BPIFB1 loss alters airway mucus properties and diminishes mucociliary clearance

Lauren J. Donoghue[1,2], Matthew R. Markovetz[3], Cameron B. Morrison[3], Kathryn M. McFadden[1], Troy D. Rogers[3], Brian Button[3,4], Camille Ehre[3,5], David B. Hill[3,6], Barbara R. Grubb[3], and Samir N. P. Kelada[1,3*]

[1]Department of Genetics, University of North Carolina at Chapel Hill
[2]Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill
[3]Marsico Lung Institute, University of North Carolina at Chapel Hill
[4]Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill
[5]Division of Pediatric Pulmonology, University of North Carolina at Chapel Hill
[6]Department of Physics and Astronomy, University of North Carolina at Chapel Hill

*Corresponding author
Samir Kelada, PhD MPH
University of North Carolina
Department of Genetics
120 Mason Farm Road
Chapel Hill, NC 27599
919-962-2148
samir_kelada@med.unc.edu

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ABSTRACT

Airway mucociliary clearance (MCC) is required for host defense and often diminished in chronic lung diseases. Effective clearance depends upon coordinated actions of the airway epithelium and a mobile mucus layer. Dysregulation of the primary secreted airway mucin proteins, MUC5B and MUC5AC, is associated with a reduction in the rate of MCC; however, how other secreted proteins impact the integrity of the mucus layer and MCC remains unclear. We previously identified the gene *Bpifb1/Lplunc1* as a regulator of airway MUC5B levels using genetic approaches. Here, we show that BPIFB1 is required for normal mucociliary clearance *in vivo* using *Bpifb1* knockout (KO) mice. Reduced MCC in *Bpifb1* KO mice occurred in the absence of defects in sodium or chloride ion transport or reduced ciliary beat frequency. BPIFB1 loss resulted in airway mucus flakes with significantly increased complex viscosity, a key biophysical property of mucus known to impact MCC. Finally, we detected colocalization of BPIFB1 and MUC5B in secretory granules in mice and in the protein mesh of secreted mucus in human airway cultures. Collectively, our findings demonstrate that BPIFB1 is an important component of the mucociliary apparatus in mice and a key component of the mucus protein network.

INTRODUCTION

The airway mucus layer plays a critical role in airway homeostasis and host defense in part by facilitating mucociliary clearance (MCC) of inhaled particles and pathogens. Effective MCC requires a properly hydrated airway surface, an optimal balance of components in secreted mucus (i.e., proteins, salt), and coordinated beating of cilia. The importance of the MCC apparatus is highlighted by the fact that mutations in genes essential to ciliary function or ion

23 transport cause primary ciliary dyskinesia (PCD) and cystic fibrosis (CF), respectively, in which
24 respiratory infections are prominent and severe. In the context of more common obstructive
25 airway disease such as asthma and chronic obstructive pulmonary disease (COPD), changes in
26 MCC have been associated with variation in the abundance of the secreted mucin proteins
27 MUC5B and MUC5AC, which are key contributors to the gel-like nature of healthy airway
28 mucus[1]. The concentration, organization, and post-translational modifications of MUC5B and
29 MUC5AC also have been shown to alter biophysical properties of mucus that are required for
30 effective MCC[2–6]. Furthermore, the ability of mucins to form a proper gel-structure is
31 dependent upon interactions with other secreted proteins, referred to as the "mucin
32 interactome"[7], although the specifics of these dependencies remain unclear.
33
34 In contrast to MUC5AC, which is typically only observed at high levels in disease states, MUC5B
35 is constitutively expressed in the airways of healthy individuals where it has a role in regulating
36 homeostasis through airway MCC and host defense[8,9]. We previously identified the gene *Bpifb1*
37 (also known as *Lplunc1*) as a regulator of airway MUC5B protein levels using a quantitative
38 genetics approach in mice and showed that loss of BPIFB1 in a knockout (KO) mouse model led
39 to increased levels of MUC5B protein in the airways[10]. Given that MUC5B levels have been
40 shown to affect MCC[9,11], we sought to determine if *Bpifb1* also had a role in regulating MCC.
41 We observed a substantial loss of MCC in the lower airways of *Bpifb1* KO mice. To identify
42 which aspects of the MCC apparatus contributed to this defect, we assessed epithelial sodium
43 and chloride ion transport, ciliary function, and biophysical properties of mucus. Our results
44 show that alterations in the mucus layer including increased mucus viscoelasticity were

