Supplemental Material (Ornatowski et al.)

Supplemental Figures

Figure S1. CF cells produce abnormally high levels of TGFβ. (A) Total biologically active TGF-β was measured by luciferase assay using TGF-β sensitive reporter system in mink lung epithelial cells (MLEC). The conditioned medium (2, 10, and 25% final dilution) (CM) from IB3-1 (CF) or C-38 and S-9 (CFTR-corrected) cells was added to the reporter system. (B) CM from CFBE (CF) cells and 16HBE (normal) cells tested with MLEC. (C) CM from pCEP-R (over-expressing the dominant negative CFTR R domain) and pCEP (mock-transfected). (D) CM from CuFi-3 (CF) and NuLi-1 (normal) cells tested in MLEC. (E) Total TGF–β (all isoforms) in IB3-1 and S9 cells measured by ELISA. (F) The amount of TGF-β3 isoform produced. Data are presented as pg of TGF-β/ml/10^5 cells (A) and % of TGF-β (B-F). Values are representative of three experiments, means ± SEM (*$p < 0.05$, **$p \leq 0.01$).

Figure S2. Chloroquine normalizes TGF-β production in CF respiratory epithelial cells. CF cells were treated with 0.1 mM chloroquine (CQ) for 24 h. Production of TGF-β was measured using MLEC luciferase assay in conditioned media. (A) IB3-1 (CF) and S-9 (CFTR-corrected) cells. Results are presented as % of TGF-β in each cell line. 100% represents TGF-β levels in non-CF cells, (n = 3 experiments) means ± SEM; **$p \leq 0.01$, relative to IB3-1; (†) $p \geq 0.05$, relative to S9). (B) Live fluorescence microscopy images of CF and CFTR-corrected human bronchial epithelial cells expressing TGN38 fused to pH-sensitive GFP. Color ratiometric images are shown in the right column; color look-up scale shows relationship between 508 nm emission ratios upon excitation at 410 and 470 nm and pH, with mean TGN pH values of IB3-1 being 6.2 and S9 pH 6.7; TGN pH in IB3-1 cells treated with chloroquine was indistinguishable from that in S9 cells. Top row, CF cells (IB3-1); middle row, IB3-1 cells treated with chloroquine; bottom row, S9, CFTR-corrected IB3-1 cells. The external buffer had a pH of 7.4. Note that fluorescence intensity increases for 410 nm excitation, upon chloroquine treatment, reflecting less acidic pH.

Figure S3. Excess TGF-β inhibits anti-pseudomonal action of macrophages and is responsible for diminished iNOS levels in CF cells. (A) Primary human peripheral blood monocyte-derived macrophages (MDM; differentiated for 5 days by adhesion) were pre-incubated with TGF-β1 (10 ng/ml) for 24 h prior to infection with *P. aeruginosa*. Data, means ± SEM (n=3; **$p \leq 0.01$). 1 (B) CF primary respiratory epithelial cells were co-cultured with MDM and incubated with TGF-β antibodies (5 μg/ml) for 4 h, or the epithelial cells were pre-incubated with furin inhibitor decanoyl-RVKR-chloromethylketone (CMK) (50 μM) for 24 h prior to co-culture with MDM and *P. aeruginosa* infection. Bacterial survival was determined after 3 h of incubation as described in Supplementary Methods. Data, means ± SEM (**$p \leq 0.01$, † $p \geq 0.05$; n = 6). (C) iNOS immunofluorescence in IB3-1 (CF) and S9 (CFTR-corrected) cells. Cells were incubated in full medium

with 10 ng/ml TGF-β1 or in the presence of 5 μg/ml TGF-β antibodies. (D) Quantification (%) of iNOS expression visualized by immunofluorescence (values are means from blots obtained in 3 separate experiments ± SEM (*$p < 0.05$, **$p \leq 0.01$). (E) Cell lysates from S9 (CFTR-corrected) immunoblotted with antibody to iNOS. Overhead numbers represent density units, (n = 3 experiments) means ± SEM (*$p < 0.05$, **$p \leq 0.01$).

Figure S4. Colocalization of furin-GFP with TGN markers. Bronchial epithelial cells were co-transfected with furin-GFP. Immunofluorescent visualization of anti-human syntaxin-6 and anti-G58 (primary Ab) by using secondary Alexa 568-conjugated Ab (red fluorescence). GFP-furin colocalization with human syntaxin-6 or G58 (merged) in: (A) IB3-1 cells. (B) S9 cells. Appearance of IB3-1 cell was identical to S9 cells.

