Modulation of H4K16Ac levels reduces pro-fibrotic gene expression and mitigates lung fibrosis in aged mice

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Online Supplementary Materials:

Methods:

RNA sequencing

Total RNA were prepared from three different primary IPF fibroblasts after transfection with either siRNA Mof or NT as described. RNA-sequencing (RNA-Seq) was performed on the Illumina NextSeq500 following the manufacturer's protocol (Illumina Inc., San Diego, CA) at the UAB Genomics Core Facility.

Briefly, RNA quality was assessed using the Agilent 2100 Bioanalyzer. RNA with a RNA Integrity Number (RIN) of ≥7.0 was used for sequencing library preparation. RNA passing quality control was converted to a sequencing ready library using the Agilent SureSelect Strand Specific mRNA library kit as per the manufacturer's instructions (Agilent, Santa Clara, CA). The cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with the Kapa Biosystems kit for Illumina library quantitation (Kapa Biosystems, Woburn, MA) prior to cluster generation. Cluster generation was performed according to the manufacturer's recommendations for onboard clustering (Illumina, San Diego, CA). We generated between 30-35 million paired end 75bp sequencing reads per sample for transcript level abundance.

Data assessment:

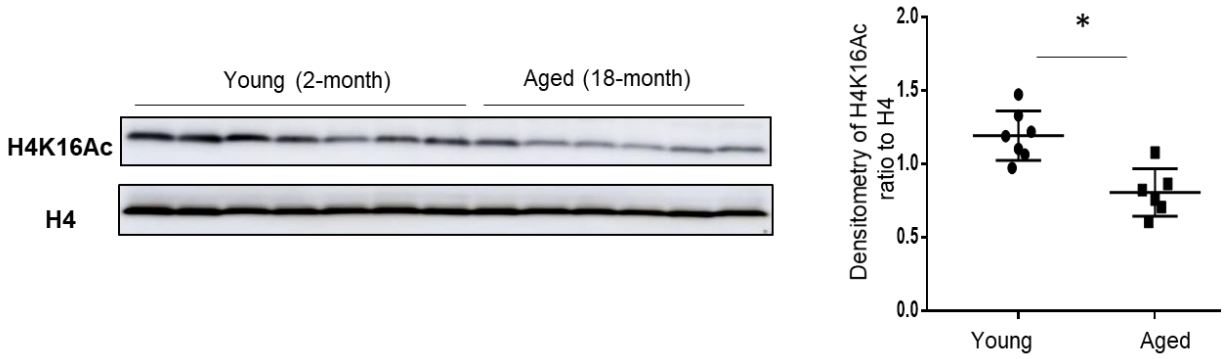
STAR (version 2.5.3a) was used to align the raw RNA-Seq fastq reads to the reference genome from Gencode [1]. Following alignment, HTSeq-count was used to count the number of reads mapping to each gene [2]. Normalization and differential expression was then applied to the count files using DESeq2 [3].

Supplementary Reference:

1. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1): p. 15-21.
2. Anders, S., P.T. Pyl, and W. Huber, *HTSeq--a Python framework to work with high-throughput sequencing data.* Bioinformatics, 2015. **31**(2): p. 166-9.
3. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.

Supplementary Figures:

A



B

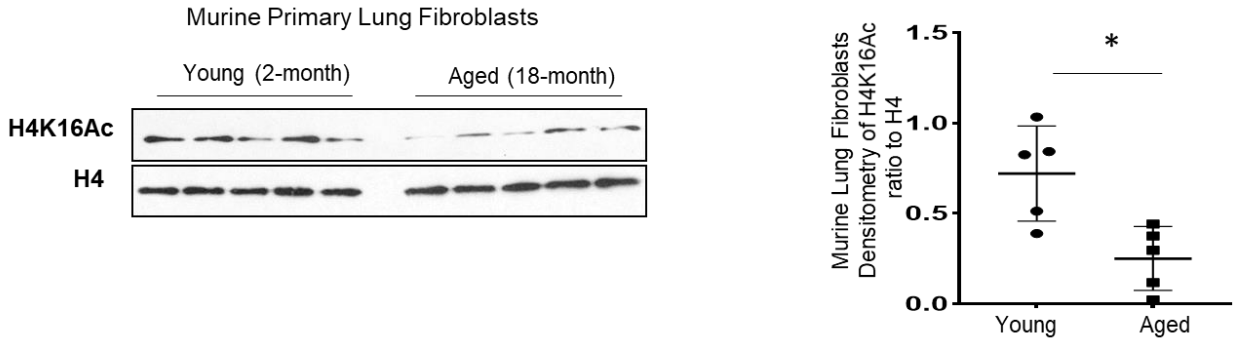


Figure S1. Histone H4K16Ac levels in young and aged mice lung tissues / primary murine lung fibroblasts.

(A) The H4K16Ac levels in young (2-month old) and aged (18-month old) mice lung tissues at baseline by western blots, H4 is the loading control. *Right,* Densitometry of H4K16Ac relative to H4, as in A. *P < 0.05, aged (n = 6) compared to young (n = 7) mice. **(B)** Baseline levels H4K16Ac and H4 by WB in lung fibroblasts isolated from young (2-month) or aged (18-month) mice. *Right,* Densitometry of H4K16Ac relative to H4 as in C. *P < 0.05, n = 5 in each group.

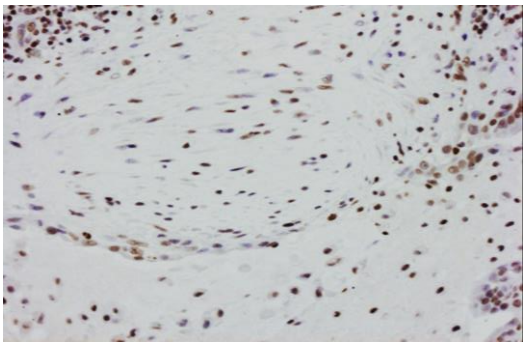
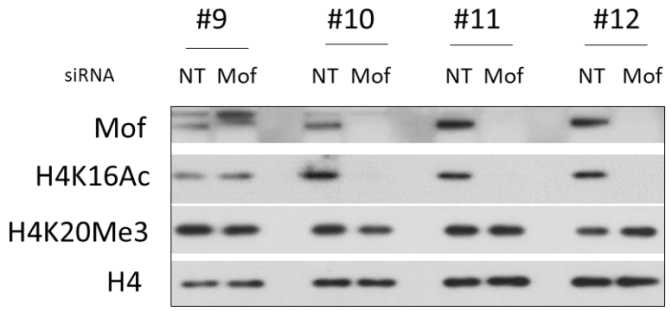


Figure S2. IPF lung fibrotic focus stain of H4K16Ac (brown), picture taken at 20 x.

A



B

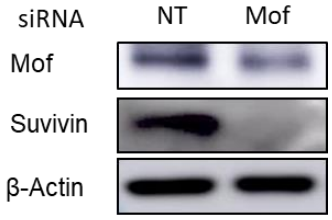


Figure S3. (A) Four different siRNA Mof from Thermo Scientific were tested for knocking down Mof in lung fibroblasts. # 12 was used in the rest of the experiments. Western blots demonstrated the Mof, histone H4K16Ac, and H4K20me3 levels in nuclear extracts. H4 is the loading control. **(B)** IPF fibroblasts transfected with siRNA or NT, western blots demonstrated the Mof knockdown for the blot show in Figure 3B for survivin expression by western blot.

Figure S4. Pathways of IPF fibroblasts transfected with siRNA Mof vs NT by RNA-Seq from 3 different IPF fibroblast cell lines.

