

FOXF1 transcription factor promotes lung regeneration after partial pneumonectomy.

Craig Bolte^{1,2*}, Hannah M. Flood^{1,2}, Xiaomeng Ren^{1,2}, Artem Barski³, Jagannathan Sajjiev³, Tanya V. Kalin², Vladimir V. Kalinichenko^{1,2*}

¹Center for Lung Regenerative Medicine, Cincinnati Children's Research Foundation, Cincinnati, Ohio, USA.

²Division of Pulmonary Biology, Cincinnati Children's Research Foundation, Cincinnati, Ohio, USA.

³Division of Allergy and Immunology, Director of Epigenomics Data Analysis Core, Cincinnati Children's Research Foundation, Cincinnati, Ohio, USA.

***Correspondence** to: Dr. Vladimir V. Kalinichenko (Vladimir.Kalinichenko@cchmc.org) or Dr. Craig Bolte (Craig.Bolte@cchmc.org), Center for Lung Regenerative Medicine, Division of Pulmonary Biology, Cincinnati Children's Hospital Research Foundation, 3333 Burnet Ave., MLC 7009, Cincinnati, OH 45229.

Gene name	Assay number
<i>Ccnd3</i>	Mm01612362_m1
<i>Cd44</i>	Mm01277161_m1
<i>Cdkn1a</i>	Mm04205640_g1
<i>Cdkn2b</i>	Mm00483241_m1
<i>Col1a1</i>	Mm00801666_g1
<i>Col3a1</i>	Mm00802331_m1
<i>Foxf1</i>	Mm00487497_m1
<i>Hdac7</i>	Mm00469527_m1
<i>Mmp14</i>	Mm00485054_m1
<i>Pecam1</i>	Mm01242584_m1
<i>Sox17</i>	Mm00488363_m1
<i>Sftpc</i>	Mm00488144_m1
<i>Spry4</i>	Mm00442345_m1
<i>Timp3</i>	Mm00441826_m1

Supplemental Table 1. List of Taqman probes used in qRT-PCR analysis

Gene	TSS	Binding Sites						
<i>Adamts9</i>	Chr6: 92,901,441	-9727- -9869	5308- 4898	75129- 74923				
<i>Ccnd3</i>	Chr17: 47,505,051	-3284- -2886	-1864- -1691	-271- 599	2956- 3150	3373- 3538	5781- 6659	16799- 16942
<i>Cd44</i>	Chr2: 102,901,655	-494- -1014	6058- 5857	57180- 56969	72281- 72137			
<i>Cdkn1a</i>	Chr17: 29,093,771	-151- 394	1447- 1627					
<i>Cdkn2b</i>	Chr4: 89,311,032	897- 1073	1866- 2032					
<i>Cenpj</i>	Chr14: 56,571,846	-595- -452	-288- -64					
<i>Flt1</i>	Chr5: 147,725,988	-19162- 18975	-11484- -10784	18242- 18462	23564- 23733			
<i>Hdac5</i>	Chr11: 102,230,172	-15282- -15651	13879- 13534	28512- 28307				
<i>Hdac7</i>	Chr15: 97,844,502	-6818- -6982	-5580- -5833	-5335- -5476	3354- 3141	13065- 12025	23382- 22718	30891- 30600
		31253- 31111						
<i>Itgb3</i>	Chr11: 104,607,999	-18006- -17212	3414- 4095	6936- 7158	9768- 10229	14636- 15158	18040- 18494	32848- 33257
		40507- 40755						
<i>Itgb4</i>	Chr11: 115,974,725	-9928- -9027	-4597- -3793	28280- 28423				
<i>Kdr</i>	Chr5: 75,978,428	-66836- -67420	-9369- -9689					
<i>Notch2</i>	Chr3: 98,013,535	-19287- -19625	5019- 5435	12548- 12704	18780- 19111	25510- 25793	27865- 28407	29481- 29866
		40235- 40732	43281- 44062	48295- 48669	53592- 53764			
<i>Pdgfb</i>	Chr15: 80,014,808	-30792- -31301	-9457- -9969					
<i>Pecam1</i>	Chr11: 106,715,281	12291- 12605	48504- 48351	55814- 55563				
<i>Ptgs1</i>	Chr2: 36,230,426	-30722- -30316	-29650- -29233	-16305- -16038	3753- 4746	16979- 17756		
<i>Ptgs2</i>	Chr1: 150,100,124	-2432- -2276	-879- -584	92-235	5971- 6152	7444- 7854	7936- 8418	
<i>S1pr1</i>	Chr3: 115,715,055	1187- 1497						
<i>Spry4</i>	Chr18: 38,601,268	-30692- -30112	1553- 1860	90810- 91267	82852- 83415			

<i>Stat1</i>	Chr1: 52,119,437	-6144- -6002	-132- 151					
<i>Tek</i>	Chr4: 94,739,288	-30059- 30202	-15760- -15960					
<i>Timp3</i>	Chr10: 86,300,412	-9986- -9756	2439- 2648	6952- 7095	10701- 10844	10135- 10458	14306- 14459	27695- 27965
		36701- 36987	39295- 39438	41933- 42092	44983- 45256	45876- 46135	46954- 47097	48314- 48500
<i>Tubb4a</i>	Chr17: 57,087,782	-1092- -914						

Supplemental Table 2. Genes to which FOXF1 binds as identified by ChIPseq. Sites of FOXF1 binding are listed relative to the transcriptional start site (TSS).

