

1 The UIP honeycomb airway cells are the site of mucin biogenesis with deranged cilia

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14 **Conflict of Interests:** The authors have declared that no conflict of interests exists.

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22 **Abstract:**

23 Honeycombing (HC) is a histological pattern consistent with Usual Interstitial Pneumonia  
24 (UIP). HC refers to cystic airways (HC airways) located at sites of dense fibrosis with  
25 marked mucus accumulation. Utilizing laser capture microdissection coupled mass  
26 spectrometry (LCM-MS), we interrogated the fibrotic HC airway cells and fibrotic  
27 uninvolved airway cells (distant from sites of UIP and morphologically intact) in 10 UIP  
28 specimens; 6 non-fibrotic airway cell specimens served as controls. Furthermore, we  
29 performed LCM-MS on the mucus plugs found in 6 UIP and 6 mucinous adenocarcinoma  
30 (MA) specimens. The mass spectrometry data were subject to both qualitative and  
31 quantitative analysis and validated by immunohistochemistry. Surprisingly, fibrotic  
32 uninvolved airway cells share a similar protein profile to HC airway cells, showing  
33 deregulation of SLITs and ROBO pathway as the strongest category. We find that BPIFB1  
34 is the most significantly increased secretome-associated protein in UIP, whereas  
35 MUC5AC is the most significantly increased in MA. We conclude that spatial proteomics  
36 demonstrates that the fibrotic uninvolved airway cells are abnormal. In addition, fibrotic  
37 HC airway cells are enriched in mucin biogenesis proteins with a marked derangement in  
38 proteins essential for ciliogenesis. This unbiased spatial proteomic approach will generate  
39 novel and testable hypotheses to decipher fibrosis progression.

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44 **Introduction:**

45 Usual Interstitial Pneumonia (UIP) is a fibrotic disease that is associated with a variety of  
46 fibrotic entities (Idiopathic Pulmonary Fibrosis – IPF, sarcoidosis, non-specific interstitial  
47 pneumonia - NSIP, connective tissue disease - CTD, and hypersensitivity pneumonitis -  
48 HP) [1-3]. The UIP histological pattern is patchy with regions of relatively normal-  
49 appearing lung adjacent to dense fibrosis and honeycombing (HC). HC refers to the  
50 clustering of airspaces within dense fibrotic tissue and is associated with the thickening  
51 of airway walls. Accumulation and plugging of mucus and other airway debris within the  
52 HC airways likely negatively impacts lung function.

53

54 Our current understanding of fibrotic airway pathogenesis has been improved with the  
55 advancement of structural, genetic, and molecular analyses. Structurally, UIP/IPF lung  
56 have reduced numbers of terminal bronchioles in both regions of minimal and established  
57 fibrosis [4-6]. Genetically, sequence changes affecting alveolar cells (*MUC5B*, *SFTPC*,  
58 and *SFTPA2*) have been reported [7-9]. Cells comprising the HC airways present as  
59 either multi-layer or as a single layer [1], with cellular subtypes including; basal, ciliated,  
60 columnar, pseudostratified and secretory epithelium; while there are variable reports on  
61 the presence of alveolar type II (ATII) cells [10-12]. Functionally, single-cell RNA  
62 sequencing of IPF epithelial cells identify marked cellular heterogeneity as compared to  
63 control [13]. Collectively, these factors are believed to lead to airway homeostasis  
64 impairment and facilitate disease progression.

65

66 An important function of the airway is to produce mucus. Not only does mucus serve as  
67 a physical barrier but mucus has antimicrobial properties to protect distant airways [14].  
68 During the fibrotic process, mucus fills and plugs the HC airways, which affects pathogen  
69 clearance and blood-oxygen exchange. The secreted mucus hydrogel is underpinned by  
70 two gel-forming mucins, of which, mucin 5B (MUC5B) is the most abundant in health,  
71 whereas MUC5AC is also detected but at a lower level [10]. A gain-of-function *MUC5B*  
72 polymorphism is amongst one of the highest risk factors associated with lung fibrosis [10,  
73 15-18]. However, knowledge on the molecular composition of the mucus plug in UIP is  
74 incomplete.

75

76 We have recently created a tissue atlas of the fibrotic front of UIP/IPF utilizing laser  
77 capture microdissection coupled mass spectrometry (LCM-MS) [19, 20]. In this study, we  
78 used the same approach to define the composition and provide mechanistic themes of  
79 the fibrotic HC airway cells with the aim to identify key targets and pathways to intercept  
80 fibrosis progression. In addition, we identify the composition of mucus in HC airways in  
81 lung fibrosis (UIP) and compare this to lung cancer (mucinous adenocarcinoma [MA]) to  
82 determine if mucus heterogeneity exists in varying disease states where mucus plugs are  
83 found in the airways.

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85 **Results:**

86 **Laser capture microdissection of fibrotic and non-fibrotic airway cells**

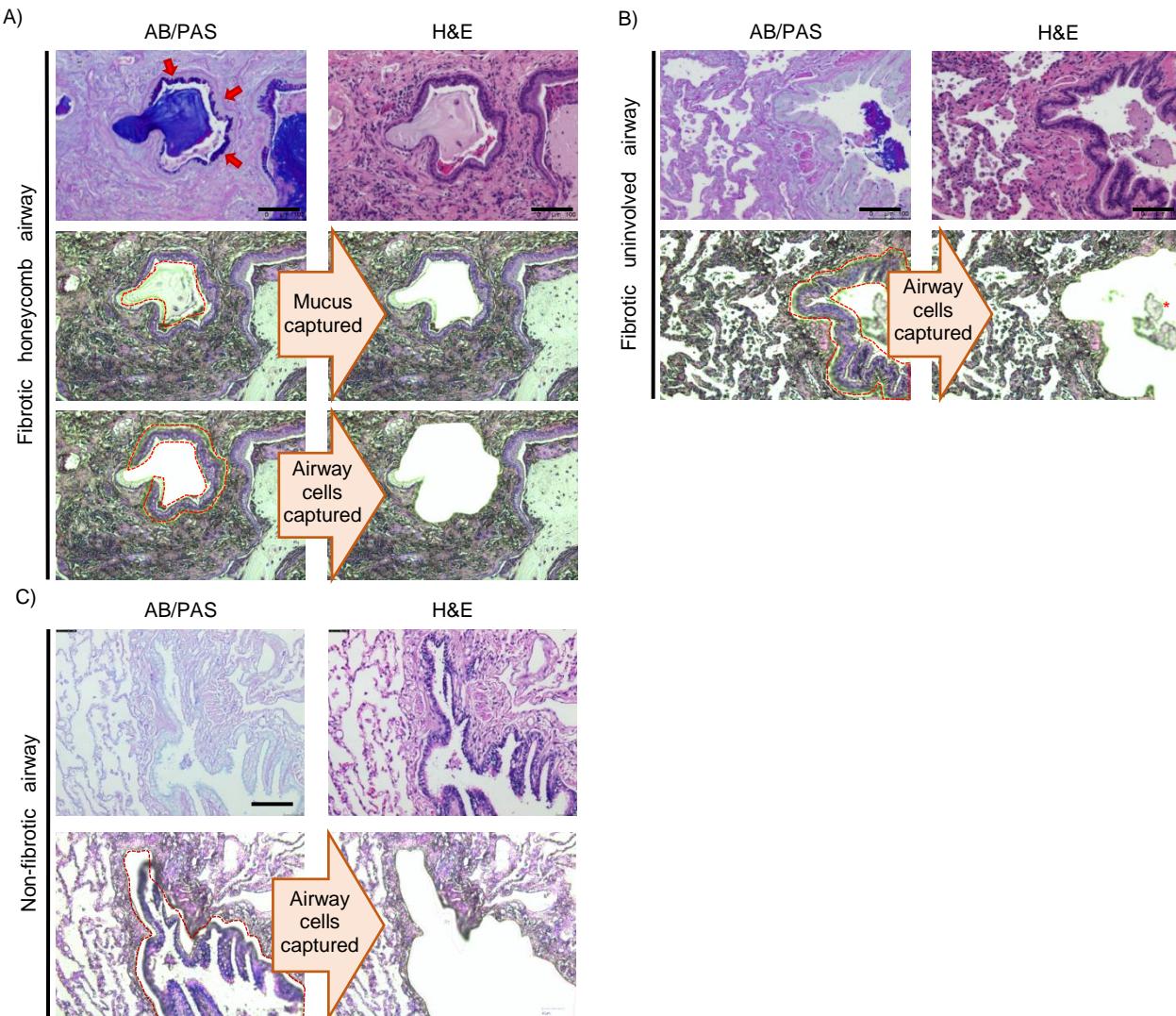
87 **Figure 1** shows our approach to laser capture microdissection (LCM). Using alcian blue/  
88 periodic acid Schiff's (AB/PAS) stain, mucus is visualized as purple in color within the  
89 fibrotic HC airway (**Figure 1A, upper row**); note how the AB/PAS stain lines the airway  
90 cells (red arrows) in a manner that suggests mucin is being secreted centrally into the  
91 airway lumen. We show that we precisely captured the mucus in a fibrotic specimen,  
92 including its cellular infiltrates (**Figure 1A, middle row**). In addition, we captured the  
93 mucin-rich epithelial lining of HC airways (**Figure 1A, lowest row**). We also captured  
94 fibrotic uninvolved airway cells defined as being in distant regions demonstrating minimal  
95 fibrosis (**Figure 1B**). Our LCM capabilities allow us to precisely isolate this region while  
96 leaving behind mucus associated in uninvolved airways, denoted with a red asterisk. To  
97 serve as a control, we performed LCM on airway cells from non-fibrotic specimens  
98 (**Figure 1C**). In total, we performed LCM on 10 fibrotic specimens ( $n = 10$  fibrotic HC  
99 airway cells and  $n = 10$  fibrotic uninvolved airway cells) and on 6 non-fibrotic airway cell  
100 controls (a total of 26 samples).

101

102 **The fibrotic HC and uninvolved airway cells are similar in protein composition**

103 We prepared our samples for mass spectrometry (MS) following our established protocol  
104 [19, 20] and performed a qualitative analysis to determine which proteins are present per  
105 group: non-fibrotic, fibrotic uninvolved, and fibrotic HC airway cells. We define a protein  
106 present if it is detected in 3 of the 6 non-fibrotic airway cell samples or 5 of the 10 fibrotic  
107 airway cell samples. We detected 2,668 proteins in human lung airway cells (**Figure 2A**)  
108 and provide a complete list of these proteins (**Supplemental File 1**). We found that more

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**Figure 1: Laser capture microdissection of the mucus, fibrotic honeycomb, fibrotic uninvolved and non-fibrotic airway cell controls.** Formalin-fixed paraffin-embedded specimens were serially sectioned at 5 microns and stained with alcian blue/periodic acid Schiff's (AB/PAS) stain or Hematoxylin & Eosin (H&E). (A) A representation of laser capture microdissection in a fibrotic specimen. AB/PAS (Top left) or H&E (the other 5 panels). Mucus (purple) was visualized with AB/PAS stain; notice how the mucin lines the inner airway consistent with these cells producing mucin centrally into the airspace [red arrows]. We individually captured the mucus and fibrotic honeycomb airway cells for mass spectrometry preparation. (B) In the same fibrotic patient, we found uninvolved airways in the morphologically intact regions of the fibrotic lung and captured the airway cells for mass spectrometry preparation (notice how the mucus plug is left behind— depicted with red asterisk). (C) A representation of non-fibrotic airway cells captured for mass spectrometry preparation. Scale bar represents 100 microns.

