

## Supplemental Methods, Figures, Tables, and References

## Supplemental Methods

*FOXM1 adenovirus transduction.* CCL210 cells were infected with recombinant adenovirus expressing FOXM1 (AdFOXM1) (1) (a gift from Guy Adami, University of Illinois at Chicago-Chicago College of Dentistry, Chicago, Illinois, USA) in DMEM containing 10% FBS and 2.5 µg/ml of polybrene. At 6 hours after infection, culture medium was changed and replaced with fresh medium without polybrene. To study the inhibitory actions of PGE<sub>2</sub>, cells were treated with and without PGE<sub>2</sub> followed by stimulation with and without FGF2. After 24 hours of incubation, cells were processed for ChIP assay.

*Quantitative reverse-transcriptase PCR.* mRNA expression in cell lysates or lung homogenates was analyzed by qPCR. Briefly, cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems) with 800 ng of total RNA. cDNA was amplified with Fast SYBR Green Master Mix, and analyzed on a StepOne real time PCR system (Applied Biosystems). PCR was performed using a Fast SYBR Green master mix (Applied BioSystems) and the primers used are listed in Supplemental Table 1.

*siRNA transfection.* CCL210 cells were plated at 2 x 10<sup>6</sup> cells / 6 cm plate in DMEM supplemented with 10% FBS and incubated overnight. Cells were transiently transfected with 50 nM siRNA targeting FOXM1, FOXO3A and scrambled siRNA

negative control, all purchased from Dharmacon. The siRNA sequences are listed in Supplemental Table 2. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Briefly, both RNAiMax and siRNA were diluted in Opti-MEM and mixed at 1:1 ratio and incubated for 10 minutes at room temperature. siRNA-lipid complexes were then added to the cultures in serum free medium. After 24 hours of transfection, cells were treated as described. Knockdown of FOXM1 and FOXO3A mRNA levels was confirmed by qPCR.

*Quantitative chromatin immunoprecipitation assay.* ChIP assay was performed as previously described (2) using the ChIP-IT Express kit (Active Motif) to measure FOXM1 binding to promoters of target genes *CCNB1*, *PLK1*, and *BIRC5*. In brief, cross-linked chromatin was sonicated for 15 minutes using a Bioruptor Pico (Diagenode) to shear chromatin fragments to ~ 200 - 500 bp in length. Cross-links in a portion of sheared chromatin were reversed at 65 °C for 4 hours, and cross-linked DNA was purified by the QIAquick PCR purification kit (Qiagen). Sonicated chromatin was immunoprecipitated with 4 µg of rabbit anti-FOXM1 polyclonal antibody (catalog sc-502 from Santa Cruz Biotechnology); immunoprecipitation with 4 µg of rabbit IgG isotype antibody was used as a negative control. Immune complexes were recovered with Magna ChIP protein A magnetic beads (Millipore). DNA was purified and qPCR was performed to quantify FOXM1 binding to target gene promoter fragments. The primers used to detect the promoter regions for

FOX M1 binding are listed in Supplemental Table 1. Chromatin binding was calculated as the percentage of immunoprecipitated DNA relative to the amount of input.

*Immunofluorescence microscopy.* Cells were seeded into sterile two-chamber slides (Nunc). On reaching 60-70% confluence, cells were starved in serum-free DMEM for 24 hours. Cells were then treated as described. For immunofluorescence studies, chambers were washed twice with chilled PBS and fixed with freshly prepared 4% formaldehyde for 20 minutes and quenched with 50 mM NH<sub>4</sub>Cl for 15 minutes. Slides were blocked and permeabilized with 10% goat serum in 0.1% Triton X100 for 1 hour. Cells were incubated with FITC-conjugated  $\alpha$ -SMA mAb (1:500) overnight at 4°C. DAPI was used to stain the nuclei. Slides were examined using a Leica DC 500-fluorescence microscope equipped with a digital camera.

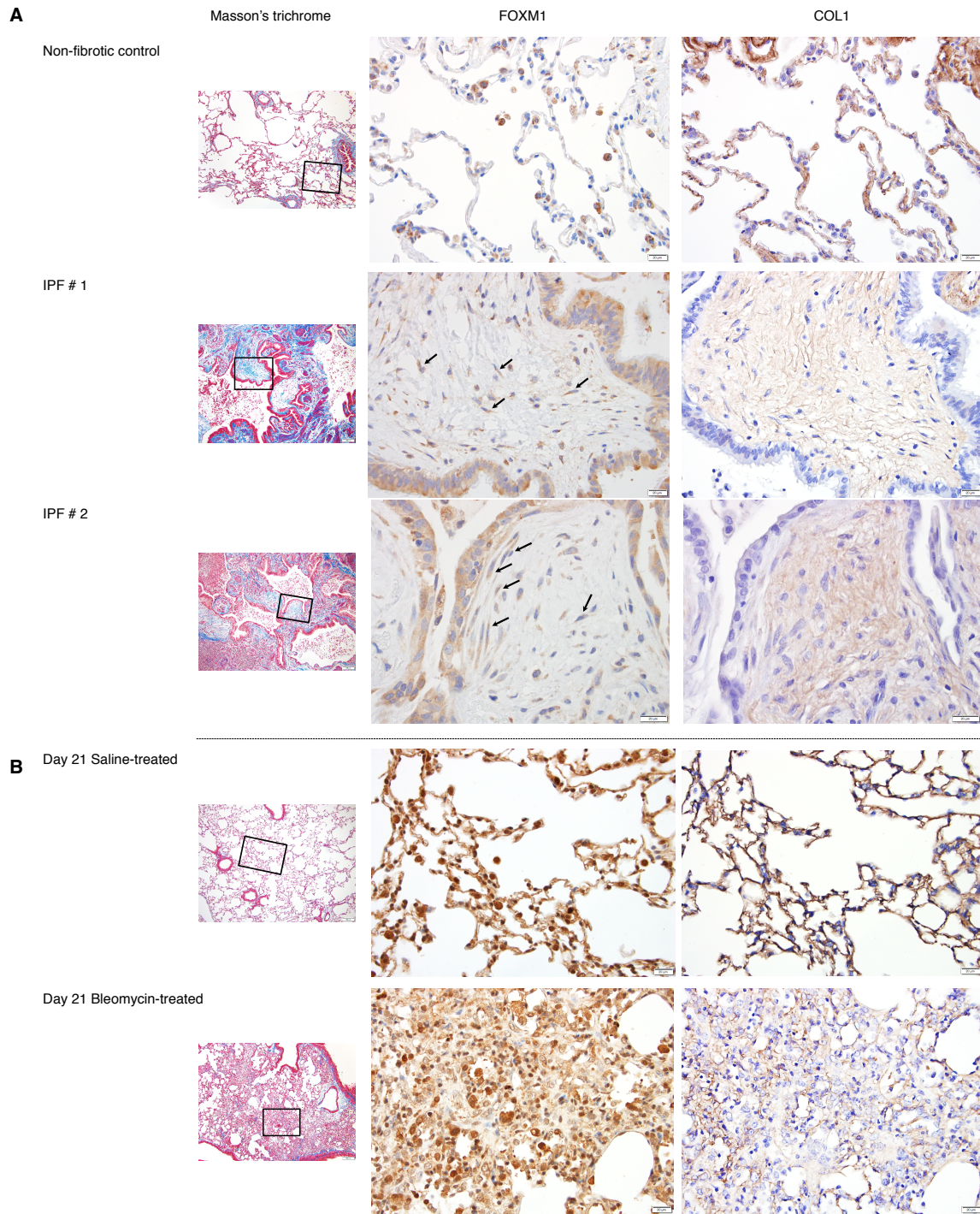
*Immunohistochemistry.* After deparaffinization and blocking of nonspecific binding, lung sections were washed with PBST (0.1 M phosphate buffer, pH 7.4, 0.1% (v/v) Tween 20), and then incubated overnight at 4°C with anti-FOX M1 antibody (1:100; catalog sc-502 from Santa Cruz Biotechnology), anti-collagen 1 antibody (1:100; catalog ab34710 from Abcam) or anti-cleaved casp-3 antibody (1:100; catalog 9661 from Cell Signaling Technology). The sections were washed twice with PBST and detection was performed by a biotin-free polymer based commercial detection

system (Biocare Rabbit-on-Rodent HRP Polymer) with the chromogen diaminobenzidine and a hematoxylin nuclear counterstain.

*Viability assays.* Cell viability assays were performed using CCL210 fibroblasts and BEAS-2B cells. Briefly, cells were plated at  $1 \times 10^4$  cells/well in a 96-well plate in complete medium (DMEM with 10% FBS). After 24 hours, cells were treated with concentrations of Sio A ranging from 1.25 to 10  $\mu$ M. ATP content was measured by the CellTiter-Glo luminescent cell viability kit (Promega).

