Inhibition of MRTF activation as a clinically achievable anti-fibrotic mechanism for Pirfenidone

Supplementary material

Figure S1. Flow sorting strategy for human lung mesenchymal cells.

(**A**) Representative flow plot showing sorting strategy and percentage of populations in normal human lungs. (**B**) COL1A1, EPCAM and CD31 expression in freshly sorted mesenchymal cells (CD45$^-$EPCAM$^-$CD31$^-$), epithelial cells (EPCAM$^+$) and endothelial cells (CD31$^+$) from normal and IPF lungs.

Figure S1

A

CD45⁻ live cells

B

• Mesenchymal cells (CD45⁻ EPCAM⁻ CD31⁻)   ▪ Epithelial cells (EPCAM⁺)   ▼ Endothelial cells (CD31⁺)
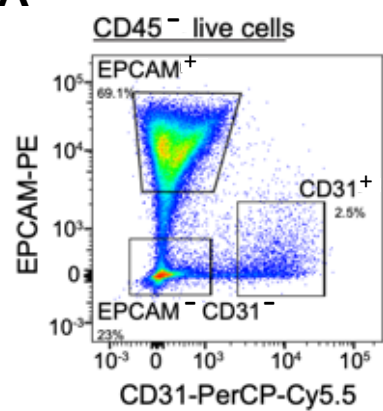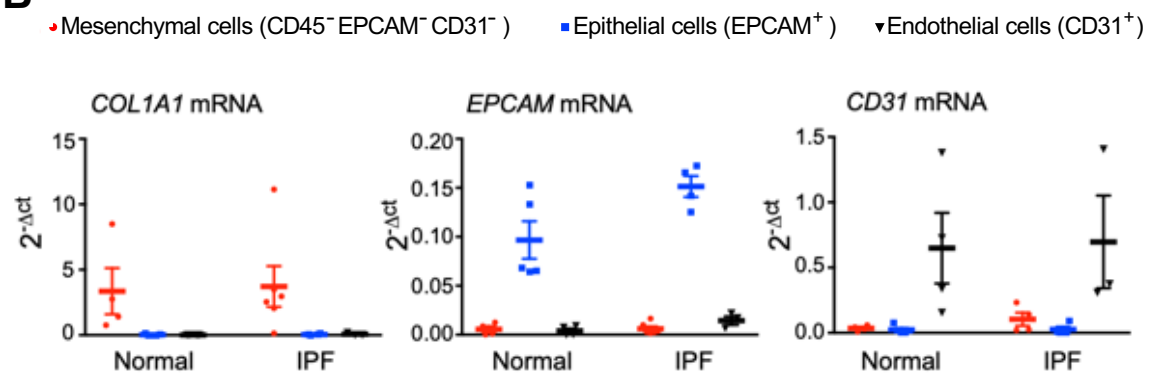
Figure S2. Enrichment of MRTF signature in a larger IPF cohort.

