AWARD NUMBER: W81XWH-19-1-0007

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

PRINCIPAL INVESTIGATOR: Harry Karmouty-Quintana, PhD

CONTRACTING ORGANIZATION: University of Texas Health Science Center, Houston, TX

REPORT DATE: March 2022

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2022	Final	15May2019-14Nov2021

4. TITLE AND SUBTITLE
Disrupting Six/Eya Signalling as New Therapy for Lung Fibrosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0007

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Harry Karmouty-Quintana, PhD

E-Mail: Harry.Karmouty@uth.tmc.edu

5d. PROJECT NUMBER
0011297565

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Health Science Center – Houston

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Idiopathic pulmonary fibrosis (IPF) is the most common type of interstitial lung disease, with a median survival of 2-4 years from the time of diagnosis [1]. It is estimated that the prevalence of IPF in the US is approximately 10-60 cases per 100,000 people, with limited pharmacological therapies available [2, 3]. IPF is a chronic, progressive disease characterized by alveolar injury, increased extracellular matrix (ECM) deposition and resultant alveolar destruction. Macroscopically, this leads to poor lung compliance, impaired trans-alveolocapillary membrane gas exchange and ultimately, end-stage respiratory failure, necessitating lung transplantation [2, 4, 5]. Several non-genetic risk factors, such as male sex, older age, and smoking, increase the risk of developing IPF [4, 6]. More recently, several genetic risk factors for IPF have also been discovered, including a single-nucleotide polymorphism (rs35705950) in the promoter region of MUC5B [7-9], which codes for an essential protein for airway clearance and innate immune response, along with genes associated with telomere maintenance, such as telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT) [1, 10].

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			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	20	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

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:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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?

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?

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?

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:

What was the impact on the development of the principal discipline(s) of the project?

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?

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?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

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

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

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

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:

- **Publications, conference papers, and presentations**

Journal publications.

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.

Nothing to report

Other publications, conference papers and presentations.

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)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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?

Nothing to Report

What other organizations were involved as partners?

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

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

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

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.

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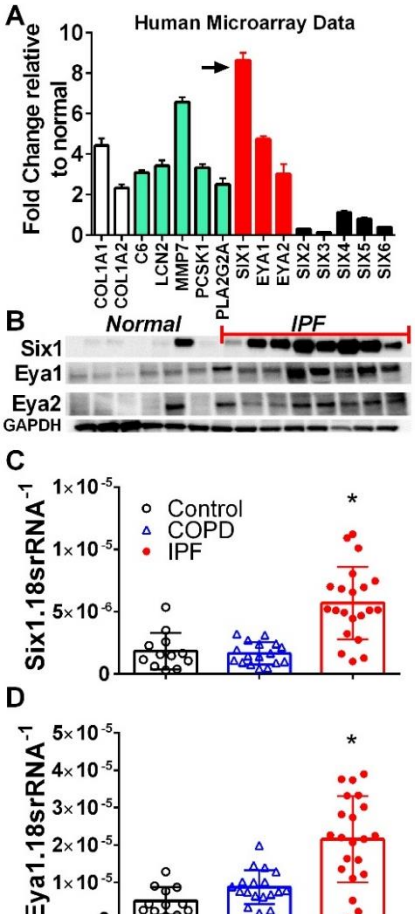
2. Naikawadi RP, Disayabutr S, Mallavia B, et al. Telomere dysfunction in alveolar epithelial cells causes lung remodeling and fibrosis. *JCI Insight* 2016; **1**(14).
3. Liu Y, Han N, Zhou S, et al. The DACH/EYA/SIX gene network and its role in tumor initiation and progression. *International journal of cancer* 2016; **138**(5): 1067-75.

