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Supplemental information

Loss of IGFBP2 mediates alveolar type 2 cell senescence and promotes lung fibrosis

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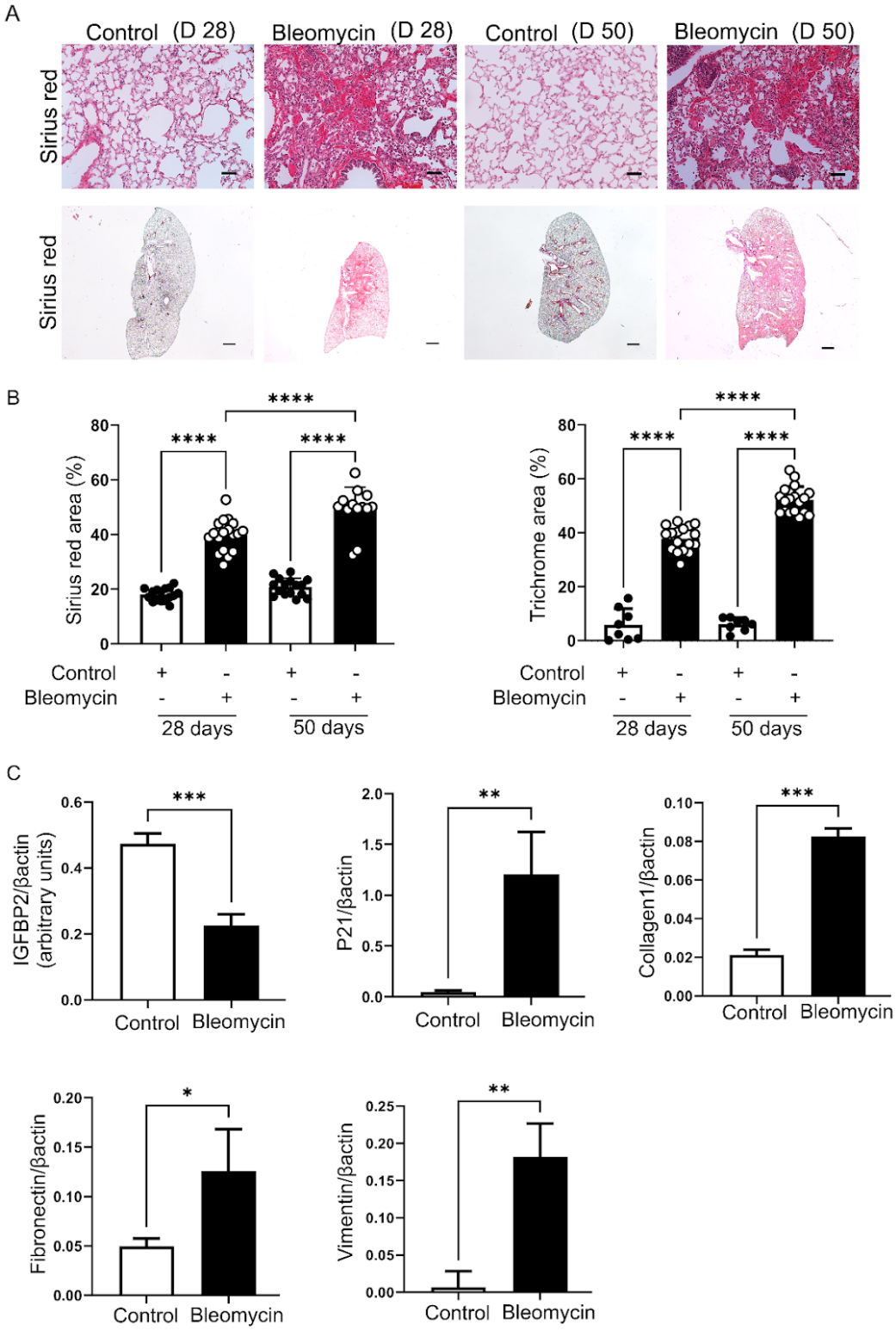
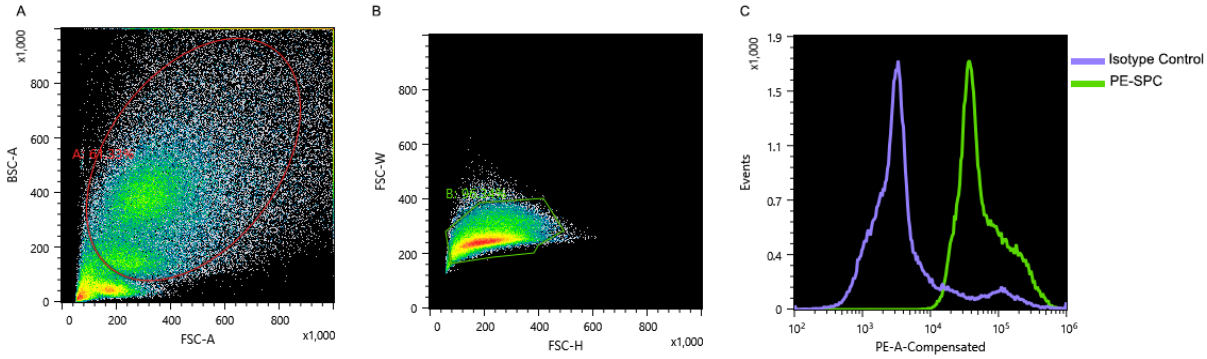
Supplementary Figures