110 proteins are detected in fibrotic HC airway cells, which may be attributed to the  
111 metabolically demanding process of mucin production [21].

112

113 We next performed a quantitative analysis, which compares the relative abundance of the  
114 detected proteins, to create a 3-dimensional (3-D) principal component analysis (PCA)  
115 (**Figure 2B**). Firstly, we showed that non-fibrotic airway cells (red dots) separate from  
116 both the fibrotic HC (green dots) and fibrotic uninvolved airway cells (yellow dots).  
117 Surprisingly, we found that both the fibrotic HC airway cells and fibrotic uninvolved airway  
118 cells closely cluster with some deviation. This analysis suggests that the fibrotic  
119 uninvolved airway cells (found in morphologically intact lung) display an abnormal protein  
120 profile as the mucin-rich HC airway cells (found within the densely fibrotic region of the  
121 lung).

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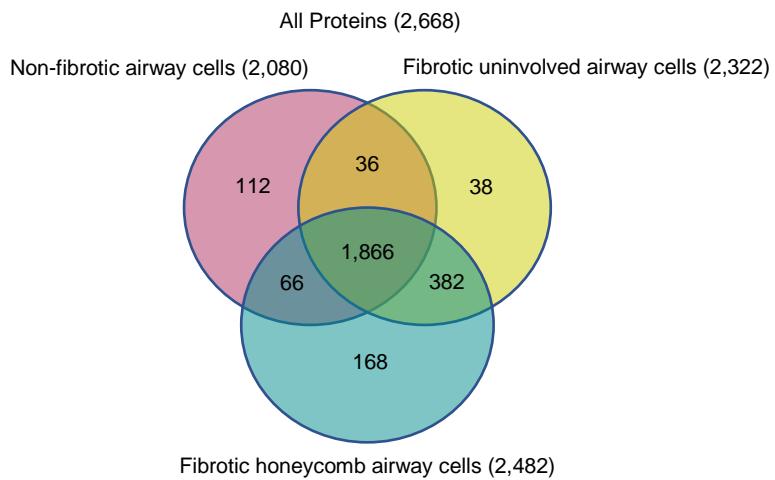
123 **Honeycomb airway cells are enriched in proteins involved in mucus biogenesis**  
124 **and have decreased cilia-associated proteins**

125 Although the fibrotic HC and fibrotic uninvolved airway cells cluster by PCA, we sought to  
126 further compare these groups. Of the 2,957 proteins detected, we found that there are  
127 101 proteins significantly increased in fibrotic HC airway cells while 18 are statistically  
128 increased in the fibrotic uninvolved airway cells (**Figure 3A**, a full list in **Supplemental**  
129 **File 2**). A list of the highest and lowest proteins is provided in **Table 1**. Consistent with  
130 our approach of capturing mucin-rich HC airway cells, we found that MUC5B is

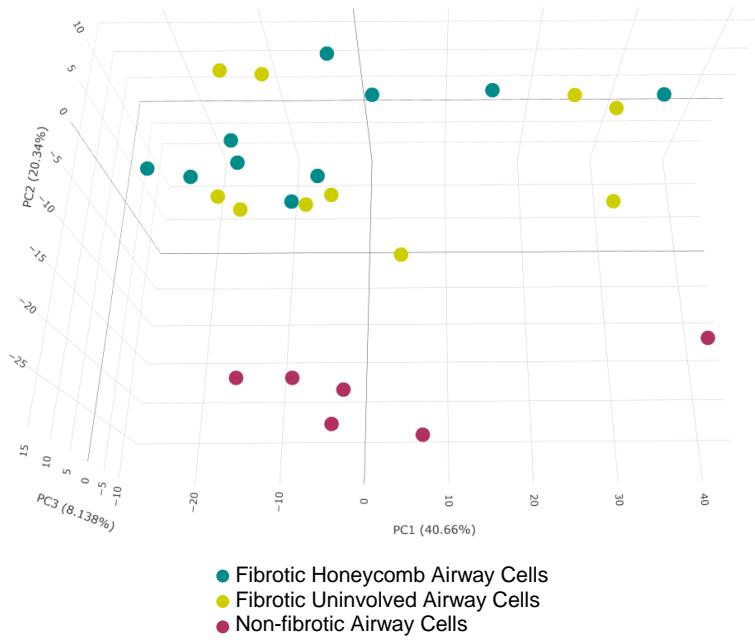
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A)



B)



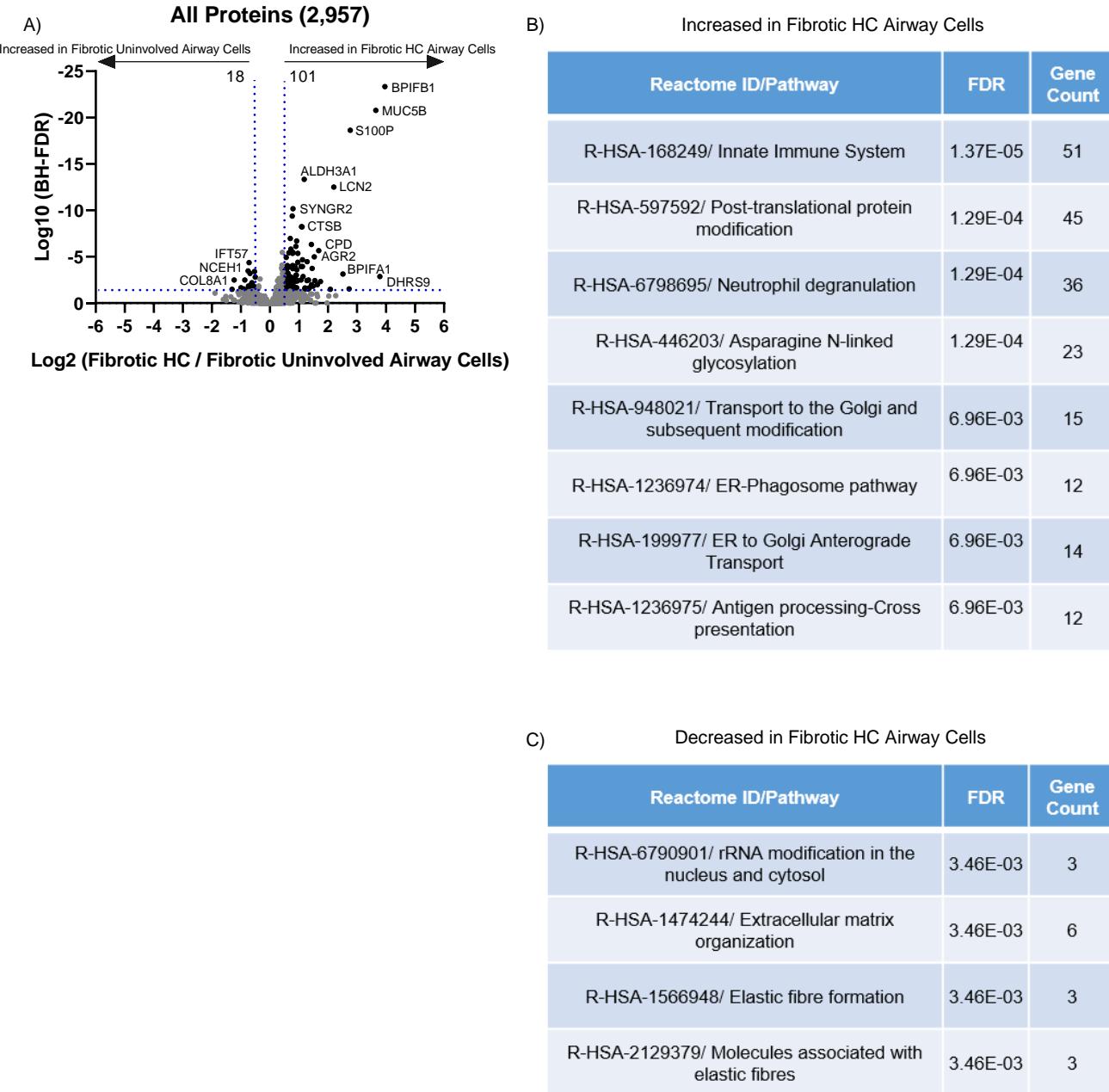
**Figure 2. Spatial proteomic analysis of the fibrotic airway cells.** Fibrotic and non-fibrotic specimens were subjected to laser capture microdissection coupled mass spectrometry (LCM-MS) to collect non-fibrotic airway cells ( $n = 6$  specimens), fibrotic uninvolved airway cells ( $n = 10$  specimens), and fibrotic honeycomb (HC) airway cells ( $n = 10$  specimens). (A) Venn diagram showing the number of proteins found in each airway cell type. (B) 3-D Principal component analysis showing that the non-fibrotic airway cells (red dots) cluster away from the fibrotic airway cells (the other dots). Surprisingly, fibrotic uninvolved airway cells (yellow dots) and HC airway cells (green dots) cluster together.

134 significantly increased in the fibrotic HC airway cells and not in the fibrotic unininvolved  
135 airway cells.

