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TITLE: Disrupting Six/Eya Signaling as New Therapy for Lung Fibrosis

PRINCIPAL INVESTIGATOR: Harry Karmouty-Quintana, PhD

CONTRACTING ORGANIZATION: University of Texas Health Science Center, UT HEALTH

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14. ABSTRACT

Pulmonary fibrosis is frequently encountered in the United States Veteran population. Among the different types of pulmonary fibrosis, Idiopathic pulmonary fibrosis (IPF) remains a disease with a dire prognosis with patients progressing to respiratory failure and death within a few years of diagnosis. Unfortunately, limited curative options are available, and prognosis remains extremely poor. This research proposal aims to evaluate the capacity of pharmacological and gene therapy aimed at a novel molecular target for lung fibrosis. This proposal is highly relevant to the FY18 PRMRP Pulmonary Fibrosis and Respiratory Health Topic Areas. IPF is a lethal lung disease that affects about 200,000 people in the United States. Studies have shown that most patients die within 5 years after diagnosis. Despite its prevalence, the pathogenesis of pulmonary fibrosis is poorly understood due to a lack of knowledge of the mechanisms governing its progression. Evidence suggests that pulmonary fibrosis results from aberrant repair responses in the damaged lung. Thus, there is a critical need to identify the mechanisms that lead to overactive repair processes contributing to the development of fibrosis in order to develop new therapies to interrupt the progression of disease and reduce mortality.

Sine Oculis Homeobox Homolog 1 (Six1) is a transcription factor that is essential for normal lung morphogenesis in utero and is expressed in both mice and humans. The Six family encompasses Six1-6, all of them are expressed in mice and humans. Studies have shown that Six1 is important in the development of the lung where its absence results in lung hypoplasia. In these experiments Six1 was shown to modulate expression of the developmental genes sonic hedgehog (SHH) and the epithelial cell markers surfactant protein B and C. However it is important to mention that the activation of Six family proteins and its transcription targets is modulated by its activity with its co-factors eyes absent homolog (EYA), Dachschund (Dach) or the CREB binding protein (CBP) family.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
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TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The prevalence of IPF in the US has increased 2-fold in the last 10 years affecting ~180,000 Americans. Despite the recent approval of nintedanib and pirfenidone for IPF, these agents do not completely halt or reverse the progression of disease[1] and their efficacy in non-resolvable COVID-19 is unknown. This underscores the need to identify novel therapies for the treatment of pulmonary fibrosis where the etiology is complex; implicating mutations in genes involved in the maintenance of telomere length, expression of cilium-associated genes, proteostatic dysregulation and enhanced cellular senescence[1]. Central to the pathogenesis of IPF, is the reprograming of alveolar AEC2, concomitant with mesenchymal cell activation and immune cell dysregulation resulting in enhanced extracellular matrix (ECM) deposition and lung remodeling[1].
Through an initial unbiased micro-array, we have identified a novel developmental transcription factor that is up-regulated in IPF and other presentations of lung fibrosis: Six1 (**Figure 1A**). The Six family encompasses Six1-6, in mice and humans[3]. Six proteins are necessary for the development of many organs and are usually turned-off in adulthood. The transcriptional function of Six1 is modulated by the formation of a complex with the co-factors absent homolog (Eya) family[3]. Our microarray and western blot results (**Figure 1 A, B**) revealed increased Six1/Eya1/Eya2 levels. Our results also demonstrate upregulated Six1, Eya1 and Eya2 transcript levels in IPF lungs compared to controls and age-matched tissue from patients with a diagnosis of chronic obstructive pulmonary disease (COPD) (**Figure 1 C-E**). Immunohistochemistry for Six1 revealed increased signals in AEC2 (**Figure 1F**). In the intra-peritoneal (IP) -BLM model of lung fibrosis, we also showed increased Eya1 and Eya2 expression (**Figure 2**). Our central hypothesis was that***: Increased expression of Six1 promotes lung fibrosis.***

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Idiopathic Pulmonary Fibrosis, Interstitial pulmonary disease, Six1, Eya1, Eya2, MIF, alveolar type II epithelial cells, COVID-19, non-resolvable COVID-19 ARDS

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1: Evaluate whether drugs targeting the Six1/EYA complex are able to treat experimental lung fibrosis.

