# **Supplementary Information**

# **Detecting the Molecular System Signatures of Idiopathic Pulmonary Fibrosis**

# **through Integrated Genomic Analysis**

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# **Supplementary Methods**

## **High throughput data and processing**

Each microarray experiment's data was categorized into two classes, Normal and IPF individuals, while excluding all other specific condition. P-values were adjusted using Benjamini & Hochberg FDR. As in practice, genes with significant P-value  $(<0.05$ ) and  $-1 <$ LogFC  $>1$  were considered as differentially expressed genes. Genes which displayed inconsistent and opposite expression status across the samples for any given condition were removed and only those genes were considered which exhibited single consistent trend in any given experiment for the given condition. This removed noise in the micro-array data. Z-score transformation of the expression data was done for all kind of platforms, in order to maintain a single comparable scale.

### **Classification of IPF genes**

For classification purpose, different datasets were created for training and testing using a support vector machine approach. A total of 159 IPF cases worked as positive samples while a total of 112 normal, sarcoidosis and NSIP cases were considered as negative non-IPF cases. For each considered gene set, samples were randomly distributed into training and testing model 10 times, each time yielding different training and testing set pair and model. This was done to ensure that the performance of the classifier was not biased towards only certain combination of samples.

Initially, 271 individuals were considered from five different IPF experiments (169 from Yang et al., 23 from Meltzer et al., 17 in Cho et al., 8 in Sanders et al. and 15 in Nance et al.), 27 from Sarcoidosis (Crouser et al. and Lockstone et al.) and 12 from NSIP (Yang et al.), making a total of 159 IPF and 112 non-IPF individuals. While building the training models, the non-IPF set also included 11 different Sarcoidosis individuals, and 6 NSIP individuals as a part of the non-IPF training dataset along with the control and normal individuals. The remaining 10 Sarcoidosis and 6 NSIP instances would go to the non-IPF test set. This way, 136 individuals having 80 IPF and 56

non-IPF individual were considered for building each model in a mutually exclusive manner in order to avoid any bias. To build random model for each, both the sets (A1 and A), 80 IPF samples were picked randomly as the positive set for the training set. The remaining 79 IPF instances were used as test set. Same was followed for non-IPF instances where 56 individuals were used as negative set for the training and remaining 56 non-IPF instances for testing. This kind of random selection based set pairs were created 10 times to build 10 different models using Radial basis function (RBF) kernel with optimized cost and gamma factors. Each of these models was tested over the corresponding test set.

LibSVM with RBF kernel and optimized cost and gamma factor was run. The models were checked for accuracy, sensitivity and specificity on different randomly generated test sets. Receiver Operator Characteristics plots with Area Under curve were made using  $ROCR<sup>1</sup>$  package of R. Matthews correlation coefficient (MCC) was calculated to measure perfection of SVM based classification. Following equations were used to measure the classifier's performance:

 $Sensitivity=[TP/(TP+FN)]*100$ *Specificity=*(*TN* /(*TN+FP* ))∗100  $Accuracy = (TN+TP)/(TP+TN+FP+FN)$ *MCC=*(*TP*∗*TN* )−( *FP*∗*FN* )/√(*TP+FP*)∗(*TP+FN* )∗(*TN+FP*)∗(*TN+FN* )

where TP= True Positive, TN= True Negative, FP=False Positive and FN= False Negative Further system wide analysis was done for three datasets (SetA and SetB) as they were found to be most confident differentially expressed sets.

AUCs for above mentioned sets for each of the 10 instance-set pairs were evaluated and scored in the range of 0.92 to 0.99 (Supplementary Figure S6), clearly suggesting robust modeling of IPF by these gene-sets.