136

137 Strikingly, many of the proteins increased in the fibrotic HC airway cells are involved in  
138 mucin biogenesis and/or regulation. For instance, bactericidal/permeability-increasing  
139 (BPI) fold-containing family B member 1 (*BPIFB1*) is a negative regulator of *MUC5B*  
140 expression [22] and is at the top of the list. Similarly, secretory leukocyte protease inhibitor  
141 (*SLPI*) reduces mucin expression *in vitro* and is enriched in the fibrotic HC airway cells  
142 [23]. This suggests that negative regulators of mucin expression in lung fibrosis are  
143 insufficient to stop mucin production. Retinoic acid signalling induces mucin gene  
144 expression and secretion [24, 25]; Dehydrogenase reductase SDR family member 9  
145 (*DHRS9*) and cellular retinoic acid-binding protein 2 (*CRABP2*) both modulate retinoic  
146 acid synthesis and are increased in the fibrotic HC airway cells. Recently, CRABP2 was  
147 shown to be increased in IPF airway cells [26]. In accord with mucin biogenesis, anterior  
148 gradient homolog 2 (*AGR2*) has been shown to be essential for *MUC2* production and  
149 FK506-binding protein 11 (*FKBP11*) has been demonstrated to have a mucin secretory  
150 function [27]. Both *AGR2* and *FKBP11* are increased in the fibrotic HC airway cells. Some  
151 unique proteins to the fibrotic HC airway cells are *GALNT12*, *GALNT3*, *ST6GAL1*, and  
152 *GALNT6*, which are associated with O-linked glycosylation of mucins. In accord with  
153 these findings, Reactome pathway analysis demonstrated that a variety of pathways  
154 pertaining to mucin production are increased, such as 'Post-translational protein  
155 modification', 'Transport to the Golgi and subsequent modification', and 'ER-phagosome  
156 pathway' (**Figure 3B**); unfortunately, mucin biogenesis is not an established Reactome

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**Figure 3: The fibrotic honeycomb airway cells have a pro-mucin protein signature. (A)** Volcano plot comparing the fibrotic honeycomb (HC) airway to fibrotic uninvolved airway cells showing the negative natural log of the false discovery values (FDR) values plotted against the base 2 log (fold change) for each protein. Reactome pathway showing the most **(B)** increased or **(C)** decreased for the fibrotic HC airway cells compared to fibrotic uninvolved airway cells.

**Table 1: Highest and lowest 15 significantly changed proteins in the fibrotic HC airway cells compared to fibrotic uninvolved airway cells.**

<i>Increased in fibrotic HC airway cells</i>			<i>Decreased in fibrotic HC airway cells</i>		
<i>Protein</i>	<i>Log<sub>2</sub></i>	<i>FDR</i>	<i>Protein</i>	<i>Log<sub>2</sub></i>	<i>FDR</i>
BPIFB1	3.97	4.60E-24	CEP135	-1.30	3.18E-02
DHRS9	3.79	1.40E-03	COL8A1	-1.23	3.28E-03
MUC5B	3.65	1.70E-21	FBLN2	-1.00	2.16E-02
S100P	2.77	2.40E-19	LRRC45	-0.90	2.88E-02
FAM3D	2.73	2.90E-02	TTC21B	-0.86	3.29E-03
BPIFA1	2.52	7.40E-04	LAMB2	-0.75	3.40E-04
LCN2	2.20	3.13E-13	APCS	-0.74	4.45E-02
CRABP2	2.08	3.22E-02	CGN	-0.74	1.45E-02
DMBT1	1.74	4.82E-03	FBN1	-0.72	2.24E-02
CPD	1.68	2.30E-06	IFT57	-0.72	4.30E-05
ST6GAL1	1.64	1.02E-02	NCEH1	-0.69	6.30E-04
IGJ	1.55	3.763E-03	PLG	-0.63	8.00E-03
AGR2	1.53	1.00E-05	H1F0	-0.62	6.25E-03
FKBP11	1.49	1.53E-02	CYP51A1	-0.60	2.90E-02
SLPI	1.46	1.80E-04	CERS2	-0.59	2.59E-02

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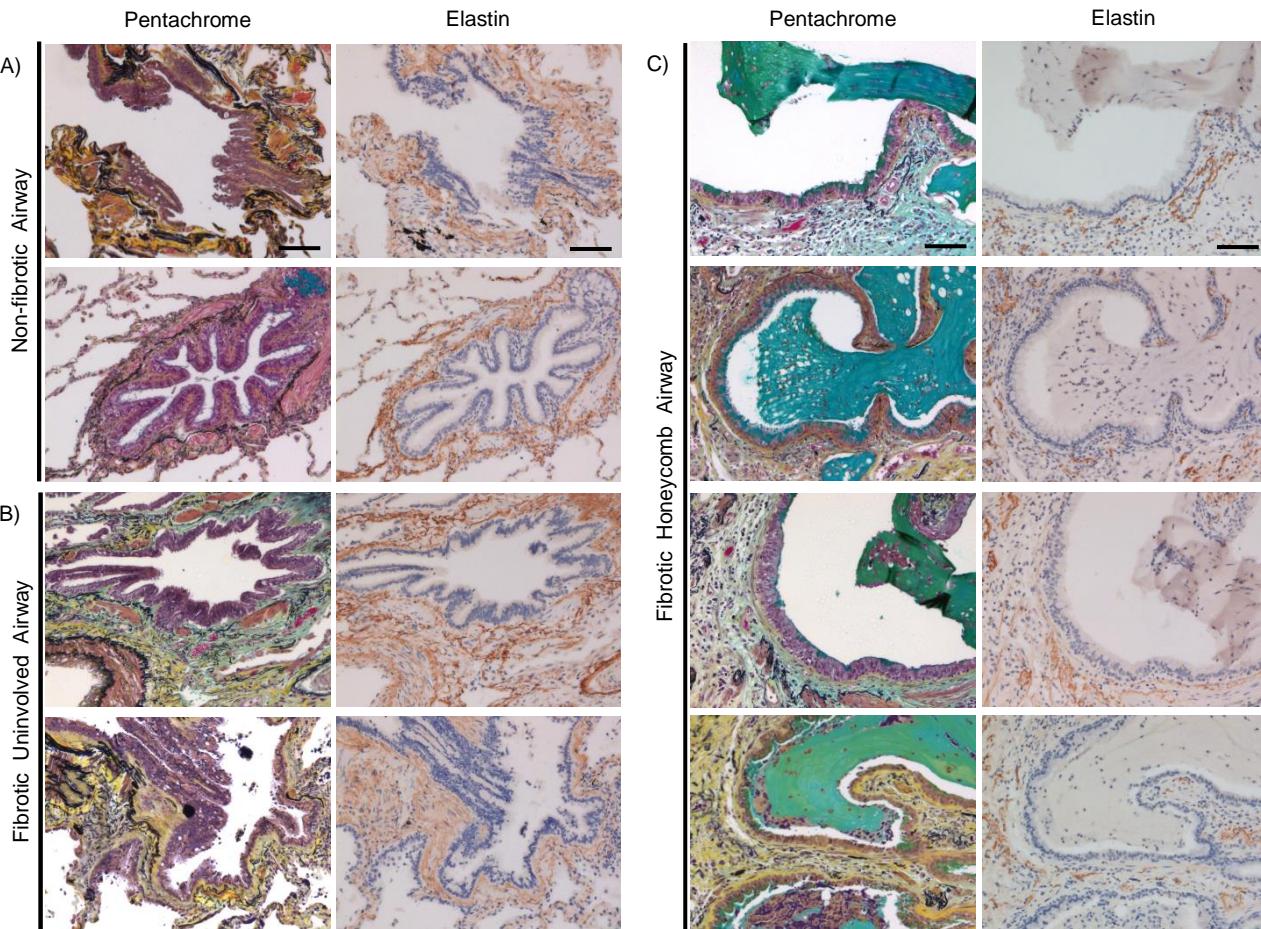
160 pathway. Reactome pathway analysis also demonstrated that 'extracellular matrix  
161 organization' and 'elastic fibre formation' are decreased in the fibrotic HC airway cells  
162 [**Figure 3C**]. We confirm that there are disorganized elastic fibres in the HC airways,  
163 which is in accord with the loss of airway structure and increased fibrosis in this region  
164 (**Supplemental Figure 1**). Thus, our spatial proteomic approach identifies a pro-mucin  
165 protein signature associated with the fibrotic HC airway cells as compared to fibrotic  
166 uninvolved airway cells.

167

168 Cilia are conserved organelles that function to clear airway mucus and associated debris.  
169 Herein, we demonstrated that multiple proteins associated with ciliogenesis are  
170 decreased in the fibrotic HC airway cells. For instance, Centrosomal protein 135  
171 (CEP135) is the most decreased protein and is required for ciliogenesis initiation [28].  
172 Ciliogenesis relies on a variety of proteins, including intraflagellar transport (IFT) proteins  
173 [29]. Intraflagellar transport protein 57 (IFT57) is required for cilia maintenance and is  
174 decreased in the fibrotic HC airway cells; other intraflagellar transport proteins (IFT81,  
175 IFT46) are not expressed in the fibrotic HC airway cells but expressed in the non-fibrotic  
176 or fibrotic uninvolved airway cells. Leucine-rich repeat protein 21B (*LRRC45*) and  
177 tetratricopeptide repeat protein 21B (*TTC21B*) are critical for ciliogenesis and are also  
178 decreased in the fibrotic HC airway cells [30, 31]. A variety of proteins that are not  
179 expressed in the fibrotic HC airway cells include proteins associated with cilia function,  
180 such as CEP131, CCP110, KIF3A, CYB5D1, DYNLRBR2, RPGR, and WRD66 [32-38].  
181 These data suggest that the loss of proteins associated with ciliogenesis within the fibrotic  
182 HC airways may be part of the mechanism of fibrosis progression.

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184



**Supplemental Figure 1. Elastin disorganization in the fibrotic honeycomb airway.** 2 Non-fibrotic and 4 fibrotic specimens were stained for pentachrome or immunostained for elastin. Shown are representative images for (A) non-fibrotic airway, (B) fibrotic uninvolved airways, and (C) fibrotic honeycomb airways. Note that elastin fibres (black in color in the pentachrome) surround airways in the non-fibrotic airway and fibrotic uninvolved airways, but is disorganized in the honeycomb airways.