Aim 2: Determine the capacity of gene therapy approaches to silence the Six1/EYA axis

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We were able to confirm elevated levels of Sine Oculis Homebox Homolog 1 (Six1) and its co-factors eyes absent (EYA) 1 and 2 in lung explants from patients with a diagnosis of Idiopathic pulmonary fibrosis (IPF), in addition to other presentations of lung fibrosis, including interstitial lung disease (ILD) in patients with Systemic Sclerosis (SSc) and in patients with non-resolvable Coronavirus Disease 2019 (COVID-19) that required lung transplantation (**Figure3**). Using RNA-scope, we were also able to localize increased SIX1 signals in type II alveolar epithelial cells (AEC2). Furthermore, using a clinically relevant model of experimental lung fibrosis where mice lacking the telomere shelterin protein, telomere repeat binding factor 1 (TRF1) in AEC2 develop spontaneous lung fibrosis[2], we were able to show increased levels of Six1 in this model (**Figure 4**). Thus, taken together, an important accomplishment of the previous award is the demonstration that elevated Six1 signals are a *bona fide* target for pulmonary fibrosis that is observed in many presentations, including IPF, SSC-ILD and non-resolvable COVID-19.

Intriguingly, our experiments using a selective protein/protein inhibitor targeting Six1/Eya2 did not show an improvement in lung function, or reduction of fibrotic markers in our model of bleomycin (BLM) induced lung injury (data not shown). These results contrasted with experiments where selective Six1 deletion in AEC prevented or halted the progression of experimental lung fibrosis. To address this challenge, further experiments using transgenic mice lacking AEC2-Six1 were performed. These experiments revealed that deletion of Six1 is protective against lung fibrosis (**Figure 5**). Next, using a mouse lung epithelial cell line (MLE12) where Six1 was upregulated, we aimed to identify a potential mechanism for the deleterious effects of Six1 on lung fibrosis. These experiments revealed increased expression of ribosomal proteins (RP, **Figure 6**). In addition, elevated macrophage migration inhibitory factor (MIF) as a downstream target of Six1 was identified (**Figure 7**). *In vitro* and *in vivo* inhibition of MIF was also able to inhibit the profibrotic effects of elevated Six1 (**Figure 8**). Taken together, we have demonstrated beyond reasonable doubt that Six1 deletion is protective in experimental lung fibrosis and that the potential mechanism involves activation of MIF which does not require Eya2. These results are significant and warrant further studies that aim to inhibit Six1 (a misexpressed developmental gene) for the treatment of lung fibrosis. An ideal approach is to silence Six1 as initially proposed however, of the short mRNA sequence of Six1, commercial siRNA approaches for Six1 did not possess the necessary efficacy *in vitro* in lung cells, thus *in vivo* studies were not performed.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Cory Wilson an MD/PhD student in my lab has performed the majority of the research. The results discussed herein were an integral part of his thesis. Thank to this work, we has able to secure a highly competitive Physician Scientist Training Pathway (PTSP) residency at the University of Iowa Health Care at the Department of Internal Medicine. In addition, Nancy Wareing another MD/PhD student in my lab has participated in the study design/analysis and interpretation of results.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Importantly, a manuscript from our findings on Six1-MIF is currently under revision in *JCI Insight (IF:8.3)* where we are responding to the reviewers' comments. Portions of our research has also been presented at the 2020 and 2021 (submitted and accepted for a poster presentation) MHSRS, the 2018, 2019 American Thoracic Society Annual (ATS) congress, the 2019 Gordon Research Conference on Lung Development and Regeneration. In addition, I have been invited to give seminar talks on my research on Six1 in lung fibrosis at: "De novo developmental gene expression in Idiopathic Pulmonary Fibrosis" Translational Lung Research Center Heidelberg, The German Center for Lung Research (DZL), Heidelberg, Germany (2018); Six1 Expression in the Alveolar Epithelium Promotes Lung Fibrosis" TMC Lung Biology Seminar Series, Houston, TX. (2019), Novel developmental gene targets for Idiopathic Pulmonary Fibrosis" Ionis Pharmaceuticals, Carlsbad, CA. (2019), "Sineoculis homeobox homolog 1 (Six1) is elevated in IPF and promotes lung fibrosis" Invited lecture at the Division of Pulmonary, Critical Care and Sleep Medicine, The Ohio State University, Columbus, OH, (2020). Based on our results and new preliminary results we believe that our research qualifies for follow-on research with the title of ***"Six in lung fibrosis."***