Supplemental Figure 1. FOXF1 present throughout endothelium in regenerating lung.

Immunostaining with FOXF1 antibodies demonstrated that FOXF1 is widely distributed in the alveolar region as well as a subset of endothelium cells in arteries and veins of sham-operated and regenerating lungs. Arrows indicate FOXF1-positive cells. Scale bars are 20 μ m. Abbreviations: Br-bronchiole, Ar-artery, Ve- vein.

Supplemental Figure 2. Alveolar size is unaltered during post-PNX regeneration.

(A) Hematoxylin and eosin (H&E) staining of post-PNX lungs from control and *PDGFb-iCre/Foxf1^{fl/+}* mice demonstrate similar morphology. (B) Measurement of air space showed no change in alveolar size following PNX in *PDGFb-iCre/Foxf1^{fl/+}* lungs. (C) Lung tissue resistance was altered by PNX but did not differ between control and *PDGFb-iCre/Foxf1^{fl/+}* mice. (D) Tamoxifen administration did not affect lung regeneration as there was no difference in lung volumes in *Foxf1^{fl/+}* mice with or without tamoxifen or *PDGFb-iCre/Foxf1^{fl/+}* mice in the absence of tamoxifen. (E) qRT-PCR analysis of *Col1a1* and *Col3a1* showed no difference in expression between regenerating control and *PDGFb-iCre/Foxf1^{fl/+}* lungs. (F) Masson's trichrome stain showed no difference in collagen localization between control and *PDGFb-iCre/Foxf1^{fl/+}* mice in regenerating lung. #p<0.05 compared to sham. Scale bars are 20 μ m.

Supplemental Figure 3. ChIPseq data shows FOXF1 binding to genes regulating lung regeneration.

Schematic of FOXF1 binding regions in *Adamts9*, *Ccnd3*, *Hdac5*, *Itgb4*, *Ptgs1*, *Ptgs2*, and *Spry4* DNA regulatory regions as determined by ChIPseq. Regions of significant FOXF1 binding to chromatin are shown by blue box, transcriptional start sites and direction of translation is shown with a green arrow.

Supplemental Figure 4. Verification of endothelial and epithelial cell populations isolated by FACS.

(A) qRT-PCR analysis of endothelial cells (CD45⁻CD31⁺CD326⁻) isolated by FACS. Endothelial cells had high *Pecam-1* expression but did not express surfactant protein C (*Sftpc*). (B) qRT-PCR analysis showed that FACS-sorted epithelial cells (CD45⁻CD31⁻CD326⁺) expressed *Sftpc* but not *Pecam-1*.

Supplemental Figure 5. Epithelial cell proliferation in *Foxf1*-deficient mice.

(A) Immunofluorescence experiments were performed using ENDOMUCIN to mark the endothelial network. (B) Mean fluorescent intensity was used to quantify ENDOMUCIN staining. n=4 mice. (C) Co-immunofluorescence experiments using SPC to mark epithelial cells and PCNA to visualize proliferating cells demonstrated epithelial proliferation in control and *PDGFb-iCre/Foxf1^{fl/+}* mice following PNx. (D) No major differences in epithelial proliferation were observed between *PDGFb-iCre/Foxf1^{fl/+}* and control mice during lung regeneration. *p<0.05 compared to controls. Scale bars are 20 μ m.

Supplemental Figure 6. *Foxf1*-deficiency does not alter lung apoptosis, low endothelial proliferation in late regeneration.

(A) Immunohistochemistry for Cleaved Caspase 3 showed little apoptosis in control or *PDGFb-iCre/Foxf1^{fl/+}* mice before or after PNx. Scale bars are 20 μ m. (B) Histogram and scatter plot showing DNA content as measured using DRAQ5 in FACS-sorted endothelial cells (CD45⁺CD31⁺CD326⁻) from control and *PDGFb-iCre/Foxf1^{fl/+}* lungs 19 days after PNx. (C) Quantification of percentage of endothelial cells in S/G₂/M phases 19 days after PNx in control and *PDGFb-iCre/Foxf1^{fl/+}* lungs. n=3.

Supplemental Figure 7. *Mmp14* expression unchanged by *Foxf1*-deficiency.

Western blot analysis showed MMP14 protein levels were not affected by *Foxf1*-deficiency. Cropped gels are presented here with full gel available in Supplemental Figure 9. qRT-PCR analysis showed that *Mmp14* mRNA levels were not altered in *PDGFb-iCre/Foxf1^{fl/+}* lungs compared to control. n=4.

Supplemental Figure 8. Full images for PECAM, CDKN1A, and CDKN2B Western blots.