45 associated with decreased MCC in the absence of BPIFB1 and that BPIFB1 is a component of the
46 mucus protein network.
47
48 **RESULTS AND DISCUSSION**
49 In order to determine if *Bpifb1* has a role in MCC, we assessed MCC in tracheas of naïve *Bpifb1*
50 KO and WT mice. We quantified MCC by measuring the percentage of instilled beads that were
51 cleared by the trachea over a fixed period of time. *Bpifb1* KO mice exhibited significantly
52 decreased clearance, with some mice exhibiting no tracheal bead clearance whatsoever (Figure
53 1A). These results indicated clearly that BPIFB1 is required for MCC.
54
55 To understand the mechanism by which BPIFB1 loss leads to defective MCC, we systematically
56 tested the expression and function of key components of the MCC apparatus. First, we
57 examined the expression of key ion transporters in the airway epithelium given their
58 importance in regulating airway hydration and MCC[1]. RNA sequencing of lung tissue from
59 *Bpifb1* KO and WT mice did not reveal significant differences in expression of well-characterized
60 sodium and chloride ion transporter genes including *Cftr*, *Scnn1b*, and *Tmem16a* (Figure S1).
61 However, given that a lack of difference in ion transporter gene expression does not rule out a
62 difference in functionality, we also measured bioelectric capacities in *Bpifb1* KO and WT
63 tracheas. We found no differences in short-circuit current (I_{sc}) at baseline or following
64 stimulation with compounds targeting the major ion channels in the airway epithelium (i.e.
65 CFTR, ENaC, CaCC), (Figure 1B). This indicated that the loss of MCC in *Bpifb1* KO mice was likely
66 not driven by altered ion transport. Additionally, as ciliary beating is essential for MCC, we

67 directly measured ciliary beat frequency (CBF) in intact tracheas *in situ*. In comparison to WT
68 mice, *Bpifb1* KO mice had a slightly elevated CBF, albeit not statistically significant at $P < 0.05$
69 (Fig 1C). These data indicated that reduced MCC was not associated with reduced ciliary
70 beating.
71
72 Next, to determine if *Bpifb1* loss influenced the expression of other genes or biological
73 processes that could underlie the defect in MCC, we examined global gene expression in lung
74 tissue from *Bpifb1* KO and WT mice. Excluding *Bpifb1*, only 4 protein coding genes and 2 long
75 non-coding RNAs were differentially expressed by genotype ($|\text{fold change}| \geq 2$ and adjusted *P*-
76 value ≤ 0.05, Figure S1). Of these genes, none have been previously annotated for a direct role
77 in MCC or related pathways. Furthermore, genes related to mucin production, including *Muc5b*,
78 *Muc5ac*, *Spdef*, and *Foxa3,* were not differentially expressed. These gene expression results
79 suggested the reduction in MCC in *Bpifb1* KO mice was not mediated by differential expression
80 of key MCC or mucus production genes.
81
82 Given the lack of differences in hallmark causes of reduced MCC, we investigated if there were
83 differences in the airway mucus *per se* that could be contributing to the observed loss of MCC.
84 Using microbead rheology, we measured the complex viscosity (η^*), a measure of both viscosity
85 and elasticity, of mucus flakes (i.e. the insoluble portions of airway mucus) in bronchoalveolar
86 lavage (BAL) fluid from *Bpifb1* KO vs WT mice. For these measurements, mice were intranasally
87 exposed to house dust mite allergen for three weeks to increase the levels of mucus in BAL, and
88 there were no differences in the degree of eosinophilic inflammation by genotype

