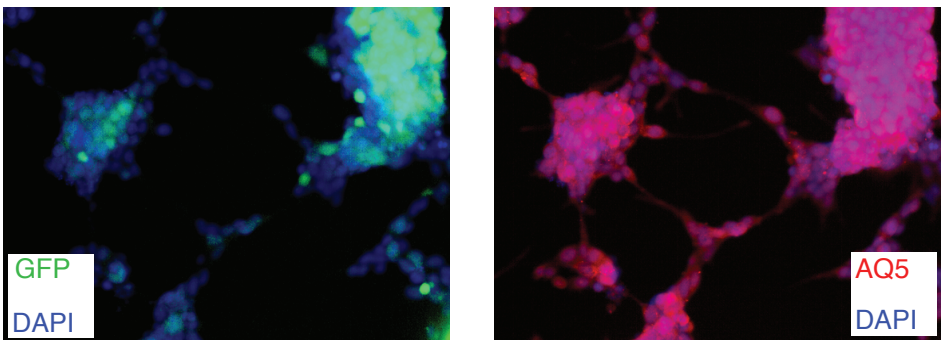


E

E-Cad/Lgr6^{+} from Patients	Cam1	Cam2	Cam3	Cam4
Number of Colonies	23/40	20/43	19/45	16/38

F

Laminin



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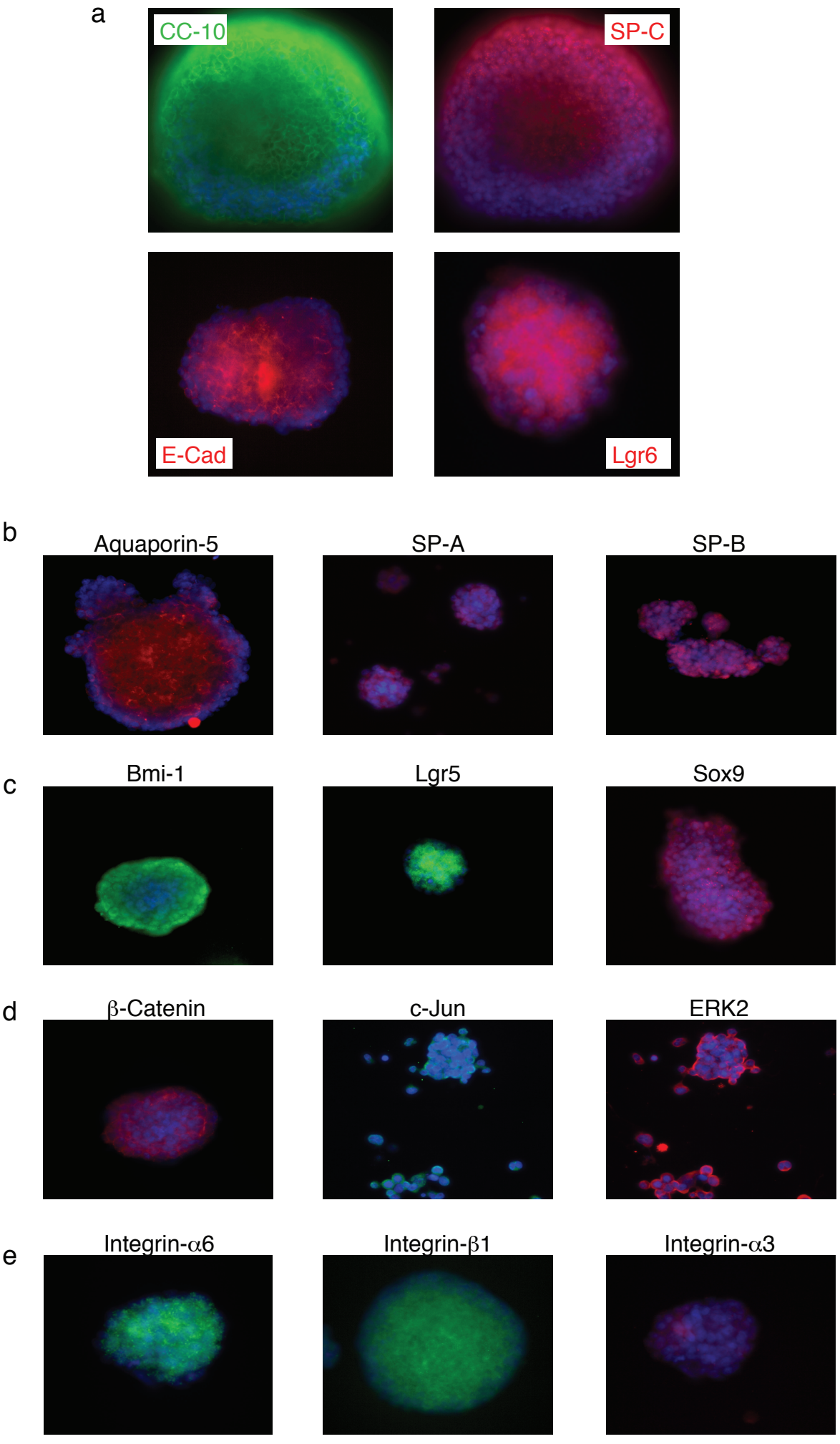