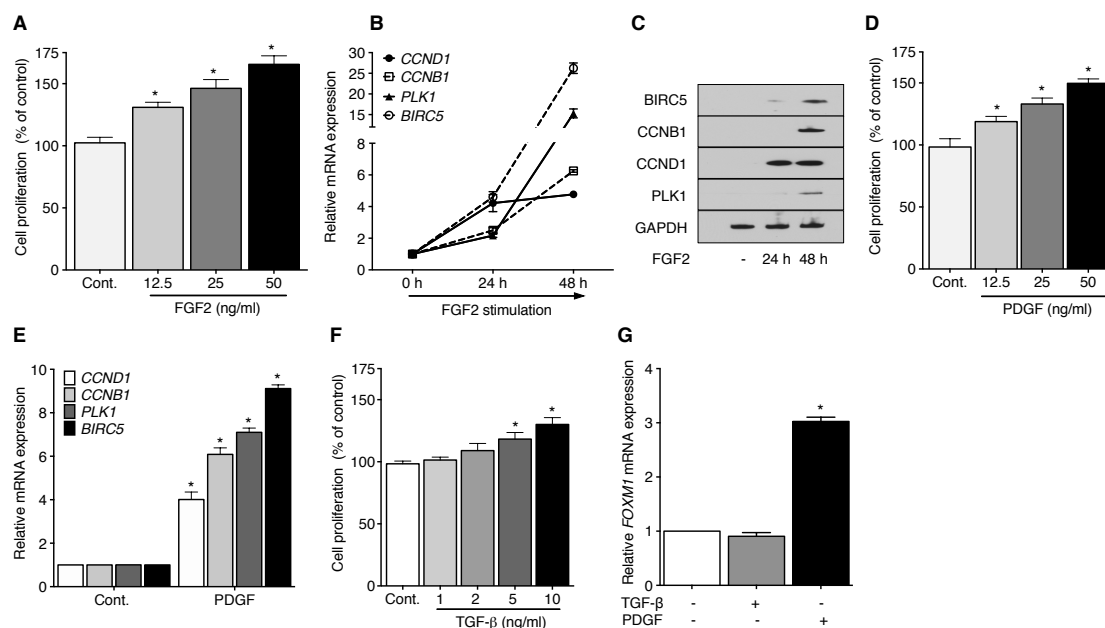
*TGF- $\beta$  ELISA.* The levels of active TGF- $\beta$  in murine BAL fluid were measured using the LEGEND MAX Mouse Latent TGF- $\beta$  ELISA kit (Biolegend). Briefly, 50  $\mu$ L BAL fluid was used for each assay and the assay was performed in triplicate according to the manufacturer's guidelines. Color development was quantified by measuring the absorbance measured at 450 nm using an ELISA plate reader (Microplate AutoReader EL311; Bio-Tek Instruments).

## Supplemental Figures



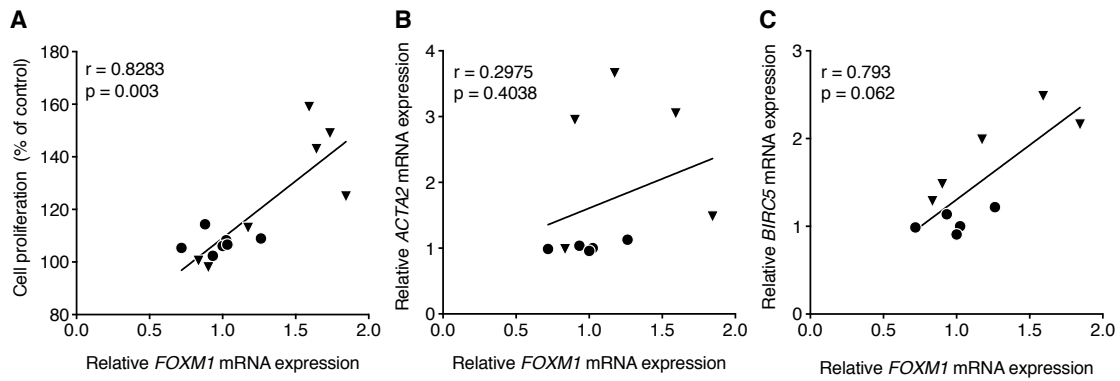
**Supplemental Figure 1. Greater FOXM1 expression in sections of fibrotic than control lung. (A, B) Digital images of Masson's trichrome,**

immunohistochemical (IHC) staining of FOXM1 and COL1 in serial lung sections derived from control and IPF patients (**A**) and saline-treated and bleomycin-treated murine lung sections (**B**). In (**A**), #1 and #2 refer to distinct patients, and arrows indicate spindle-shaped cells in fibrotic foci that stain positively for both FOXM1 and COL1. Original IHC images at 200x objective magnification, scale bars = 100  $\mu\text{m}$  (for Masson's trichrome images) and 20  $\mu\text{m}$  (for IHC images).

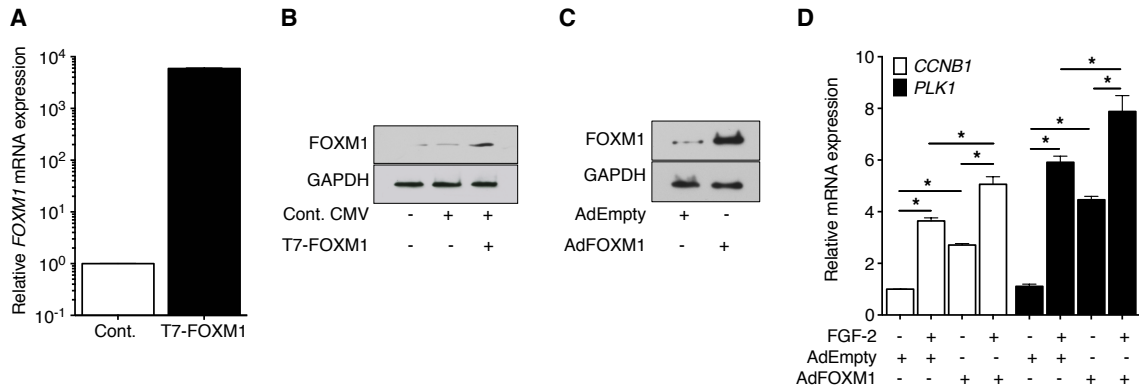


**Supplemental Figure 2. Effects of growth factors on fibroblast proliferation and expression of FOXM1 and cell cycle-regulated genes. (A, D, F)** Dose-related effects of FGF2-, PDGF-, or TGF- $\beta$  on CCL210 fibroblast proliferation as determined by the CyQUANT NF Cell Proliferation Assay at 72 hours. **(B, C)** Effect of FGF2 stimulation on cell cycle-regulated gene expression evaluated by qPCR **(B)** and by Western blot **(C)**. **(E)** Effect of PDGF stimulation on cell cycle-regulated gene expression evaluated by qPCR. **(G)** Effect of TGF- $\beta$  stimulation on *FOXM1* expression evaluated by qPCR **(G)**. In **A, D, F**, the control value represents the fluorescence value of cells initially seeded. The bar graphs in **A, D, F** represent the percentage change from a single experiment representative of a total of 3 independent experiments, each performed in quadruplicate. Bar graphs in **B, E, G** represent the mean  $\pm$  s.e.m. of 3 independent experiments and the Western blot data in **E** is representative of 1 of 3 independent experiments. \* $P < 0.05$ , 2-way ANOVA.

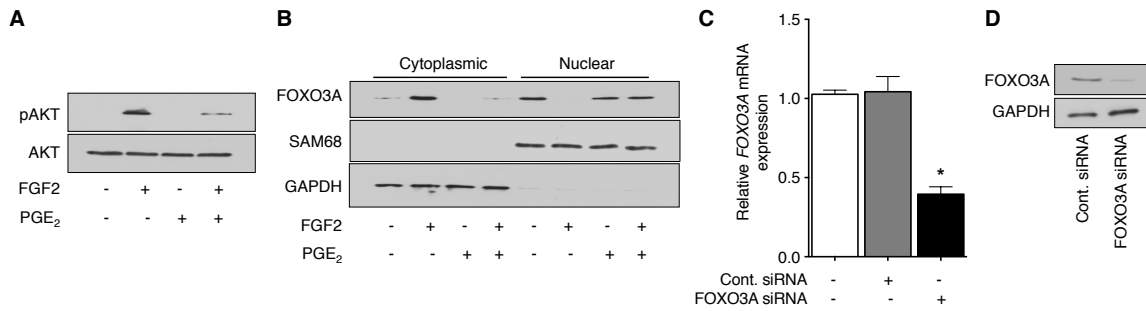




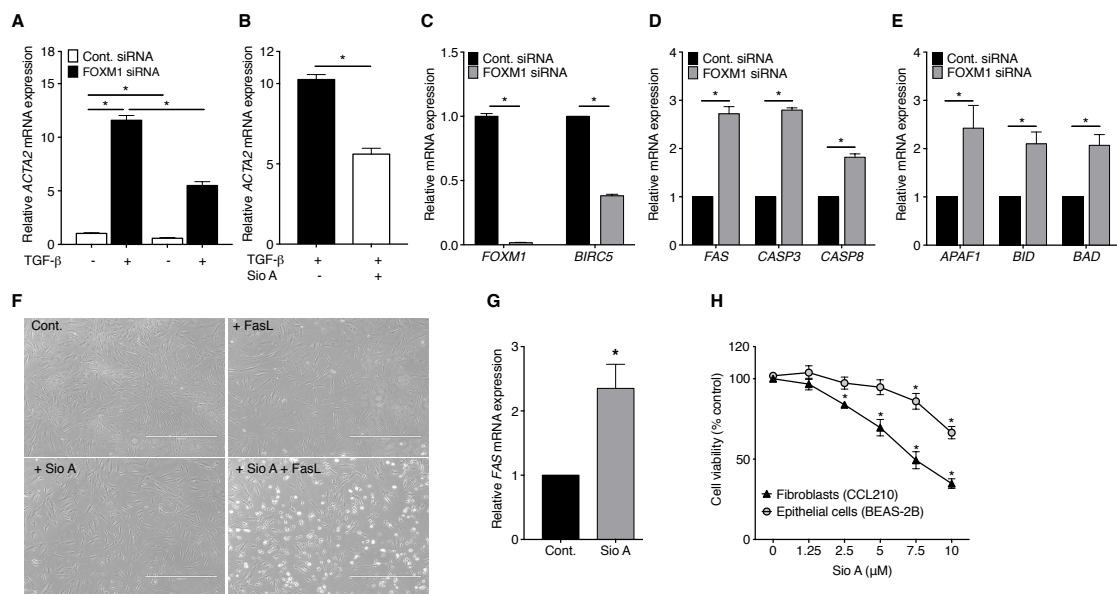
**Supplemental Figure 3. Correlation between *FOXM1* mRNA expression and patient-derived fibroblast activation phenotypes.** Correlation analysis was performed between the basal *FOXM1* mRNA expression and cell proliferation rate (**A**), *BIRC5* (encodes survivin) mRNA expression (**B**), and *ACTA2* (encodes  $\alpha$ -SMA) mRNA expression (**C**) in non-fibrotic (circles) and IPF (triangles) fibroblasts. Expression of *FOXM1*, *BIRC5* and *ACTA2* were determined by qPCR. Proliferation of fibroblasts was quantified by the CyQUANT NF Cell Proliferation Assay at 72 hours, and the control value represents the fluorescence value of cells initially seeded. 2-way ANOVA.