(**A**) Density fit of $\log_2$ fold-change distributions of IPF vs normal lungs. Separate distributions are shown for MRTF response genes (MRTF signature) and all other genes with expression levels passing quality control (Background). The displayed density fit is truncated at the bounds of the observed $\log_2$ fold-change values within each category of genes. $p < 0.0001$, MRTF signature ≠ Background. (**B**) Violin plot of MRTF signature in normal and IPF lungs (Normal lungs, n = 26; IPF lungs, n = 46). p value is calculated using one-way ANOVA. (**C**) Unsupervised hierarchical clustering analysis of MRTF signature gene expression in IPF (n = 46) and normal lungs (n = 26).
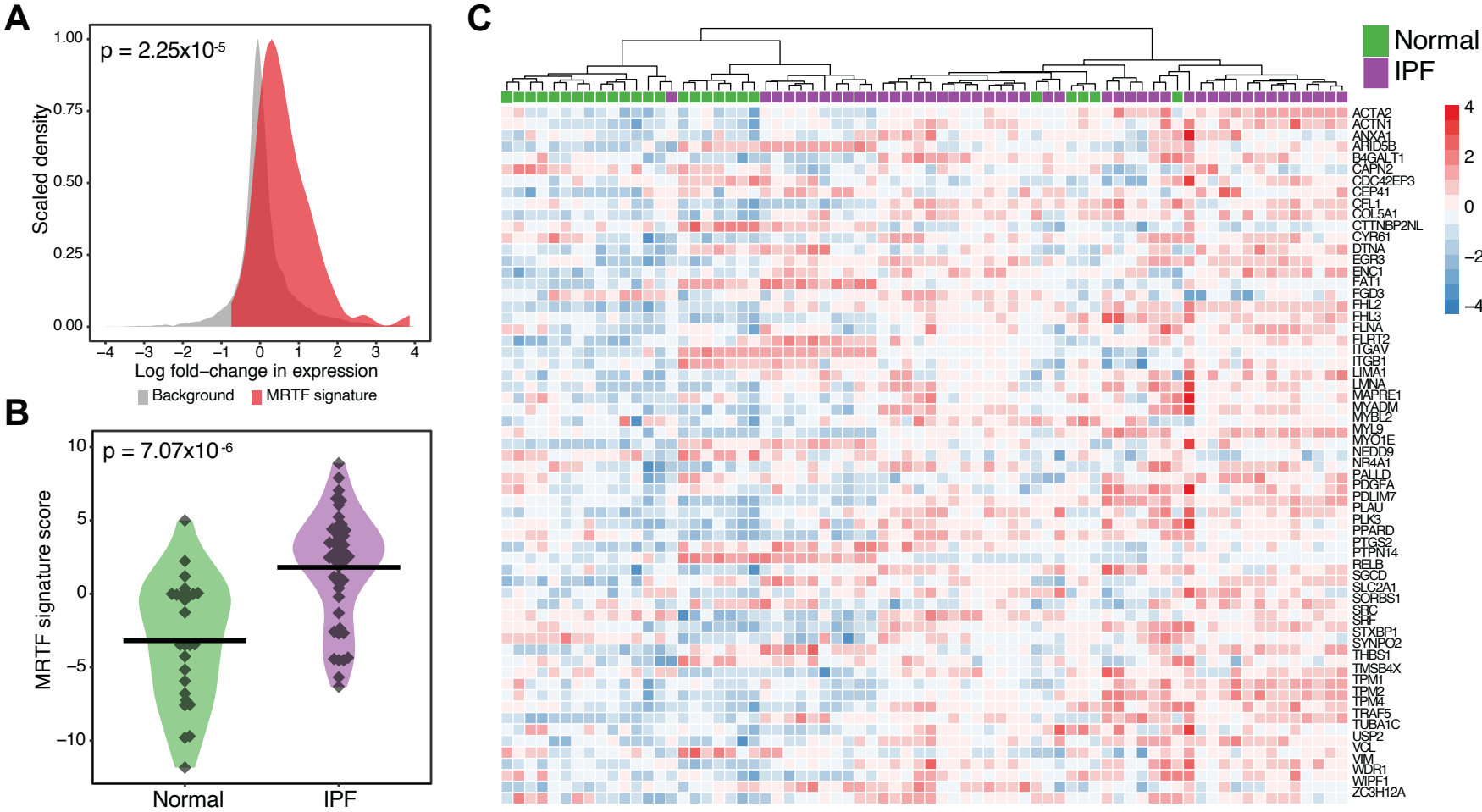
Figure S2

Figure S3. PFD does not affect collagen expression and cell viability in hLFs.