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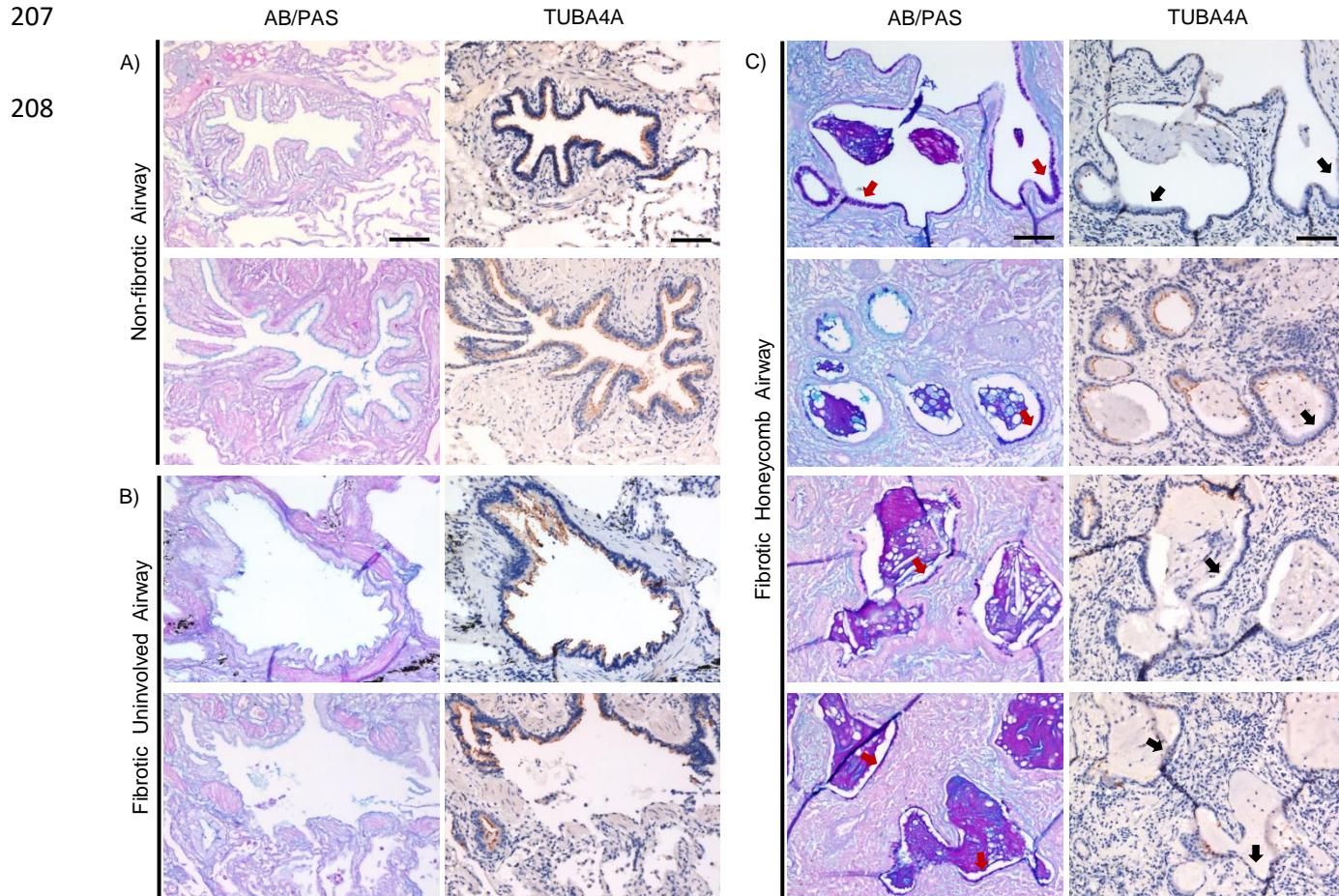
186 A previous study demonstrated that ciliary microtubules are morphologically disorganized  
187 in UIP/IPF, which will have profound effects on cilia structure and function [39]. To  
188 demonstrate that abnormal ciliogenesis is a potential mechanistic theme in the fibrotic HC  
189 airway cells, we immunostained for tubulin alpha 4a (TUBA4A; a marker of cilia) in 4 UIP  
190 specimens and 2 controls. We found that the cilia marker is widely expressed in cells  
191 lining the airway cells of non-fibrotic and fibrotic uninvolved airways (**Figure 4A – 4B**). In  
192 contrast, the mucin-rich regions of the HC airway (red arrows) are largely devoid of cilia  
193 (black arrows) (**Figure 4C**).

194

### 195 **The fibrotic uninvolved airway cells have an abnormal protein signature**

196 We next compared both the fibrotic airway cells to the non-fibrotic airway cell controls.  
197 We showed that 333 proteins are significantly increased in the fibrotic HC airway cells,  
198 whereas 157 proteins are significantly increased in the non-fibrotic airway cell controls  
199 (**Figure 5A**; a full list in **Supplemental File 2**). Reactome pathway analysis demonstrated  
200 that 'regulation of expression of *SLITs* and *ROBOs*' and 'Signaling by *ROBO* receptors'  
201 are the strongest categories increased in the fibrotic HC airway cells as compared to non-  
202 fibrotic controls (**Figure 5B**). The Slit/Robo pathway is implicated in a variety of cellular  
203 processes, including neurogenesis, cell proliferation, and migration [40]. Slit/Robo has  
204 been shown to be involved in airway development [41, 42], and can improve alveolar  
205 regeneration in lung-injury models [43].

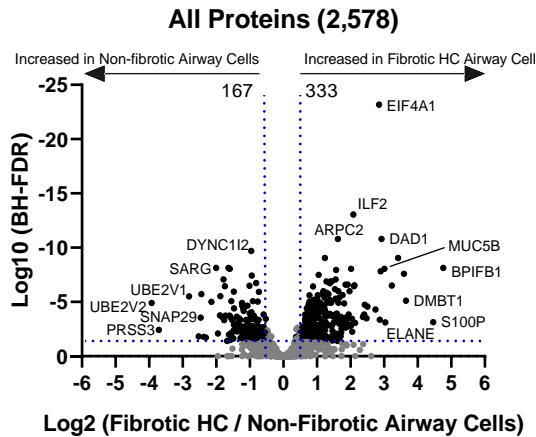
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**Figure 4. Cilia expression in the fibrotic honeycomb airway cells.** Two Non-fibrotic and 4 fibrotic specimens were stained for alcian blue/periodic acid Schiff's (AB/PAS) or immunostained for TUBA4A (a marker of cilia). Shown are representative images for (A) non-fibrotic airway, (B) fibrotic uninvolved airways, and (C) fibrotic honeycomb (HC) airways. Note that regions of mucin positivity (red arrows) are absent of cilia (black arrows) in the fibrotic HC airway cells. Scale bar represents 100 microns.

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A)



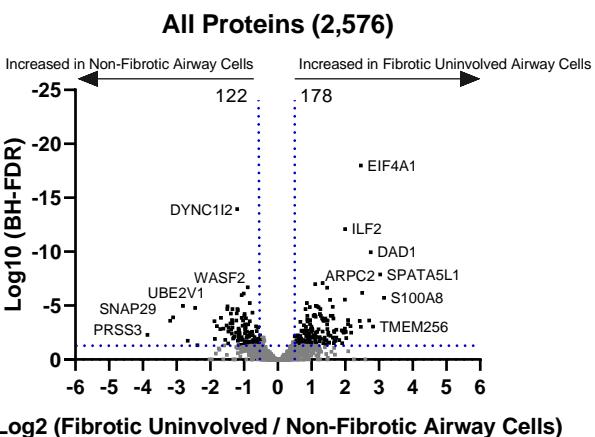
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B)

Up-regulated in Fibrotic HC Airway Cells

Reactome ID/ Pathway	FDR	Gene Count
R-HSA-9010553/ Regulation of expression of SLITs and ROBOs	3.85E-05	42
R-HSA-376176/ Signaling by ROBO receptors	7.95E-05	43
R-HSA-71291/ Metabolism of amino acids and derivatives	5.54E-04	50
R-HSA-422475/ Axon guidance	5.54E-04	56
R-HSA-4086400/ PCP/CE pathway	5.54E-04	21
R-HSA-168249/ Innate Immune System	5.54E-04	84
R-HSA-9675108/ Nervous system development	5.54E-04	56
R-HSA-5663205/ Infectious disease	5.54E-04	73

C)



D)

Up-regulated in Fibrotic Uninvolved Airway Cells

Reactome ID/Pathway	FDR	Gene Count
R-HSA-9010553/ Regulation of expression of SLITs and ROBOs	2.38E-06	30
R-HSA-376176/ Signaling by ROBO receptors	1.03E-05	30
R-HSA-422475/ Axon guidance	1.49E-05	39
R-HSA-9675108/ Nervous system development	1.72E-05	39
R-HSA-1266738/ Developmental Biology	1.72E-05	43
R-HSA-156842/ Eukaryotic Translation Elongation	7.23E-05	20
R-HSA-71291/ Metabolism of amino acids and derivatives	1.86E-04	32
R-HSA-72766/ Translation	2.55E-04	28

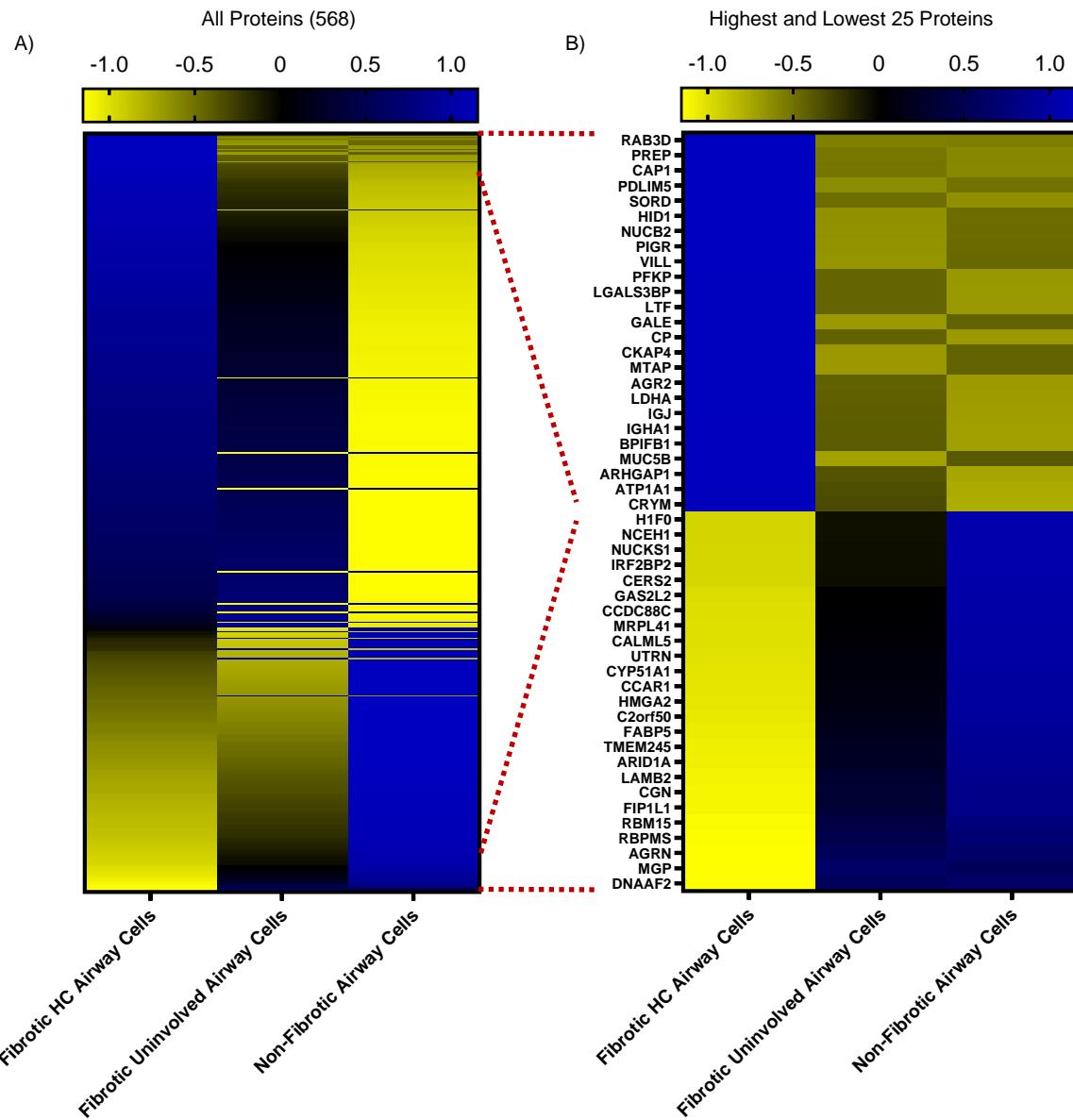
**Figure 5: The fibrotic uninvolved airway cells have an abnormal protein signature. (A)** Volcano plot comparing the fibrotic honeycomb airway cells to non-fibrotic airway cells showing the negative natural log of the false discovery values (FDR) values plotted against the base 2 log (fold change) for each protein. **(B)** Reactome pathway showing the most increased pathway for the fibrotic honeycomb airway cells compared to non-fibrotic airway cells. **(C)** Volcano plot comparing the fibrotic uninvolved airway cells to non-fibrotic airway cells showing the negative natural log of the FDR values plotted against the base 2 log (fold change) for each protein. **(D)** Reactome pathway showing the most increased pathway for the fibrotic uninvolved airway cells compared to non-fibrotic airway cell controls.