Figure 1



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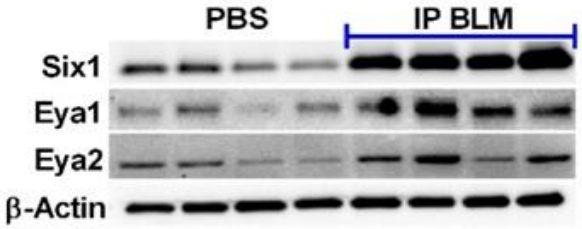


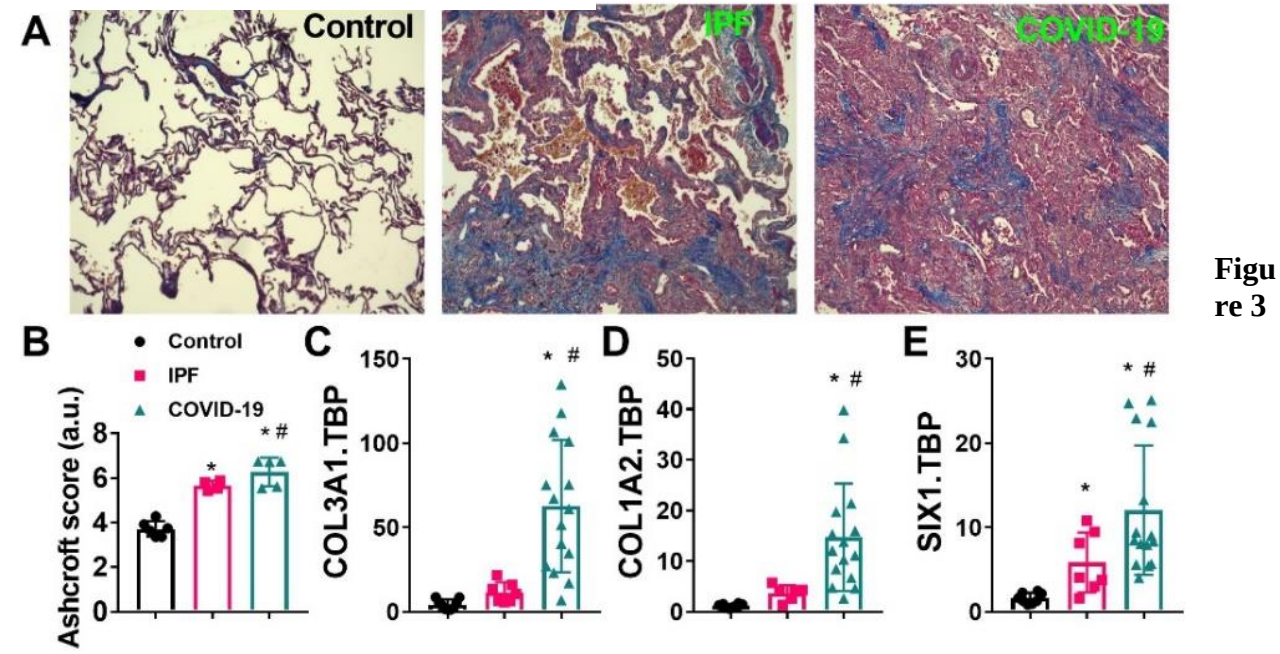
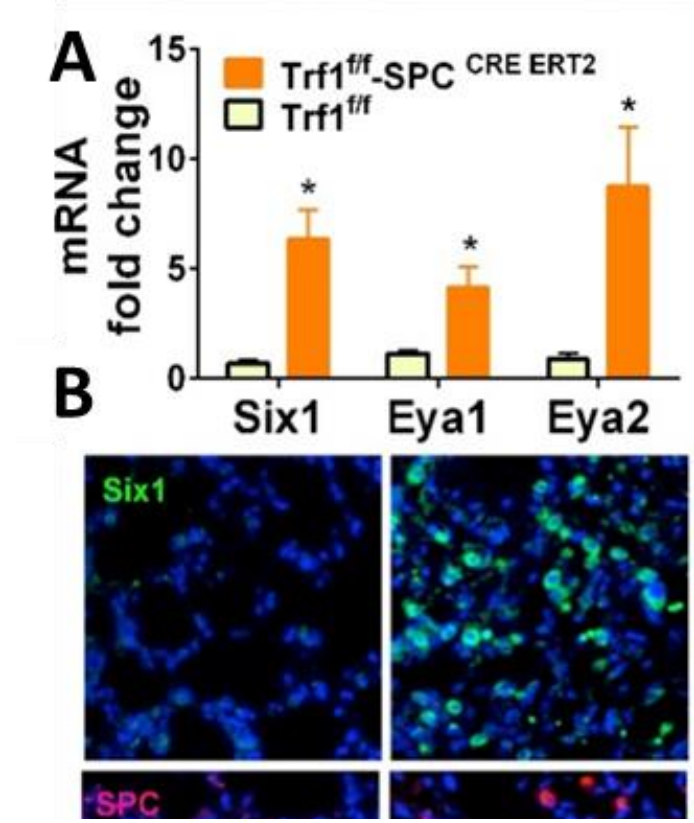