(**A**) RT-qPCR validation of PFD inhibition on COL1A1, COL1A2 and COL3A1 expression in normal and IPF human lung fibroblasts. (**B**) Normal and IPF lung fibroblasts were treated with PFD at the indicated concentrations for 48 hours followed by CellTiter-Glo assay. PFD concentration: 1mM, n = 3 biological replicates.
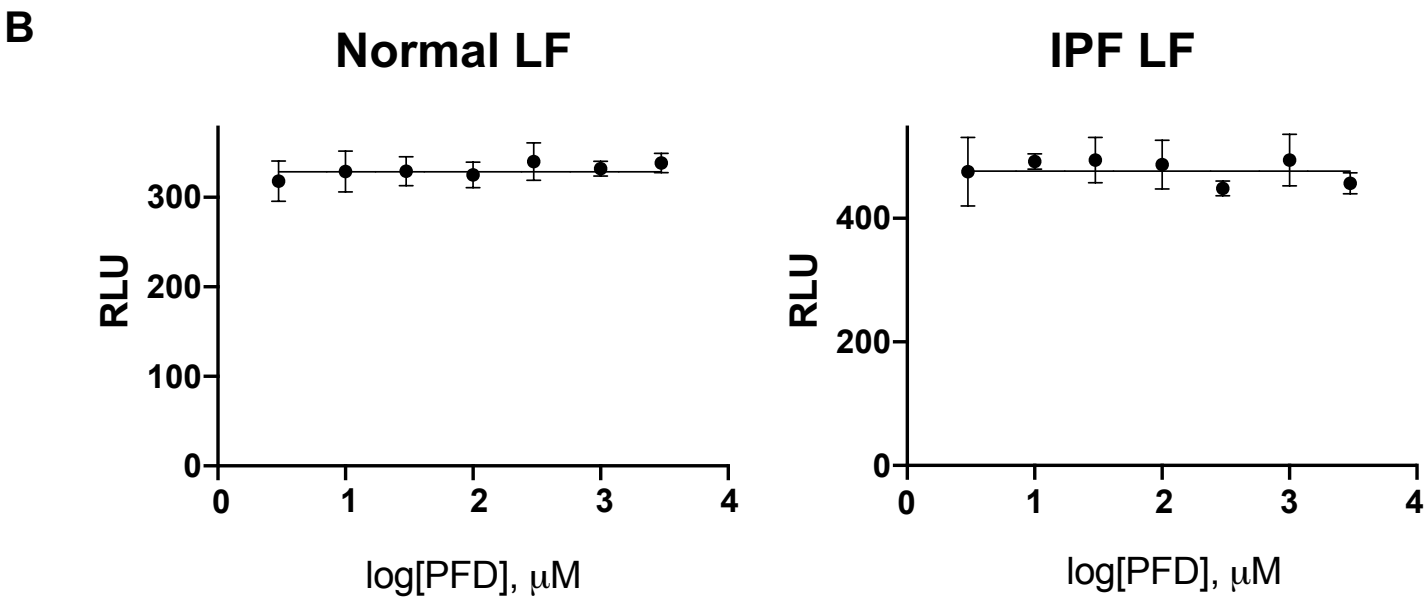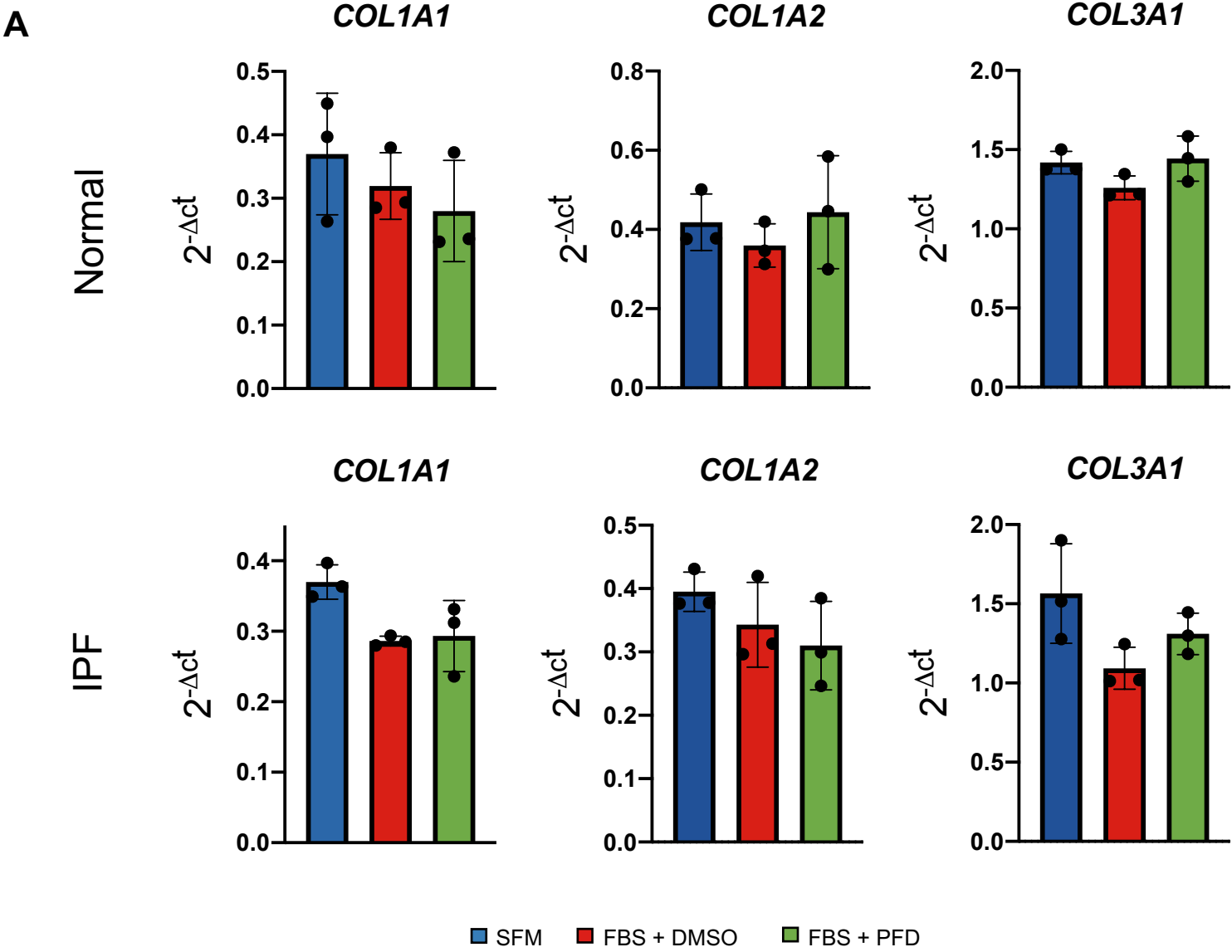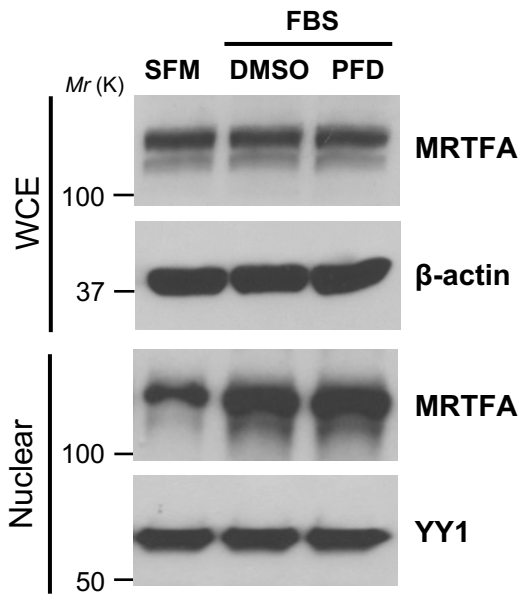
Figure S3