(A) Full blots for PECAM-1 and corresponding β -ACTIN along with digital image of blot showing ladder. PECAM-1 Western blot using Abcam 28364 antibody has been previously published¹. Western blot using Santa Cruz β -ACTIN antibody SC-1615 has also been previously published². (B) Full blots for CDKN1A (P21^{Cip1}), CDKN2B (P15^{INK4b}), and corresponding β -ACTIN. Membrane was cut prior to staining so ladder is not shown. CDKN1A Western blot using Cell Signaling Technology antibody 2947 has been previously published³. CDKN2B Western blot using Aviva Systems Biology antibody AVARP03047 has been previously published⁴.

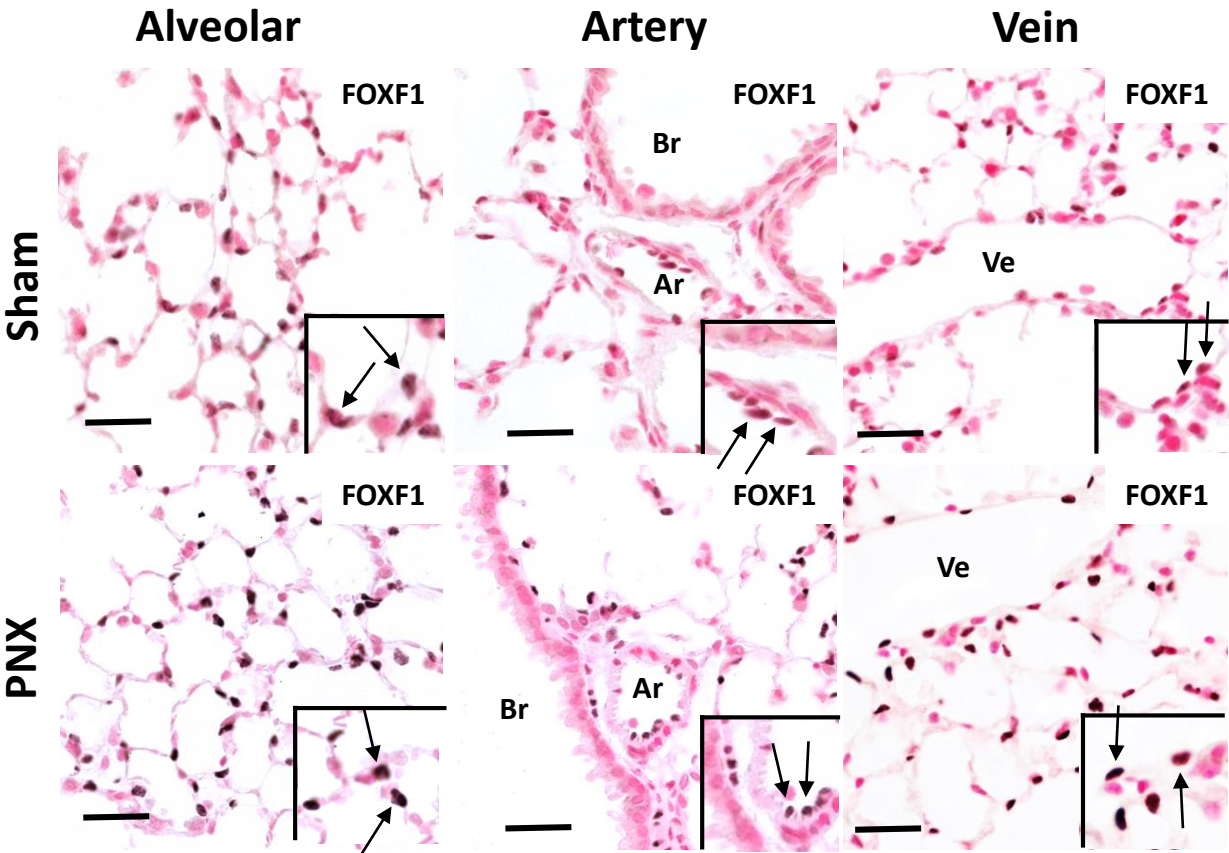
Supplemental Figure 9. Full images for Western blots and zymography.

(A) Full zymography gel used for analysis of MMP14 activity. (B) Full blots for MMP14 and corresponding β -ACTIN. MMP14 Western blot using Abcam 51074 antibody has been previously published⁵.