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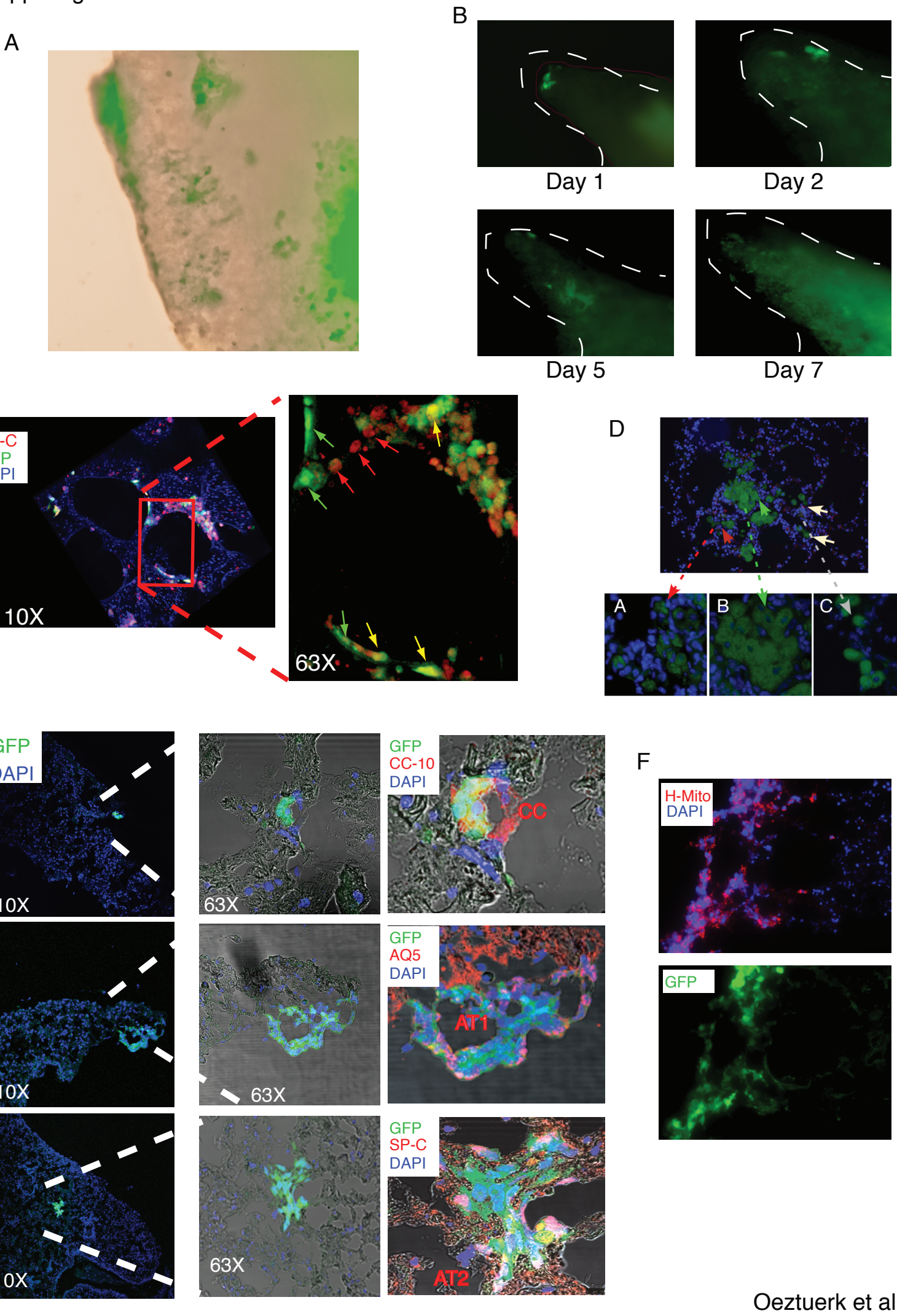

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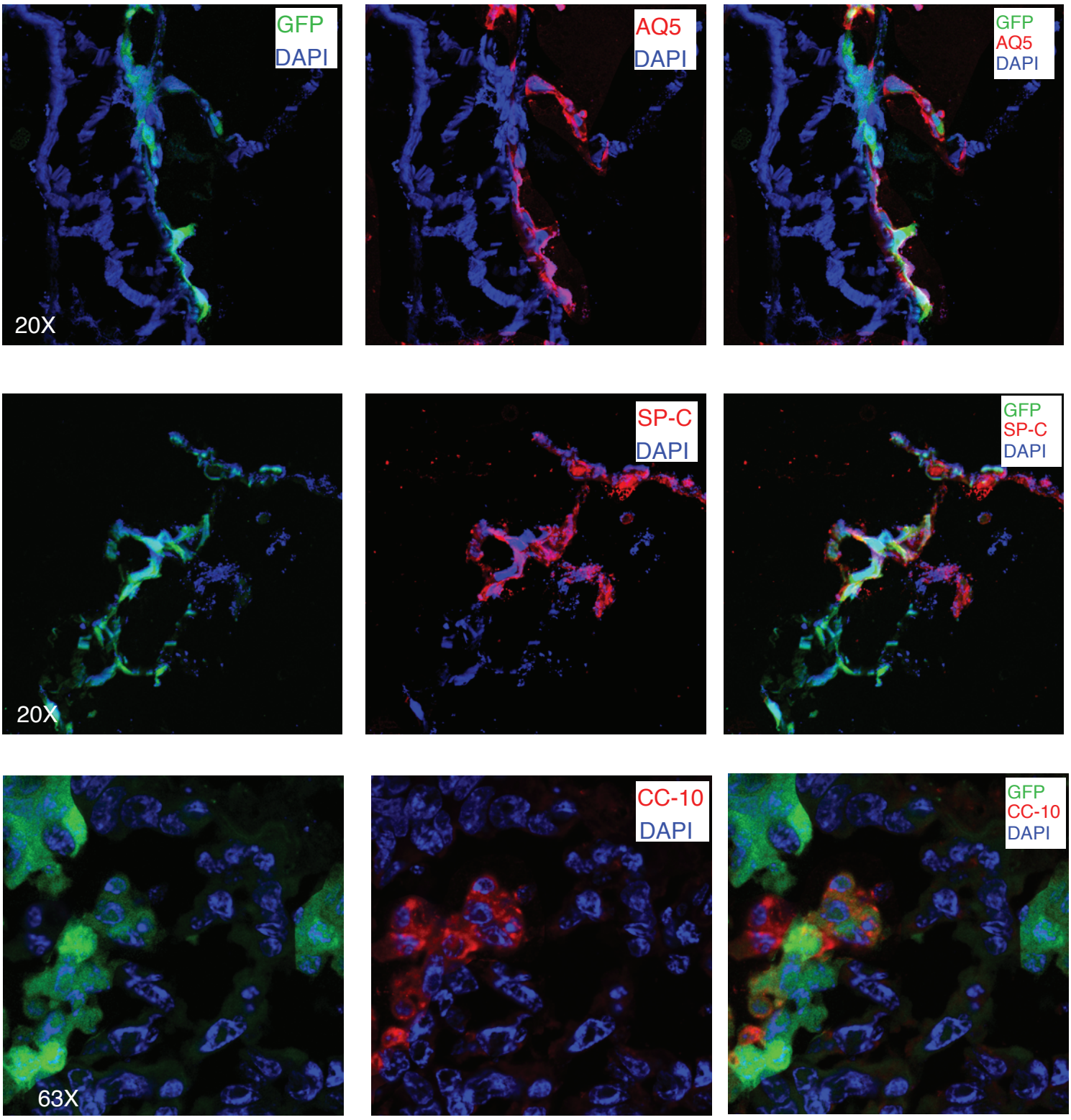
Suppl. Fig. S5