A

Normal

COL1A1
COL1A2
COL3A1

IPF

COL1A1
COL1A2
COL3A1

■ SFM ■ FBS + DMSO ■ FBS + PFD

B

Normal LF

IPF LF

Figure S4. PFD does not inhibit serum induced MRTFA nuclear translocation in non-fibroblastic cells.

(**A**-**F**) Representative western blot of MRTFA in WCE (top panel) and nuclear fractions (bottom panel) in A549 cells (**A**), MLE12 mouse alveolar epithelial cells (**B**), HL60-derived neutrophil-like cells (**C**), THP1-derived DC-like cells (**D**), U937-derived macrophage-like cells (**E**) and primary human lung artery endothelial cells (**F**) under indicated conditions. PFD concentration: 1mM. YY1, loading control for nuclear fractions. β-actin, loading control for WCE. Similar results were seen in three independent experiments.
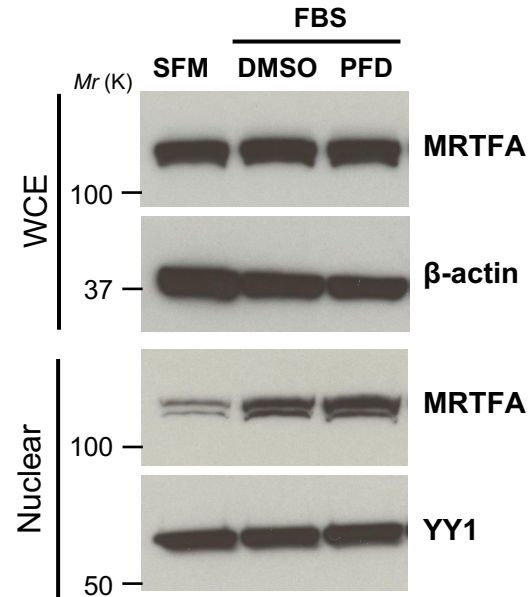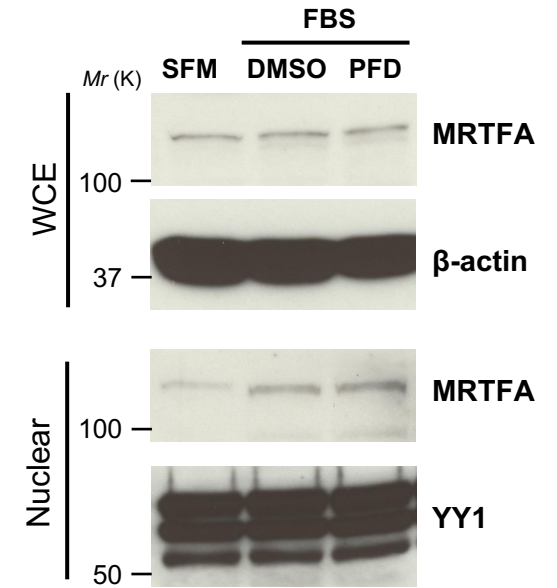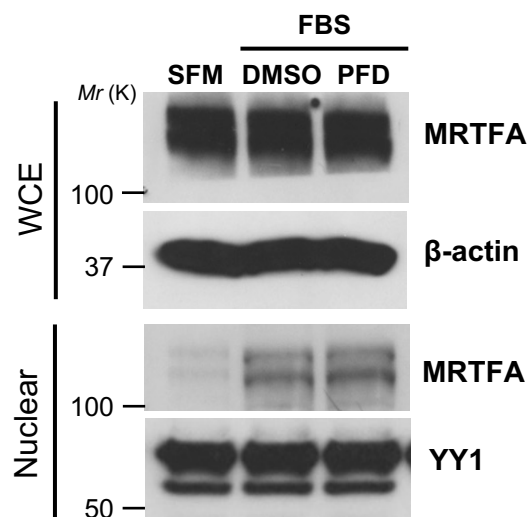
Figure S4