#### **TRANSFAC and CHIP-Seq identified TF-target interactions**

Besides the manual curation, the approach for the identification of transcription factors involved two more methods: Scanning through 1) TRANSFAC v8.3 and 2) CHIP-seq data. Identification was done for both sets (SetA, B). The first method includes the identification of potentially TF regulated differentially expressed genes, their 2kb upstream promoter regions up to first exon were identified and were searched for transcription factor binding sites using PROMO 3.0. This tool incorporates TRANSFAC database (v8.3) having largest available collection of eukaryotic factor specific weight matrices. TFBS and transcription factor associated with them were identified on the basis of sequence similarity to the matrix. Similarity threshold of 85% was applied to obtain high quality results. The Second method was based on the availability of TF peaks data from regulatory map of transcription factor binding sites (ReMap) repository as it contains validated transcription factor CHIP-seq peak data from well-established repositories ENCODE and GEO. Available transcription factor peak bed files for all 237 transcription factors were downloaded from this repository and their binding was checked across the target genes in both the data sets using bedtools. Details related to the TF-target interactions in the regulation of differentially expressed genes is covered in supplementary Table S6 and list of identified TF is given in supplementary Table S7.

### **miRNA data**

So far three microarray studies have been published for miRNAs (GSE27430, GSE21394 and GSE32538). The study has been done using all these high-throughput studies and a RNA-seq study data. Details are covered in the main text.

# **Statistical significance of potential Feed-Forward loops**

Let suppose two edges E1 and E2 are connecting nodes N0 to N1 and N4 to N5, respectively. These

were chosen randomly and rewired such that nodes were swapped constructing edges E1 =(N0-N5) and E2= (N4-N1). P-value of significance was calculated as proportion of randomized networks having particular FFL count greater than or equal to its number in real network to the total number of randomized networks (1000 in our study).

### **Network properties of gene regulatory network in IPF**

Degree of a vertex/node in the network is the number of edges connecting to it. High degree nodes are characterized as hub nodes in the network. Centrality estimates the importance of node in a network. Betweenness centrality of a node is defined as the total occurrences of node in form of a bridge across shortest path of two other nodes. Farness of a node is a measure of sum of distances of the node with all other nodes and reciprocal of it is defined as the closeness centrality of the node. Tendency of a node in a graph to remain clustered together is referred as clustering coefficient whereas average shortest path length is described as mean value of steps through shortest paths of all potential node pairs of network. Indegree and outdegree terms are defined for directed networks in form of edges converging towards the node and diverging from the node respectively.

# **Supplementary discussion**

### **IPF specific GRN**

IPF associated TF-miRNAs mediated regulatory networks were reconstructed via merging of significant potential FFLs. 15 differentially downregulated genes and 18 differentially upregulated genes were observed regulated by both TFs and miRNAs, forming network motifs in Set A. 22 upregulated miRNAs and 19 downregulated miRNAs in IPF were found regulating IPF DEGs via association with 42 TFs incorporating 16 DETFs in IPF (CSRNP1, NFE2, AFF3, BHLHE22, HOPX, ID1, JUN, KLF6, MYB, MYOCD, SIX1, SIX4, SMAD6, SOX2, TFAP2A and TP63) (Supplementary Figure S11). Upregulated DEGs forming significant potential FFLs in these sets

were enriched in ECM receptor interaction, p53 signaling pathway, ABC transporters and cell adhesion molecules (CAMs). The downregulated DEGs were found enriched in fatty acid metabolism and AMPK signaling pathways. Enhanced extracellular matrix deposition and cell adhesion was observed as a characteristic feature of IPF lungs. Previous studies had reported p53 upregulation causing G1 arrest of cell cycle combining chronic DNA damage in IPF disease condition<sup>33</sup>. Fatty acid metabolism affects function of alveolar epithelial cells, which are responsible for maintaining lung homeostasis. Its deficiency stimulates lung injury in bleomycin induced mice resulted in induction of apoptosis and TGF- $\beta$  expression<sup>34</sup>. All these previous observations concur with the findings here.