211 We next compared the fibrotic uninvolved airway cells to the non-fibrotic airway cell  
212 controls. We detected 178 proteins significantly increased in fibrotic uninvolved airway  
213 cells, whereas we found 202 proteins significantly increased in non-fibrotic airway cell  
214 controls (**Figure 5C**; a full list in **Supplemental file 2**). Surprisingly, the 15 highest  
215 proteins increased in the fibrotic uninvolved airway cells are also increased in the fibrotic  
216 HC airway cells. Reactome pathway analysis again show that 'Regulation of expression  
217 of *SLTs* and *ROBOs*' and 'Signaling by *ROBO* receptors' are the strongest categories in  
218 the fibrotic uninvolved airway cells as compared to non-fibrotic controls (**Figure 5D**);  
219 Reactome pathway analysis did not find any significantly decreased pathway enrichment  
220 in either fibrotic airway groups. Given that similar pathways and proteins are increased in  
221 the fibrotic uninvolved airway cells as the fibrotic HC airway cells, suggests that the fibrotic  
222 uninvolved airway cells are abnormal.

223

224 A heatmap of the 568 significantly changed proteins across the groups: fibrotic HC,  
225 fibrotic uninvolved, and non-fibrotic airway cell controls is shown in **Figure 6A** (a full list  
226 in **Supplemental File 3**). The fibrotic HC airway cells share similarities to the fibrotic  
227 uninvolved airway cells, but with substantial deviations. We next show the 25 highest and  
228 lowest changed proteins (**Figure 6B**). Ras-related protein 3D (RAB3D) was the most  
229 increased in the fibrotic HC airway cells and is involved in the biogenesis of secretory  
230 granules [44]. MUC5B and MUC5AC are both packaged in the secretory granules of  
231 airway cells [45]. Similarly, prolyl endopeptidase (PREP) is found within exosomes in  
232 airway cells and released upon LPS stimulation [46]. Cyclase associated protein 1 (CAP1)  
233 is associated with lung cancer and post-translational modification promotes proliferation

234



**Figure 6: Fibrotic airway cells differ than controls.** Shown are heatmaps of (A) all 568 statistically changed proteins or (B) the highest and lowest 25 proteins in the fibrotic honeycomb, fibrotic uninvolved, and non-fibrotic airway cells. Proteins are arranged by increasing abundance with reference to the honeycomb airway cells.

235 and migration [47]. At the bottom of the list are dynein axonemal assembly factor 2  
236 (DNAAF2), matrix gla protein (MGP), and agrin (AGRN) which are only decreased in the  
237 fibrotic HC airway cells (detected in the fibrotic uninvolved and non-fibrotic airway cell  
238 controls). *DNAAF2* is involved in cilia homeostasis and mutations in *DNAAF2* lead to cilia  
239 defects [48]. *MGP* is considered an inhibitor of calcification based on the extensive  
240 cardiovascular calcification observed in *MGP*-null mice [49]; calcification occurs in  
241 UIP/IPF patients, and is associated within regions of honeycombing [50]. *AGRN* is a  
242 proteoglycan that serves a variety of biological functions, including the promotion of  
243 regeneration [51]. Further work interrogating the collective roles of these changed  
244 proteins will help decipher the mechanism of fibrosis progression.

245

#### 246 **The composition of fibrotic lung mucus**

247 We previously demonstrated our capacity to microdissect the mucus plugs in fibrotic  
248 specimens (**Figure 1A**, middle panels). Utilizing our MS approach, we detected 650  
249 proteins in the fibrotic/UIP mucus plugs (detected in 3 or more of the 6 samples;  
250 **Supplemental File 4**). Using intensity Based Absolute Quantification (iBAQ; a measure  
251 of protein abundance) [52], we provide a list of the most abundant proteins in UIP mucus  
252 (**Supplemental File 5**). We found that the mucus is enriched with immunoglobulins (Ig)  
253 which is in accord with increased protein expression of polymeric Ig receptor (PIGR) in  
254 the fibrotic honeycomb airway cells and mucus. In epithelial cells, PIGR mediates the  
255 transcytosis of Igs into the airway, which serves as a mucosal defence mechanism [53].  
256 Given that fibrotic mucus is enriched with cellular infiltrates, we next focused our list using  
257 the ‘secretome’ (secreted proteins) dataset [54] and show that BPIFB1 was the most

258 abundant secretome-associated protein found in fibrotic mucus whereas MUC5B is the  
259 ninth most abundant (**Figure 7A**; a full list in **Supplemental File 6**). This is consistent  
260 with BPIFB1 and MUC5B being amongst the most significantly expressed proteins in the  
261 fibrotic HC airway cells (**Figure 3A**). To validate some the most abundant protein hits, we  
262 show immunoreactivity for MUC5B, BPIFB1, PIGR, and TF within the UIP mucus plugs  
263 (**Figure 7B**). We also included the other gel-forming mucin, MUC5AC (60<sup>th</sup> on the  
264 abundance list), which showed a patchy/incomplete staining pattern.

265

266 Using the entire fibrotic mucus proteome, Reactome pathway enrichment analysis  
267 demonstrates that the mucus plug is defined by 'neutrophil degranulation' as the strongest  
268 category (**Figure 7C**). Neutrophil degranulation pathway is also implicated in SARS-CoV-  
269 2 lung infection models [55] and in chronic obstructive pulmonary disease (COPD) [56];  
270 in IPF bronchoalveolar lavage fluid (BALF), proteins associated with neutrophil granules  
271 are amongst the most abundant [57].

272

273 **Fibrotic-derived lung mucus is distinct from cancer-derived lung mucus**

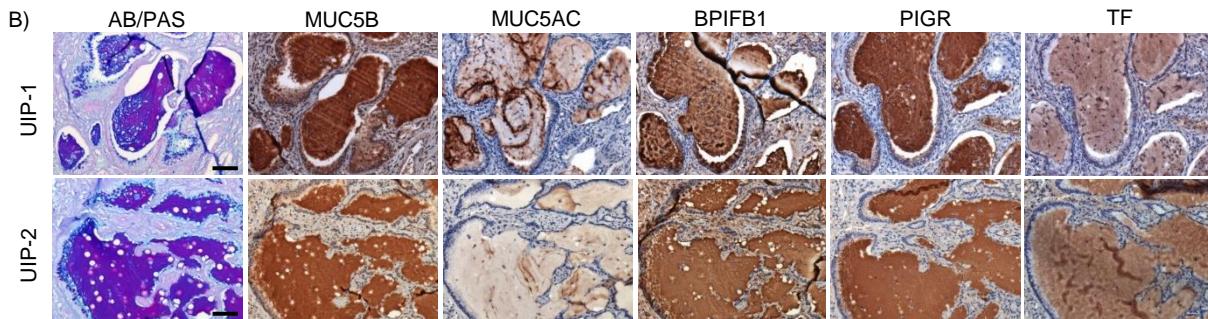
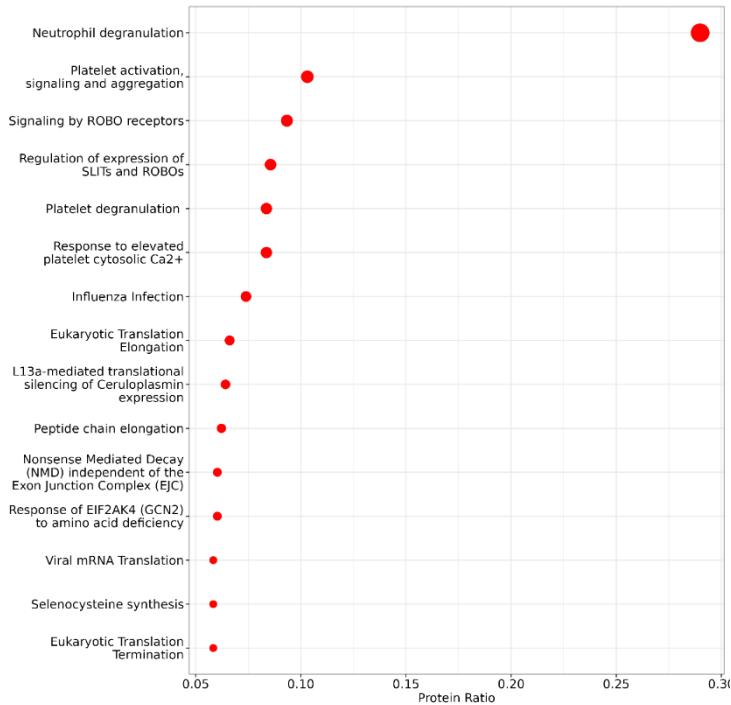
274 To further understand mucus in the context of lung disease, we additionally performed  
275 LCM-MS on 6 mucinous adenocarcinoma (MA) specimens (**Supplemental Figure 2A**).  
276 MA is a lung cancer with pronounced mucus accumulation within the alveolar space [58,  
277 59]. We detected a total of 535 proteins in MA mucus (found in 3 or more of the 6 samples;  
278 a full list in **Supplemental File 4**). Consistent with *MUC5AC* being the most abundantly  
279 expressed transcript in MA [60], MUC5AC protein is the second most abundant

280

A) **The 10 most abundant secretome-associated proteins in the fibrotic mucus. (172 proteins total)**