A



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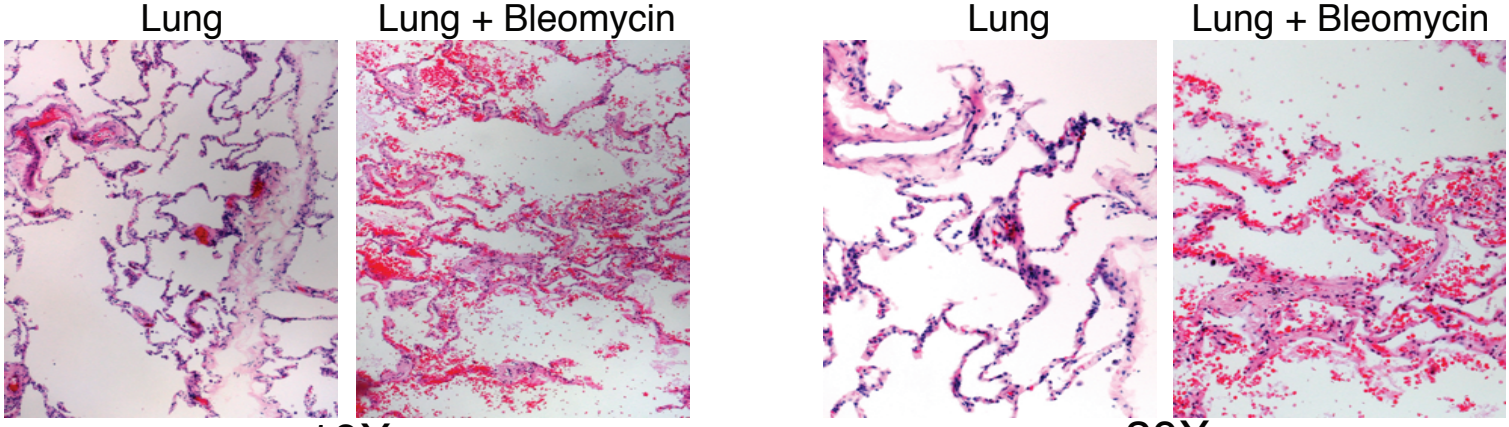