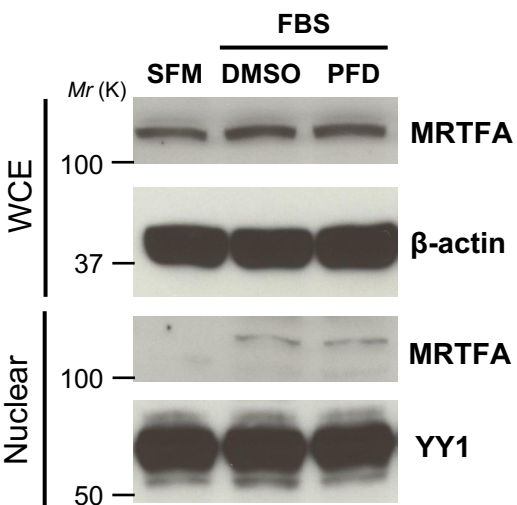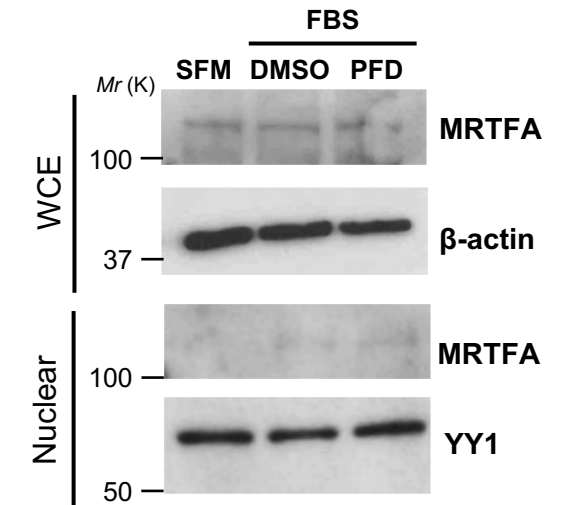**Figure S5. Transcriptomic analysis of PFD inhibitory effects on MRTF signaling in IPF LFs.**

(**A**) Heatmap showing relative expression of MRTF, TCF and Hippo target genes in IPF human lung fibroblasts treat with or without 1mM PFD. n = 3/group. (**B**) Top GO biological processes enriched in the genes suppressed by PFD in 20% FBS stimulated IPF human lung fibroblasts.