Figure S1



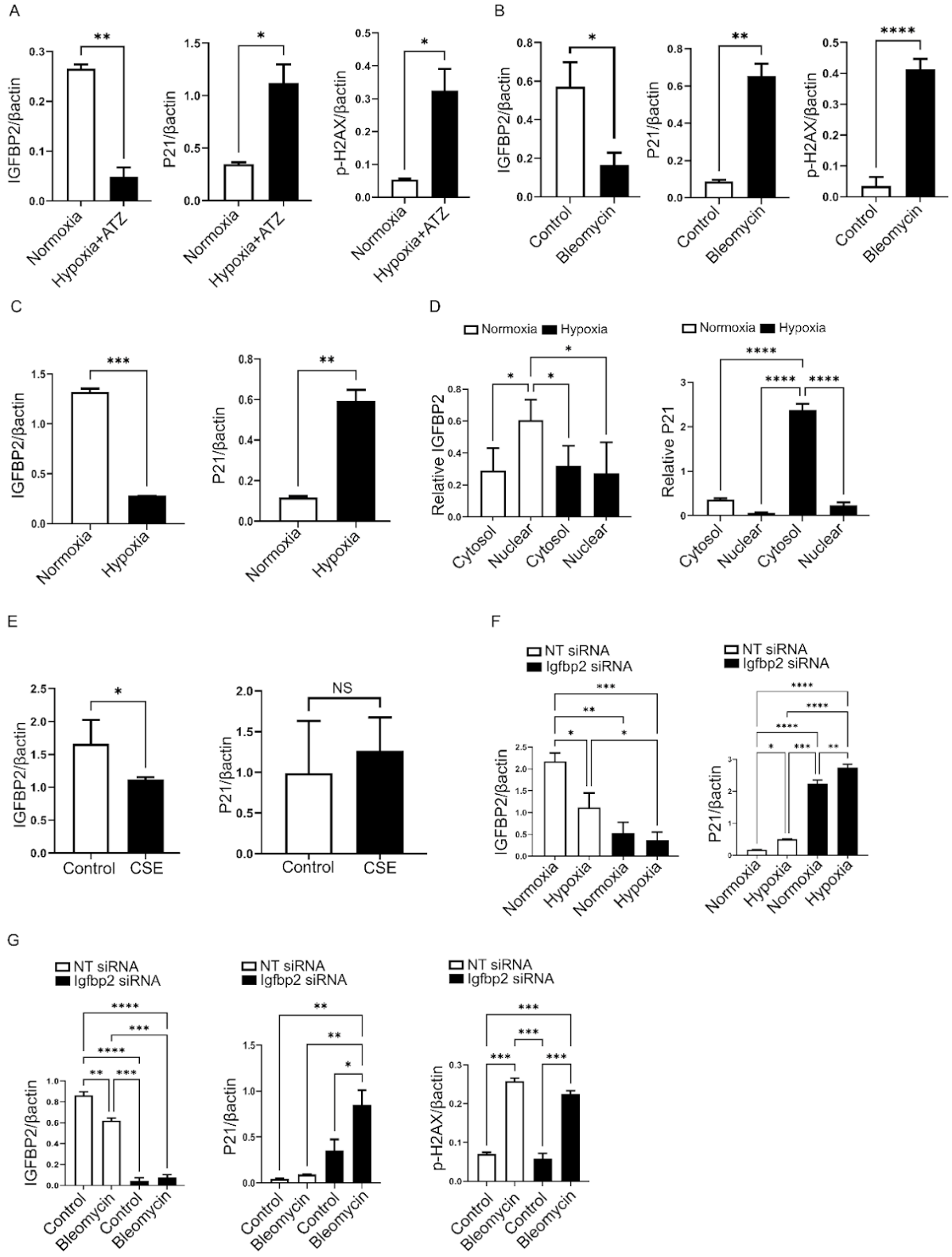
S1. Related to Figure 1B. Low-dose bleomycin induces persistent lung fibrosis in aged mice. (A) Representative images of Sirius red stained lung sections of aged mice 28 or 50 days after intratracheal administration of bleomycin (1U/Kg body weight). Scale bars, 50 μm (top); 1 mm (bottom). (n = 8 WT saline; n = 8 WT bleomycin) (B) Quantification of Sirius red (left panel) and trichrome (right panel) staining positive areas (%) in aged mice after bleomycin challenge at 28 and 50 days. ****P < 0.001 one way ANOVA with Tukey post-hoc test. (C) Related to Figure 1C. Densitometric analysis of the Western blot images from Figure 1C. Quantification by densitometric analysis through normalization to β-actin, expressed as fold change relative to control. Data are shown as mean $\pm$ s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, Student's unpaired two-tailed *t* test.