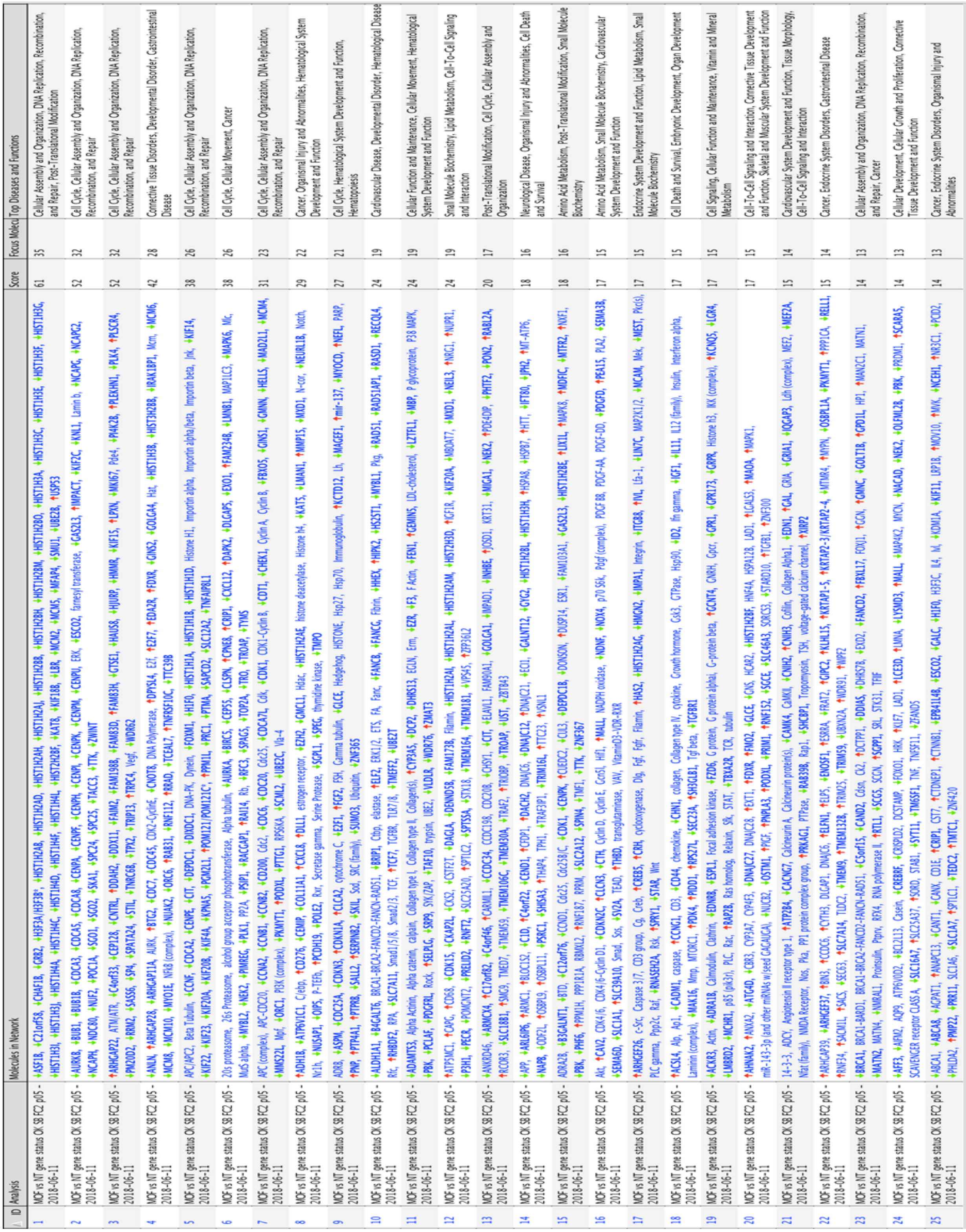


Figure S5. RNA-Seq data for related network changes in IPF fibroblasts transfected with siRNA Mof vs NT.

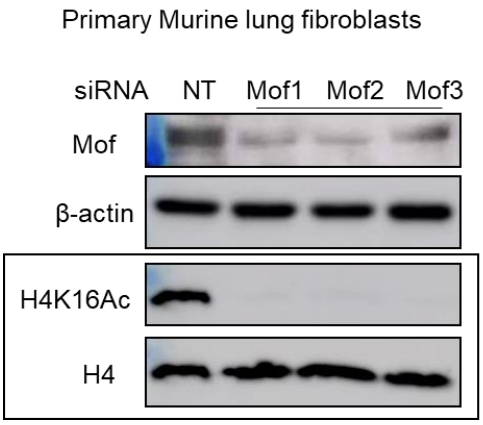


Figure S6. Testing of three different siRNA mMof for knocking down Mof in primary murine lung fibroblasts. Western blots showing Mof in whole cell lysate, or H4K16Ac in histone extracts by western blots. The loading control is β-actin for whole cell lysate, or H4 for histone extracts. Mof2 was used in the animal studies; sequence of siRNA mMof is in Table 2S.