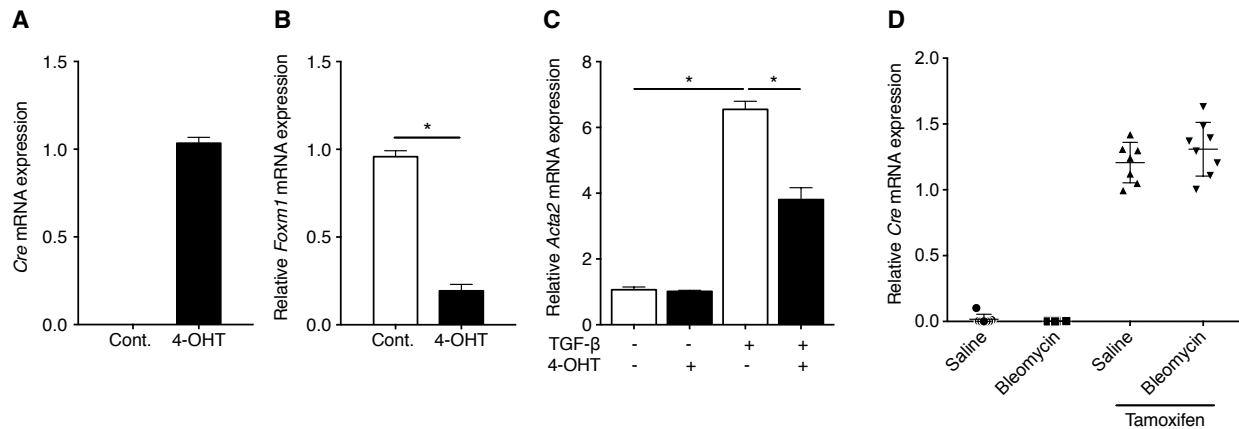
**Supplemental Figure 4. Expression of FOXM1 and cell cycle-regulated genes driven by CMV T7-FOXM1 and AdFOXM1.** (A, B) Expression of *FOXM1* in CCL210 cells transfected with CMV T7-FOXM1 overexpression plasmid or control plasmid determined by qPCR (A) and by Western blot (B). (C, D) Expression of FOXM1 by Western blot (C) and cell cycle-regulated genes *CCNB1* and *PLK1* by qPCR (D) in CCL210 cells infected with AdFOXM1 or control adenovirus (AdEmpty) with and without FGF2 for 24 hours. Bar graphs in A represent 1 of 2 independent experiments and the graphs in D represent the mean  $\pm$  s.e.m. of 3 independent experiments. The Western blots in B, C are representative of 1 of 3 independent experiments. \* $P < 0.05$ , 2-way ANOVA.



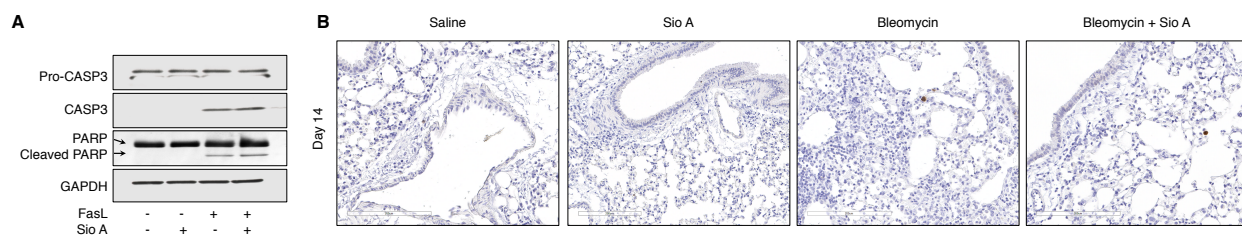
**Supplemental Figure 5. PGE<sub>2</sub> effects on AKT phosphorylation and characterization of FOXO3A knockdown.** (A) Inhibition of FGF2-induced AKT phosphorylation by PGE<sub>2</sub> in CCL210 cells as determined by Western blot. (B) FOXO3A localization was determined by subcellular fractionation followed by Western blot analysis of nuclear and cytoplasmic fractions. (C, D) FOXO3A knockdown by FOXO3A siRNA as determined by qPCR (C) and by Western blot (D). Western blots in A, B, D are representative of 1 of 3 independent experiments and the bar graphs in C represent the mean  $\pm$  s.e.m. of 3 independent experiments. \* $P < 0.05$ , 2-way ANOVA.



**Supplemental Figure 6. Effects of FOXM1 inhibition on cell viability, differentiation and apoptosis.** (A) Effect of pretreatment with FOXM1 or control siRNA on TGF-β-induced expression of *ACTA2* as determined by qPCR in CCL210 cells. (B) Reversal by Sio A of TGF-β-generated myofibroblast differentiation as determined by qPCR analysis of *ACTA2*. (C-E) Effect of transfection with FOXM1 siRNA or scrambled (control) siRNA (for 16 hours) on TGF-β-generated myofibroblast expression of *FOXM1* and *BIRC5* (C) and pro-apoptotic genes *FAS*, *CASP3*, *CASP8* (D), *APAF1*, *BID* and *BAD* (E), as determined by qPCR analysis. (F) Effect of Sio A on morphological changes in TGF-β-generated myofibroblasts. Representative phase contrast micrographs from myofibroblasts treated with Sio A with and without FasL for 24 hours, depicting cell rounding and blebbing structures. (G) Effect of Sio A (for 16 hours) on TGF-β-generated myofibroblast expression of pro-apoptotic gene *FAS* as determined by qPCR analysis. (H) CCL210 fibroblasts and BEAS-2B epithelial cells were treated with Sio A (1.25 to 10 μM) for 48 hours and cell viability was determined using a CellTiter-Glo luminescent cell viability kit. The graphs in A, B, C, D, E, G and H represent the mean ± s.e.m. of 3 independent experiments. \**P* < 0.05, 2-way ANOVA.



**Supplemental Figure 7. Validation of conditional *FOXM1* deletion in lung fibroblasts.** Lung fibroblasts were isolated from conditional *FOXM1* knockout mice and treated for 24 hours with and without 4-hydroxy tamoxifen (4-OHT). Expression of *Cre* (**A**) and *Foxm1* (**B**) were determined by qPCR. (**C**) Cells were pretreated with and without 4-OHT for 24 hours and then stimulated  $\pm$  TGF- $\beta$  for additional 24 hours. Expression of *Acta2* was determined by qPCR. (**D**) Expression of *Cre* in lung homogenates of mice treated with and without tamoxifen with and without bleomycin, evaluated on day 21 by qPCR. Values in each group represent results from two pooled independent experiments with a total of 5-8 mice per group. The bars represent the mean  $\pm$  s.e.m. of 3 independent experiments. \* $P < 0.05$ , 2-way ANOVA.



**Supplemental Figure 8. Sio A did not induce apoptosis of lung epithelial cells *in vitro* and alveolar cells *in vivo*.** (A) Effects of a 1 hour pretreatment with 2.5  $\mu$ M Sio A on FasL-induced apoptosis in murine lung epithelial cells (MLE-12), as determined by active Caspase 3 (CASP3) and cleaved PARP expression assessed by Western blot. (B) Effect of Sio A treatment in mice treated with and without bleomycin, as determined by IHC analysis of CASP3 in paraffin sections of lungs at day 14. Images in B are representative IHC images showing CASP3 (brown) and a hematoxylin nuclear counterstain. Original magnification,  $\times 200$ . Scale bars: 200  $\mu$ m.