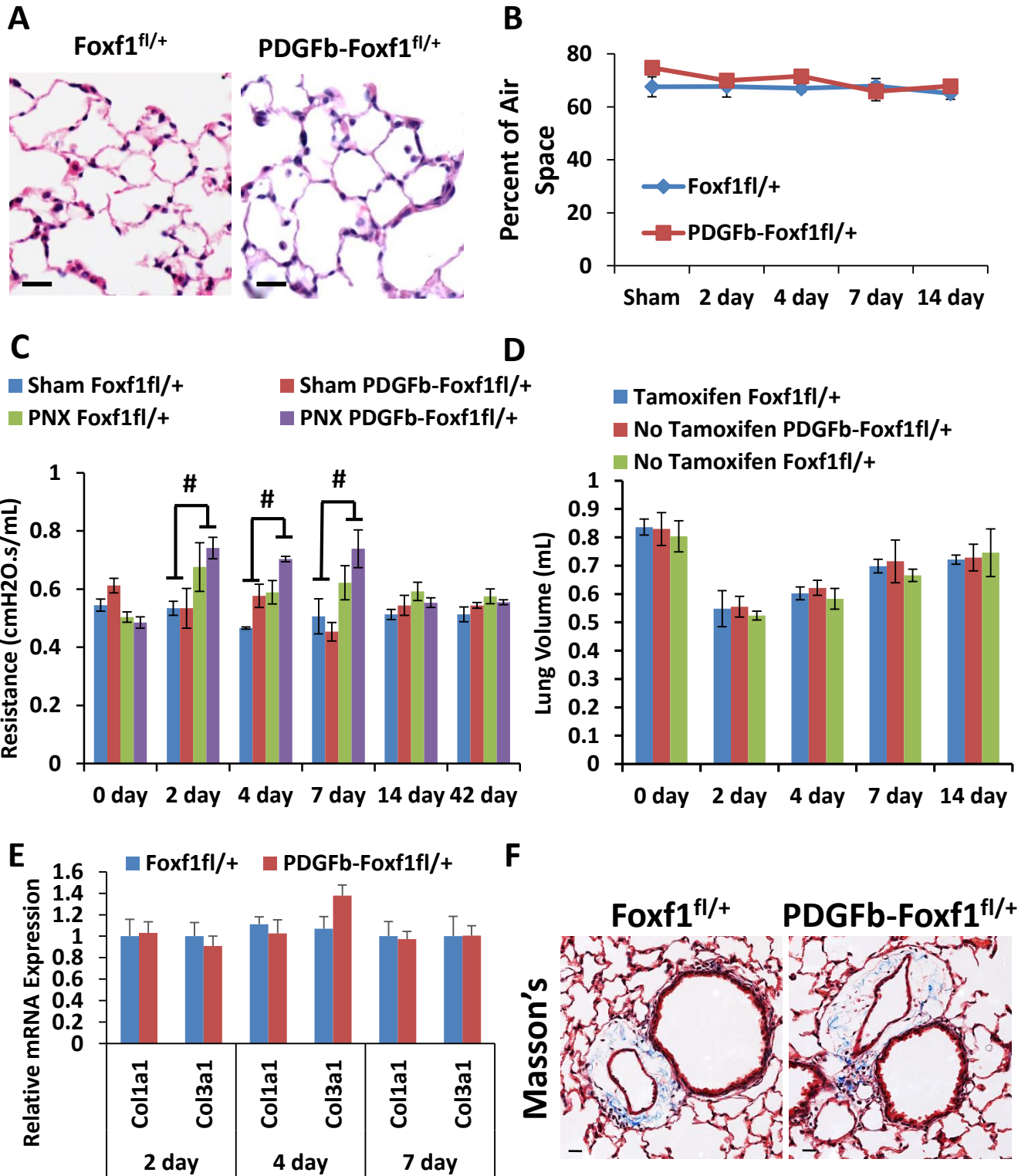
Supplemental References

- 1 Corallo, C. *et al.* Bosentan and macitentan prevent the endothelial-to-mesenchymal transition (EndoMT) in systemic sclerosis: in vitro study. *Arthritis Res Ther* **18**, 228, doi:10.1186/s13075-016-1122-y (2016).
- 2 Sato, M. *et al.* LW6, a hypoxia-inducible factor 1 inhibitor, selectively induces apoptosis in hypoxic cells through depolarization of mitochondria in A549 human lung cancer cells. *Mol Med Rep* **12**, 3462-3468, doi:10.3892/mmr.2015.3862 (2015).
- 3 Fiorentino, F. P. *et al.* Growth suppression by MYC inhibition in small cell lung cancer cells with TP53 and RB1 inactivation. *Oncotarget* **7**, 31014-31028, doi:10.18632/oncotarget.8826 (2016).
- 4 Scott, S. A. *et al.* Methylation status of cyclin-dependent kinase inhibitor genes within the transforming growth factor beta pathway in human T-cell lymphoblastic lymphoma/leukemia. *Leuk Res* **28**, 1293-1301, doi:10.1016/j.leukres.2004.03.019 (2004).
- 5 Ding, B. S., Gomi, K., Rafii, S., Crystal, R. G. & Walters, M. S. Endothelial MMP14 is required for endothelial-dependent growth support of human airway basal cells. *J Cell Sci* **128**, 2983-2988, doi:10.1242/jcs.168179 (2015).