Figure S2


A
B
C
BSC-A
FSC-A
FSC-W
FSC-H
Events
PE-A-Compensated
x1,000
Isotype Control
PE-SPC

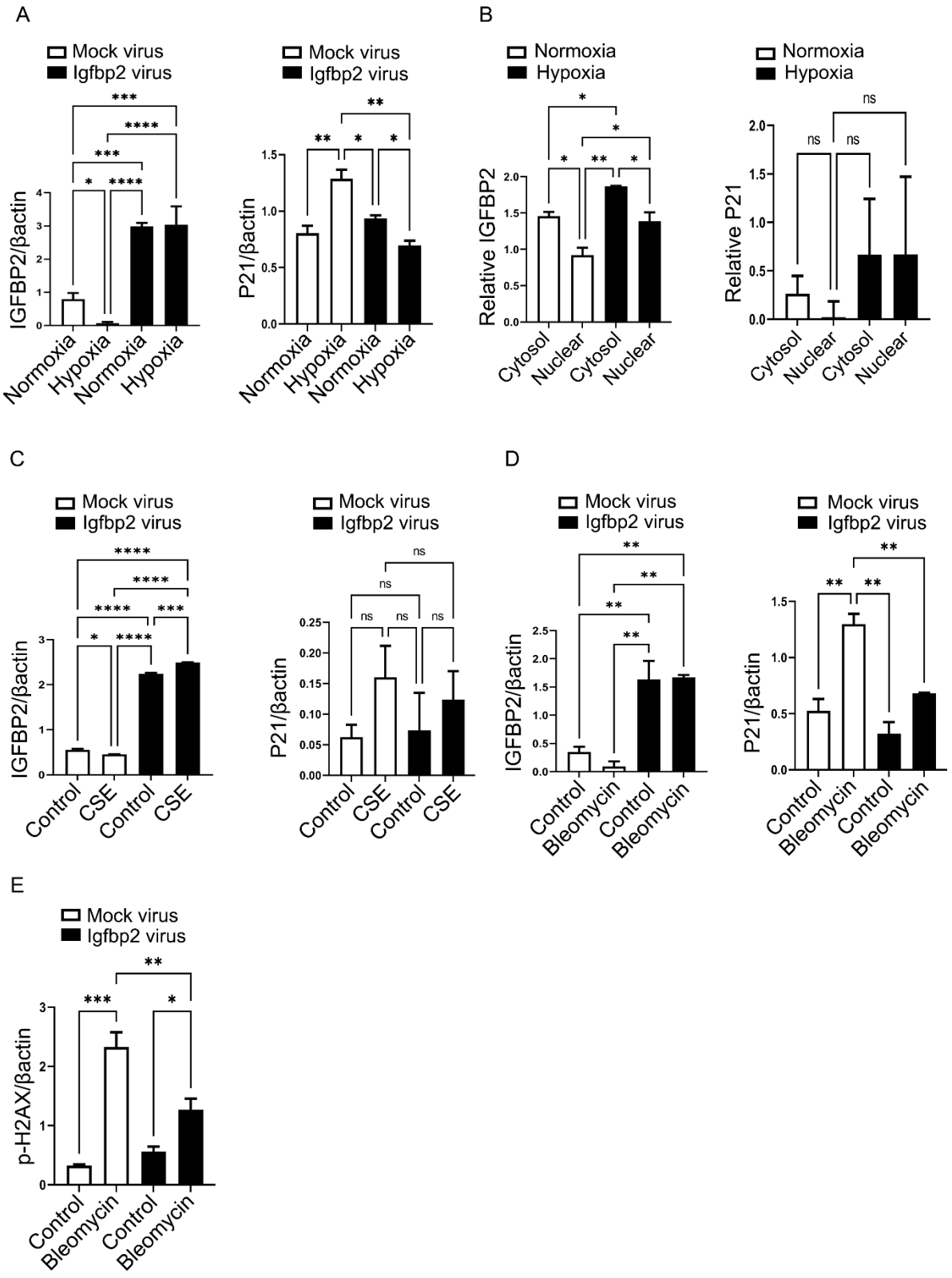
S2. Related to Figure 1G. Flow cytometry staining for Surfactant protein-C (SPC) in primary murine AEC2 cells. (A) Total murine lung AEC2 cells were isolated using differential cell strainer (20 μm) and magnetic sorting (CD326/EpCAM) followed by staining with PE-SPC antibody. Live AEC2 cells were sorted based on their forward/side scatter profile. (B) Representative flow cytometric sorting of AEC2 cells based on single cell profile. (C) The expression level of SPC was revealed based on distinct fluorescence profiles of isotype control and PE-stained AEC2 cells after overlay. Violet color indicates isotype control AEC2 cells; Green color indicates PE-SPC stained AEC2 cells.

Figure S3


A
IGFBP2/βactin
Normoxia
Hypoxia+ATZ
P21/βactin
p-H2AX/βactin
B
Control
Bleomycin
C
Normoxia
Hypoxia
D
Normoxia Hypoxia
Relative IGFBP2
Relative P21
Cytosol
Nuclear
E
Control
CSE
NS
F
NT siRNA
Igfbp2 siRNA
G