## Supplemental Tables

**Supplemental Table 1. Primer sequences**

Gene	Forward sequence (5' - 3')	Reverse sequence (5' - 3')
qPCR		
<i>GAPDH</i>	CAGCCTCAAGATCATCAGCA	ACAGTCTTCTGGGTGGCAGT
<i>FOXM1</i>	GCAGGCTGCACTATCAACAA	TCGAAGGCTCCTCAACCTTA
<i>CCNB1</i>	TGTGGATGCAGAAGATGGAG	GTGACTTCCCGACCCAGTAG
<i>CCND1</i>	CGTGGCCTCTAAGATGAAGG	CCACTTGAGCTTGTTCACCA
<i>PLK1</i>	AGTCGACCACCTCACCTGTC	CTGACCATTCCACCAAGGTT
<i>BIRC5</i>	CCACTGAGAACGAGCCAGAC	GACAGAAAGGAAAGCGCAAC
<i>PIK3CA</i>	GCTCTGTTAAAGGCCGAAAG	ACTGGCCAAAGATTCAAAGC
<i>PIK3CB</i>	GCAAGTCAGCGGGAGAGTAG	CTTGATCTTGCAGCATTCCA
<i>PIK3CG</i>	GTGATGATCTGCGCCAAGAC	CCATATGGCAGGAGGCATAG
<i>PIK3CD</i>	GTACGCCGTGATCGAGAAAG	CGGTCTTAAGCTGGTCCTTG
<i>APAF1</i>	GGAGTGCATTGGGTTTCAGT	GAGAGACCTTGGGTGTTTGC
<i>BID</i>	TGTGAACCAGGAGTGAGTCG	TCTCTGCGGAAGCTGTTGTC
<i>BAD</i>	CGGAGGATGAGTGACGAGTT	ATCCCACCAGGACTGGAAGA
<i>FAS</i>	CGGACCCAGAATACCAAGTG	TGGTGAGTGTGCATTTCCTTG
<i>CASP3</i>	GCGAATCAATGGACTCTGGA	GCTGCATCGACATCTGTACC
<i>CASP8</i>	GAAAGGGTGGAGCGGATTAT	GCTTCCTTTGCGGAATGTAG
<i>Actb</i>	GACGGCCAGGTCATCACTAT	GCACTGTGTTGGCATAGAGG
<i>Acta2</i>	ATCACCAACTGGGACGACAT	CATACATGGCTGGGACATTG
<i>Foxm1</i>	CTCCTTCTGGACCATTACC	GATTGGGTCGTTTCTGCTGT
<i>Tgfb1</i>	GGAGAGCCCTGGATACCAAC	ATCCACTTCCAACCCAGGTC
<i>Ctgf</i>	GCAGACTGGAGAAGCAGAGC	ACACTGGTGCAGCCAGAAGG
<i>Col1a1</i>	ACCTCAGGGTATTGCTGGAC	CACCACTTGATCCAGAAGGA
<i>Cre</i>	CGGTTATTCAACTTGCACCA	AGGTTGCAAGAACCTGATG
ChIP-qPCR		
<i>CCNB1</i>	CCGGAGCTTTCAGTTTGTTC	CGGAATGCCGAGACAAGG
<i>PLK1</i>	CAATGGGAAGGGAGTGAGTG	TACACCCAGCAGAAACCAAC
<i>BIRC5</i>	CCAGAGGGAGAAGATGTCCA	GTCGTTGTCCTCGAAAAAGC

**Supplemental Table 2. siRNA sequences**

Name of siRNA	Sequence (5' - 3')
siGenome Human FOXM1	GGACCACUUUCCCUACUUU
siGenome Human FOXO3A	CGAAUCAGCUGACGACAGU
	GUACUCAACUGCAAAC
	CAGUAAAGAGGUACGAAAA
	UGCAUUAACUUGCGGUAAU
Scrambled siRNA	UUCUCCGAACGUGUCACGUUU



## Supplemental References

1. Liu Y, Sadikot RT, Adami GR, Kalinichenko VV, Pendyala S, Natarajan V, et al. FoxM1 mediates the progenitor function of type II epithelial cells in repairing alveolar injury induced by *Pseudomonas aeruginosa*. *The Journal of experimental medicine*. 2011;208(7):1473-84.
2. Penke LR, Huang SK, White ES, and Peters-Golden M. Prostaglandin E2 inhibits alpha-smooth muscle actin transcription during myofibroblast differentiation via distinct mechanisms of modulation of serum response factor and myocardin-related transcription factor-A. *The Journal of biological chemistry*. 2014;289(24):17151-62.

Full unedited gel for Figure 1

Figure 1A  
raw Western blots

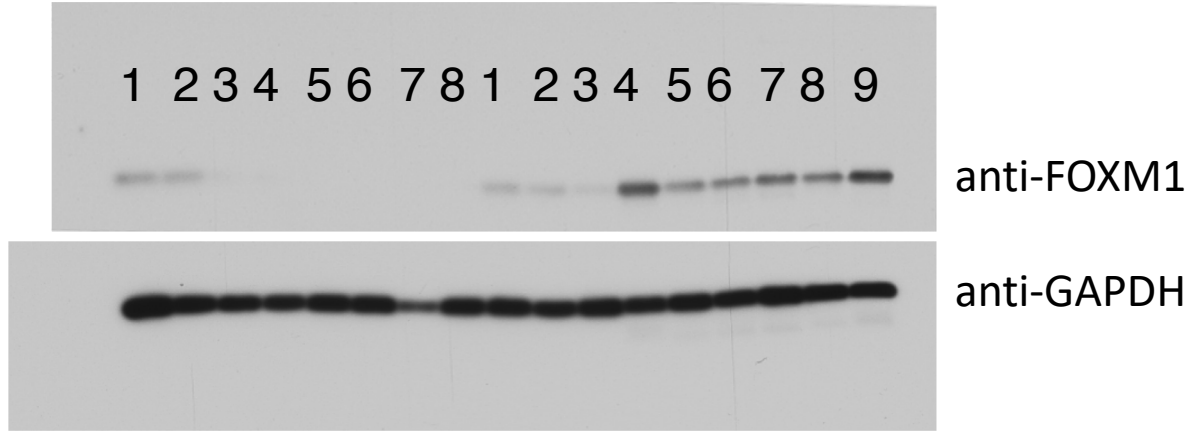
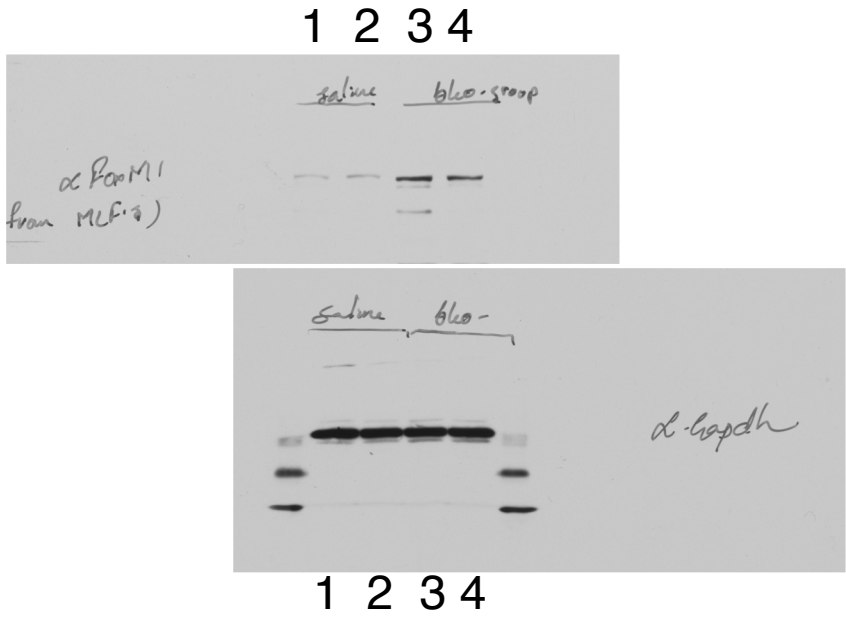
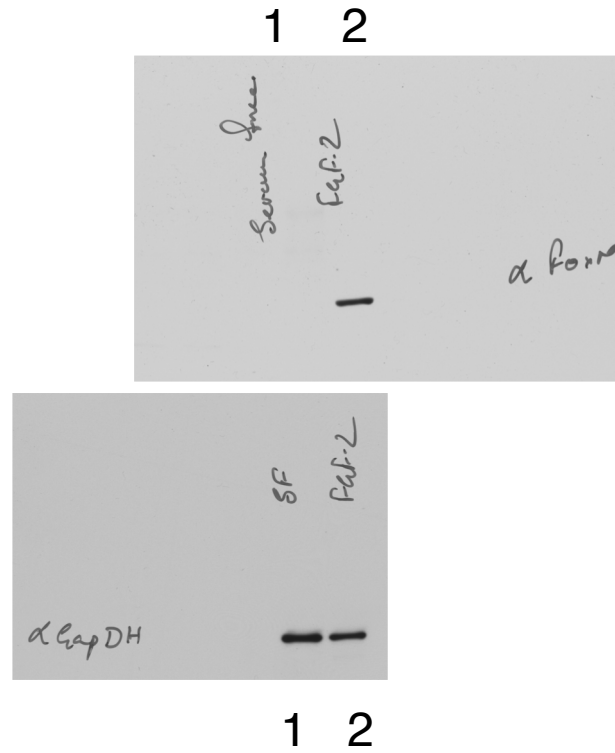