Figure S5. Increased susceptibility of primary human CF lung epithelial cells to ExoA and its production by planktonic and biofilm *P. aeruginosa* forms. (A) Normal and CF lung primary epithelial cells were incubated in media alone or media containing 100 ng/ml of ExoA for 4 h in the presence of furin inhibitor decanoyl-RVKR-chloromethylketone (CMK) (50 μM) or 100 nM bafilomycin A_1 (BA). Protein synthesis levels were determined by measuring the incorporation of [3H] leucine. Data, means ± SEM (n=3, *$p < 0.05$). (B) SDS-PAGE analysis of ExoA expression in planktonic bacteria (PAO1) and biofims, cultured as described by O'Toole and Kolter (1). Proteins in bacterial lysates were separated by SDS-PAGE, and immunoblotted with anti-ExoA as described in Supplemental Methods.

Supplementary Methods

Determination of intra-organellar pH using ratiometric pH-sensitive green fluorescent protein (GFP). Fluorescence microscopy was carried out by using an Olympus IX-70 microscope and Olympix KAF1400 charge-coupled device (CCD) camera (LSR, Olympus), using described filter sets (Chroma Technology) mounted in a Sutter Instruments (Novato) filter wheel and controlled by the MERLIN software program (version 1.89; LSR, Olympus). Fluorescence images were taken on excitation at 410 and 470 nm. The pH was calculated as previously described (2, 3).

Confocal microscopy and localization studies. Cells grown on glass slides to 60-70% confluency were transfected with 1 μg of Cellubrevin pHluorin GFP or GFP-furin by using Effectene (Qiagen). After 24 h of expression, IB3-1 and S9 cells were fixed with 1% paraformaldehyde followed by membrane permeabilization using 1% TritonX-100. The coverslips were washed three times with PBS prior to incubating with blocking solution (10% skim milk, 6% BSA fraction V, and 2% goat serum in PBS). Airway epithelial cells were incubated with a polyclonal rabbit anti-iNOS antibody (Transduction Labs) at 4°C overnight, followed by a secondary antibody. Alexa 488-conjugated to goat anti-rabbit IgG

antibody (Molecular Probes) was incubated with samples for 5 h at 37°C in blocking solution. For syntaxin-6 colocalization, GFP furin-transfected cells were fixed and visualized with mouse mAb against human syntaxin-6 or G58 (BD Transduction) followed by goat anti-mouse secondary Ab conjugated to Alexa 568. For ExoA colocalization cells incubated with ExoA conjugated to secondary Alexa 568 (red fluorescence). EEA1 was visualized using primary human anti-EEA1 antibody and Alexa 468-conjugated antibody (green). All images were taken at a magnification of x63. Collection of 1 μm thick optical sections was performed using an Axiovert 200 M microscope and Zeiss Meta Confocal System

Bacterial killing by macrophages. Primary human peripheral blood monocyte-derived macrophages were infected with 1 x 10^7 CFU/ml *P. aeruginosa*, strain PAO1, either in the presence or in the absence of human TGF-β1 (10 ng/ml) (R&D Systems) (4, 5). The bacteria were allowed to interact with the primary macrophages for 3 h at 37°C, after which the non-adherent bacteria were removed by washing. To kill the extracellular organisms, the cell cultures were treated with 300 μg/ml of gentamicin for 45 min. The cells were lysed in 0.5% Triton X-100 to release the intracellular organisms. The surviving bacteria were diluted for plating on *Pseudomonas*-isolation agar. Bacterial colonies were counted after overnight incubation at 37°C.

Co-culture of CF epithelial cells with macrophages and *P. aeruginosa* survival. MDM were differentiated as described in Methods. Primary human epithelial cells and macrophages were co-cultured by adding CF epithelial cells (1 x 10^5/plate) to a 60% confluent culture of adherent macrophages in 60 mm Petri dishes. When indicated, CF epithelial cells were pre-treated with CMK (50 μM) for 24 h prior to mixing with macrophages. TGF-β antibodies were added after epithelial cells and macrophages were mixed for 4 h. The capacity of co-cultured human respiratory epithelial cells and human monocytes derived macrophages to eliminate *P. aeruginosa* was analyzed by adding CF primary cells to macrophages after incubation with furin inhibitor or TGF-β antibodies. Co-cultures of primary cells were grown for 48 h to achieve confluency. The killing assay was performed by the method described above.

ELISA. Quantitative determination of bioactive TGF-β1 in cell culture supernatants of CF and non-CF cells were performed using an ELISA assay specific for mature and active TGF-β1, β2, and β3 (R & D Systems).