Bolte Supplemental Figure 1

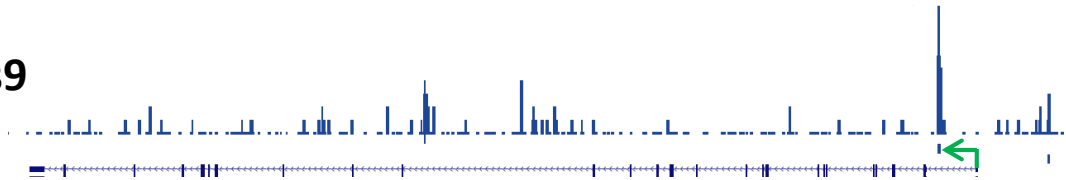


Bolte Supplemental Figure 2



Bolte Supplemental Figure 3

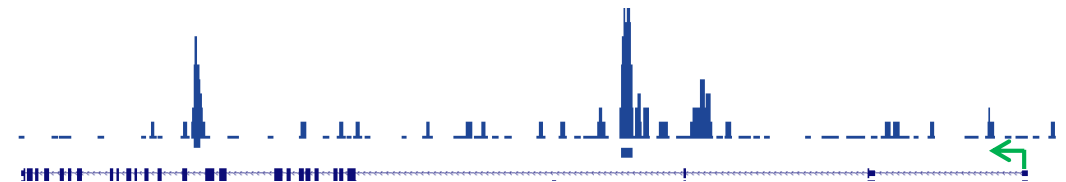
Adamts9



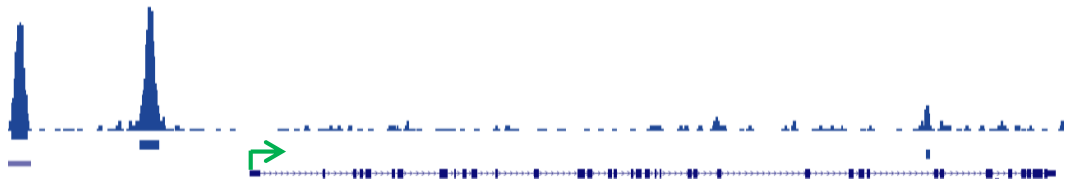