Fig S5. Transcriptomics analyses of PFD effects on IPF LFs

A
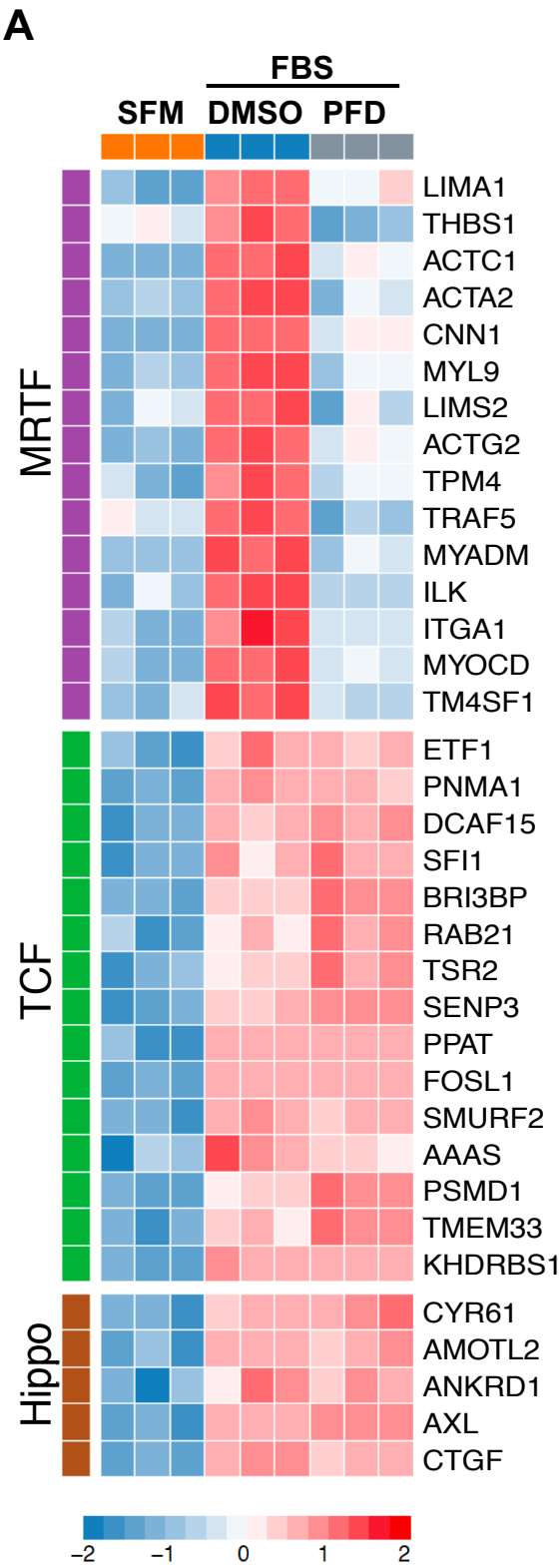
B

PFD suppressed genes (*p-adj.* <0.05)

Description	-Log10(q)
Cell motility	17.36
Biological Adhesion	15.98
Regulation of cellular component movement	13.65
Cell projection orgnization	12.34
Cell morphogenesis	12.10
Cytoskeleton orgnization	11.03
Actin filament based process	10.11
Extracellular structure orgnization	8.20

Figure S6. NTD does not inhibit serum induced MRTF target gene expression in hLFs.

MRTF target gene expression in normal and IPF lung fibroblasts treated with DMSO, PFD (1mM) or NTD (1µM) in the presence or absence of 20% FBS. n = 3 biological replicates.

Figure S6
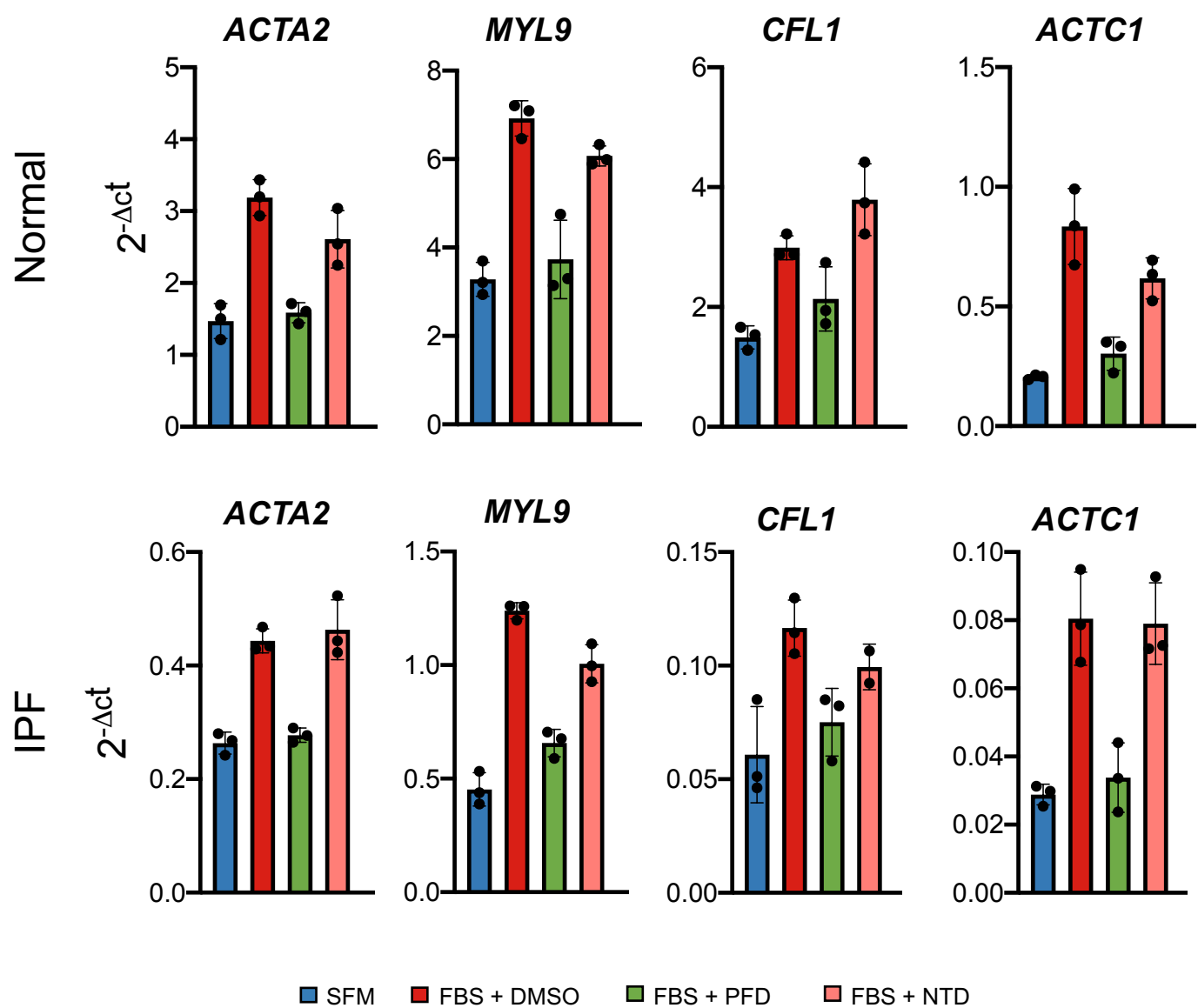
Figure S7. Neither PFD nor NTD impacts MRTF activation in EPCAM+ epithelial cells in human IPF lungs.