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Final report, nothing to report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our results will place SIX1/EYA2 as central players in the pathogenesis of lung fibrosis and establish them as a novel target. Our results have also uncovered novel mechanisms that promote lung fibrosis through increased expression of AEC2-SIX1 and subsequent increased in extracellular MIF levels that can then activate macrophages.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Importantly, our results revealed that increased SIX1 is a novel pro-fibrotic mechanism that is also present in other forms of lung fibrosis, such as in SSc-ILD and most intriguingly, in patients with non-resolvable COVID-19 who required lung transplantation. Very little is known regarding the mechanisms that lead to lung fibrosis in COVID-19 thus our research has important implications in this field.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Lack of in vitro effect of commercially available LNA that targeted SIX1/EYA1/EYA2, we initiated a collaboration with IONIS to develop anti-sense oligonucleotides as an alternative approach to commercial LNAs, however the company did not provide us the ASO to test the agents in vivo. Due to the lack of efficiency of commercial LNAs nanotechnological approaches were not developed further.
Using ATAC-seq approaches, we also surmised that chromatin from IPF-derived fibroblasts may have open reading frames for SIX1. These experiments did not reveal this, however, the results led to a publicton on ATAC-seq in the Amer J. of Respir Cell and Mol Biol. (Accepted)

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

To address the challenges created by the lack of pharmacological approaches to test the feasibility of drugs aimed at targeting SIX1/EYAs, we utilized transgenic mice that allowed for gain-of-function or loss-of-function of epithelial-specific SIX1. These studies were instrumental at identifying the patho-physiological role of SIX1, its potential mechanisms and potential to serve as a novel target for lung fibrosis.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The need to maintain transgenic mouse lines and breeding schemes necessary to generate experimental mice led to additional expenses on animal costs.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1- Sine Oculis Homeobox Homolog (Six1) Plays a Critical Role in the Progression of Pulmonary Fibrosis – *JCI Insight : response to reviewers due Aug 20, 2021*

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1- Transcriptomic and epigenetic profiling of fibroblasts in Idiopathic Pulmonary Fibrosis (IPF) – *Amer J Respir Cell Mol Biol: Accepted for publication July 2021*
2- Characterization of fulminant pulmonary fibrosis in COVID-19 patients requiring lung transplantation- *submitted to Eur Respir J*

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	*Harry Karmouty-Quintana*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-4753-9823*
Nearest person month worked:	*2*
Contribution to Project:	*Overview of the project, planning and preparation of manuscript*
Funding Support:	*NIH 1R01HL138510*

Name:	*Weizhen Bi*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Performed RT-PCR experiments and other molecular biology experiments, maintained mouse colonies*
Funding Support:	*NIH 1R01HL138510*

Name:	*Cory Wilson*
Project Role:	*Graduate Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3948-3831*
Nearest person month worked:	*1*
Contribution to Project:	*Planned and performed in vivo and in vitro experiments*
Funding Support:	*NIH F30HL147508-01 H*

Name:	*Nancy Wareing*
Project Role:	*Graduate Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-5149-3966*
Nearest person month worked:	*1*
Contribution to Project:	*Planned and performed in vivo and in vitro experiments*
Funding Support:	*NIH 1R01HL138510*

Name:	*Elvin Blanco*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-5149-3966*
Nearest person month worked:	*1*
Contribution to Project:	*Planned in vitro experiments*
Funding Support:	*DoD: W81XWH-19-1-0129*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

<u>Organization Name:</u> Houston Methodist Hospital
<u>Location of Organization:</u> Houston, TX
<u>Partner's contribution to the project</u> (identify one or more)

- *Collaboration (e.g., partner's staff work with project staff on the project);*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Figure 1. A) Microarray data for IPF vs control (collagen-white), IPF genomic signature (green), Six1/Eya1/Eya2 (red), Six2-6 (black). B) Western blots for Six1, Eya1, Eya2 and GAPDH from normal and IPF lung tissue. Transcripts for Six1(C), Eya1(D) and Eya2(E) from normal, COPD and IPF lung tissue. Double IHC for SPC (blue) and Six1(brown), arrows point at double stained cells (F). No differences in expression levels were seen between females and males.