Ccnd3



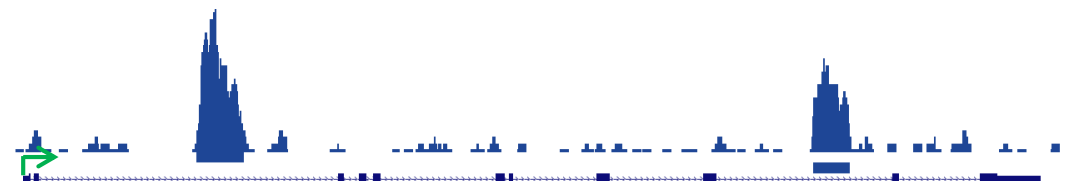
Hdac5



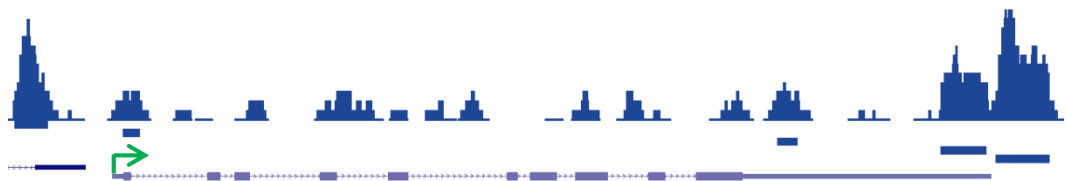
Itgb4



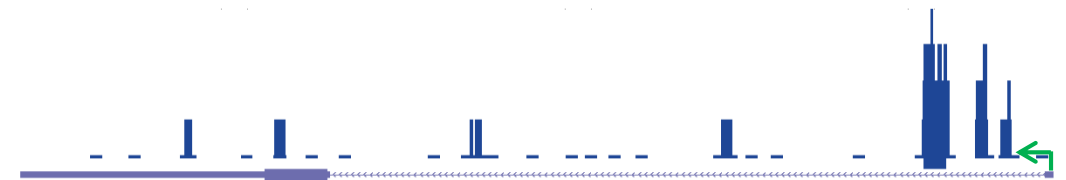
Ptgs1



Ptgs2

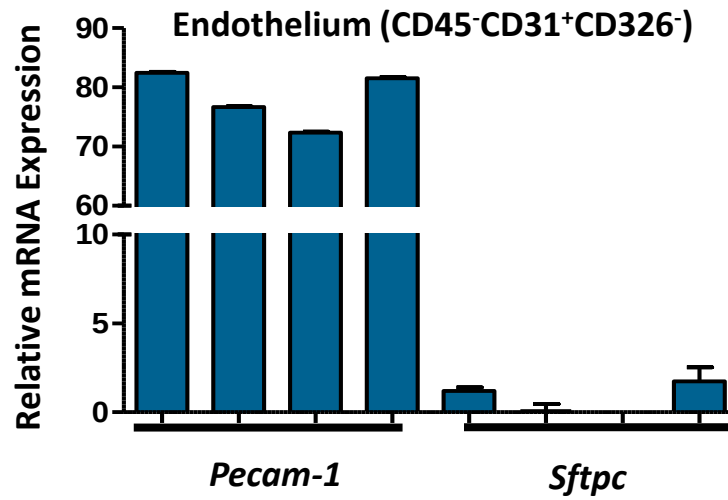