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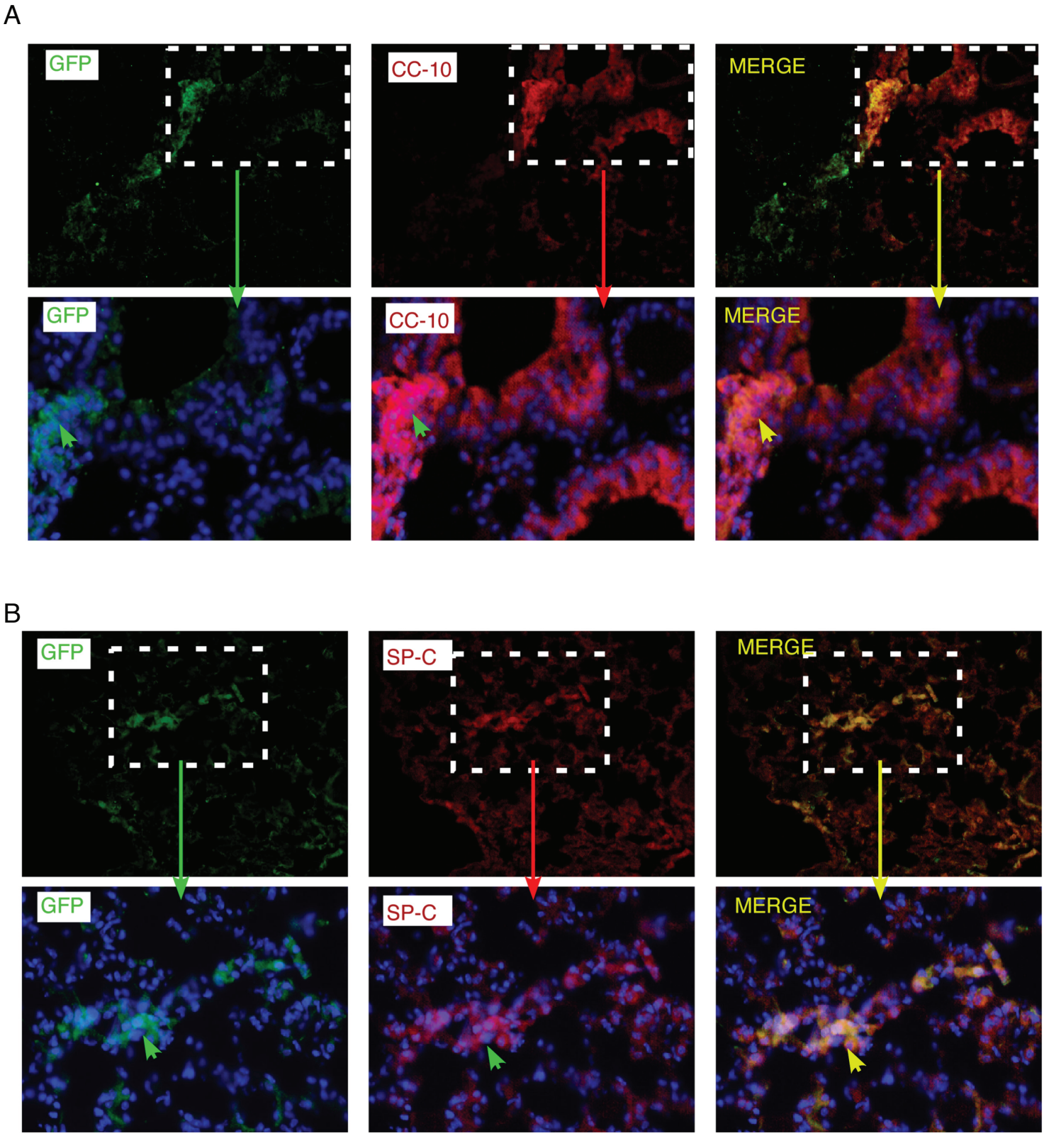

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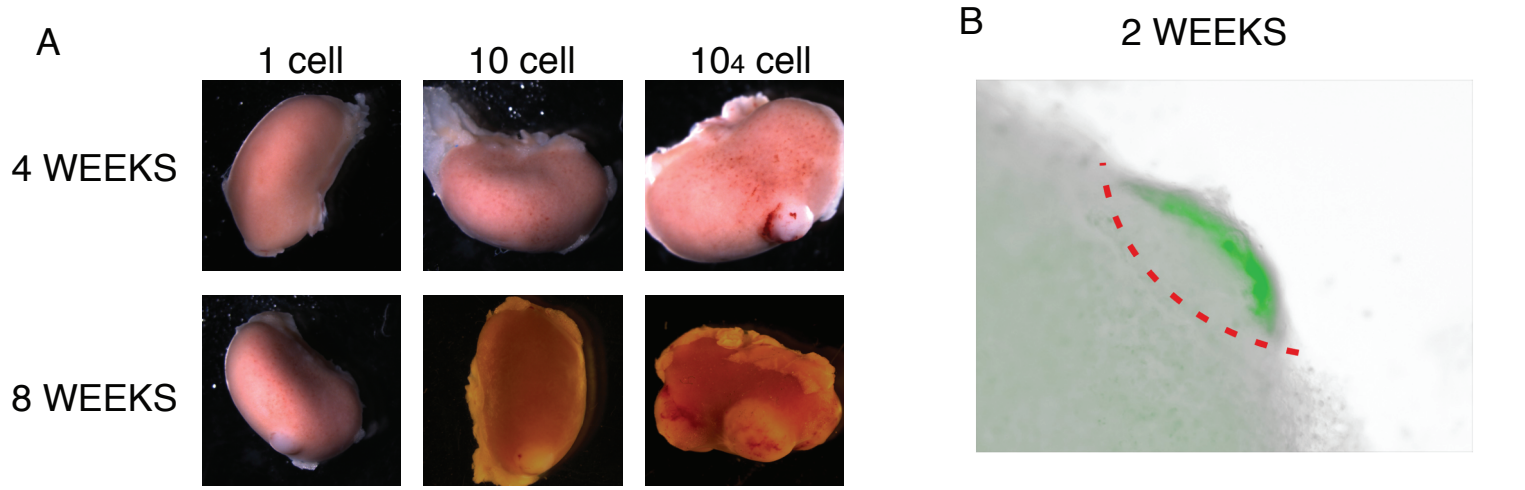