Figure 1C  
raw Western blots



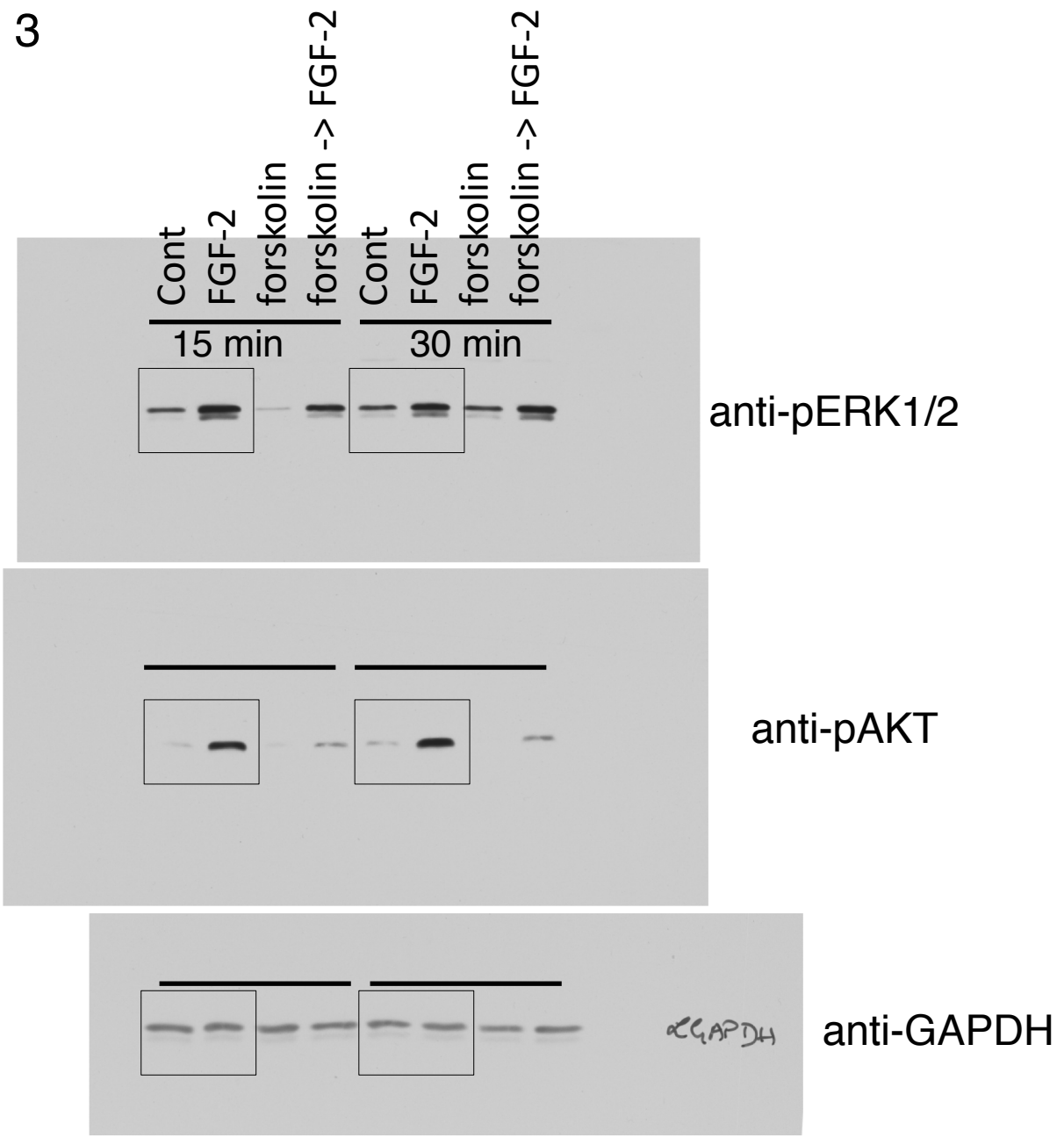
# Full unedited gel for Figure 2

Figure 1B  
raw Western blots



Full unedited gel for Figure 3

Figure 3A  
raw Westerntern blots

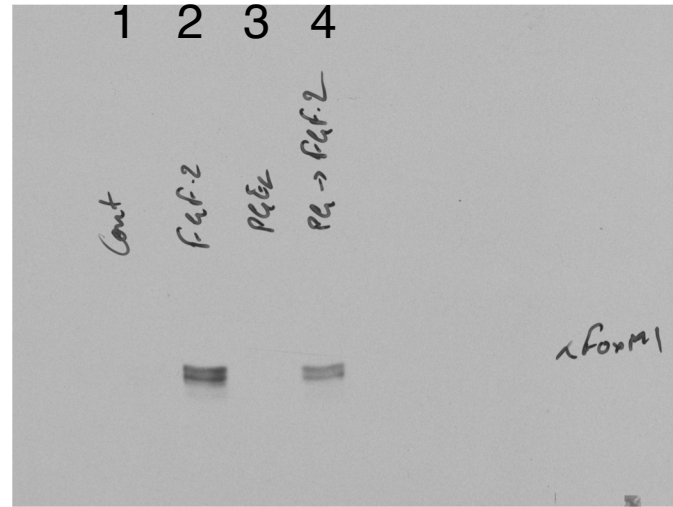


Lanes represented in square box of the unedited gel correspond to those shown in the cropped images used in the manuscript as Fig 3A.

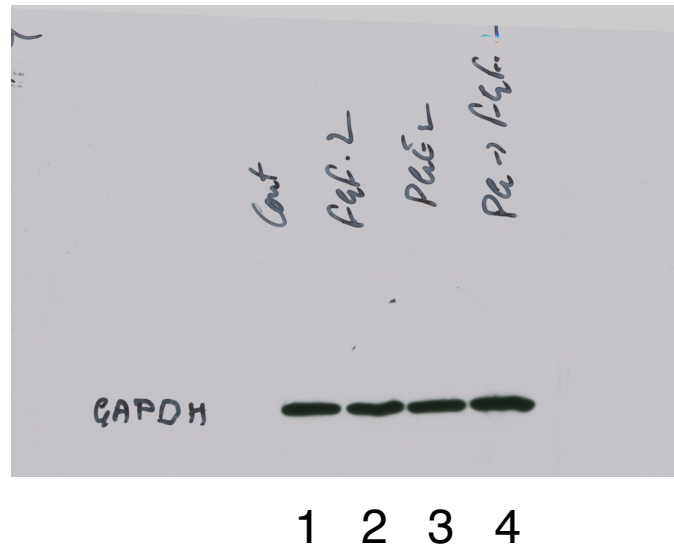
# Full unedited gel for Figure 4

## Figure 4D raw Western blots

FOXM1 →



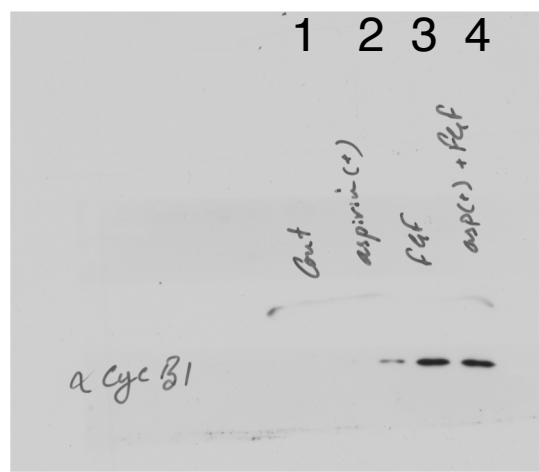
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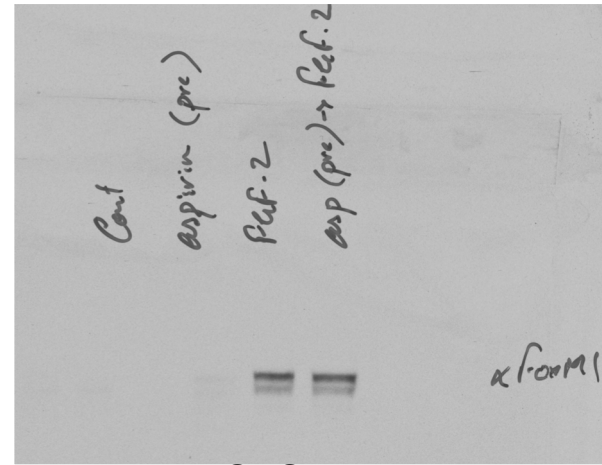
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## Figure 4G raw Western blots