Table S1: Accuracy, sensitivity, specificity and MCC values for sets A1 and A in contrast to ten



different random models









**Table S3:** Differentially expressed TF-target interaction analysis for Set A and Set B genes.







Transcri ption factor	Description	<b>Class</b>	Family	<b>Expressi</b> on in study	<b>Function</b>	Involvement in down- regulated pathways associated with IPF identified in study	GO enriched terms associate d with <b>IPF</b> identified in study	role in IPF
<b>CEBPD</b>	CCAAT/enh ancer binding protein delta	<b>Basic</b> leucine zipper factors (bZIP)	C/EBP family	down- regulated	tumor suppressor function	Metabolic pathways, Endocytosis	Response to wounding , RAGE receptor binding	enhance fibroblast proliferation <sup>21</sup>
CSRNP1	Cysteine rich nuclear protein	AXUD/ <b>CSRNP</b> domain factors	<b>CSRNP</b> factors	down- regulated	tumor suppressor function	Metabolic pathways, Endocytosis	Response to stress, response to wounding	Down-regulation inhibits tumor suppression activity <sup>22,23</sup>
FOSL <sub>2</sub>	FOSL <sub>2</sub>	<b>Basic</b> leucine zipper factors (bZIP)	Fos-related factors	down- regulated	cellular proliferation	Metabolic pathways, Endocytosis	Response to stress, response to wounding	Down-regulation inhibits its anti- proliferation $\text{activity}^{24}$
HIF3A	Hypoxia- inducible factor-3- alpha	Basic- helix- loop- helix factors (Bhlh)	PAS domain factors	down- regulated	mediates oxidative stress	Metabolic pathways, Endocytosis	Response to stress, response to wounding	Down-regulation leads to hypoxia induce damage <sup>25</sup>
<b>HOPX</b>	Homeodoma in-only protein	Homeo domain factors	Paired- related HD factors	down- regulated	developing pulmonary airway	Endocytosis, Metabolic pathways	Response to chemical stimulus, <b>RAGE</b> receptor binding	Down-regulation lead to alveolar damage <sup>26</sup>
ID1	Inhibitors of Differentiati on	Basic- helix- loop- helix factors (Bhlh)	ID family	down- regulated	inhibits differentiati on	Endocytosis, Metabolic pathways	Response to wounding , RAGE receptor binding	Down-regulation induces impairment and fibrosis $27$
<b>JUN</b>	Jun	<b>Basic</b> leucine zipper factors (bZIP)	Jun-related factors	down- regulated	cell cycle progression and apoptosis	Endocytosis, Metabolic pathways	Response to stimulus, regulation of catalytic activity	Down-regulation induces reduction in apoptosis <sup>24</sup>
KLF6	Kruppel -	C2H2	Three-zinc	down-	differentiati	Endocytosis,	Response	dysregulation of

**Table S5:** Differentially expressed down-regulated TF classification and role in IPF





**Table S6:** Transcription factor-target analysis using Promo (transfac v8.3) and ReMap

**Table S7: List of TF regulating differentially expressed genes** (A total 107 TFs were identified from both methods (TRANSFAC and CHIP-Seq). Enrichment analysis of their target genes showed the involvement of similar pathways as well as the involvement of same biological and molecular functions as identified in case of differentially expressed transcription factors.)





**Table S8:** Novel, overlapping and contradictory set of DEmiRNAs during IPF



Table S9: Top ten differentially expressed miRNAs on the basis of target gene count and their functional enrichment.









**Table S10:** Description of various potential Feed-Forward loops and interaction in IPF-mediated regulatory networks





**Table S11:** Hub components of regulatory network in terms of IPF genes, IPF miRNAs and TFs

**Table S12:** Detailed information of high-throughput data considered for study from different Data sources















































# **References:**

- 1. Sing, T., Sander, O., Beerenwinkel, N. & Lengauer, T. ROCR: visualizing classifier performance in R. Bioinformatics 21, 3940–3941 (2005).
- 2. Yoshioka, H. et al. In vivo therapeutic effect of CDH3/P-cadherin-targeting radioimmunotherapy. Cancer Immunol. Immunother. 61, 1211–1220 (2012).
- 3. He, W. et al. Exogenously administered secreted frizzled related protein 2 (Sfrp2) reduces fibrosis and improves cardiac function in a rat model of myocardial infarction. Proc. Natl. Acad. Sci. U.S.A. 107, 21110–21115 (2010).
- 4. Vuga, L. J. et al. WNT5A Is a Regulator of Fibroblast Proliferation and Resistance to Apoptosis. Am J Respir Cell Mol Biol 41, 583–589 (2009).
- 5. Burkhardt, A. M. et al. CXCL17 Is a Mucosal Chemokine Elevated in Idiopathic Pulmonary Fibrosis That Exhibits Broad Antimicrobial Activity. J Immunol 188, 6399– 6406 (2012).
- 6. P, C. & J, P. Regulatory Roles of Dclk1 in Epithelial Mesenchymal Transition and Cancer

Stem Cells. Journal of Carcinogenesis & Mutagenesis 7, (2016).