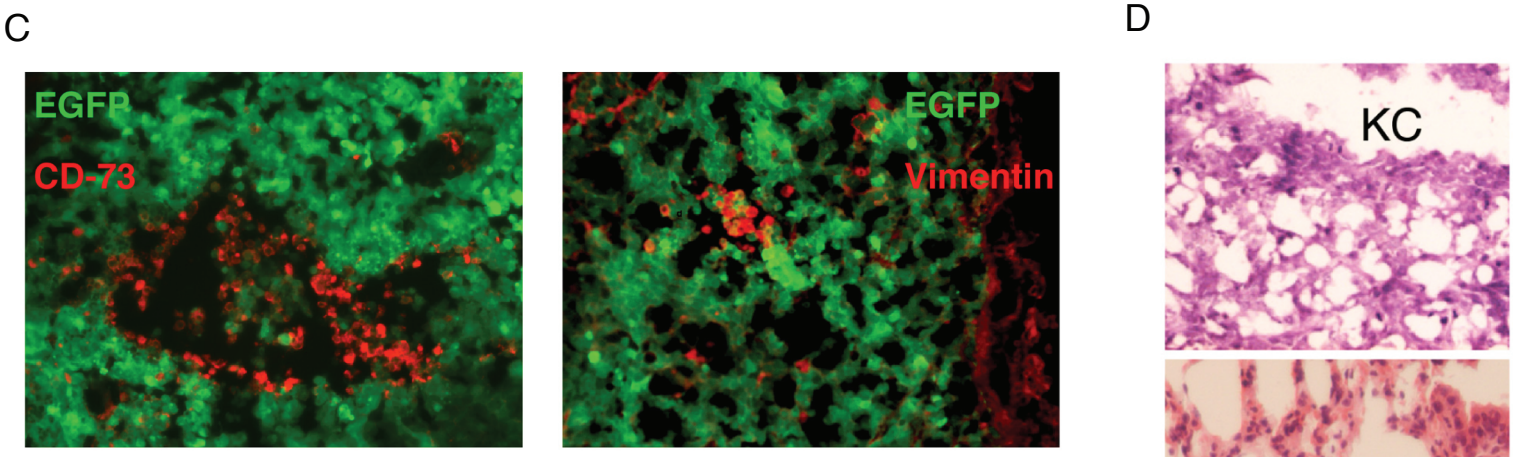

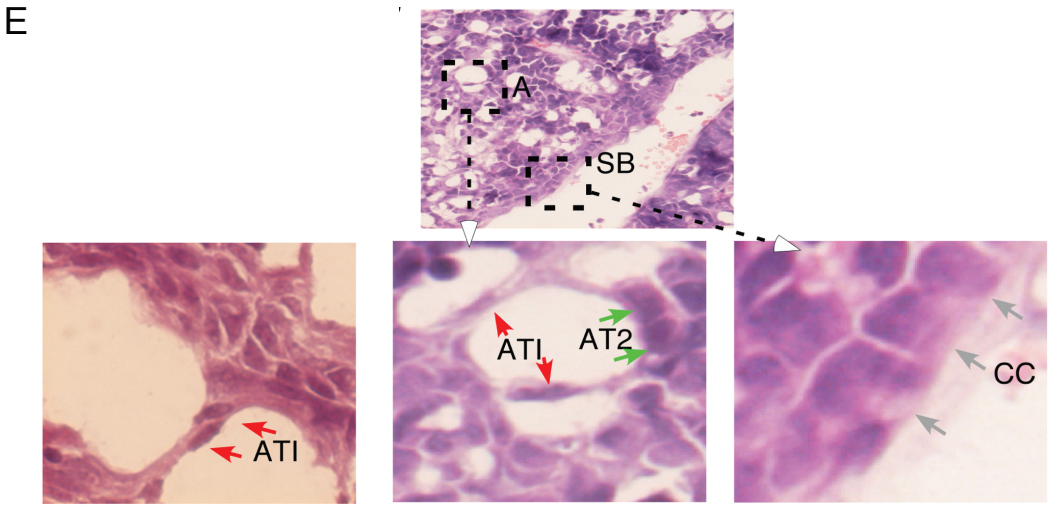

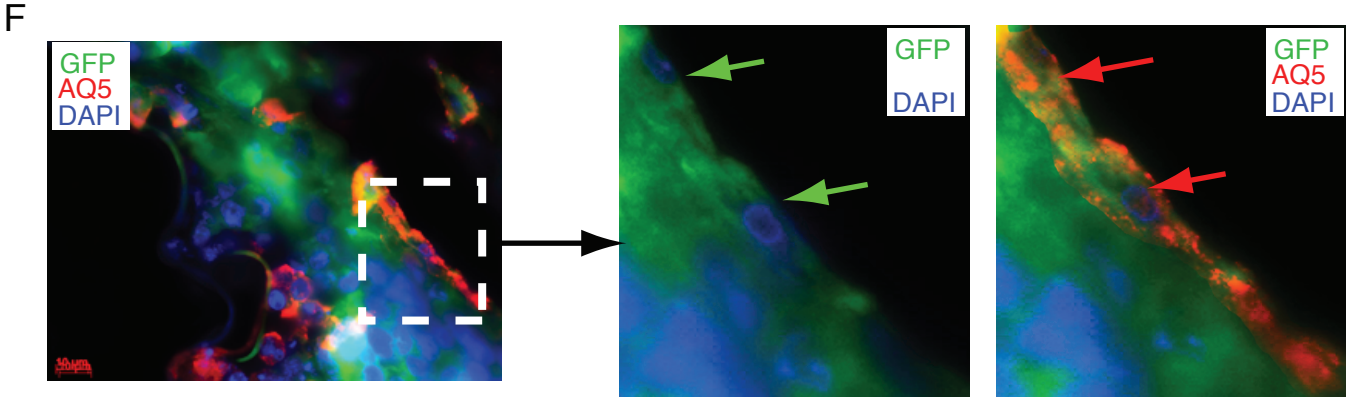