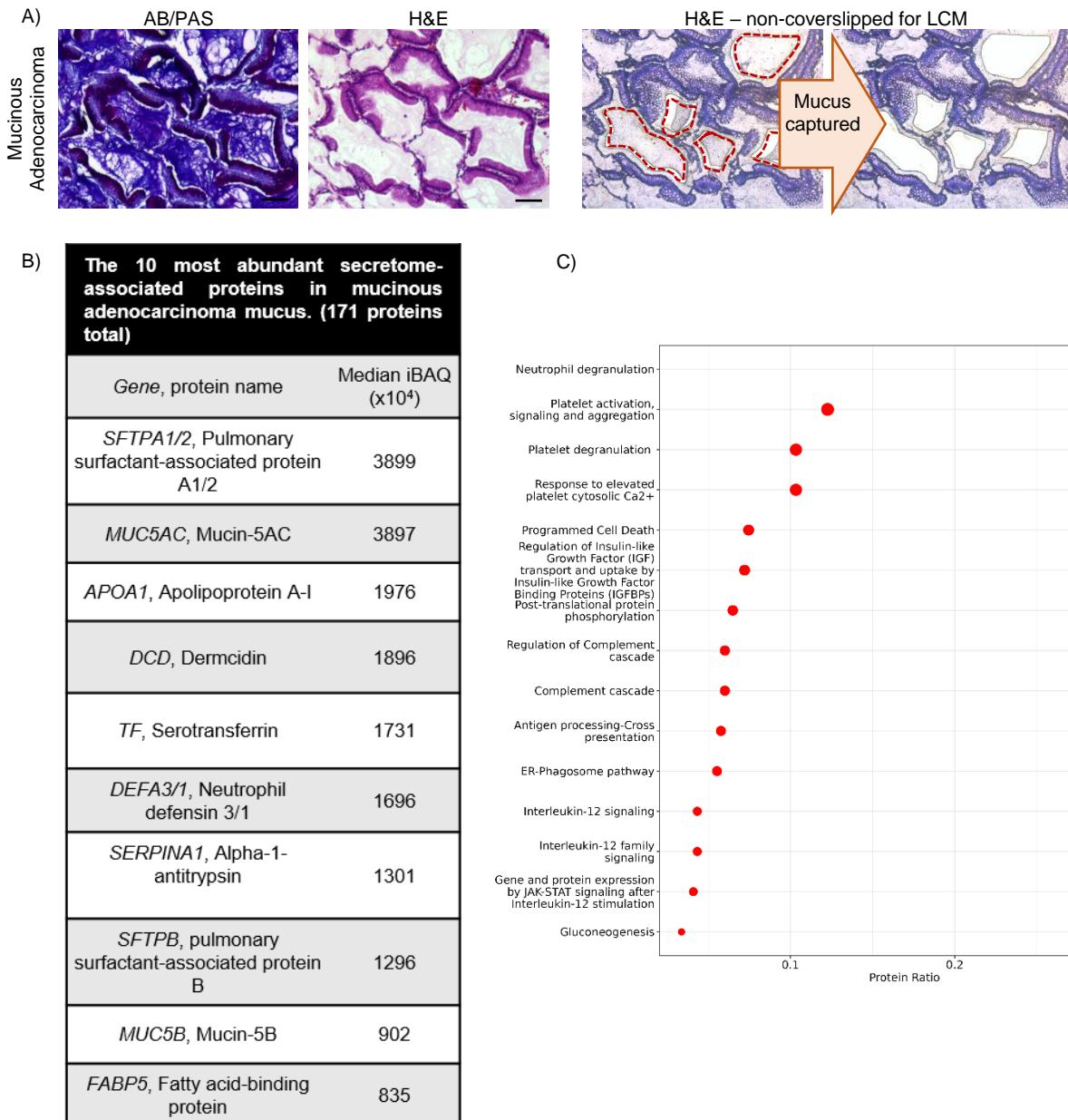
Gene, protein name	Median iBAQ (x10 <sup>4</sup> )
<i>BPIFB1</i> , BPI fold-containing family B member 1	9920
<i>DEFA3/1</i> , Neutrophil defensin 3/1	9324
<i>PIGR</i> , Polymeric immunoglobulin receptor	7718
<i>SFTPB</i> , pulmonary surfactant-associated protein B	6418
<i>SFTPA1/2</i> , Pulmonary surfactant-associated protein A1/2	3667
<i>S100A9</i> , Protein S100-A9	3361
<i>DCD</i> , Dermcidin	3111
<i>APOA1</i> , Apolipoprotein A-I	2833
<i>MUC5B</i> , Mucin-5B	2771
<i>TF</i> , Serotransferrin	2586

C)



**Figure 7: The composition of fibrotic mucus.** Laser capture microdissection coupled mass spectrometry was performed on the mucus plugs of 6 Usual Interstitial Pneumonia (UIP) specimens. (A) A list of the most abundant secretome-associated proteins found in the fibrotic mucus shown as intensity Based Absolute Quantification (iBAQ). (B) Reactome pathway enrichment of UIP mucus represented as a dotplot. (C) Serial sections of UIP specimens stained for alcian blue/periodic acid Schiff's (AB/PAS) or immunostained for MUC5B, MUC5AC, BPIFB1, PIGR, and TF (N = 4 UIP specimens with 2 representatives shown). Scale bar represents 100 microns.

281



**Supplemental Figure 2: The mucus in mucinous adenocarcinoma (MA).** (A) A MA specimen was serially sectioned at 5 microns and stained with alcian blue/periodic acid Schiff's (AB/PAS) stain or Hematoxylin & Eosin (H&E). Mucus was laser microdissected for mass spectrometry analysis. Scale bar represented 100 microns. (B) A list showing the most abundant secretome-associated proteins found in the mucus of MA. (C) Dotplot visualization of the MA mucus using Reactome pathway enrichment.

283 secretome-associated protein found in the mucus of MA (**Supplemental Figure 2B**, a full  
284 list in **Supplemental File 5**). Reactome pathway analysis demonstrates that MA mucus  
285 is defined by 'Neutrophil degranulation', like fibrotic mucus, as the strongest category  
286 (**Supplemental Figure 1C**).

287

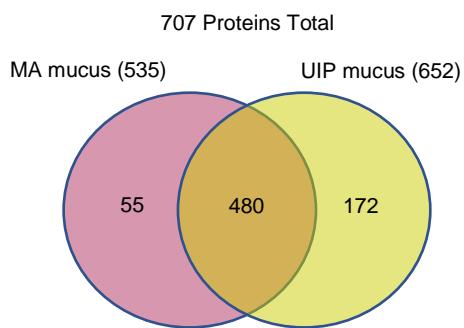
288 We next compared the mucus of MA to UIP. In total, we detected 707 lung mucus proteins  
289 (**Figure 8A**), with UIP having the most proteins detected (a full list in **Supplemental File**  
290 **4**). A 3-D PCA analysis showed that UIP mucus samples largely cluster together, whereas  
291 only one MA sample overlaps with UIP (**Figure 8B**). Quantitative analysis of our data  
292 show that 9 proteins are significantly enriched in fibrotic mucus whereas 3 are significantly  
293 enriched in cancer (MA) mucus (**Figure 8C**, a full list in **Supplemental File 7**). To validate  
294 this result, we performed immunofluorescence on both MA (n = 3) and UIP specimens (n  
295 = 4) for MUC5B, MUC5AC, and BPIFB1 (**Figure 8D**). We found that UIP mucus has  
296 variable expression of MUC5AC (white arrows mark the absence of MUC5AC where  
297 MUC5B/BPIFB1 is present) in comparison to MUC5B. Note that one mucus plug was  
298 positive for MUC5AC, which resembles the chromogenic patchy/incomplete stain in  
299 **Figure 7B**; MUC5AC has been previously reported to have variable staining [10].  
300 Inversely, MA mucus was positive for MUC5AC, whereas MUC5B and BPIFB1 are largely  
301 negative at the immunofluorescence level (**Figure 8E**, white arrows point where  
302 MUC5B/BPIFB1 are absent). Thus, a distinguishing factor for fibrotic/UIP mucus is the  
303 high abundance of MUC5B and BPIFB1, whereas MUC5AC is predominant in MA mucus.

304

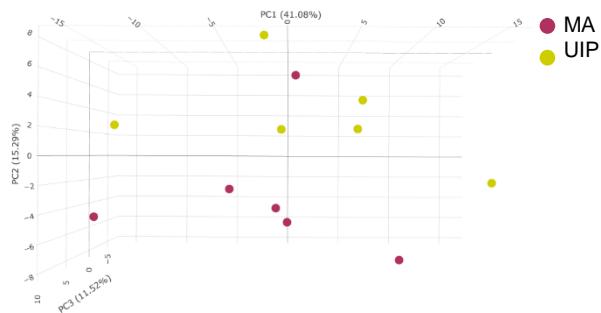
305

A)

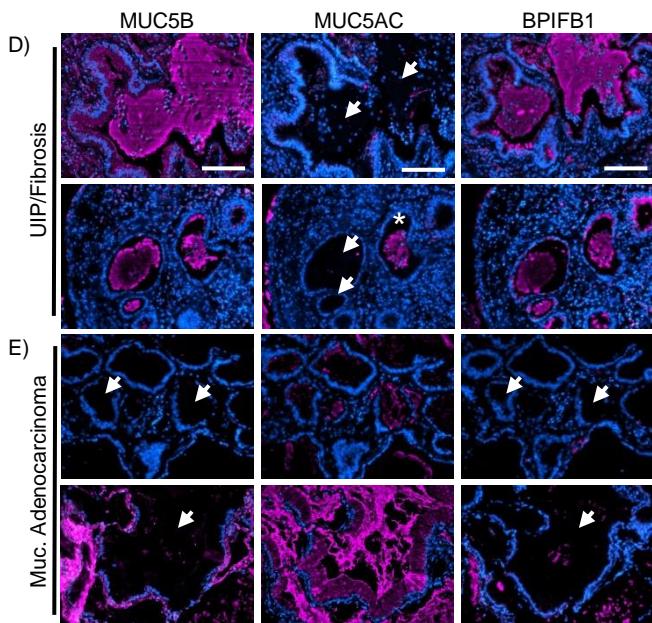
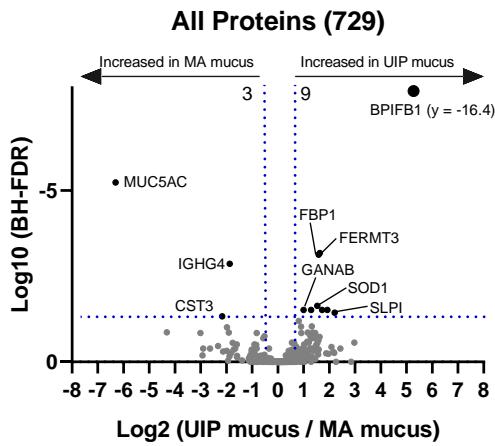
306



B)



C)



**Figure 8: The mucus in usual interstitial pneumonia has distinct features from mucinous adenocarcinoma.** (A) Venn diagram showing the proteins detected in the mucus of mucinous adenocarcinoma (MA) or usual interstitial pneumonia (UIP). (B) 3-dimensional principal component analysis for each mucus type. (C) Volcano plot comparing the UIP mucus to MA mucus showing the negative natural log of the false discovery values (FDR) values plotted against the base 2 log (fold change) for each protein. (D) Immunofluorescence for MUC5B, MUC5AC, and BPIFB1 in UIP mucus ( $n = 4$  specimens) and MA mucus ( $n = 3$  specimens) with representative images shown for each disease type. White arrows points to regions of mucus accumulation and asterisk shows positivity of MUC5AC within UIP mucus. Scale bar represents 100 microns.