89 (Supplementary Figure S2). We found that the complex viscosity of mucus flakes was
90 significantly increased in *Bpifb1* KO mice compared to WT mice (Figure 2A, B). By imaging of
91 whole BAL, we determined this increase was independent of the size and number of mucus
92 flakes, and there were no differences in these metrics by genotype (Supplementary Figure S3A-
93 E). Consistent with these findings and our previous report of increased MUC5B in BAL from
94 naïve *Bpifb1* KO mice[10], we found that MUC5B levels were elevated by genotype in the
95 supernatant fraction of centrifuged BAL from naïve mice, but not in the pelleted (i.e. insoluble)
96 fraction (Figure 2C). Thus, loss of BPIFB1 results in altered biophysical properties of mucus
97 flakes and increased levels of soluble MUC5B.
98
99 To gain insight into whether BPIFB1 colocalizes with mucin protein in the airways and, if so,
100 whether this association occurs before mucin granule release, we assessed the spatial
101 relationship between BPIFB1 and mucin protein using fluorescent immunostaining. By
102 colocalization analysis of labeled BPIFB1 and MUC5B protein in large airways of mice challenged
103 with allergen, we determined that the vast majority of MUC5B positive cells were also positive
104 for BPIFB1 (Figure 3A, B), indicating regulated cellular co-expression of MUC5B and BPIFB1.
105 Colocalization was also present in the airways of mice not challenged with allergen
106 (Supplementary Figure S4). Furthermore, MUC5B positive granules were also positive for
107 BPIFB1 and luminal mucus contained both MUC5B and BPIFB1 (Figure 3C), suggesting that
108 packaging and secretion of these proteins is likely coupled.
109

110 Finally, we performed immunogold-labeled scanning electron microscopy for BPIFB1 and
111 MUC5B in human bronchial epithelial cells (HBECs) cultured at air-liquid-interface to determine
112 if BPIFB1 closely associated with extracellular mucus. We detected BPIFB1 exclusively in mucus
113 strands coating the cellular surface (Figure 4). This finding confirmed that BPIFB1 is a
114 component of the mucus protein network produced by human airway cells.
115
116 Significantly altered levels of either *BPIFB1* gene or protein expression have been observed in
117 CF, COPD, asthma, and IPF[12–15], indicating a strong connection between *BPIFB1* and muco-
118 obstructive diseases. After identifying *Bpifb1* as a regulator of airway mucus levels through an
119 unbiased genome-wide search in the mouse[10], we sought to interrogate the specific function of
120 BPIFB1 in the airways. We found that the loss of BPIFB1 reduced MCC dramatically in naïve
121 *Bpifb1* KO mice, demonstrating the critical role of this gene in regulating MCC. Thus, in addition
122 to the strong connection between *BPIFB1* gene/protein expression and muco-obstructive
123 diseases, our findings indicate BPIFB1 may have an important role in airway host defense by
124 regulating MCC. Studies in humans have shown that there is significant interindividual variation
125 in baseline MCC, and analysis of MCC in monozygotic and dizygotic twins has implicated genetic
126 variation as a modifier of MCC[16]. Given the identification of common genetic variation
127 associated with the levels of *BPIFB1* gene expression[17], genetic variation in *BPIFB1* regulation
128 could be one such factor regulating baseline MCC in humans. Furthermore, BPIFB1 was one of
129 the top 20 proteins detected in endotracheal tube mucus from individuals without a history of
130 lung disease or smoking, highlighting its abundance in non-disease states[18].
131

132 Due to the lack of differences in basal or stimulated transport of sodium and chloride ions,
133 ciliary beating, and MCC-related gene expression between *Bpfib1* KO and WT mice, our data
134 point toward changes to the airway mucus layer as the cause of reduced MCC. More
135 specifically, the increased complex viscosity of mucus, as we observed in mucus flakes from
136 *Bpifb1* KO mice and may be the case for soluble mucus as well, is suggestive of mucus that is
137 more difficult to clear from the airways. *In vitro* studies with human airway cultures[19–22], as well
138 as earlier studies in model organisms[23–25], have demonstrated that mucus transport is directly
139 related to the rheological properties of mucus. The increased CBF that we observed in *Bpifb1*
140 KO mice, although not statistically significant, may be indicative of an attempt to compensate
141 for altered airways mucus[26]. Such a phenomenon has been demonstrated in human bronchial
142 epithelial cultures where CBF increased following a moderate increase in mucus viscosity or
143 mucus load[27].
144
145 The increased complex viscosity of mucus flakes in *Bpifb1* KO mice may be attributable to a
146 change in mucin network structure, which is a function of the density and/or nature of mucin-
147 mucin/mucin-protein interactions[7]. In muco-obstructive airway disease contexts, for example,
148 heightened mucus viscoelasticity due to oxidation-dependent mucin cross-linking[5] or increased
149 mucin concentration[28] has been described. Direct measurement of mucin concentration was
150 not possible in our experiments due to the high dilution factor of mucus in lavage fluid.
151 However, the lack of increased MUC5B in pelleted BAL from *Bpifb1* KO mice provides some
152 evidence against the hypothesis that mucin concentration is increased in mucus flakes and is
153 the driver of changes in mucus rheology. That said, we detected elevated levels of soluble