Spry4

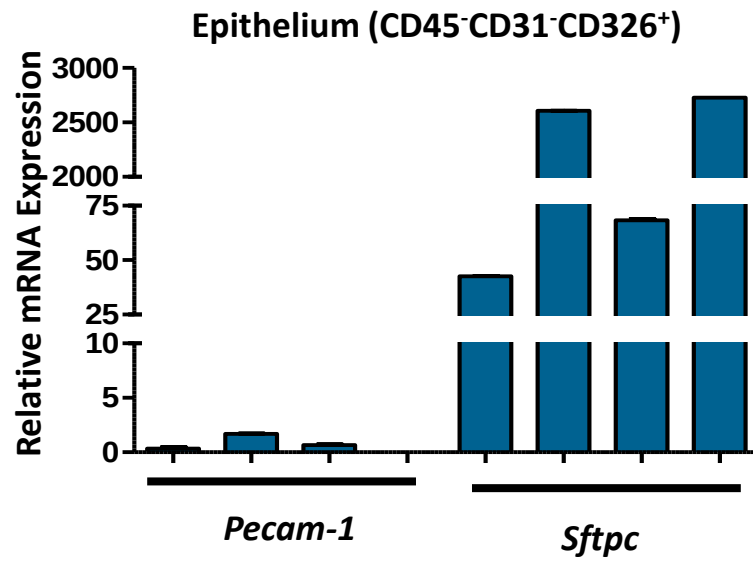


Bolte Supplemental Figure 4

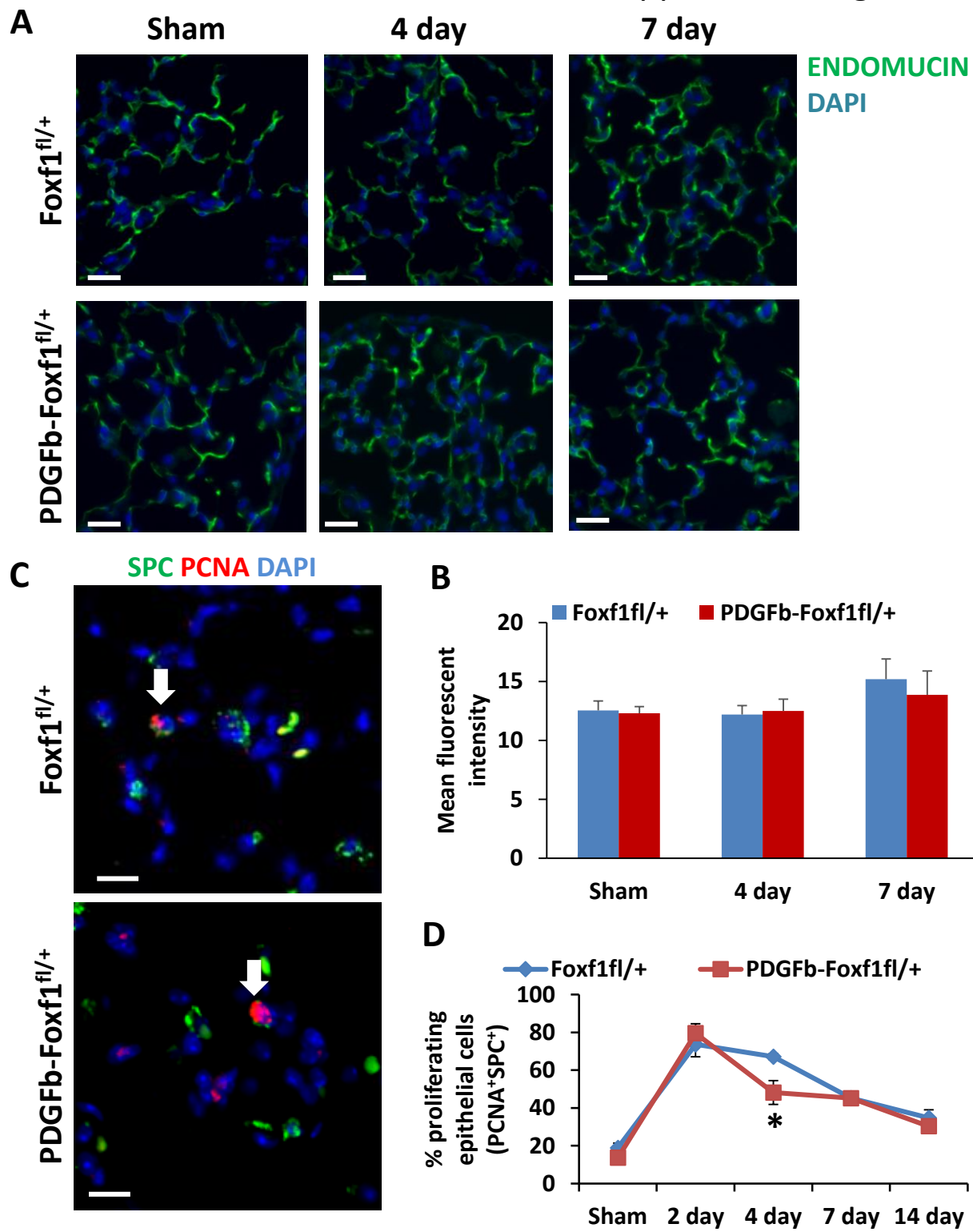
A



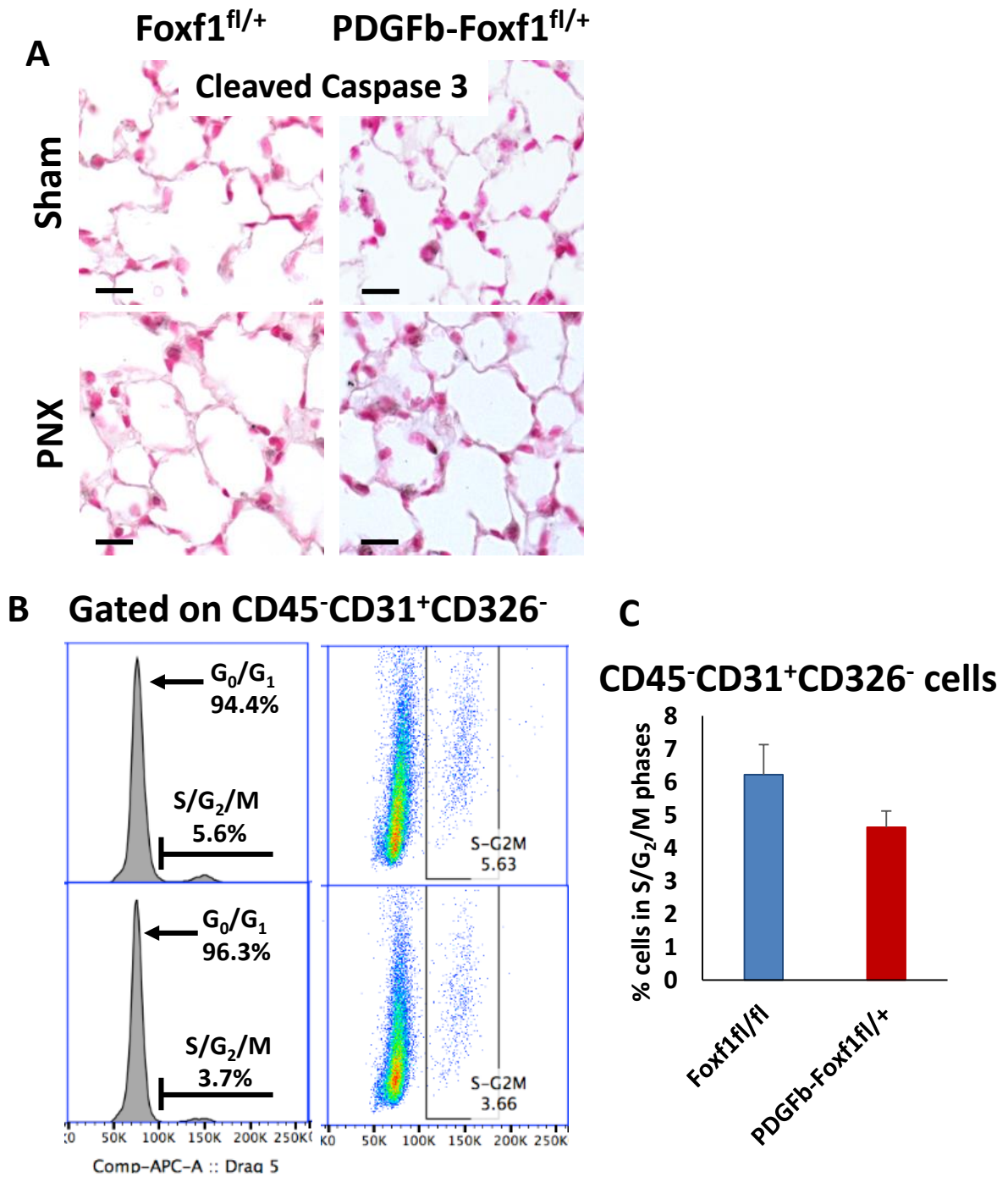
B



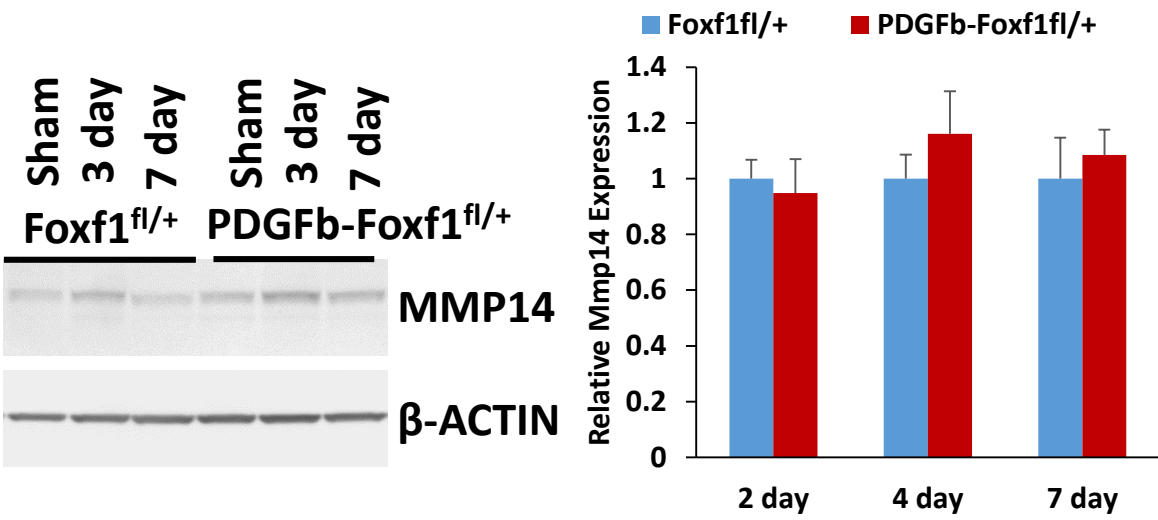