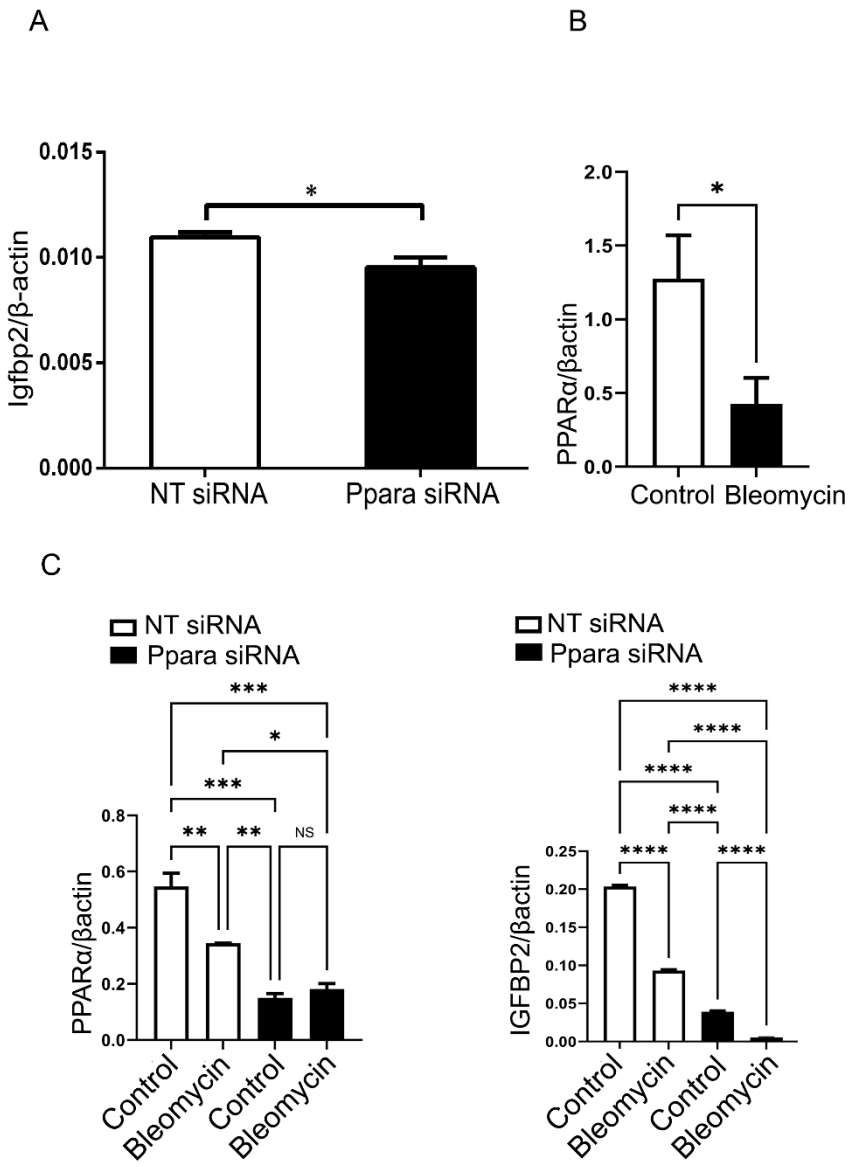
S3A – S3G. Related to Figure 3. Densitometric analysis of the Western blot images from Figures 3A – 3G. The quantification by densitometric analysis through normalization to β-actin or Tubulin or Histone H3, expressed as fold change relative to control. Data are shown as mean $\pm$ s.e.m. NS, not significant; one-way ANOVA with Tukey post-hoc test (for multiple comparisons) or Student's unpaired two-tailed *t* test (for two comparisons). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S4


A
Mock virus
Igfbp2 virus
IGFBP2/βactin
Normoxia
Hypoxia
Normoxia
Hypoxia
P21/βactin
B
Normoxia
Hypoxia
Relative IGFBP2
Relative P21
Cytosol
Nuclear
C
CSE
Control
D
Bleomycin
E
p-H2AX/βactin

S4A – S4E. Related to Figure 4. Densitometric analysis of the Western blot images from Figures 4A – 4E. The quantification by densitometric analysis through normalization to β-actin or Tubulin or Histone H3, expressed as fold change relative to control. Data are shown as mean $\pm$ s.e.m. NS, not significant; one-way ANOVA with Tukey post-hoc test (for multiple comparisons) or Student's unpaired two-tailed *t* test (for two comparisons). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

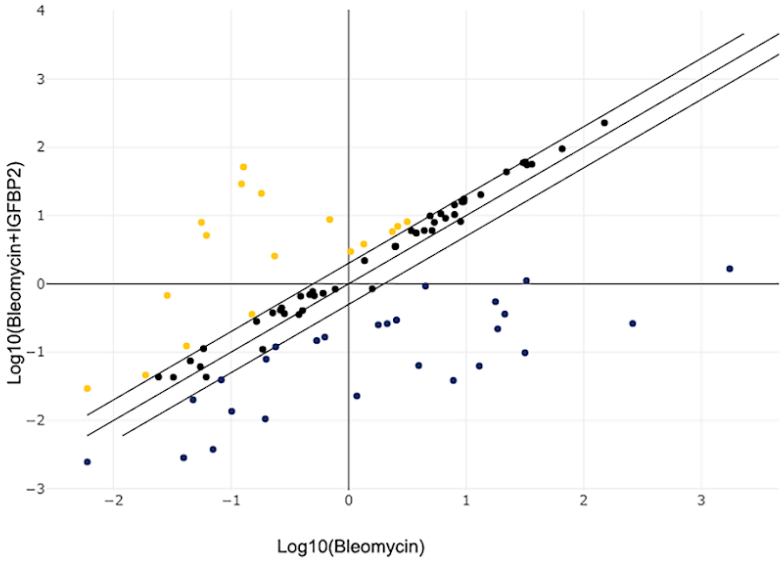
Figure S5


A
B
0.015
0.010
0.005
0.000
Igfbp2/β-actin
*
NT siRNA
Ppara siRNA
2.0
1.5
1.0
0.5
0.0
PPARα/βactin
*
Control
Bleomycin
C
NT siRNA
Ppara siRNA
***
*
***
**
**
NS
0.8
0.6
0.4
0.2
0.0
PPARα/βactin
Control
Bleomycin
Control
Bleomycin
NT siRNA
Ppara siRNA
****
****
****
****
****
****
0.25
0.20
0.15
0.10
0.05
0.00
IGFBP2/βactin
Control
Bleomycin
Control
Bleomycin