154 MUC5B in *Bpifb1* KO mice, which raises the question of whether MUC5B levels are higher in
155 *Bpifb1* KO mice simply due to decreased MCC or increased mucin concentration. *In vitro*
156 studies, in which mucus can be collected directly from the surface of epithelial cells, will be
157 required to determine specifically how BPIFB1 impacts the mucus layer.
158
159 Although the polymeric nature of gel-forming mucins is essential for the biophysical properties
160 of mucus, studies have shown that mucins alone cannot create complex networks, and thus,
161 other factors, including secreted globular proteins, are necessary for mucus formation[29]. Our
162 detection of BPIFB1 in secreted mucus from HBECs confirms BPFIB1 is indeed a component of
163 the mucus protein network and supports the conclusion that this non-mucin protein plays an
164 important role in the mucus layer. Further evidence for a direct role of BPIFB1 in the mucus
165 network comes from a finding that BPIFB1 has strong physical interactions with mucin proteins
166 in HEBC secretions. *Radicioni et al*. found that after treating mucus from HBEC cultures with
167 guanidinium hydrochloride and detergents (i.e. dissociative conditions), BPIFB1 was one of few
168 globular proteins interacting with mucins[7]. Additionally, our data demonstrating shared
169 regulatory mechanisms between BPIFB1 and MUC5B suggest strongly coordinated roles. We
170 previously found that *Bpifb1* and *Muc5b* gene expression was strongly correlated in mouse lung
171 tissue[10], and here we have shown that BPIFB1 and MUC5B protein are colocalized in airway
172 epithelial cells and present in secreted mucus. Cellular co-expression between *BPIFB1* and
173 *MUC5B* mRNA has also been demonstrated in human airways by single cell RNA sequencing
174 studies[30,31] and between BPIFB1 and MUC5AC protein in COPD[32]. Lastly, our finding that BPIFB1
175 is present in mucin secretory granules, which are tightly packed and only contain the necessary

176 proteins for mucus, implicates an additional regulatory mechanism coupling these two proteins.
177 Whether there is an optimal stoichiometry between levels of secreted BPIFB1 and mucin
178 proteins for MCC, as may be the case between MUC5B and MUC5AC[2,33], remains to be
179 determined.
180
181 In summary, we demonstrate that BPIFB1 is critical for MCC *in vivo*. These findings establish
182 that mucociliary dysfunction can be driven by non-mucin protein components of airway mucus,
183 highlighting their importance in health and disease.
184
185
186 **METHODS**
187 A detailed descriptions of the methods used in this study can be found in the Supplementary
188 Methods (Supplementary File 1).
189
190 *Study Approval.*
191 All experiments conducted with mice in this study were compliant with an Institutional Animal
192 Care and Use Committee protocol at an animal facility approved and accredited by the
193 Association for Assessment and Accreditation of Laboratory Animal Care International. Primary
194 cells were provided by the Cystic Fibrosis (CF) Center Tissue Core Facility of the University of
195 North Carolina at Chapel Hill under the auspices of protocols approved by the Institutional
196 Committee on the protection of the rights of human subjects.
197

198 *Data availability.* Complete raw data and counts generated by RNA sequencing were deposited
199 in the NCBI's Gene Expression Omnibus (GEO) database under entry GSE150452.
200
201
202 **AUTHOR CONTRIBUTIONS**
203 LJD, MRM, CBM, BB, CE, DBH, BRG, and SNPK conceived experiments and interpreted data. LJD,
204 MRM, CBM, KMM, TDR, CE, and BRG performed experiments. LJD and SNPK wrote and revised
205 the manuscript. All authors edited and reviewed the manuscript.
206
207
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308

309 **FIGURE LEGENDS**

310 **Figure 1. Decreased mucociliary clearance in *Bpifb1* KO mice.** (A) Percent bead clearance in
311 tracheal mucociliary clearance assay in naive WT and *Bpifb1* KO mice. Mean ± SEM depicted.
312 n=6/genotype. Significance of two-sided Student's *t*-test denoted as **, $P < 0.01$. (B) Ion
313 transport measured in Ussing chambers. Mean ± SEM depicted. n=6/genotype. No differences
314 between genotypes by two-sided Student's *t*-test. (C) Ciliary beat frequency by *in situ* tracheal
315 assay. Mean ± SEM depicted. n=7-8/genotype. Difference between genotypes not significant by
316 two-sided Welch's *t*-test, P = 0.1.