Bolte Supplemental Figure 5



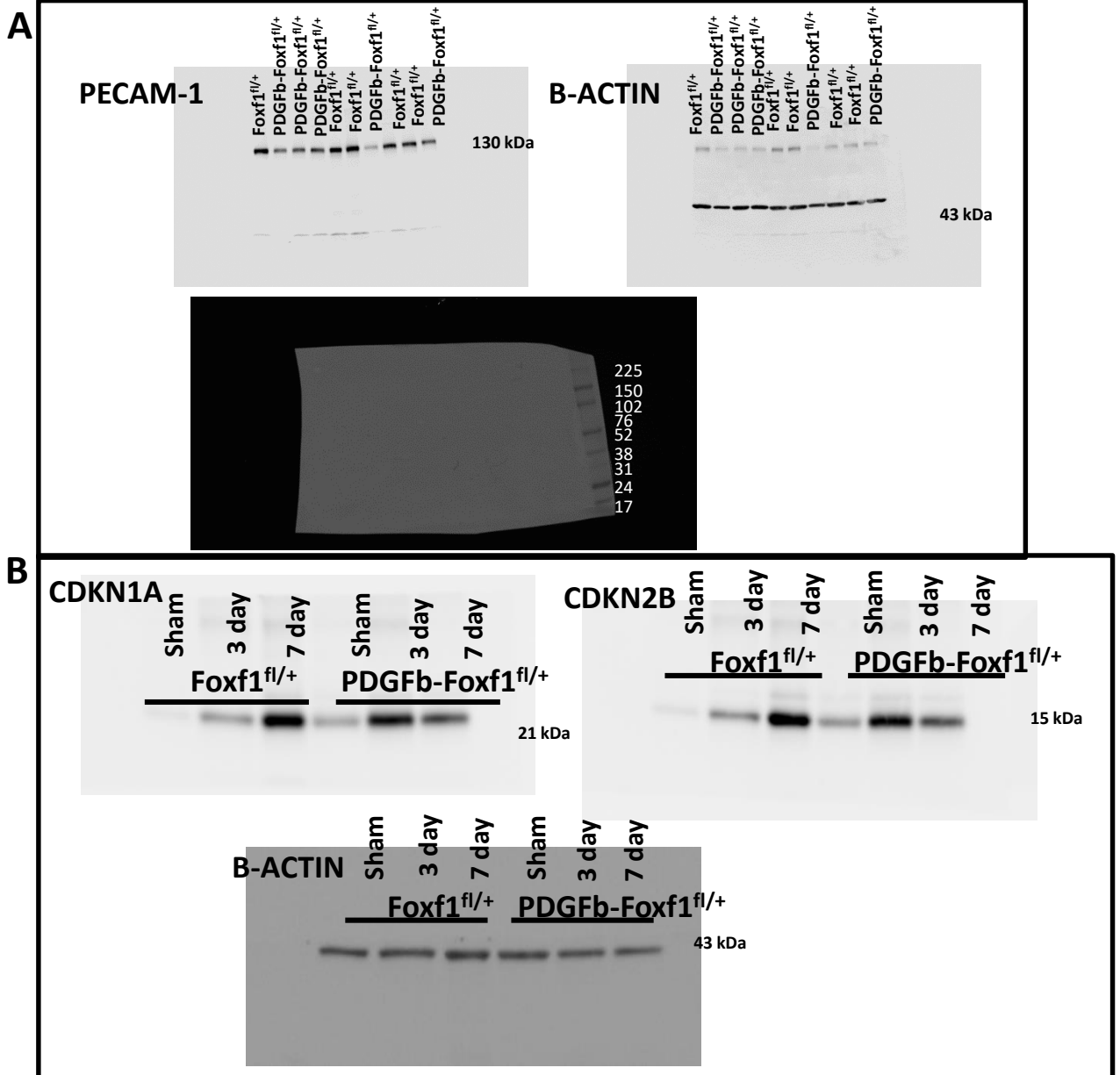
Bolte Supplemental Figure 6



Bolte Supplemental Figure 7



Bolte Supplemental Figure 8



Bolte Supplemental Figure 9