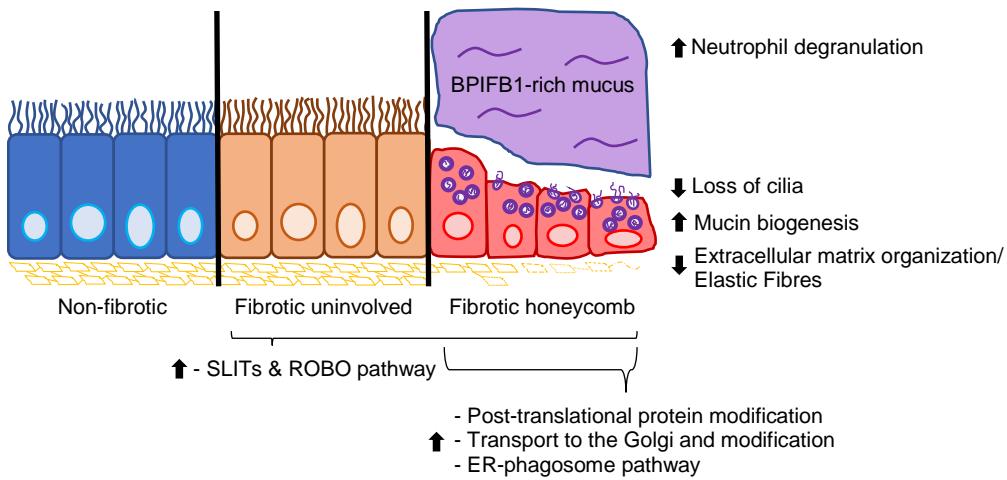
307 **Discussion**

308 In this work, we produced an unbiased spatial proteomic profile of the non-fibrotic, fibrotic  
309 uninvolved and honeycomb airway cells to create a tissue map that defines pathway  
310 changes along the progression of lung fibrosis (**Figure 9**). We showed that the structurally  
311 intact low-in-mucus airway cells in uninvolved regions of the fibrotic lung have an  
312 abnormal protein signature with increased Slits/Robo pathway as the strongest category;  
313 Slits/Robo pathway is also increased in the fibrotic HC airway cells. We confirmed that  
314 the fibrotic honeycomb airways are the site of mucin biogenesis with other categories  
315 related to protein modification and transport increased. Importantly, many proteins  
316 associated with ciliogenesis are decreased or absent from the fibrotic HC airways. In  
317 addition, honeycombing is associated with decreased extracellular matrix organization  
318 and elastic fibre formation. Lastly, the fibrotic mucus is enriched with immune defence  
319 proteins, including BPIFB1 and MUC5B, and is enriched with neutrophil degranulation  
320 pathway.

321

322 Other groups support our results that demonstrate that the fibrotic uninvolved airway cells  
323 are abnormal at the structural and genetic level. Lung fibrosis is associated with a variety  
324 of genetic risk factors affecting epithelial cells, which may abnormally prime lung airway  
325 cells to fibrosis initiation [61]. Structurally, regions without microscopic fibrosis are shown  
326 to have reduced numbers of terminal airways and have an increase of airway wall areas  
327 [4-6], suggesting that early lung airway perturbations precede fibrotic extracellular matrix  
328 remodelling. Thus, it is plausible that airway cell dysfunction is an early event in the fibrotic  
329 process. Further LCM-MS studies with precise distance registration and patient

330



**Figure 9: The fibrotic honeycomb airway.** Spatial proteomics reveal that the fibrotic uninvolved airway cells (found in regions of structurally intact lung) have an abnormal protein signature. The fibrotic uninvolved airway cells, like the honeycomb (HC) airway cells (a pathological feature of lung fibrosis), are over-represented in proteins associated with SLITs and ROBO pathway. The fibrotic HC airway cells are further defined by increased pathways associated with mucin biogenesis, and a loss of both cilia and extracellular matrix organization/elastic fibres. We find that the mucus proteome is enriched with neutrophil degranulation pathway, with a marked increase of BPIFB1 protein.

331 genotyping will inform whether there exists a transition zone where a normal airway  
332 proteome is present, or perhaps it may be that the entire airway proteome is abnormal.

333

334 Aberrant ciliogenesis has previously been described in UIP/IPF. Whole transcriptomic  
335 studies demonstrate an elevation of cilium gene expression [62]. In contrast, our results  
336 demonstrate a reduced cilia-associated protein profile within the fibrotic HC airway cells.  
337 This likely reflects the advancement of our spatial proteomic capability or that previously  
338 observed increases in ciliary gene expression represent a compensatory mechanism  
339 occurring in response to loss of ciliated epithelial cells in fibrosis. Structurally,  
340 transmission electron microscopy demonstrates that the UIP/IPF distal airways display  
341 defects in microtubule organization, which will have detrimental effect on cilia function  
342 [39]. In the context in cystic fibrosis, it is reported that airway epithelial also have  
343 decreased ciliated cells with enhanced mucin expression [63]. Thus, future studies  
344 determining the mechanism of deranged ciliogenesis is warranted.

345

346 Current literature suggests that basal airways cells differentiate into either mucin  
347 producing cells or cilia-containing cells [64]. Our spatial proteomic data fits the notion that  
348 the HC airway microenvironment directs the differentiation of basal airway cells into mucin  
349 producing cells whereas the uninvolved airway microenvironment favors ciliated cells.  
350 Given that extracellular matrix governs cell differentiation and function [65], we speculate  
351 that changes to ECM properties (mechanical, composition, and topography) may play a  
352 role in airway cell differentiation and function. Prior work utilizing decellularized COPD

353 airway tissue as a scaffold for cell-matrix interactions (as compared to donor) show that  
354 COPD matrix dramatically affects cilia gene expression in epithelial cells [66]. Other  
355 studies using decellularized UIP/IPF tissue confirm that fibrotic matrix is a driver of fibrosis  
356 progression [67]. Therefore, the changes in mucin and cilia-associated proteins may be  
357 reflective, or a consequence of the changes in airway ECM properties.

358

359 Our spatial proteomic data characterizing fibrotic HC airway cells (MUC5B-positive) are  
360 in agreement with sc-RNAseq data characterizing *MUC5B*-positive secretory cells in  
361 human lung. In one study, secretory airway cells have increased RNA expression of  
362 *MUC5B*, *LCN2*, *BPIFB1*, *SERPINB3*, *S100P*, *RARRES1*, *TSPAN8*, *CP*, and *FAM3D* [68],  
363 which are also increased or uniquely expressed at the protein level in fibrotic HC airway  
364 cells. Other mRNAs increased in *MUC5B*-positive secretory cells include *TSPAN1*,  
365 *AKR1C1*, *ZG16B*, *GSTA1*, and *SCGB1A1*, which are unchanged at the protein level in  
366 the fibrotic HC airway cells. A separate study showed that *MUC5B*-positive cells by sc-  
367 RNAseq have increased mRNA expression of *SCGB1A1*, *SCGB3A1*, *SLPI*, *BPIFB1*,  
368 *LCN2*, and *WFDC2* [69]. At the protein level, *SLPI*, *BPIFB1*, *LCN2*, and *WFDC2* are  
369 increased or uniquely expressed in the fibrotic HC airway cells (*SCGB1A1* and *SCGB3A1*  
370 are unchanged at the protein level). Thus, the fibrotic HC airway cells represent a  
371 secretory cell phenotype. Future work integrating spatial multi-omic analysis (RNA and  
372 protein) will further our understanding of lung fibrosis.

373

374 To our knowledge, we are the first to determine the composition of UIP mucus plugs by  
375 using an LCM-MS approach. This approach allows precise capture of the entirety of  
376 mucus plugs without the introduction of contaminants (salivary and upper airway proteins)  
377 as seen by traditional BALF. Proteomic analysis of BALF (an unfixed or stained sample)  
378 from lung fibrosis patients show agreement with our findings. Several reports utilizing  
379 mass spectrometry approaches show increases of immunoglobulins, complement C3,  
380 transferrin, Apolipoprotein A1, plastin-2, annexin A2, and CCL18 in fibrotic lung BALF  
381 (summarized in [70]); all of which are detected in our LCM-MS dataset. In accord with our  
382 findings, Foster et al. demonstrated that MUC5B is an abundant protein in IPF BALF [57].  
383 S100A9, detected by LCM-MS, is a potential BALF biomarker in IPF [71]. In addition, IPF  
384 patients with acute exacerbations show increased PIGR, LRG1, and SERPINA1 in BALF,  
385 which are also detected in our LCM-MS dataset [72]. Our LCM-MS approach is therefore  
386 a useful tool to determine the protein composition of mucus in archived FFPE specimens.

387

388 Our results demonstrate that the mucus found in lung cancer (mucinous adenocarcinoma;  
389 MA) has elevated levels of MUC5AC as compared to UIP mucus. A likely explanation is  
390 that the mucin-secreting cells comprising the UIP/IPF HC airway differ than the mucin-  
391 secreting cells in MA and/or that the environmental/immune signals controlling mucin  
392 production differ. For instance, reports show that there are 5X more MUC5B-positive cells  
393 versus MUC5AC-positive cells in the honeycomb airways of UIP/IPF, suggesting marked  
394 cell type heterogeneity [73]. In contrast, the morphology of MA cells are distinct and  
395 composed of goblet and/or columnar cells [74]. Another explanation is that *MUC5AC*  
396 gene expression is differentially regulated as compared to *MUC5B* [75]. For instance,

397 *MUC5AC* gene expression is increased by IL-13. In other disease settings, *MUC5AC*  
398 mRNA is increased in asthma, whereas *MUC5B* levels are decreased [76]. Further  
399 studies determining the functional consequence of varying *MUC5AC* to *MUC5B* protein  
400 ratios on fibrosis progression are needed.

401

402 Increases of BPIFB1 in both the UIP mucus and HC airway cells is of interest. BPIFB1 is  
403 a secretory protein that is implicated in immune regulatory functions and shown to have  
404 anti-tumor effects (reviewed in [77]). In other lung disorders, BPIFB1 is increased in cystic  
405 fibrosis, COPD, asthma, and IPF [78]. It is decreased in nasopharyngeal carcinoma,  
406 gastric cancer, and lung cancer, which agrees with our findings that mucinous  
407 adenocarcinoma mucus has low expression of BPIFB1. Understanding of its function in  
408 lung fibrosis is currently incomplete.