317

318 **Figure 2. Biophysical properties of airway mucus are altered in *Bpifb1* KO mice.**
319 (A) Distribution of rheological signal in BAL from a representative allergen exposed WT and
320 *Bpifb1* KO mouse. (B) Complex viscosity of BAL mucus fraction. Boxplots depict median and
321 interquartile ranges. n=13/genotype. Significant difference between genotypes by ANCOVA
322 modeling denoted as *, $P < 0.05$. (C) MUC5B in pellet and supernatant fractions of BAL from
323 naive WT and *Bpifb1* KO mice by mucin western blotting. Mean ± SEM depicted. Significant
324 difference between genotypes in supernatant fraction by two-sided Welch's *t*-test denoted as
325 **, $P < 0.01$. n=5/genotype.

326

327 **Figure 3: BPIFB1 and MUC5B colocalize in murine airway epithelial cells.** (A) BPIFB1 (green)
328 and MUC5B (red) immunostaining in airway epithelial cells from allergen challenged mice. Scale
329 bar = 10 µm. (B) Percentage of cells positively stained for BPIFB1 only, MUC5B only, or both in
330 allergen challenged mice. n = 7 mice. (C) BPIFB1 (green) and MUC5B (red) immunostaining in

331 large airways of allergen challenged mice demonstrating BPIFB1 staining in luminal mucus.

332 Scale bar = 50 µm.

333

334 **Figure 4: BPIFB1 is part of mucus network in human bronchial epithelial cell cultures.**

335 Immunogold-labeled scanning electron microscopy for (A) negative control, (B) MUC5B, and (C)

336 BPIFB1. Scale bar = 1 µm.

337

338 Supplementary Figure Legends provided in Supplementary File 1.

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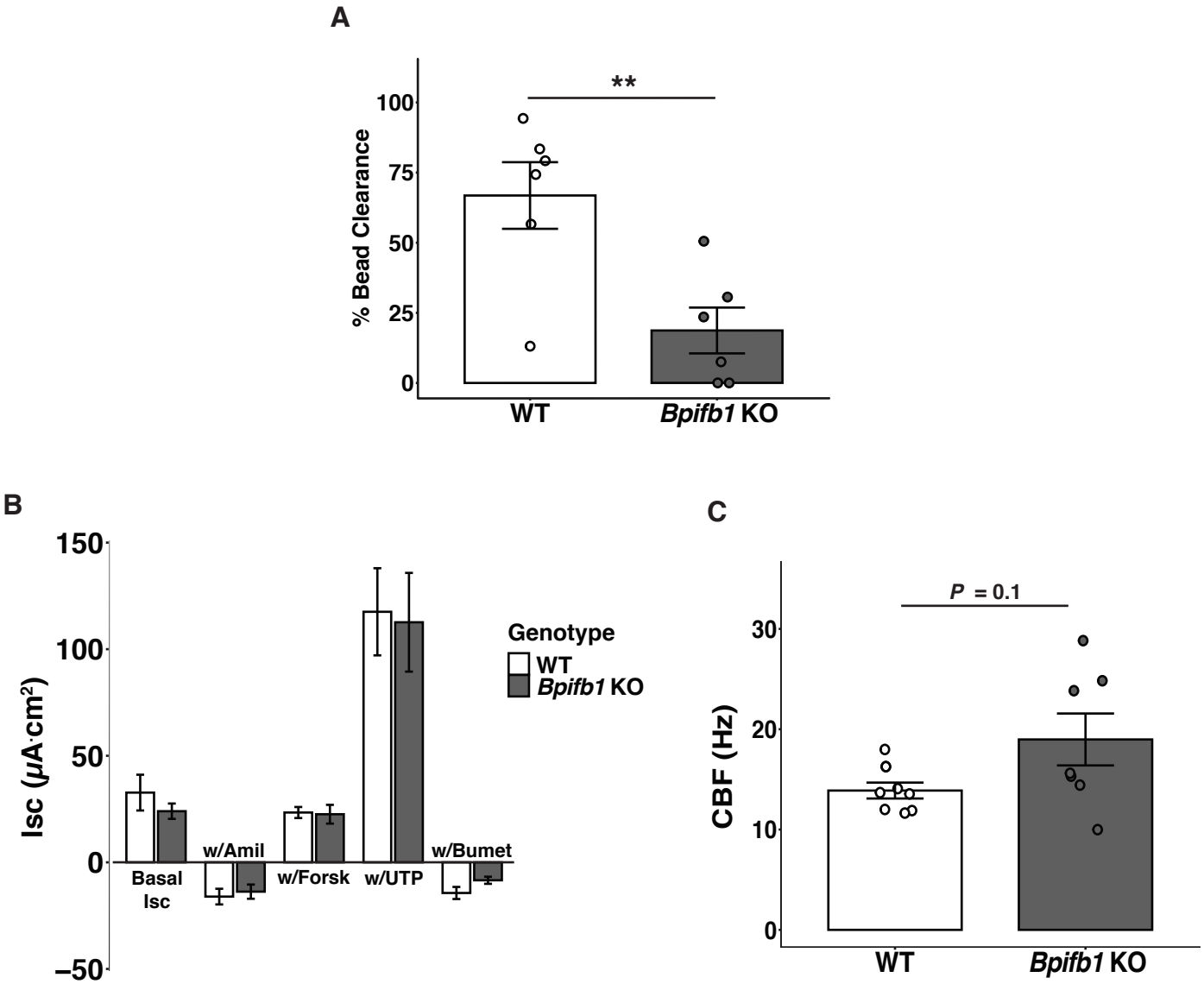