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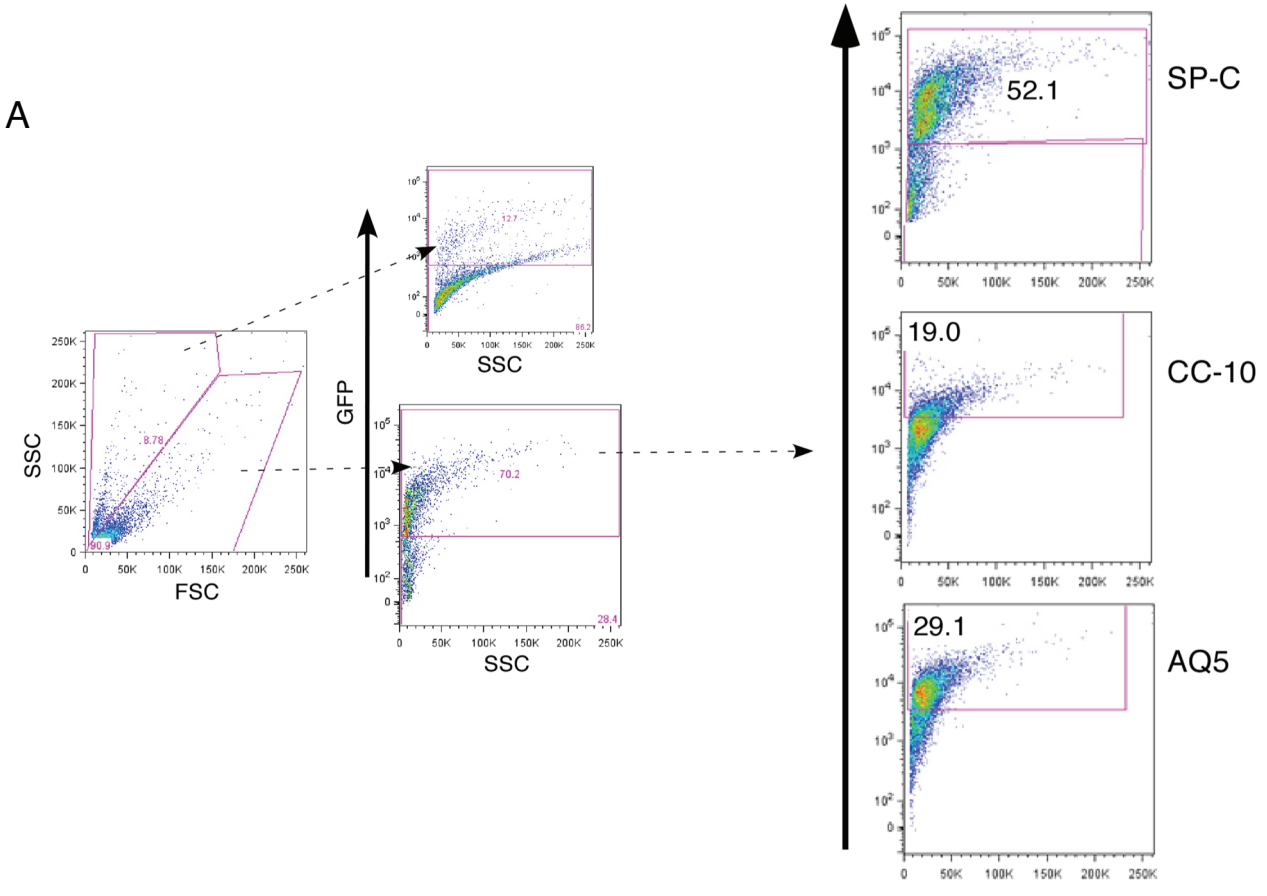