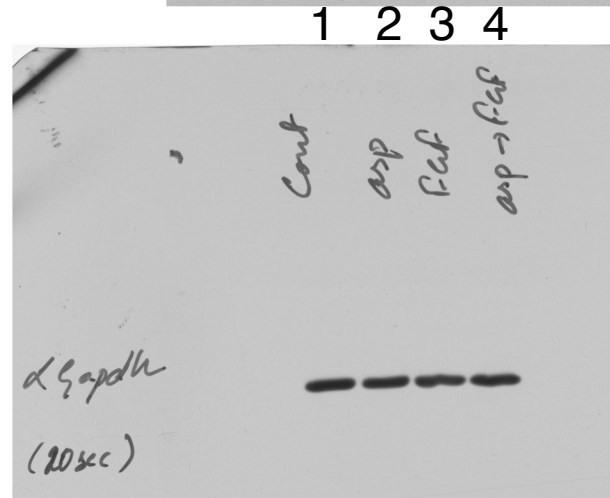
CCNB1 →



FOXM1 →

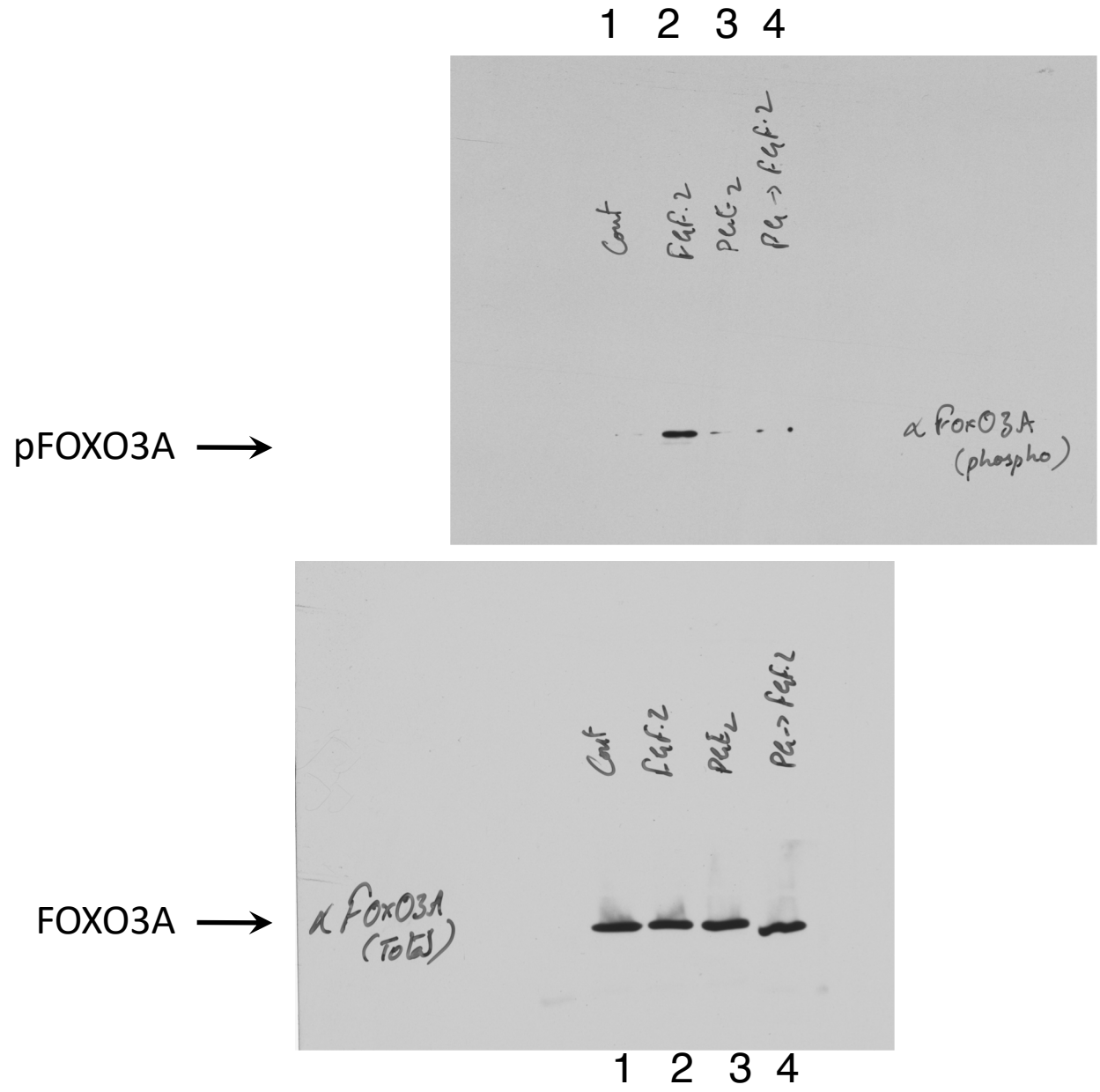


GAPDH →



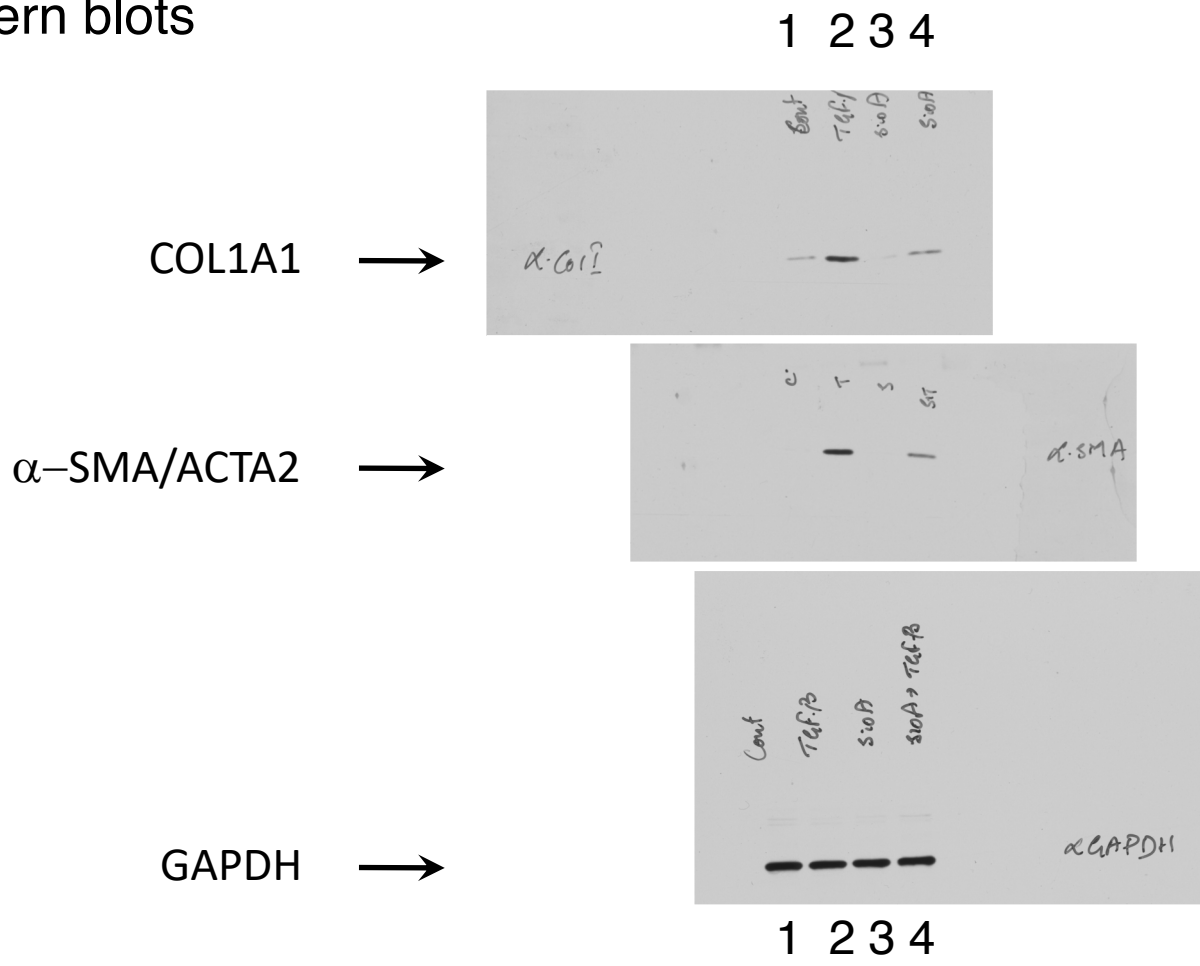
Full unedited gel for Figure 5

Figure 5E  
raw Western blots



# Full unedited gel for Figure 6

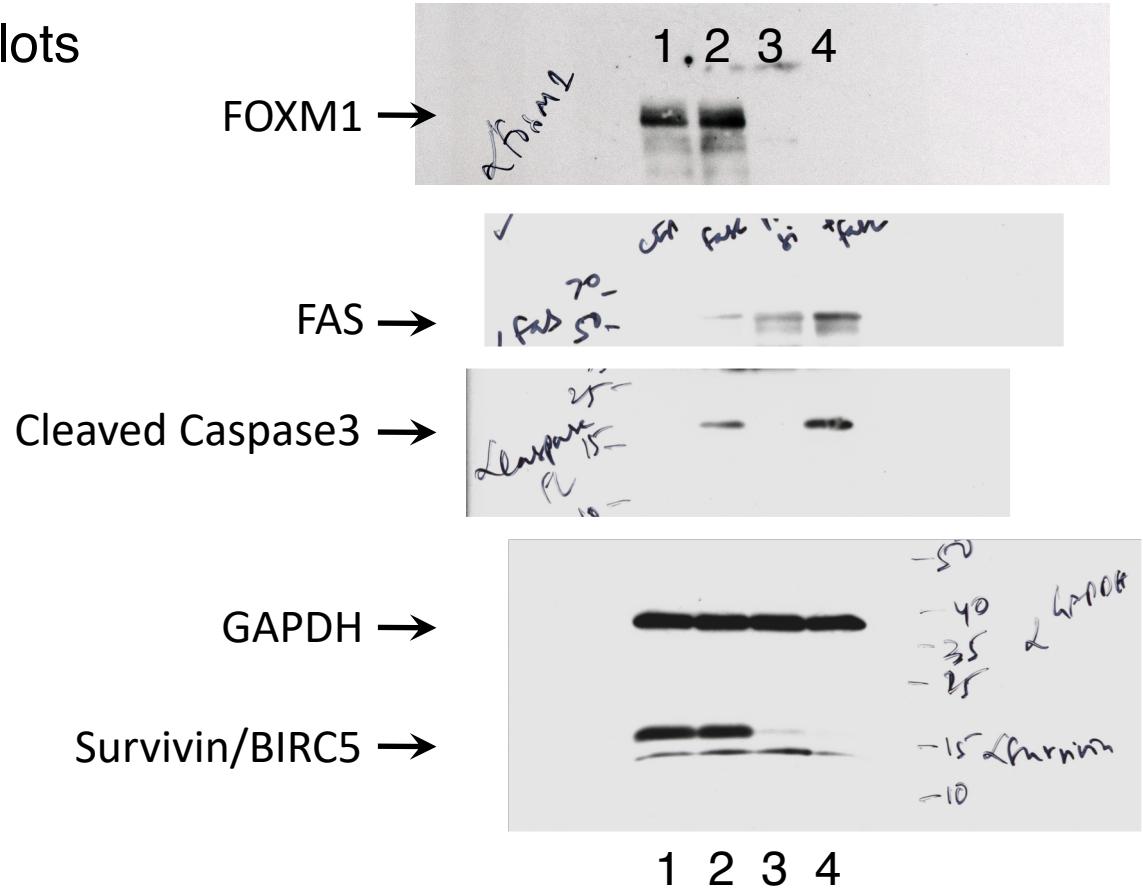
## Figure 6B raw Western blots





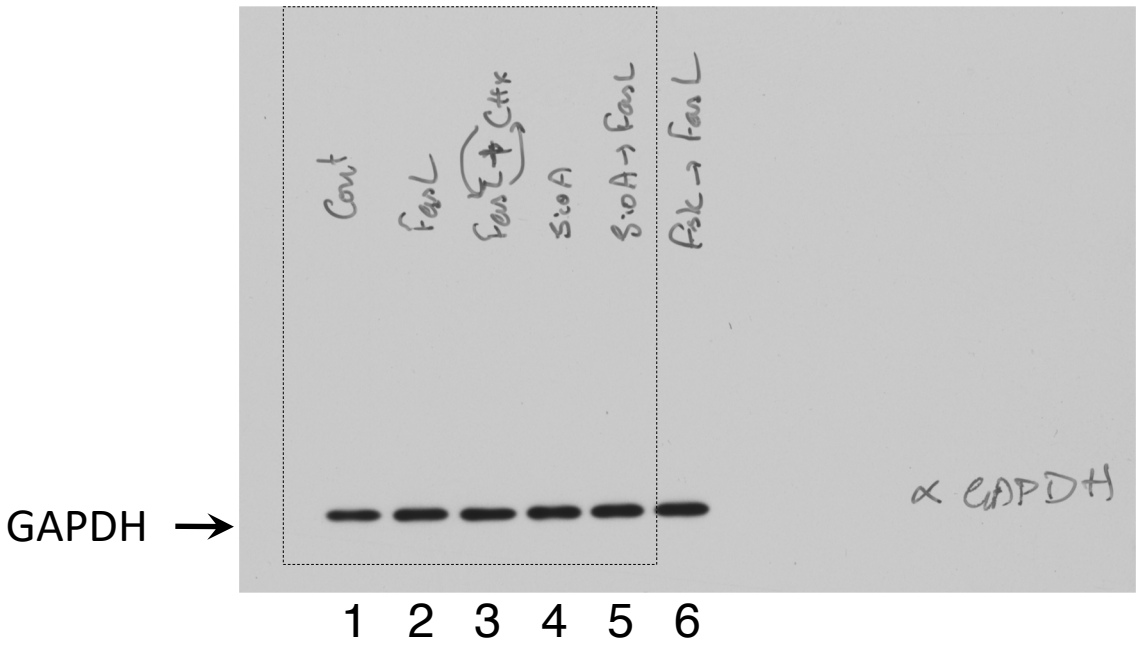
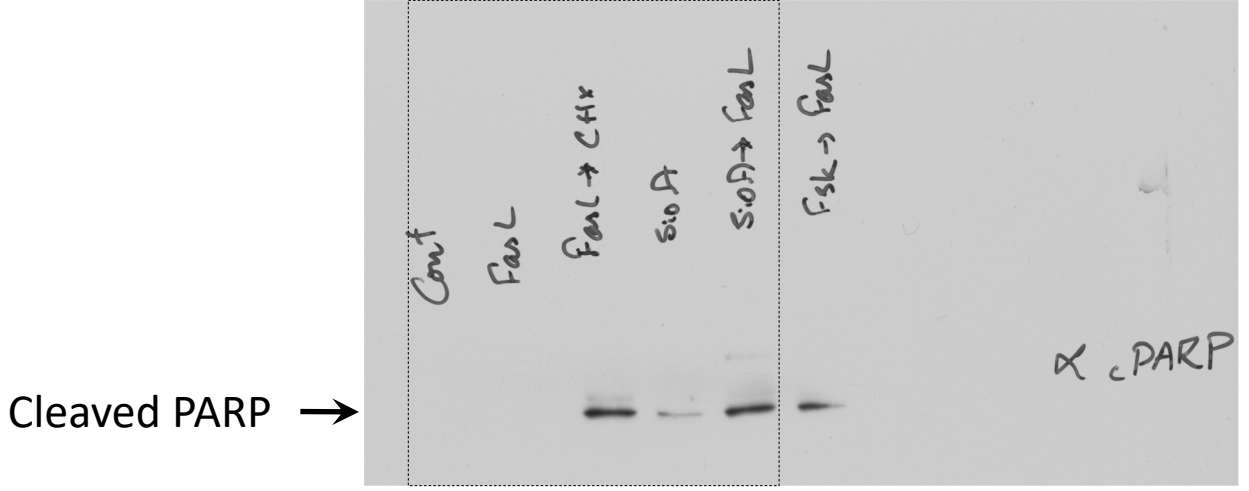