S5A – S5C. Related to Figure 5. Densitometric analysis of the Western blot images from Figures 5A – 5B. (A) Quantitative PCR analysis of Igfbp2 mRNA expression in MLE-12 cells treated with non-targeting or Ppara siRNA. (B - C) Densitometric analysis of the Western blot images from Figures 5A – 5B. The quantification by densitometric analysis through normalization to β-actin, expressed as fold change relative to control. Data are shown as mean $\pm$ s.e.m. NS, not significant; one-way ANOVA with Tukey post-hoc test (for multiple comparisons) or Student's unpaired two-tailed *t* test (for two comparisons). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure S6

A

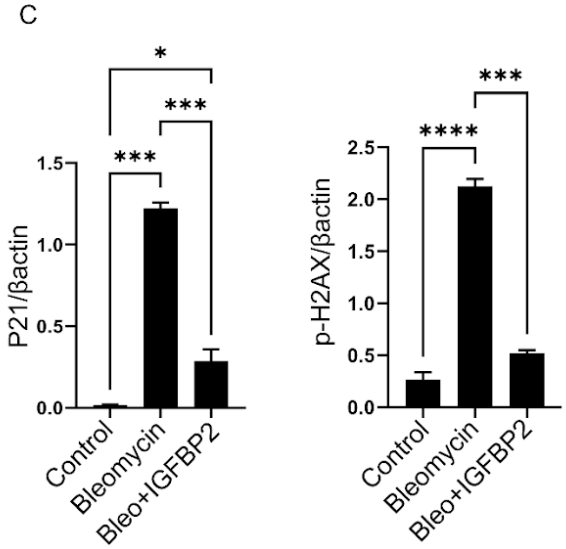


B

Gene Symbol	Up-Regulation
Abl1	2.46
Akt1	236.58
Ccna2	2.95
Ccnd1	2.38
Cdkn2a	23.59
Cdkn2c	2.86
Cdkn2d	2.46
Ets1	82.91
Igfbp7	2.66
Irf7	2.58
Mapk14	116.55
Myc	141.95
Prkcd	402.99
Rb1	12.66
Serpine1	10.88
Twist1	4.89

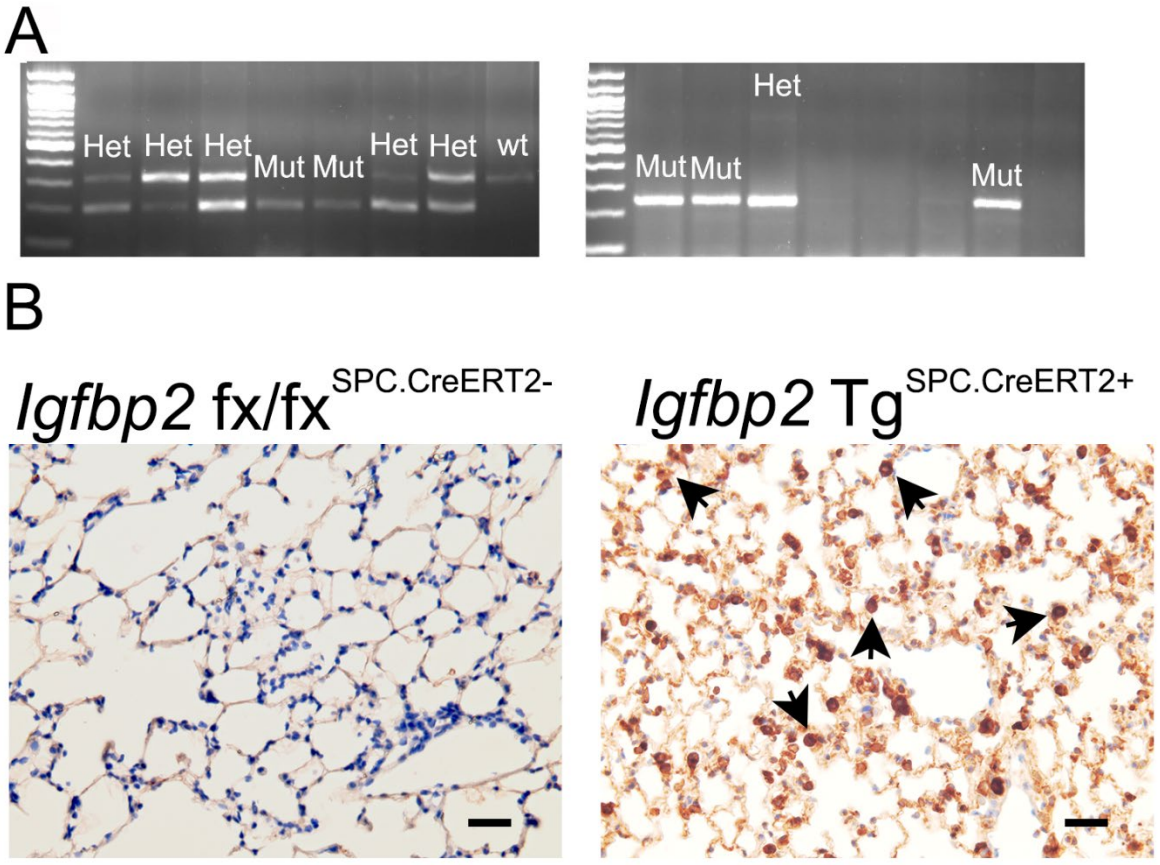
Gene Symbol	Down-Regulation
Aldh1a3	-2.37
Ccnb1	-7.13
Cdkn1b	-59.08
E2f3	-51.58
Egr1	-8.67
Gadd45a	-8.16
Gsk3b	-2.00
Id1	-62.09
Igf1	-202.20
Igf1r	-2.10
Igfbp5	-206.52
Irf5	-32.46
Mdm2	-84.71
Nox4	-2.43
Pcna	-3.78
Pik3ca	-2.52
Plau	-18.56
Sod1	-323.24
Tbx2	-18.66
Tbx3	-13.90
Tgfb1	-29.27
Tgfb1i1	-7.44
Trp53	-3.63
Vim	-994.82