- 7. Guoying Yu et al. in C68. OLD AND NEW PATHWAYS TO PULMONARY FIBROSIS A4932–A4932 (American Thoracic Society, 2012).
- 8. Bauer, Y. et al. A novel genomic signature with translational significance for human idiopathic pulmonary fibrosis. Am. J. Respir. Cell Mol. Biol. 52, 217–231 (2015).
- 9. Arderiu, G., Espinosa, S., Peña, E., Aledo, R. & Badimon, L. Monocyte-secreted Wnt5a interacts with FZD5 in microvascular endothelial cells and induces angiogenesis through tissue factor signaling. J Mol Cell Biol 6, 380–393 (2014).
- 10. Leppäranta, O. et al. Regulation of TGF-β storage and activation in the human idiopathic pulmonary fibrosis lung. Cell Tissue Res. 348, 491–503 (2012)
- 11. Plantier, L. et al. Ectopic respiratory epithelial cell differentiation in bronchiolised distal airspaces in idiopathic pulmonary fibrosis. Thorax 66, 651–657 (2011).
- 12. Vervoort, S. J., Lourenço, A. R., van Boxtel, R. & Coffer, P. J. SOX4 mediates TGF-βinduced expression of mesenchymal markers during mammary cell epithelial to mesenchymal transition. PLoS ONE 8, e53238 (2013).
- 13. Györy, I. et al. Transcription factor Ebf1 regulates differentiation stage-specific signaling, proliferation, and survival of B cells. Genes Dev. 26, 668–682 (2012).
- 14. Micalizzi, D. S. et al. The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial-mesenchymal transition and metastasis in mice through increasing TGF-beta signaling. J. Clin. Invest. 119, 2678–2690 (2009).
- 15. Liu, Y., Chu, A., Chakroun, I., Islam, U. & Blais, A. Cooperation between myogenic regulatory factors and SIX family transcription factors is important for myoblast differentiation. Nucleic Acids Res 38, 6857–6871 (2010).
- 16. Koinuma, D. *et al.* Chromatin immunoprecipitation on microarray analysis of Smad2/3

binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor beta signaling. *Mol. Cell. Biol.* **29,** 172–186 (2009).