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## Figure 6G raw Western blots



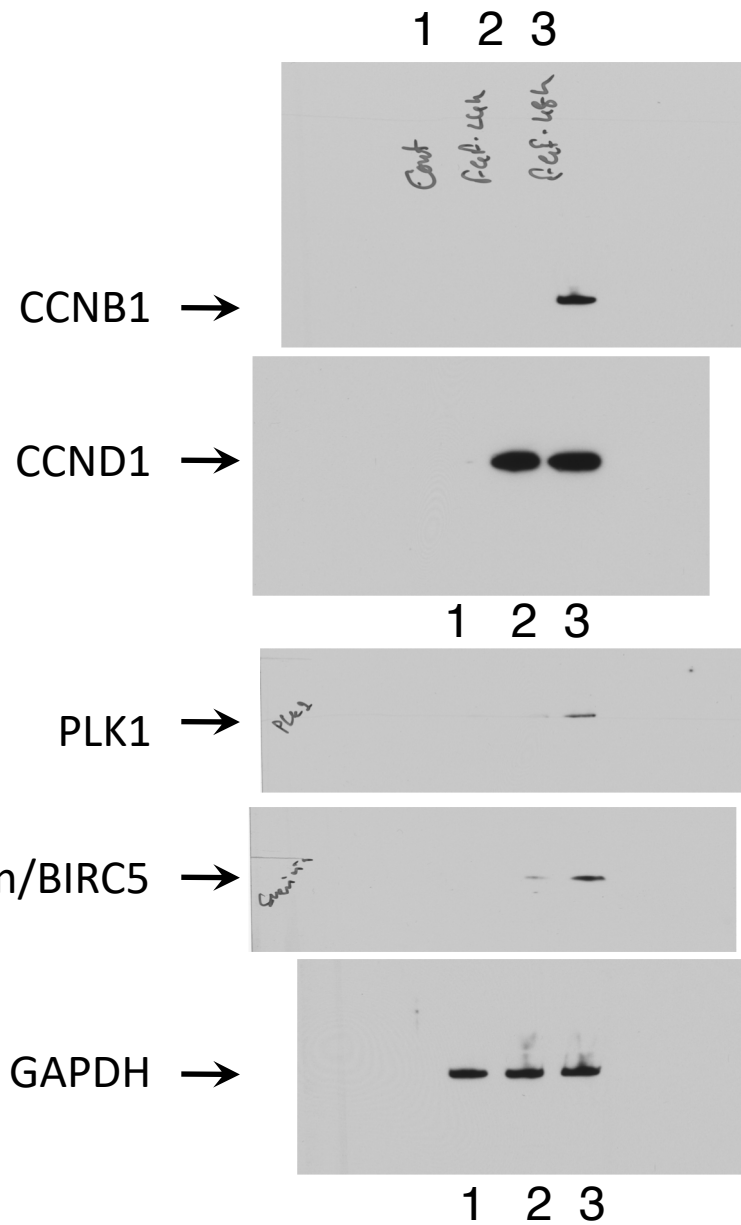
Full unedited gel for Figure 6

Figure 6H  
raw Western blots



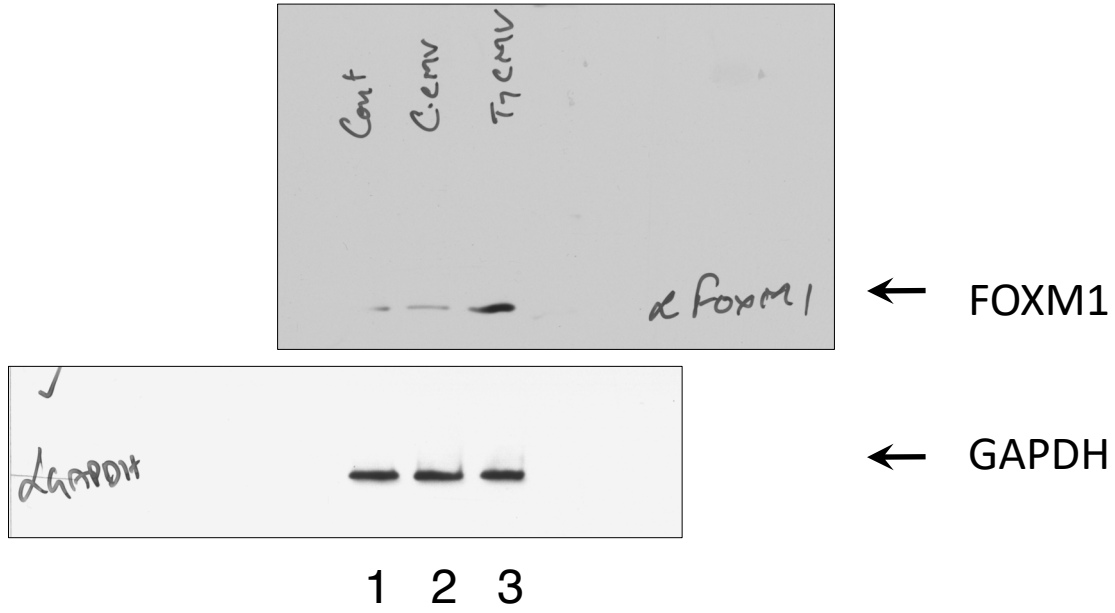
# Full unedited gel for Supplemental Figure 1

## Supplemental Figure 1B raw Western blots



# Full unedited gel for Supplemental Figure 3

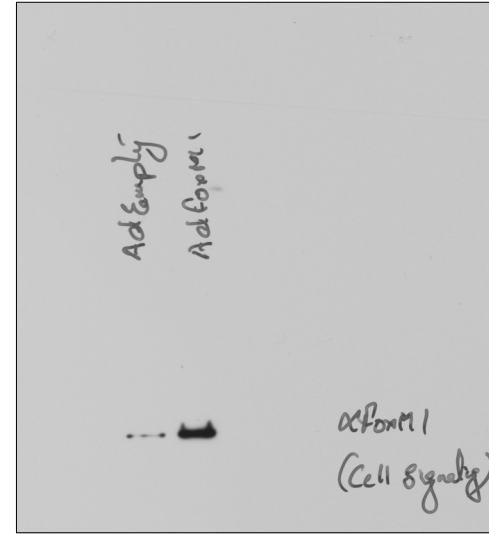
## Supplemental Figure 3B raw Western blots