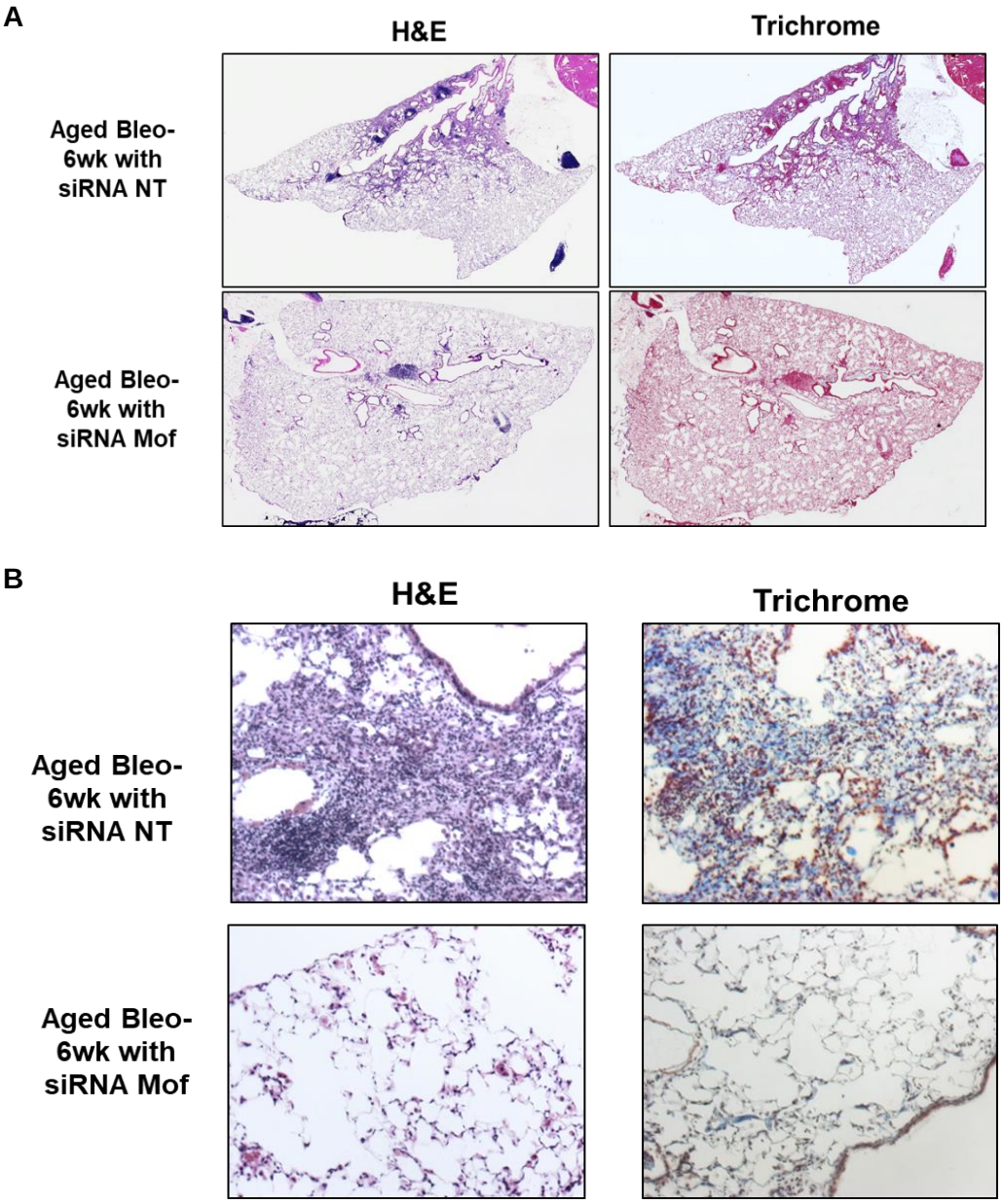


Figure S7. 18-month old mice were subjected to bleomycin injury, then treated with siRNA NT or Mof by nasal from 3-6week after bleomycin injury. Slides are stained with H&E or with trichrome. Pictures were taken at 4 x **(A)**, or at 20 x **(B)**.