Figure 1. Decreased mucociliary clearance in *Bpifb1* KO mice.
(A) Percent bead clearance in tracheal mucociliary clearance assay in naive WT and *Bpifb1* KO mice. Mean ± SEM depicted. n=6/genotype. Significance of two-sided Student's *t*-test denoted as **, $P < 0.01$. (B) Ion transport measured in Ussing chambers. Mean ± SEM depicted. n=6/genotype. No differences between genotypes by two-sided Student's *t*-test. (C) Ciliary beat frequency by *in situ* tracheal assay. Mean ± SEM depicted. n=7-8/genotype. Difference between genotypes not significant by two-sided Welch's *t*-test, $P = 0.1$.

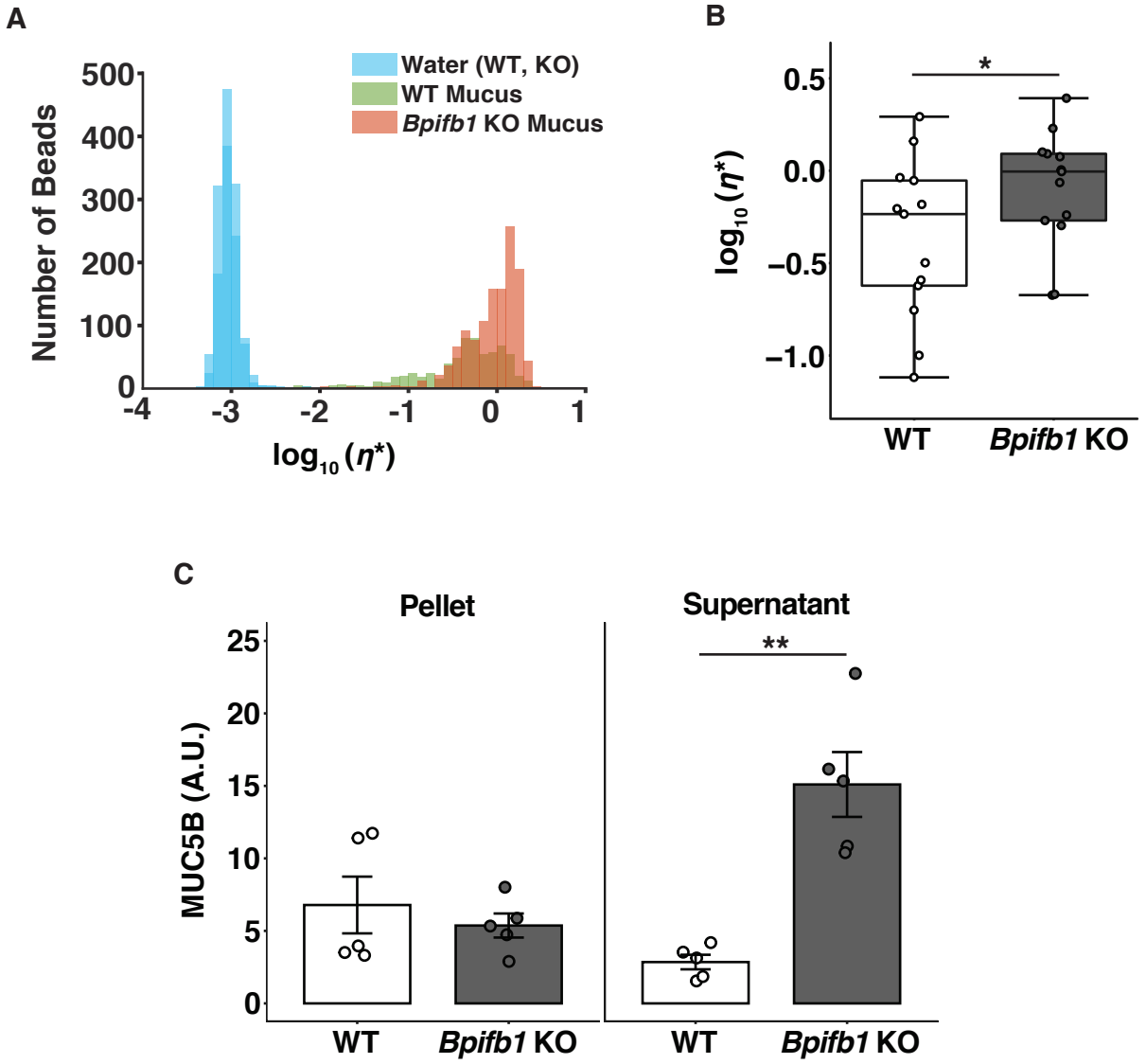

Figure 2. Biophysical properties of airway mucus are altered in *Bpifb1* KO mice.
(A) Distribution of rheological signal in BAL from a representative allergen exposed WT and *Bpifb1* KO mouse. (B) Complex viscosity of BAL mucus fraction. Boxplots depict median and interquartile ranges. n=13/genotype. Significant difference between genotypes by ANOVA modeling denoted as *, $P < 0.05$. (C) MUC5B in pellet and supernatant fractions of BAL from naive WT and *Bpifb1* KO mice by mucin western blotting. Mean ± SEM depicted. Significant difference between genotypes in supernatant fraction by two-sided Welch's *t*-test denoted as **, $P < 0.01$. n=5/genotype.