C



S6. Cellular senescence gene profiling in primary AEC2 cells of aged wild-type mice treated with recombinant IGFBP2 protein after bleomycin injury. (A) Scatter plot showing the RT2 Profiler PCR Array for 84 genes related to cellular senescence pathways performed in the primary AEC2 cells of aged WT mice exposed to bleomycin treated with or without recombinant IGFBP2, containing curosurf by intranasal instillation at 14 days. (B) Table showing the list of fold change upregulated and down regulated genes in the primary murine AEC2 cells. (n = 3 bleomycin; n = 3 bleomycin + IGFBP2) (C) Related to Figure 6. Densitometric analysis of the Western blot images from Figure 6F. The quantification by densitometric analysis through normalization to β-actin, expressed as fold change relative to control. Data are shown as mean $\pm$ s.e.m. *P < 0.05, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey post-hoc test.

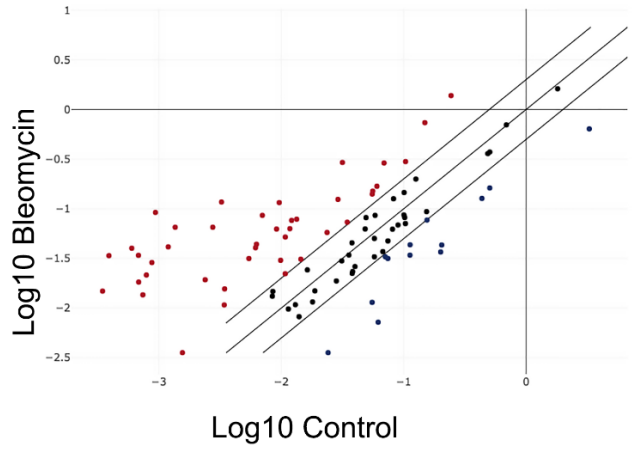
Figure S7


A
Het Het Het Mut Mut Het Het wt
Het
Mut Mut Mut
B
Igfbp2 fx/fx SPC.CreERT2-
Igfbp2 Tg SPC.CreERT2+

S7. Related to Figure 7A – 7G. Validation of Igfbp2 transgenic mice. (A) Genotyping of Igfbp2 transgenic mice (B) Immunohistochemistry of lung sections from Igfbp2 fx/fx and Igfbp2 Tg mice showing IGFBP2 staining in AEC2 cells after tamoxifen administration. Scale bars, 50 μm. Black arrowheads highlight IGFBP2 expression in AEC2 cells.

Figure S8

A



B

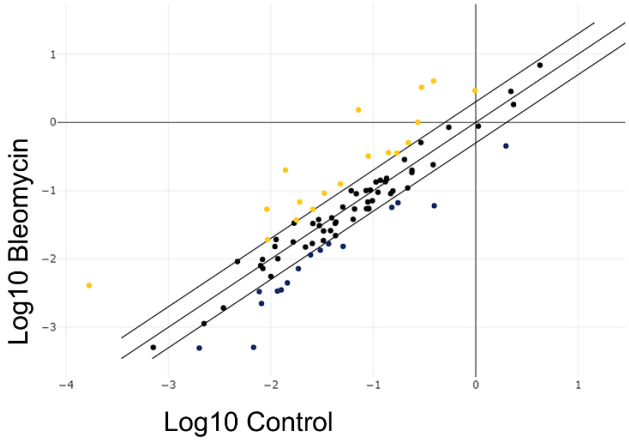
Gene Symbol	Upregulation
ABL1	12.00
ALDH1A3	66.70
ATM	2.28
CCNA2	5.80
CCNB1	34.64
CCNE1	47.93
CDC25C	86.17
CDK2	2.06
CDK4	4.19
CDKN2A	26.72
CDKN2B	9.28
CHEK1	27.21
CHEK2	18.37
COL1A1	2.89
COL3A1	4.95
E2F1	36.05
E2F3	4.53
GLB1	6.58
GSK3B	2.79
HRAS	2.70
IFNG	42.76
IGF1	12.25
IGF1R	2.54
IRF5	4.81
IRF7	3.06
MAP2K6	3.14
MYC	23.64
PLAU	8.07
PRKCD	2.12
RB1	2.44
RBL1	5.36
RBL2	2.15
SERPINB2	32.77
TBX3	7.01
TERF2	6.28
TERT	97.93
TP53	6.83
TP53BP1	5.87
TWIST1	49.83