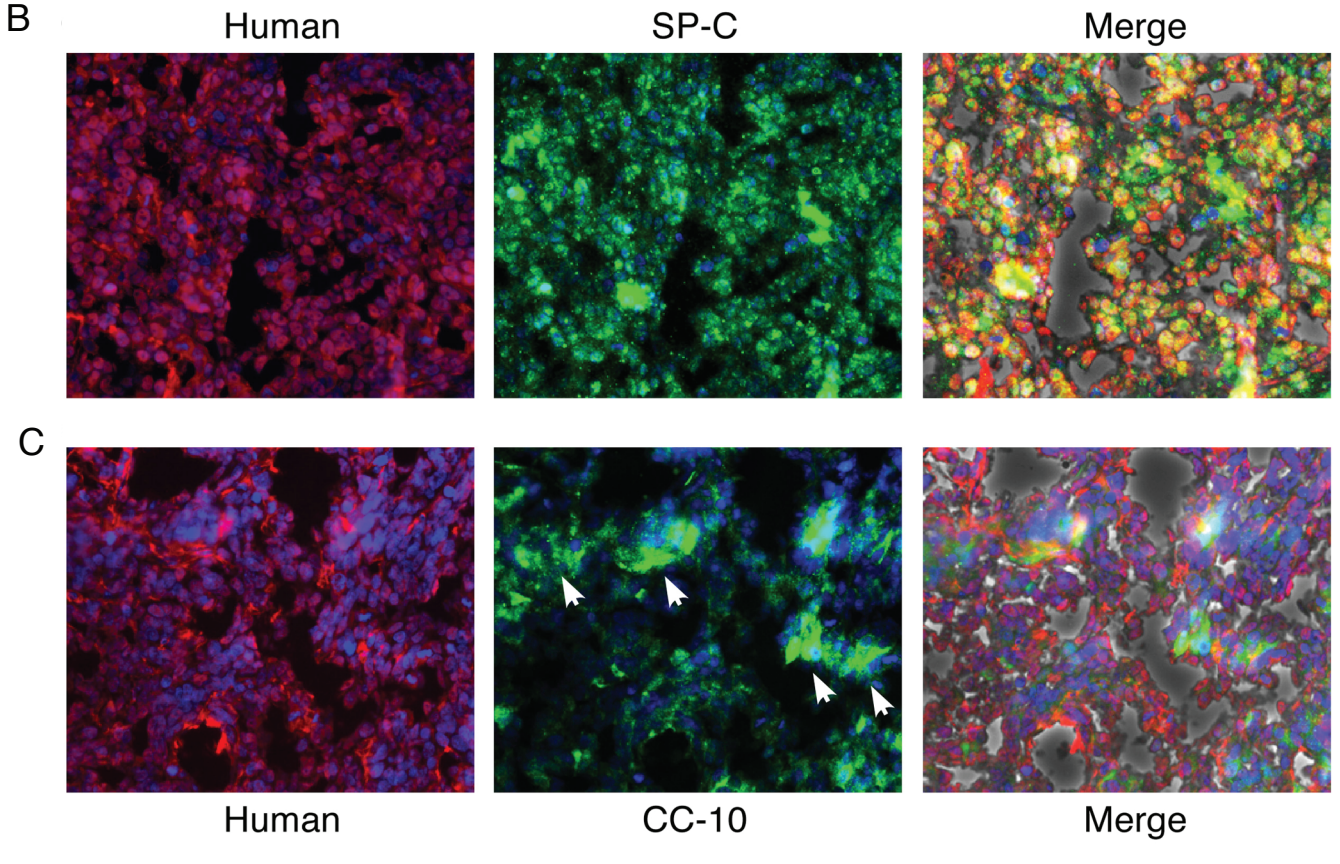

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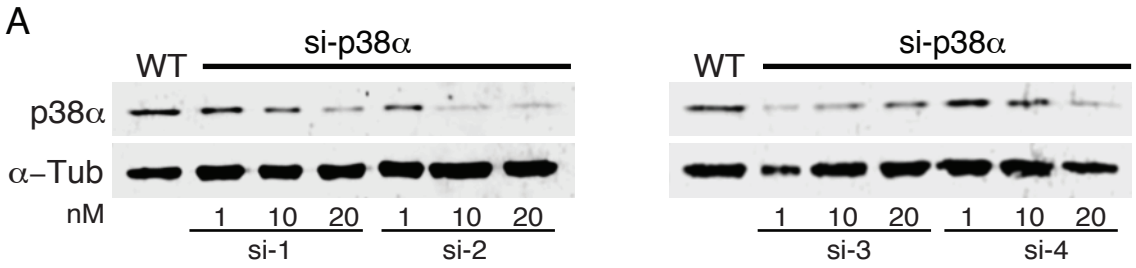