Bacterial biofilms. For analysis of ExoA protein production by *P. aeruginosa*, the standard laboratory strain of *P. aeruginosa* PAO1 was grown in Luria broth (LB). Overnight cultures were grown in LB medium and diluted to an OD_{600} of 0.1, and reincubated for a further 24 h in the same medium. For comparative assessment of ExoA expression by planktonic vs. biofilm forms of the same strain, the assay for biofilm formation was adapted from the procedure previously described by O'Toole and Kolter (1). Two milliliters of the inoculum were plated

on 6-well plates and incubated at 37°C for 48 h (biofilm culture). Proteins were extracted from the pellets of planktonic cultures and from collected plastic-adherent bacterial biofilms.

Supplemental References

1. O'Toole, G.A., and Kolter, R. 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Mol Microbiol* 30:295-304.
2. Miesenbock, G., De Angelis, D.A., and Rothman, J.E. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192-195.
3. Poschet, J.F., Boucher, J.C., Tatterson, L., Skidmore, J., Van Dyke, R.W., and Deretic, V. 2001. Molecular basis for defective glycosylation and Pseudomonas pathogenesis in cystic fibrosis lung. *Proc Natl Acad Sci U S A* 98:13972-13977.
4. Chen, S., Tuttle, D.L., Oshier, J.T., Knot, H.J., Streit, W.J., Goodenow, M.M., and Harrison, J.K. 2005. Transforming growth factor-beta1 increases CXCR4 expression, stromal-derived factor-1alpha-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunology* 114:565-574.
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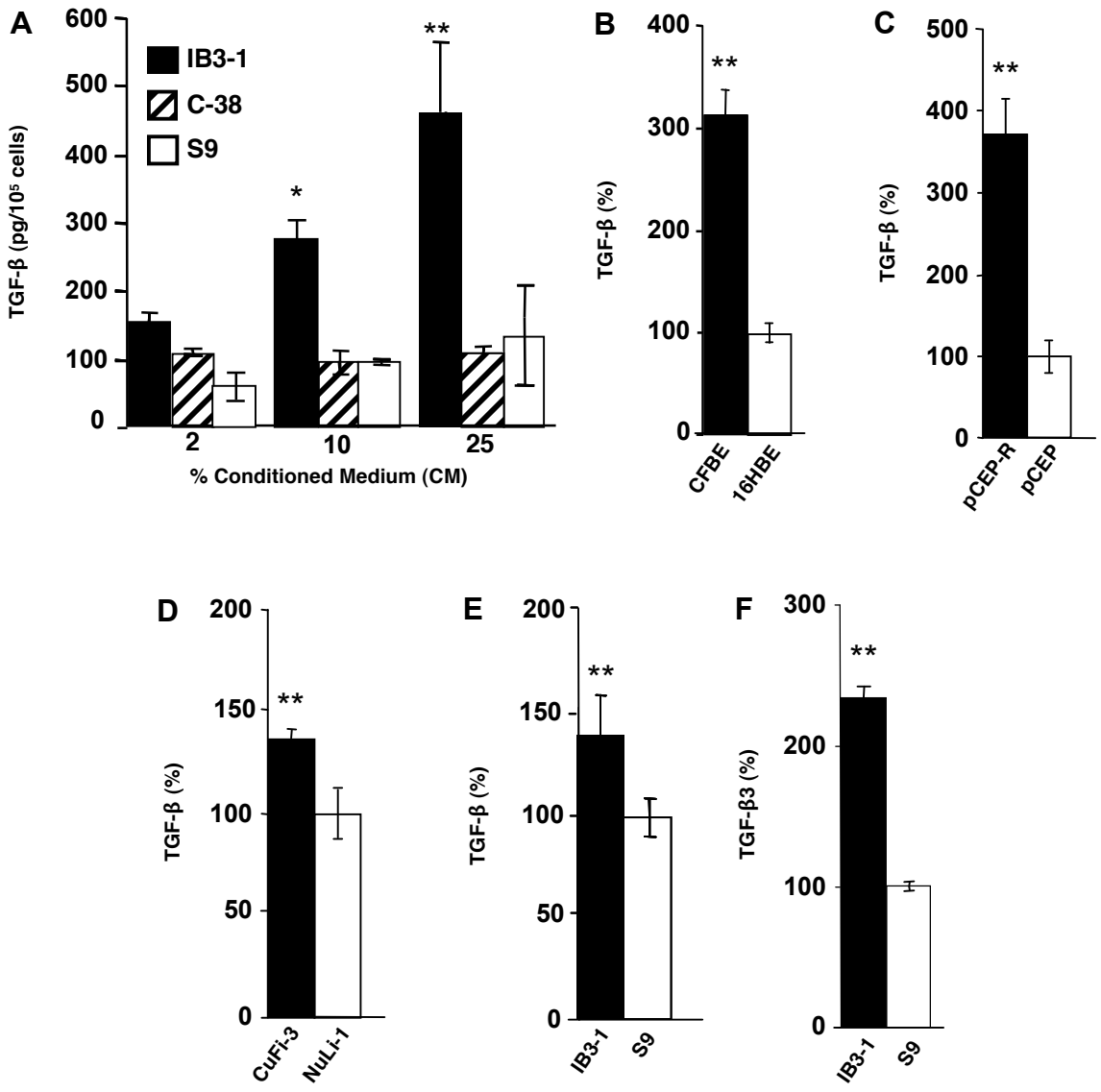
A
B
C
D
E
F
IB3-1
C-38
S9
TGF-β (pg/10⁵ cells)
% Conditioned Medium (CM)
TGF-β (%)
TGF-β3 (%)
CFBE
16HBE
pCEP-R
pCEP
CuFi-3
NuLi-1