409

410 **Conclusion:**

411 Spatial proteomics has allowed us to create an unbiased protein tissue map of the  
412 fibrotic/UIP lung airway cells. We show that the fibrotic honeycomb airway cells are the  
413 active site of mucin biogenesis affiliated with a loss of cilia. Importantly, we show that the  
414 fibrotic uninvolved airway cells have an abnormal protein signature. Therapeutic  
415 intervention of the fibrotic uninvolved airway cells may therefore slow fibrosis progression.

416

417 **Materials and Methods:**

418 **Histological staining:** Five micron sections of formalin-fixed and paraffin-embedded  
419 (FFPE) specimens were H&E-stained by using an automated stainer (Leica XL) at the  
420 University of Manchester Histology Core Facility as previously described [19]. Importantly,  
421 slides were stored at 4°C for up to one week while laser capture microdissection (LCM)  
422 was being performed. Captured material was stored at -80°C until all samples were ready  
423 for mass spectrometry processing. Alcian Blue/Periodic Acid Schiff (AB-PAS) was  
424 performed as follows. De-paraffinized slide sections were incubated for 5 minutes in 1%  
425 alcian blue 8GX (Sigma; A5268), 3% acetic acid. Slides were then washed in tap water  
426 followed by a 5-minute incubation in 1% periodic acid (Sigma; 375810). Finally, slides  
427 were washed in tap water and incubated in Schiff's reagent (Sigma – 3952016) for 15  
428 minutes. After extensive washing in tap water, slides were coverslipped without  
429 counterstain. For pentachrome, we followed a protocol as previously described [19].

430

431 For immunohistochemistry (IHC), we utilized the Novolink Polymer Detection Systems  
432 (Leica, RE7200-CE) as previously described in detail [79]. We used the following  
433 antibodies anti-BPIFB1 (Abcam; ab219098, titre 1:60,000), anti-elastin (Proteintech;  
434 15257-1-AP; titre 1:16,000) anti-PIGR (Abcam; ab224086, titre 1:8,000), anti-  
435 serotransferrin (Abcam; ab268117, titre 1: 30,000). Anti-MUC5B (titre 1:10,000) and anti-  
436 MUC5AC (titre 1:12,000) was previously purified and used here [80]. For all samples, we  
437 used antigen heat retrieval using citrate buffer pH 6.0 (Sigma, C9999), with the exception  
438 of EDTA pH 9.0 antigen heat retrieval for serotransferrin and elastin. Slides were  
439 hematoxylin counterstained and coverslipped using permount (ThermoScientific, SP15).

440

441 For MUC5B, immunostains followed a modified protocol. After citrate buffer pH 6.0  
442 antigen heat retrieval, the sections underwent reduction and alkylation. Sections were  
443 reduced by incubation at 37°C for 30 minutes in 10 mM DTT, 0.1 M Tris/HCl pH 8.0.  
444 Sections are washed in water and then incubated in 25 mM Iodoacetamide, and 0.1 M  
445 Tris/HCl pH 8.0 for 30 minutes at room temperature (kept in the dark). Lastly, sections  
446 were washed in water followed by blocking and primary antibody incubation.

447

448 For immunofluorescence, dewaxed slides were subjected to citrate buffer pH 6.0 antigen  
449 heat retrieval and probed overnight with anti-MUC5AC (titre 1:100), anti-MUC5B (post  
450 reduction/alkylation; titre 1:100), or BPIFB1 (Abcam; ab219098, titre 1:100). Sections  
451 were then incubated with secondary anti-mouse fluorophore 680 (Invitrogen, A21058,  
452 1:500) or anti-rabbit fluorophore 680 (Invitrogen; A21109; 1:500) for 1 hour. Sections were  
453 coverslipped using ProLong antifade with DAPI (Invitrogen; P36931).

454

455 **Laser Capture Microdissection:** The MMI CellCut Laser Microdissection System  
456 (Molecular Machines & Industries) was used to capture regions of interest on MMI  
457 membrane slides (MMI, 50102) as previously described [19, 20]. For this set of  
458 experiments, we collected a volume 0.03 mm<sup>3</sup> of tissue per sample.

459

460 **Histological Imaging:** For fluorescence microscopy, all stains were performed at the  
461 same time. In addition, images were taken at the same intensity utilizing EVOS FL

462 imaging system (ThermoScientific). For light microscopy, we used a DMC2900 Leica  
463 instrument with Leica Application Suite X software.

464

465 **Data Availability:** We have deposited the raw mass spectrometry data files to  
466 ProteomeXchange under the identifier of PD036465.

467

468 **Mass spectrometry sample preparation:** Samples were prepared as described [19, 20].  
469 In short, samples underwent a series of steps to maximize protein yield, including high  
470 detergent treatment, heating, and physical disruption.

471

472 **Liquid chromatography coupled tandem mass spectrometry:** The separation was  
473 performed on a Thermo RSLC system (ThermoFisher) consisting of a NCP3200RS nano  
474 pump, WPS3000TPS autosampler and TCC3000RS column oven configured with buffer  
475 A as 0.1% formic acid in water and buffer B as 0.1% formic acid in acetonitrile. An injection  
476 volume of 4  $\mu$ l was loaded into the end of a 5  $\mu$ l loop and reversed flushed on to the  
477 analytical column (Waters nanoEase M/Z Peptide CSH C18 Column, 130 $\text{\AA}$ , 1.7  $\mu$ m, 75  
478  $\mu$ m X 250 mm) kept at 35  $^{\circ}$ C at a flow rate of 300 n $\mu$ l/min with an initial pulse of 500 n $\mu$ l/min  
479 for 0.1 minute to rapidly re-pressurize the column. The separation consisted of a  
480 multistage gradient of 1% B to 6% B over 2 minutes, 6% B to 18% B over 44 minutes,  
481 18% B to 29% B over 7 minutes and 29% B to 65% B over 1 minute before washing for  
482 4 minutes at 65% B and dropping down to 2% B in 1 minute. The complete method time  
483 was 85 minutes.

484

485 The analytical column was connected to a Thermo Exploris 480 mass spectrometry  
486 system via a Thermo nanospray Flex Ion source via a 20  $\mu\text{m}$  ID fused silica capillary. The  
487 capillary was connected to a fused silica spray tip with an outer diameter of 360  $\mu\text{m}$ , an  
488 inner diameter of 20  $\mu\text{m}$ , a tip orifice of 10  $\mu\text{m}$  and a length of 63.5 mm (New Objective  
489 Silica Tip FS360-20-10-N-20-6.35CT) via a butt-to-butt connection in a steel union using  
490 a custom-made gold frit (Agar Scientific AGG2440A) to provide the electrical connection.  
491 The nanospray voltage was set at 1900 V and the ion transfer tube temperature set to  
492 275  $^{\circ}\text{C}$ .

493

494 Data was acquired in a data dependent manner using a fixed cycle time of 1.5 sec, an  
495 expected peak width of 15 sec and a default charge state of 2. Full MS data was acquired  
496 in positive mode over a scan range of 300 to 1750 Th, with a resolution of 120,000, a  
497 normalized AGC target of 300% and a max fill time of 25 mS for a single microscan.  
498 Fragmentation data was obtained from signals with a charge state of +2 or +3 and an  
499 intensity over 5,000 and they were dynamically excluded from further analysis for a period  
500 of 15 sec after a single acquisition within a 10-ppm window. Fragmentation spectra were  
501 acquired with a resolution of 15,000 with a normalized collision energy of 30%, a  
502 normalized AGC target of 300%, first mass of 110 Th and a max fill time of 25 mS for a  
503 single microscan. All data was collected in profile mode.

504

505 **Mass spectrometry data analysis and statistics:** Raw data for regional samples were  
506 processed using MaxQuant [81] version 1.6.17.0 against the human proteome obtained  
507 from uniprot (May 2021) [82]. Raw data for UIP and MA mucus samples were processed  
508 using MaxQuant [81] version 2.0.3.0 against the human proteome obtained from uniprot  
509 (May 2022) [82]. All Maxquant processing were performed with a fixed modification of  
510 carbamidomethylation of cysteine, with variable modifications of methionine oxidation and  
511 protein N-terminal acetylation. Precursor tolerance was set at 20ppm and 4.5pm for the  
512 first and main searches, with MS/MS tolerance set at 20ppm. A false discovery rate (FDR)  
513 of 0.01 was set for PSM and protein level, up to two missed cleavages were permitted  
514 and “match-between-runs” was selected.

515

516 Statistical analysis was carried out in R (v4.1.2) [83] using the MSqRob package (v0.7.7)  
517 [84]. Significantly changing proteins were taken at a 5% false discovery rate (FDR).  
518 Pathway analysis utilising Reactome Pathways was performed on significantly changing  
519 proteins using the R package ReactomePA (1.38.0) [85]

520

521

522 **Study Approval:** For this study, we utilized a variety of Research Ethics Committee  
523 (REC) protocols to obtain patient-consented lung tissue: REC#14/NW/0260 (provided by  
524 JFB and RVV, Manchester, United Kingdom) for transplanted fibrotic lung;  
525 REC#20/NW/0302 (provided by MAM and FG, Manchester, United Kingdom) for non-  
526 fibrotic lung specimens; REC#18/NW/0092 (provided by Manchester Cancer Research

527 Centre Biobank, Manchester, United Kingdom) for mucinous lung adenocarcinoma.  
528 Usual Interstitial Pneumonia (UIP) specimens were defined by current guidelines [1, 86].  
529 Non-fibrotic controls were collected from morphologically normal lung tissue distal to  
530 tumor during resection (fibrotic and control patient demographics may be found in  
531 **Supplemental Figure 3**). Mucinous adenocarcinoma (MA) was defined by current  
532 guidelines (MA patient demographics may be found in **Supplemental Figure 4**) [59]. In  
533 this study, we utilized 10 UIP, 6 non-fibrotic, and 6 MA specimens.

534

535

536 **Author Contributions:** J.A.H. designed and conducted all LCM-MS experiments and  
537 C.L. performed the associated analyses. L.D. and S.P. performed the  
538 immunohistochemistry/immunofluorescence and imaging. M.A.M. assisted in  
539 characterizing the histological stains and identification of clinical morphologies associated  
540 with Usual Interstitial Pneumonia and Mucinous Adenocarcinoma. R.V.V., J.F.B., M.A.M.  
541 and F.G. contributed to reagents. J.A.H. wrote the manuscript with all author inputs.  
542 J.A.H. and D.J.T conceived and supervised the project.

543

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555 expressed in this publication are those of the authors and not necessarily those of  
556 ManARTS, MCRC, NHS, or NWLC. In addition, we thank the study participants for their  
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558

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