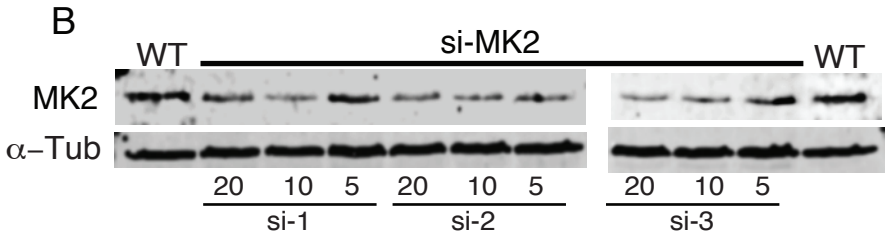

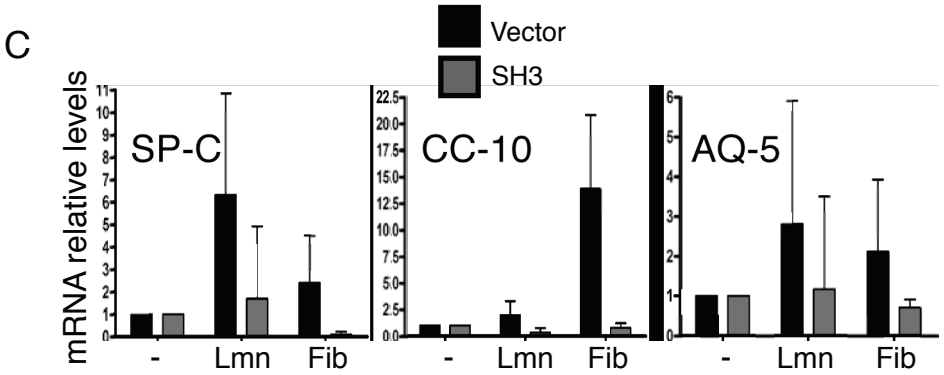

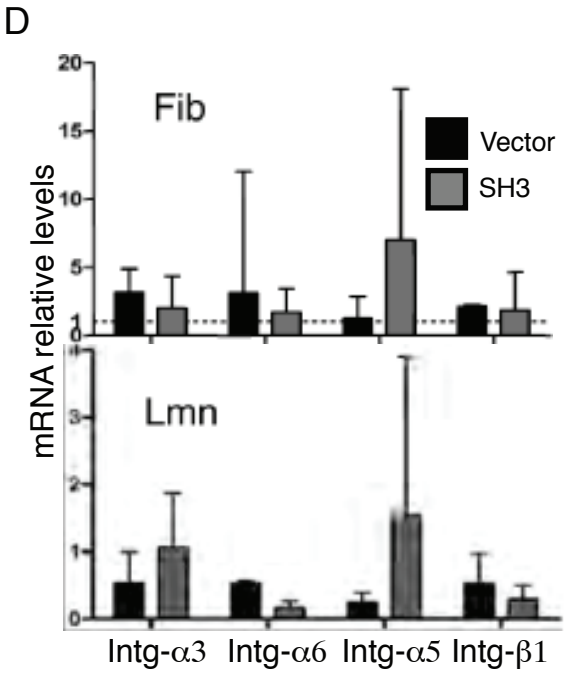

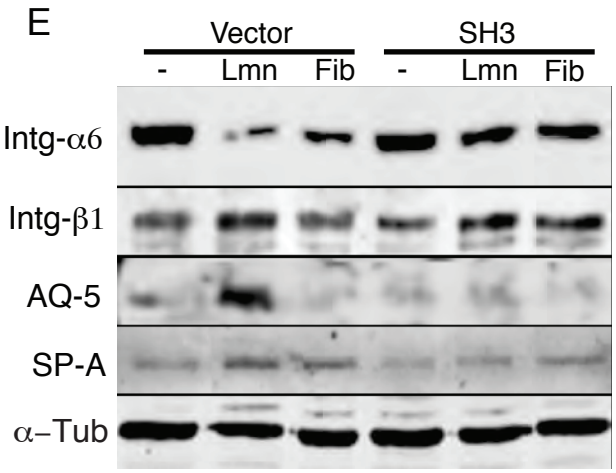

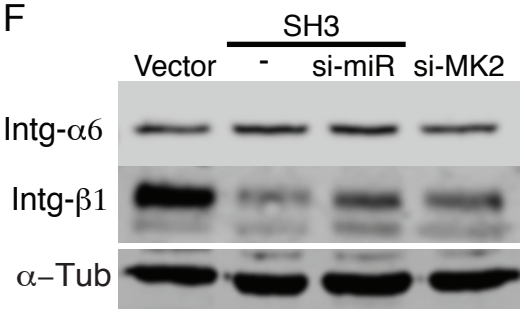

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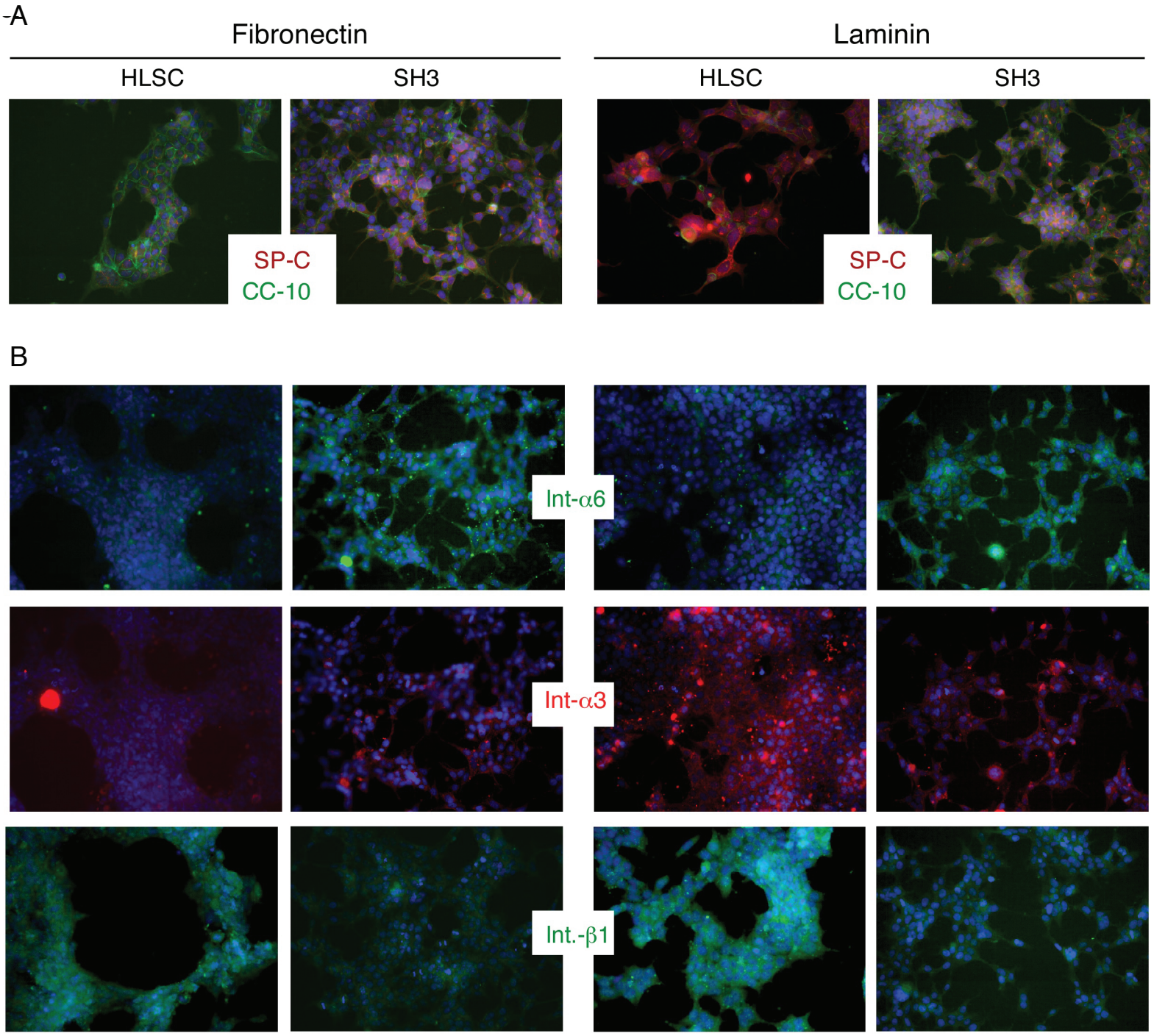

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