(**A**) Representative images of MRTFA and EPCAM immunofluorescence signal in naïve and PFD- or NTD-treated IPF lung sections, scale bar 20μm. (**B**) Nuclear/cytoplasmic distribution of MRTFA in EPCAM+ epithelial cells (n=10 for IPF, n=10 for PFD-treated, n=10 for NTD-treated). Data represents mean ± standard deviation (SD) error. Statistical analysis using one-way ANOVA. ns, not significant.

Figure S7

A

B

MRTFA

Table S1

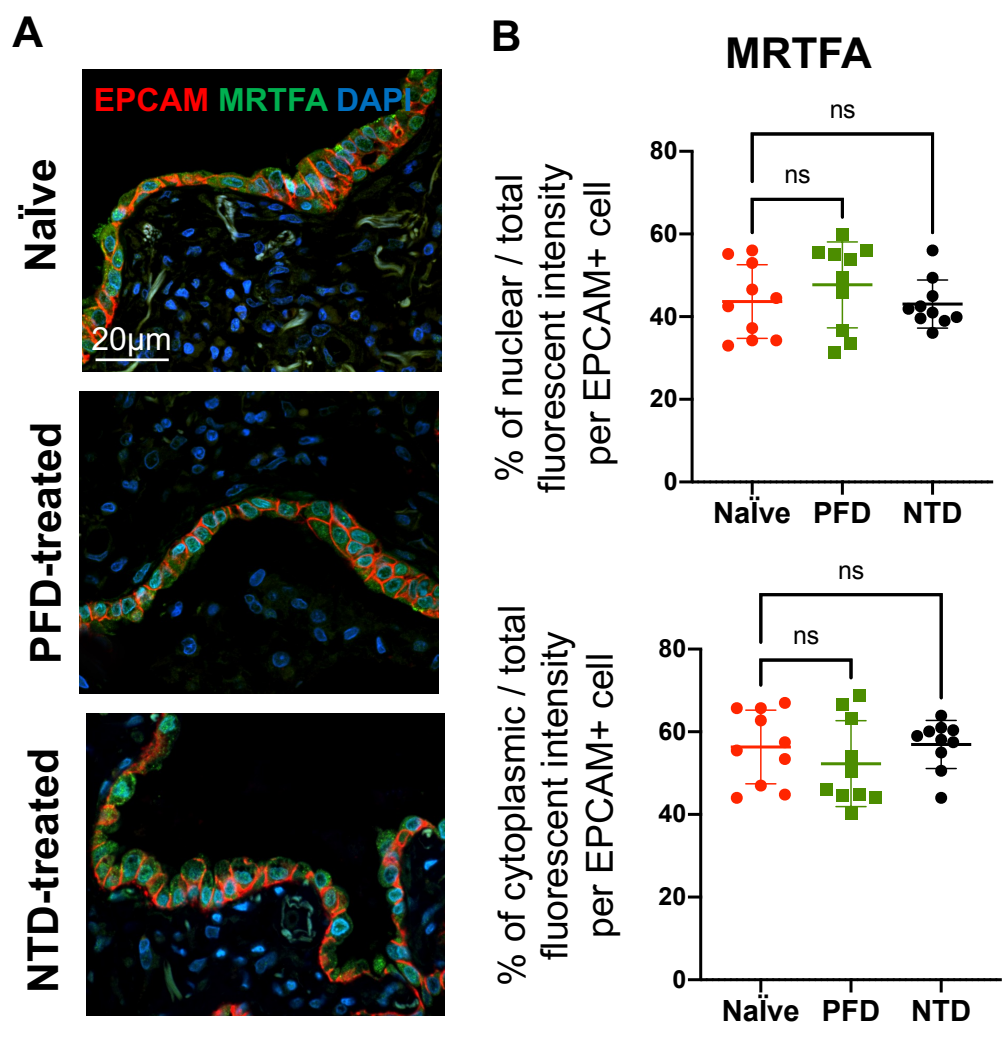| Sample	Diagnosis	Gender	Age	Ethnicity	Drug therapy
N1	Normal	Male	61	Caucasian	NA
N2	Normal	Male	76	Caucasian	NA
N3	Normal	Male	34	Caucasian	NA
N4	Normal	Female	60	Caucasian	NA
I1	IPF	Male	63	Caucasian	None
I2	IPF	Male	68	Hispanic	Nintedanib
I3	IPF	Female	58	Caucasian	None
I4	IPF	Male	69	Caucasian	None
I5	IPF	Female	56	Caucasian	None
I6	IPF	Male	70	Caucasian	Pirfenidone
I7	IPF	Male	65	Caucasian	None
I8	IPF	Male	71	Caucasian	Nintedanib
I9	IPF	Male	59	Hispanic	None
I10	IPF	Female	66	Caucasian	Pirfenidone