Figure 3

Figure 4



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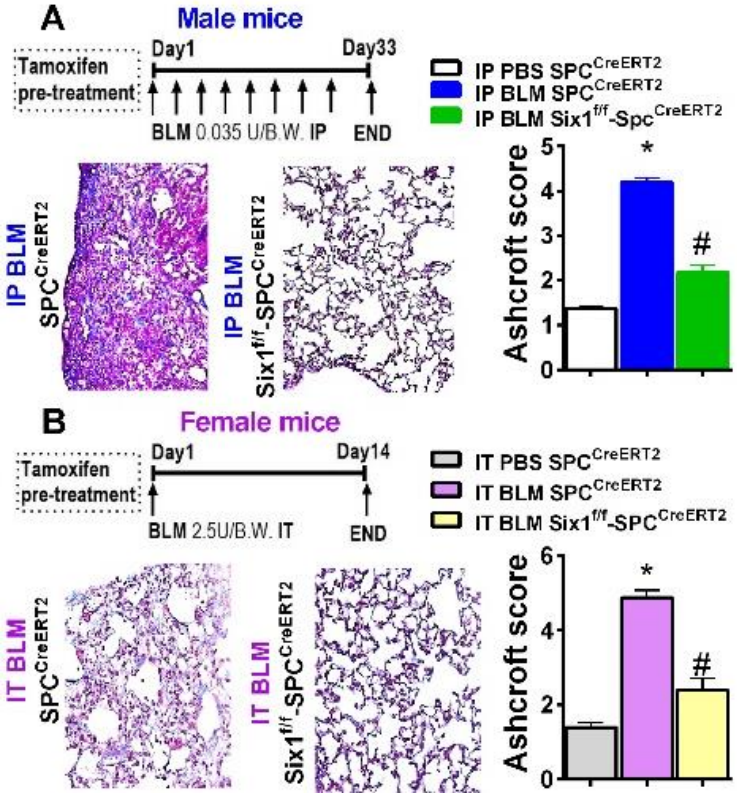

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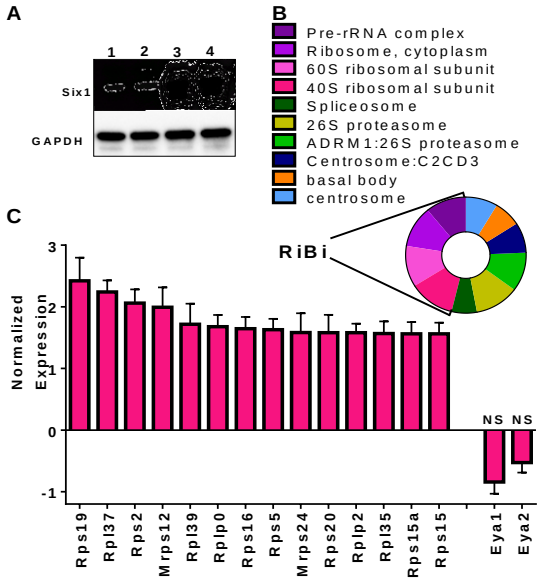