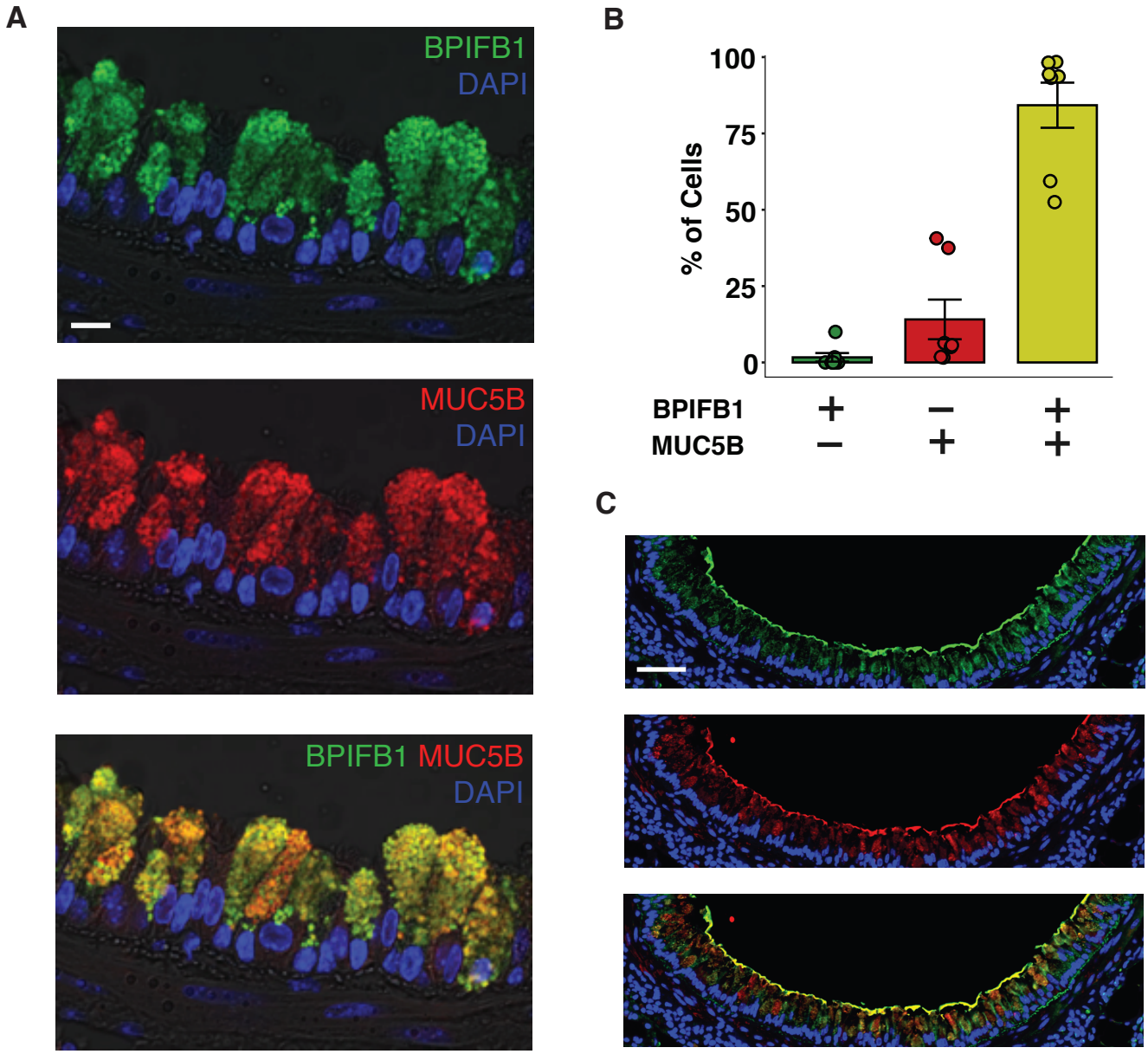

Figure 3: BPIFB1 and MUC5B colocalize in murine airway epithelial cells.
(A) BPIFB1 (green) and MUC5B (red) immunostaining in airway epithelial cells from allergen challegned mice. Scale bar = 10 μm. (B) Percentage of cells positively stained for BPIFB1 only, MUC5B only, or both in allergen challenged mice. n=7 mice. (C) BPIFB1 (green) and