Table S1. Information of the de-identified subjects of the non-IPF or IPF samples used in this study.

Non-IPF # 1	Non-IPF # 2	Non-IPF # 3	Non-IPF # 4	IPF-a	IPF-b	IPF-c	IPF-d	IPF-e	IPF-f
48yr, male	20yr, female	32yr, white, male	61yr, white, female	64yr, male	60yr, male	56yr, white, male	48yr, black, male	54yr, white, male	61yr, white, female

Table S2. siRNA sequences and Primers sequences for PCR

Name		Sequence
Mof (human) for cell culture (from Thermo Scientific, cat # J-014800-12)	siRNA	5'-ACUUUGACGUGGAGCCGUU-3'
Non-targeting (NT) (for cells culture)	siRNA	Sense: 5'-UAAGGCUAUGAAGAGAUACUU-3', Anti-sense: 5'-GUAUCUCUUCAUAGCCUUAUU-3'.
mMof (mouse, for *in vivo*)	siRNA	5'-UGAGGUGUUCCUCUACCAGCUUAGG -3'
NT Negative Control (used in vivo, mouse)	siRNA	5'-AGCUACACUAUCGAGCAAUUAACUU-3'
Mof (human) (ENSG00000103510)	RT-PCR	F: 5'-AATGGCACAGCTGGGACTAGAACT-3' R: 5'-GCTTGGCTATAGCAACTGCCGAAT-3'
a-SMA (human)	RT-PCR	F: 5'-ATGGCTCTGGGCTCTGTAA-3' R: 5'-GGAACCTAATCTGTGTCCTGTTATG-3'
	ChIP-PCR	Set A F: 5'- CTTTCTTCTTTGCATGCTACCG R: 5'- GCTGGAATTTCAGGCCATTTC Set B F: 5'-GAGGTCCCTATATGGTTGTGTTAG-3' R: 5'-AGCTGAAAGCTGAAGGGTTAT-3'
Nox4 (human) (ENSG00000086991)	RT-PCR	F: 5'- AGATGTTGGGGCTAGGATTG-3' R: 5'- TCTCCTGCTTGGAACCTTCT-3'
	ChIP-PCR	Set A F: 5'-ATCTCCTGACTCCGTGATCC-3' R: 5'-GCGTGTTAGCACTCTCTCACTTTA-3' Set B F: 5'-GAACAGCAGCAGCCACAAC-3' R: 5'-CTACCCAGAGCCGGTTTTC-3'
Col1A1 (human) (ENSG00000108821)	RT-PCR	F: 5'-TCGAGGGCCAAGACGAAGAC-3' R: 5'- CGCACAACACCTTGCCGTTG-3'
	ChIP-PCR	Set A: F: 5'-CTCTCCATTCCAACTCCCAAA-3' R: 5'-ATGGAGAGCAGGGAGGAA-3' Set B: F: 5'- CGTGAGTTGGTGCAAGAGAGAA-3' R: 5'-GGCCTTCCTGATTGCTTCTACA-3'
Survivin (human) (ENSG000000089685)	RT-PCR	F: 5'- AGCCCTTTCTCAAGGACCAC-3' R: 5'- CAGCTCCTTGAAGCAGAAGAA-3'

	ChIP-PCR	Set A: F: 5'- TCACTTGAGGTCAGGAGTTTG-3' R: 5'- CCCGAGTAGCTGAGATTAAAGG-3' Set B: F: 5'- ACCACGCCCAGCTAATTT -3' R: 5'- CATCACTTGAGTCCTGGAGTTC -3'
β-actin (human)	RT-PCR	F: 5'-TGCTATCCAGGCTGTGCTAT-3' R: 5'- AGTCCATCACGATGCCAG T-3'

Table S3. Excel file for the gene expression in siRNA Mof vs NT cells.