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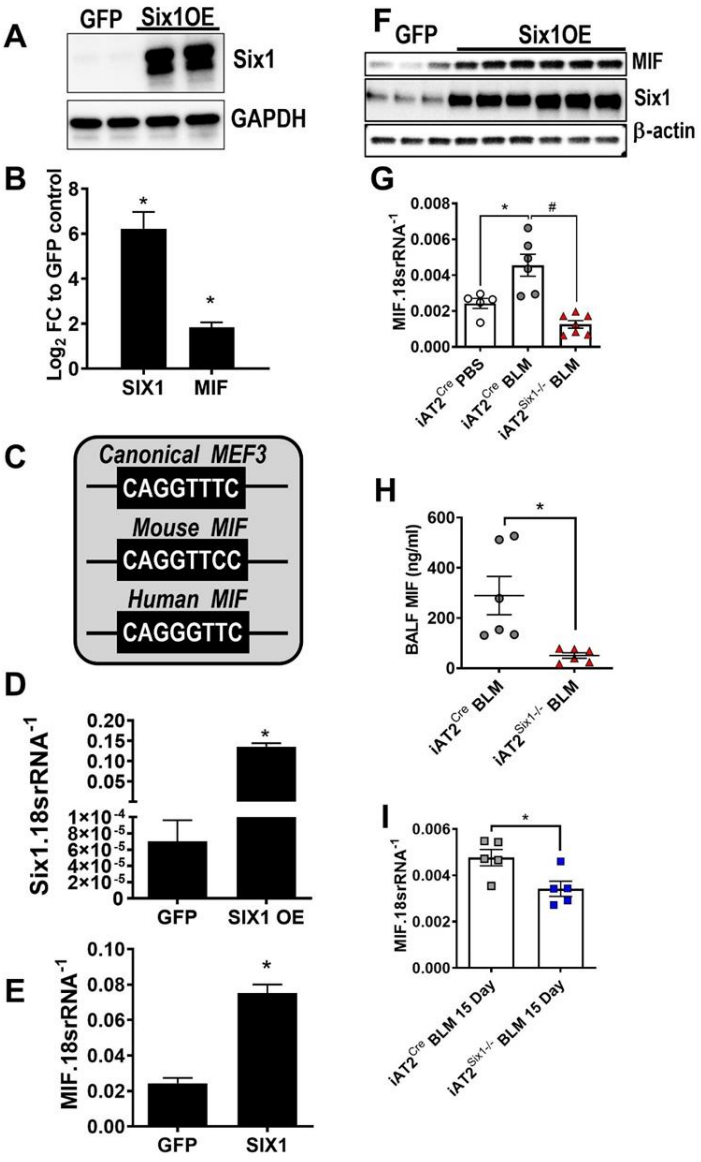
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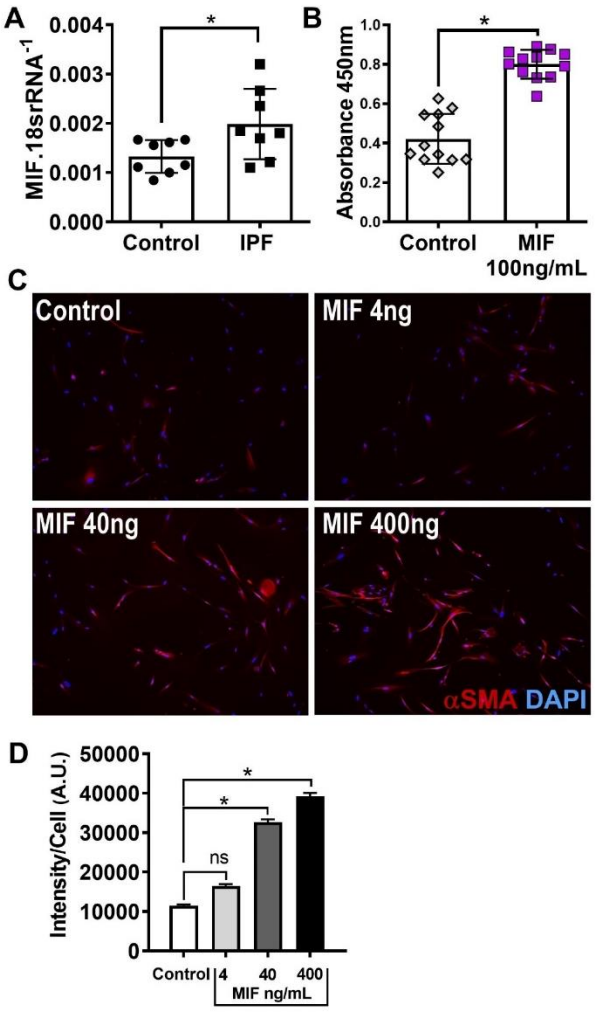
Figure 8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