Table S2

Primer	Sequence
hHPRT-F	CTCATGGACTGATTATGGACAGGAC
hHPRT-R	GCAGGTCAGCAAAGAACTTATAGCC
hCOL1A1-F	CAGACTGGCAACCTCAAGAA
hCOL1A1-R	CAGTGACGCTGTAGGTGAAG
hCOL1A2-F	GTTGCTGCTTGCAGTAACCTT
hCOL1A2-R	AGGGCCAAGTCCAACTCCTT
hCOL3A1-F	GGAGCTGGCTACTTCTCGC
hCOL3A1-R	GGGAACATCCTCCTTCAACAG
hCD31-F	AACAGTGTTGACATGAAGAGCC
hCD31-R	TGTAAAACAGCACGTCATCCTT
hEPCAM-F	AATCGTCAATGCCAGTGTACTT
hEPCAM-R	TCTCATCGCAGTCAGGATCATAA
hACTA2-F	GACCGAATGCAGAAGGAGAT
hACTA2-R	CACCGATCCAGACAGAGTATTT
hMYL9-F	TCTTCGCAATGTTTGACCAGT
hMYL9-R	GTTGAAAGCCTCCTTAAACTCCT
hACTA1-F	GGCATTCACGAGACCACCTAC
hACTA1-R	CGACATGACGTTGTTGGCATA
hACTG2-F	GCTGTAGCACCTGAAGAG
hACTG2-R	GAATGGCGACGTACATGGCA
hCFL1-F	GAGACCAAGGAG AGCAAGAAG
hCFL1-R	GTCCTTGGAGCTGGCATAAA
hTPM4-F	CTGAGACCCGTGCTGAAT TT
hTPM4-R	AGCCCACGTTCTCTTCTTTG
hCTGF-F	GCCCAGACCCAACTATGATTAG
hCTGF-R	GGAGGCGTTGTCATTGGTAA
hFOSL1-F	CAGGCGGAGACTGACAAACTG
hFOSL1-R	TCCTTCCGGGATTTTGCAGAT
hAAAS-F	GGCCTTTTTGGGGTGCTAAAT
hAAAS-R	TGGGCAAATTCAGCGATCAGA
hETF1-F	ATACAGAGGCTCTTACAGCACT
hETF1-R	AATTTGTGCAGGACTTCTCTTGT
hANKRD1-F	AGTAGAGGAACTGGTCACTGG
hANKRD1-R	TGTTTCTCGCTTTTCCACTGTT
hAXL-F	CCGTGGACCTACTCTGGCT
hAXL-R	CCTTGGCGTTATGGGCTTC