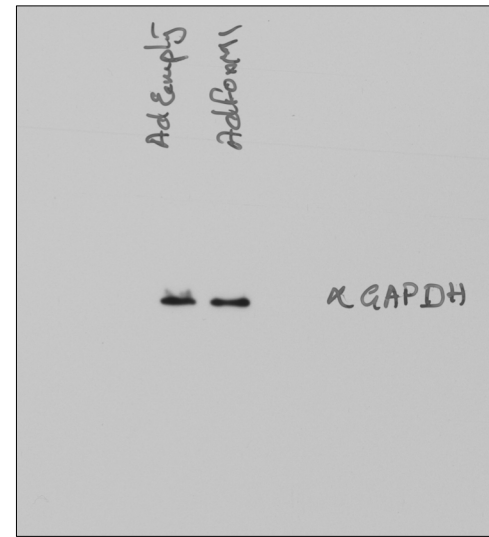
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## Supplemental Figure 3C raw Western blots

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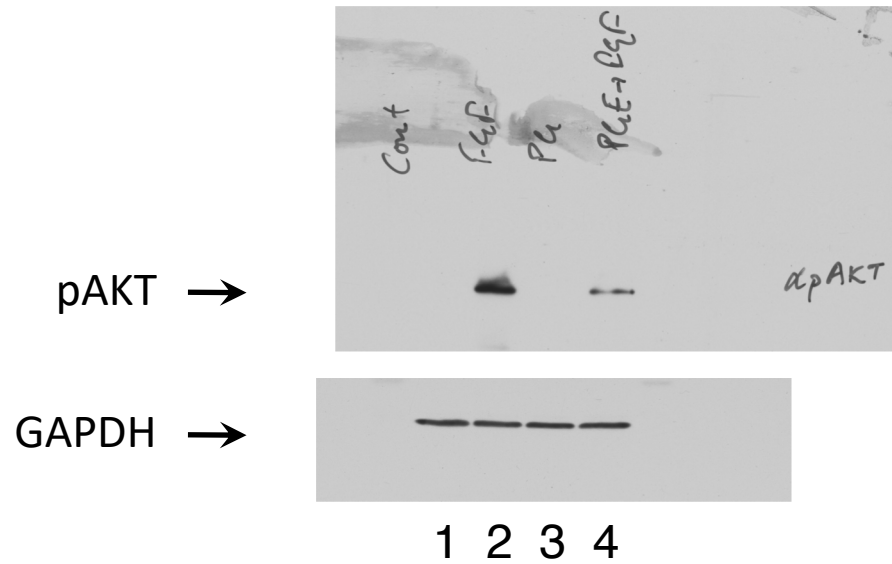
GAPDH →



1 2

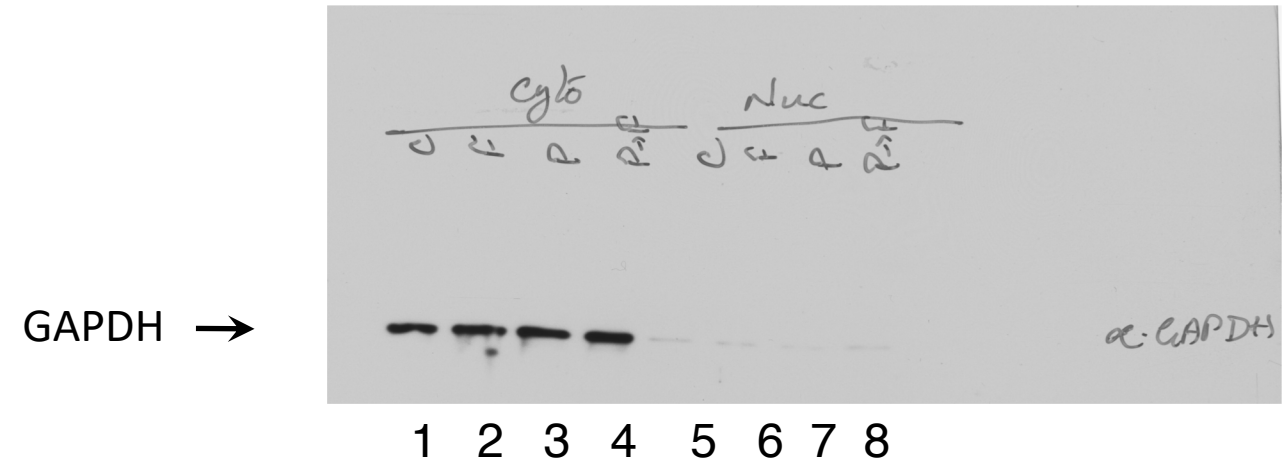
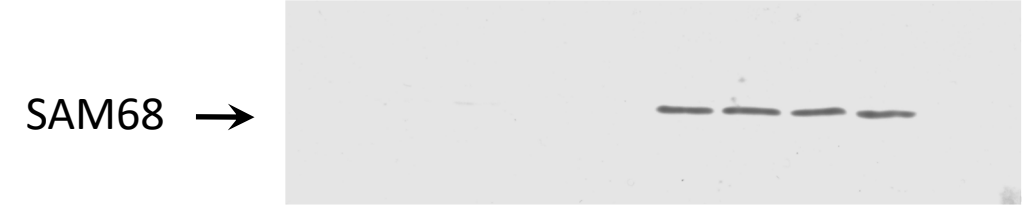
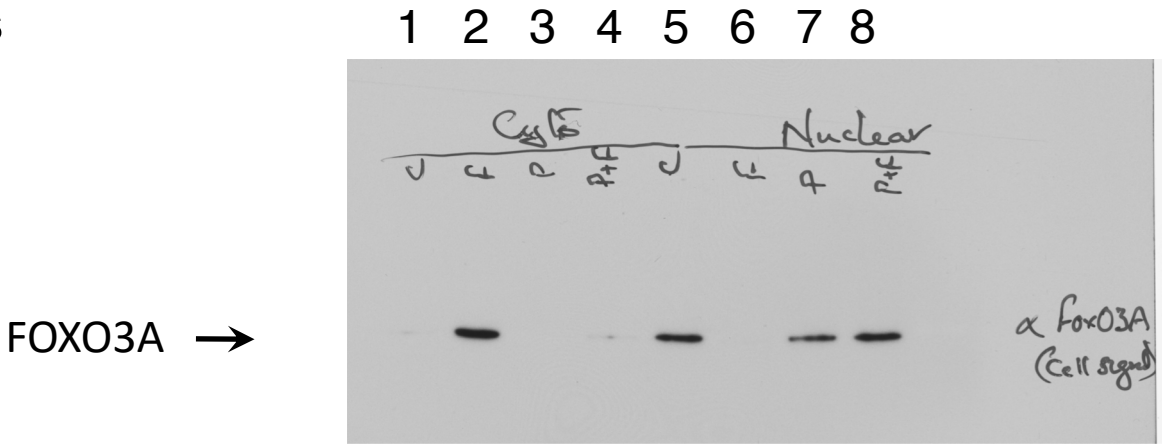
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## Supplemental Figure 4A raw Western blots



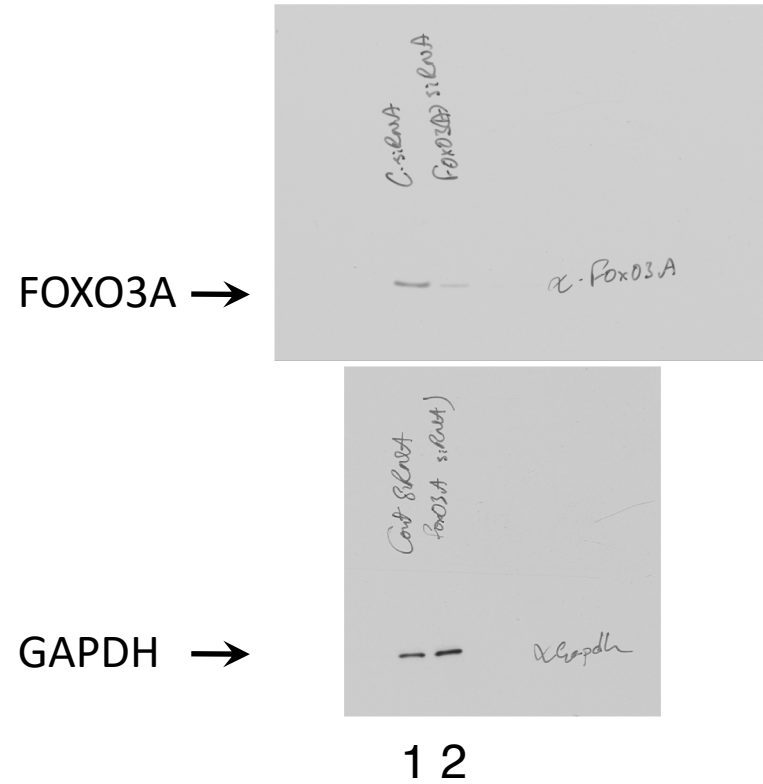
# Full unedited gel for Supplemental Figure 4

## Supplemental Figure 4B raw Western blots



# Full unedited gel for Supplemental Figure 4

## Supplemental Figure 4C raw Western blots





# Full unedited gel for Supplemental Figure 8

## Supplemental Figure 8A raw Western blots