- 17. Luchsinger, L. L., Patenaude, C. A., Smith, B. D. & Layne, M. D. Myocardin-related transcription factor-A complexes activate type I collagen expression in lung fibroblasts. J. Biol. Chem. 286, 44116–44125 (2011).
- 18. Murata, K. et al. p63 Key molecule in the early phase of epithelial abnormality in idiopathic pulmonary fibrosis. Exp. Mol. Pathol. 83, 367–376 (2007).
- 19. Sho, T. et al. TRIM29 negatively regulates p53 via inhibition of Tip60. Biochim. Biophys. Acta 1813, 1245–1253 (2011).
- 20. Lee, K. S., Buck, M., Houglum, K. & Chojkier, M. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression. J. Clin. Invest. 96, 2461–2468 (1995).
- 21. Yan, C. *et al.* CCAAT/enhancer-binding protein δ is a critical mediator of lipopolysaccharide-induced acute lung injury. *Am. J. Pathol.* **182,** 420–430 (2013).
- 22. Nakamura, T. et al. Axin, an inhibitor of the Wnt signalling pathway, interacts with betacatenin, GSK-3beta and APC and reduces the beta-catenin level. Genes Cells 3, 395–403 (1998).
- 23. Königshoff, M. et al. Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS ONE 3, e2142 (2008).
- 24. Rajasekaran, S., Vaz, M. & Reddy, S. P. Fra-1/AP-1 transcription factor negatively regulates pulmonary fibrosis in vivo. PLoS ONE 7, e41611 (2012).
- 25. Li, Q. F., Wang, X. R., Yang, Y. W. & Lin, H. Hypoxia upregulates hypoxia inducible factor (HIF)-3alpha expression in lung epithelial cells: characterization and comparison with HIF-1alpha. Cell Res. 16, 548–558 (2006).
- 26. Yin, Z. et al. Hop functions downstream of Nkx2.1 and GATA6 to mediate HDACdependent negative regulation of pulmonary gene expression. Am. J. Physiol. Lung Cell Mol. Physiol. 291, L191-199 (2006).
- 27. Myllärniemi, M. et al. Gremlin-mediated decrease in bone morphogenetic protein signaling promotes pulmonary fibrosis. Am. J. Respir. Crit. Care Med. 177, 321–329 (2008).
- 28. Patel, N. M. et al. Pulmonary arteriole gene expression signature in idiopathic pulmonary fibrosis. Eur. Respir. J. 41, 1324–1330 (2013).
- 29. Lu, Y. et al. Kruppel-like factor 15 regulates smooth muscle response to vascular injury- brief report. Arterioscler. Thromb. Vasc. Biol. 30, 1550–1552 (2010).
- 30. Fujita, R. *et al.* NF-E2 p45 is important for establishing normal function of platelets. *Mol. Cell. Biol.* **33,** 2659–2670 (2013).
- 31. Selman, M. *et al.* Accelerated Variant of Idiopathic Pulmonary Fibrosis: Clinical Behavior and Gene Expression Pattern. *PLoS ONE* **2,** (2007).
- 32. Singh, D. *et al.* Altered gene expression in blood and sputum in COPD frequent exacerbators in the ECLIPSE cohort. *PLoS ONE* **9,** e107381 (2014).
- 33. Kuwano, K. *et al.* P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 154, 477–483 (1996).
- 34. Sunaga, H. *et al.* Deranged fatty acid composition causes pulmonary fibrosis in Elovl6 deficient mice. *Nat Commun* 4, 2563 (2013).



**Figure S1:** Overlap of DEGs among different experiments **(A)** SRP033095 and GSE21411, **(B)** previously reported 82 genes and 176 genes from our study, **(C)** Down regulated genes of GSE35147, GSE32537, GSE21411, GSE24206 and SRP033095, **(D)** Up regulated genes of GSE35147, GSE32537, GSE21411, GSE24206 and SRP033095



**Figure S2:** Clustering results of Set A1 for different experiments **(A)** SRP033095, **(B)** GSE21411, **(C)** GSE24206, **(D)** GSE32537, **(E)** GSE35147



**Figure S3:** Clustering results of Set A for different experiments **(A)** SRP033095, **(B)** GSE21411, **(C)** GSE24206, **(D)** GSE32537, **(E)** GSE35147



**Figure S4:** Accuracy, Sensitivity and Specificity plot for Set A and Set A1 on 10 randomized dataset models



**Figure S5:** ROC plots of two gene sets against ten random models **(A)** Set A **(B)** Set A1



**Figure S6:** NSIP and Sarcoidosis individuals classification on 10 different models of Set A **(A)** Sarcoidosis (10 Individuals) **(B)** NSIP (6 Individuals)



**Figure S7: (A)** Ranking of differentially expressed upregulated transcription factors regulating DEGs, **(B)** Ranking of differentially expressed downregulated transcription factors regulating DEGs specific to IPF



**Figure S8:** Workflow of miRNA analysis from microarray and RNA-seq study, miRNA target

finding, support analysis and involvement of miRNA in different sets of genes



**Figure S9: (A)** Workflow for generation of various potential Feed-Forward loops and construction of TF-miRNA mediated regulatory network in IPF, **(B)** Statistics of three and four node potential FFLs for the three sets of DEGs in IPF.



Figure S10: The framework and workflow of IPF portal. User input query is processed in

background through PHP and MySQL and retrieved results are visualized in HTML.



Figure S11: Graphical view of IPF regulatory networks generated for Set A DEGs representing important nodes in the form of IPF genes and miRNAs at each stage (down and up regulated genes and miRNAs are shown in red and green color, respectively). Edges are highlighted based on the edge betweenness and nodes size is adjusted based on outdegree. TFs are shown in sea green color and hexagon shape.