Figure 2. Western blots at day 33 for Six1, Eya1, Eya2 and β-actin from PBS and IP BLM treated male mice

Figure 3. Masson's trichome stained lung sections (A), Aschroft scores (B), COL3A1 (C), COL1A2 (D) and SIX1 (E) transcript levels from control, IPF and non-resolvable COVID-19 lung explant tissue. Significance levels * refers to comparisons against the control group and # refers to comparisons between IPF vs COVID-19.

Figure 4. (A) Lung Six1, Eya1, Eya2 transcript levels at 3 months of tamoxifen and (C) IF for Six1 (green), SPC (red) and merge (yellow) histo-pictographs from Trf1f/f and Trf f/f-SPC Cre-ERT2 8 months after tamoxifen.

Figure 5. Treatment regimen, Masson's Trichrome stained sections and Ashcroft scores for SPCCreERT2 or Six1 f/f-SPCCreERT2 mice treated with PBS or BLM. (A) Depicts IP BLM in male mice. (B) Depicts IT BLM in female mice. All mice were pre-treated with tamoxifen. * refers to comparisons vs PBS; # refers to comparisons vs BLM SPCCreERT2

Figure 6. A) Western blot for Six1 and GAPDH from control pcDNA-treated (1,2) or Six1pcDNA-treated MLE-12 (3,4) cells. B) Top 10 Enriched protein complex-based sets determined using the ConsensusPathDB: cpdb.molgen.mpg.de and graphical representation identifying Ribosome Biogenesis (RiBi) as central to Six1 overexpression in MLE-12 cells. C) Expression levels of upregulated genes encoding for RP, Eya1 and Eya2.

Figure 7. (A) Western blot showing Six1 protein overexpression (Six1OE) compared to control MLE12 cells. (B) RNA-sequencing data expressed as Log2 fold-change ± SEM comparing GFP control MLE12 cells (n=3) to SixOE (n=3); * $p < 5.92E-14$. (C) Diagram depicting the MEF3 binding sites in the mouse and human MIF promoters. (D) RT-PCR showing increased expression of Six1 and (E) MIF in SixOE cells. (F) Western blot showing increase in Six1 and MIF protein levels in SixOE cells compared to GFP controls. (G) RT-PCR for MIF levels from iAT2Cre or iAT2Six1-/- mice administered with tamoxifen 14 days prior to PBS or BLM treatment. Data is shown ± s.d. * $p < 0.05$ refers to comparisons between AT2Cre mice, # $p < 0.05$ refers to comparisons among BLM-treated mice using a One-way ANOVA with Holm-Sidak post hoc test. (H) MIF protein levels in BALF from iAT2Cre or iAT2Six1-/- mice administered with tamoxifen 14 days prior to BLM treatment. Data is shown ± s.d. Significance levels, * $p < 0.01$ refers to a Mann-Whitney test. (I) MIF transcript levels from BLM-treated iAT2Cre or iAT2Six1-/- mice where tamoxifen was administered on day 15 of BLM. Data is shown ± s.d. Significance levels, * $p < 0.01$ refers to a Mann-Whitney test.

Figure 8. (A) MIF transcript levels in IPF (n=8) compared to control (n=8) patient samples. (B) Absorbance values of WST-1 assay at 24 hrs read at 450 nm for control human lung fibroblasts (n=12 (4 donors in triplicate)) with or without 100 ng/mL recombinant human MIF. Data shown ± s.d. *$p < 0.05$ using two-tailed, unpaired Student's t test with Welch's correction. (C) Human lung fibroblasts treated with a dose response (4-400 ng/mL) of MIF in vitro for 48 hrs stained with alpha-smooth muscle actin (αSMA; red signal) and counter-stained with DAPI. (D) Quantification of αSMA fluorescent signal using integration of per cell fluorescence pixel intensity using an automated fluorescence cell cytometer. Data shown ± s.d. * $p < 0.05$ using One-way ANOVA with Holm-Sidak post hoc test.

References

1. Lederer DJ, Martinez FJ. Idiopathic Pulmonary Fibrosis. *New England Journal of Medicine* 2018; **378**(19): 1811-23.
2. Naikawadi RP, Disayabutr S, Mallavia B, et al. Telomere dysfunction in alveolar epithelial cells causes lung remodeling and fibrosis. *JCI Insight* 2016; **1**(14).
3. Liu Y, Han N, Zhou S, et al. The DACH/EYA/SIX gene network and its role in tumor initiation and progression. *International journal of cancer* 2016; **138**(5): 1067-75.