MUC5B (red) immunostaining in large airways of allergen challegned mice demonstrating BPIFB1 staining in luminal mucus. Scale bar = 50 μm.

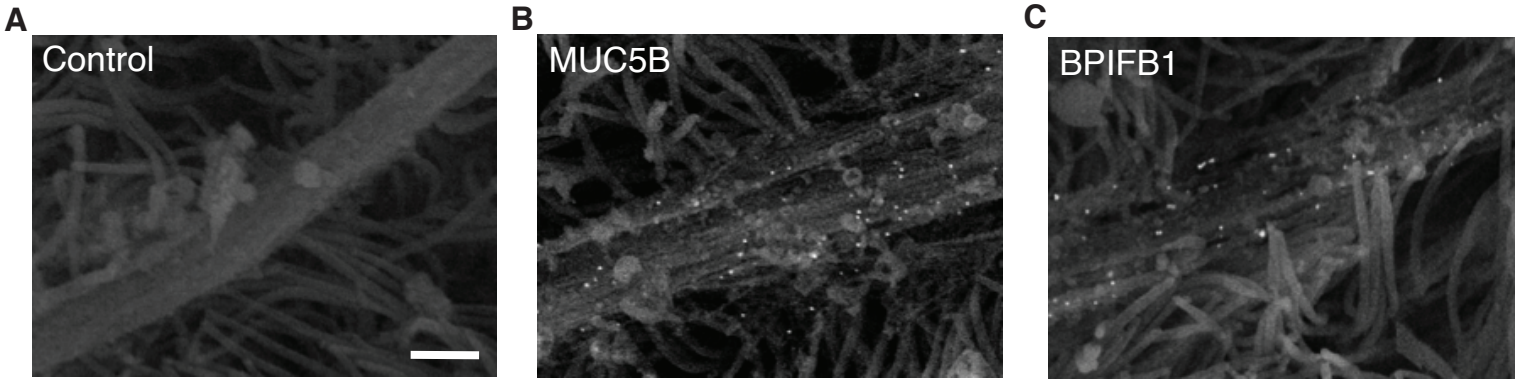

Figure 4: BPIFB1 is part of mucus netwrok in human bronchial epithelial cell culutures. Immunogold-labeled scanned electron microscopy for (A) negative control, (B) MUC5B, and (C) BPIFB1. Scale bar = 1 μm.