Gene Symbol	Downregulation
CDKN1B	-2.15
CITED2	-4.84
ETS1	-2.60
ETS2	-2.35
ID1	-4.74
IGFBP5	-5.46
IGFBP7	-3.11
ING1	-6.79
MAP2K3	-8.58
SOD1	-2.01
SOD2	-3.30
VIM	-3.42

S8. Related to Figure 7. Cellular senescence gene profiling in lungs of aged Igfbp2 floxed mice subjected to bleomycin injury. (A) Scatter plot showing the RT2 Profiler PCR Array for 84 genes related to cellular senescence pathways performed in the lungs of aged Igfbp2 floxed mice subjected to low-dose bleomycin injury. Red dots indicate upregulated genes; black dots indicate no change; blue dots indicate downregulated genes. (B) Table showing the list of fold upregulated and down regulated genes in the total lungs challenged with bleomycin compared to normal saline (n = 3 saline control; n = 3 bleomycin).

Figure S9

A

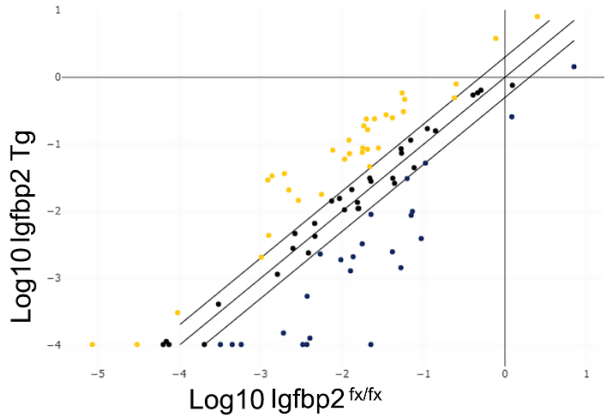


B

Gene Symbol	Up Regulation
Ccnd1	2.3
Cdkn1a	2.09
Cdkn2b	14.45
Chek1	24.35
Col1a1	3.69
Col3a1	10.45
Creg1	3.6
E2f1	2.06
Fn1	21.28
Glb1	2.09
Id1	2.57
Igf1	2.63
Igfbp7	2.97
Nox4	5.87
Serpine1	2.08
Sparc	11.05

Gene Symbol	Down Regulation
Abl1	-2.58
Aldh1a3	-2.32
Egr1	-2.66
Ifng	-4.04
Igfbp3	-2.62
Igfbp5	-6.55
Map2k6	-3.43
Morc3	-2.14
Myc	-2.26
Rbl2	-3.25
Serpinb2	-3.59
Tert	-13.34
Trp53	-2.2
Trp53bp1	-3.32
Twist1	-3.64

C



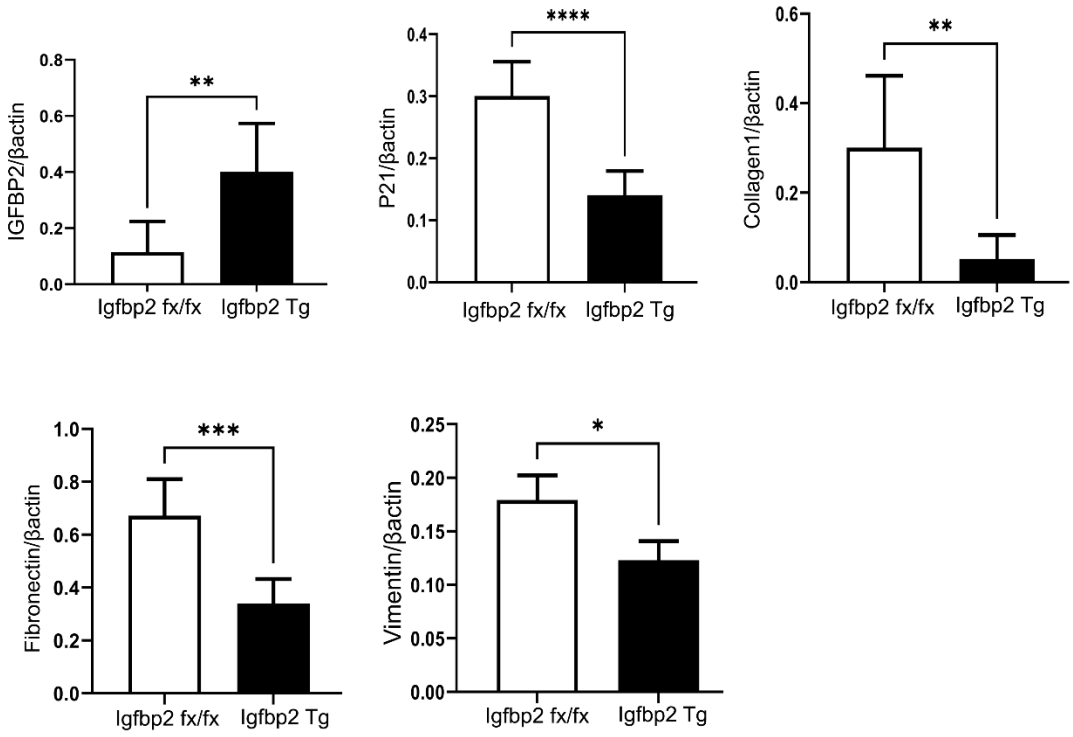
D

Gene Symbol	Up Regulation
Aldh1a3	11.95
Atm	2.03
Bmi1	9.47
Calr	4.91
Ccna2	12.04
Ccnb1	10.67
Ccnd1	4.92
Ccne1	4.99
Cdc25c	18.62
Cdk2	4.28
Cdk6	2.12
Cdkn1b	7.98
Cdkn2a	23.83
Cdkn2b	24.44
Chek1	3.47
Cited2	3.15
Creg1	3.15
Gsk3b	9.57
Igfbp3	3.38
Irf3	4.10
Map2k6	9.36
Mdm2	7.92
Nbn	3.20
Pik3ca	5.89
Pten	10.67
Rb1	10.17
Rbl1	8.03
Sod1	5.98
Sod2	5.41
Tert	3.21
Tgfb1	2.05
Trp53	5.57

Gene Symbol	Down Regulation
Akt1	-23.85
Cdkn1a	-8.09
Chek2	-4.36
E2f1	-12.49
Fn1	-4.72
Gadd45a	-5.12
Ifng	-2.34
Igf1r	-32.05
Igfbp5	-3.12
Igfbp7	-35.92
Ing1	-6.51
Irf5	-36.66
Irf7	-219.06
Map2k1	-2.04
Pcna	-2.02
Plau	-5.41
Prkcd	-7.34
Rbl2	-9.83
Serpine1	-2.52
Sparc	-31.43
Tgfb1i1	-5.62
Thbs1	-16.66
Trp53bp1	-6.90

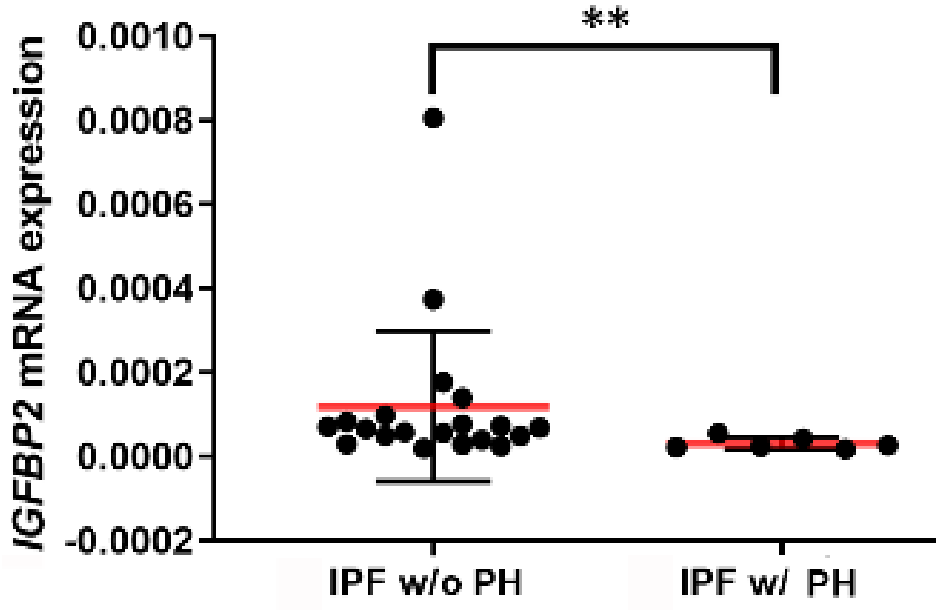
S9. Related to Figure 7. Cellular senescence gene profiling in primary AEC2 cells of aged wild-type and human-*Igfbp2* transgenic mice (A) Scatter plot showing the upregulated and downregulated genes relevant to senescence pathway in primary AEC2 cells from aged wild-type mice after 14 days of low-dose bleomycin treatment. (B) Table showing the list of relevant fold regulated genes with gene symbol. (C) Scatter plot showing the upregulated and downregulated genes relevant to senescence pathway in primary AEC2 cells from aged human-*Igfbp2* transgenic mice after 14 days of low-dose bleomycin treatment. Yellow dots indicate upregulated genes; black dots indicate no change; blue dots indicate downregulated genes. (D) Table showing the list of fold regulated genes with gene symbol. + indicates upregulated genes; - indicates downregulated genes. (n = 3 *Igfbp2* fx/fx; n = 3 *Igfbp2* Tg)

Figure S10


IGFBP2/βactin
**
Igfbp2 fx/fx
Igfbp2 Tg
P21/βactin
****
Collagen1/βactin
**
Fibronectin/βactin
***
Vimentin/βactin
*

S10. Related to Figure 7. Densitometric analysis of the Western blot images from Figure 7D. The quantification by densitometric analysis through normalization to β-actin, expressed as fold change relative to control. Data are shown as mean $\pm$ s.e.m. **P < 0.01, ***P < 0.001, ****P < 0.0001, Student's unpaired two-tailed *t* test.

Figure S11


**
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
-0.0002
IGFBP2 mRNA expression
IPF w/o PH
IPF w/ PH

S11. Related to Figure 8. IGFBP2 mRNA expression determined by qPCR in the primary AEC2 cells obtained from fibrotic lung regions of IPF patients with pulmonary hypertension (MPAP < 30 mmHg) as compared to IPF patients with pulmonary hypertension (MPAP > 30 mmHg). Data are shown as mean $\pm$ s.e.m. **P < 0.01, Student's unpaired two-tailed *t* test.