Fig. S1

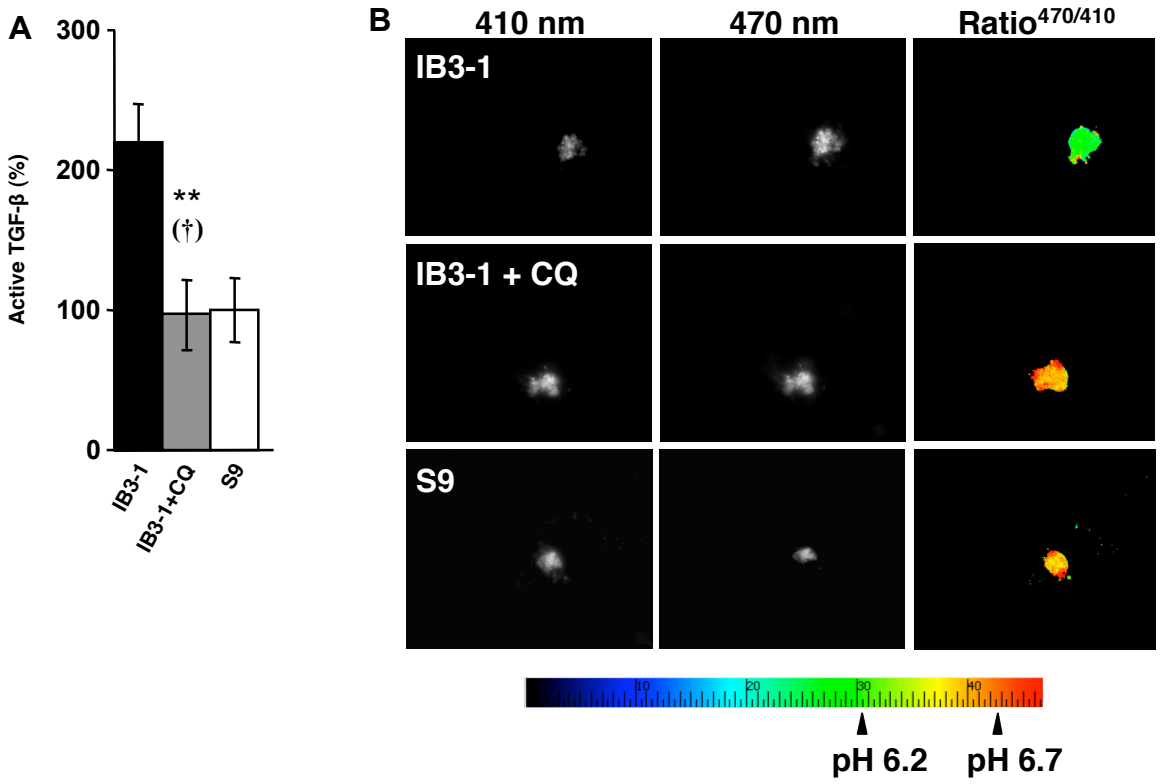
A
300
200
100
0
Active TGF-β (%)
**
(†)
IB3-1
IB3-1+CQ
S9
B
410 nm
470 nm
Ratio470/410
IB3-1
IB3-1 + CQ
S9
10
20
30
40
pH 6.2
pH 6.7

Fig. S2

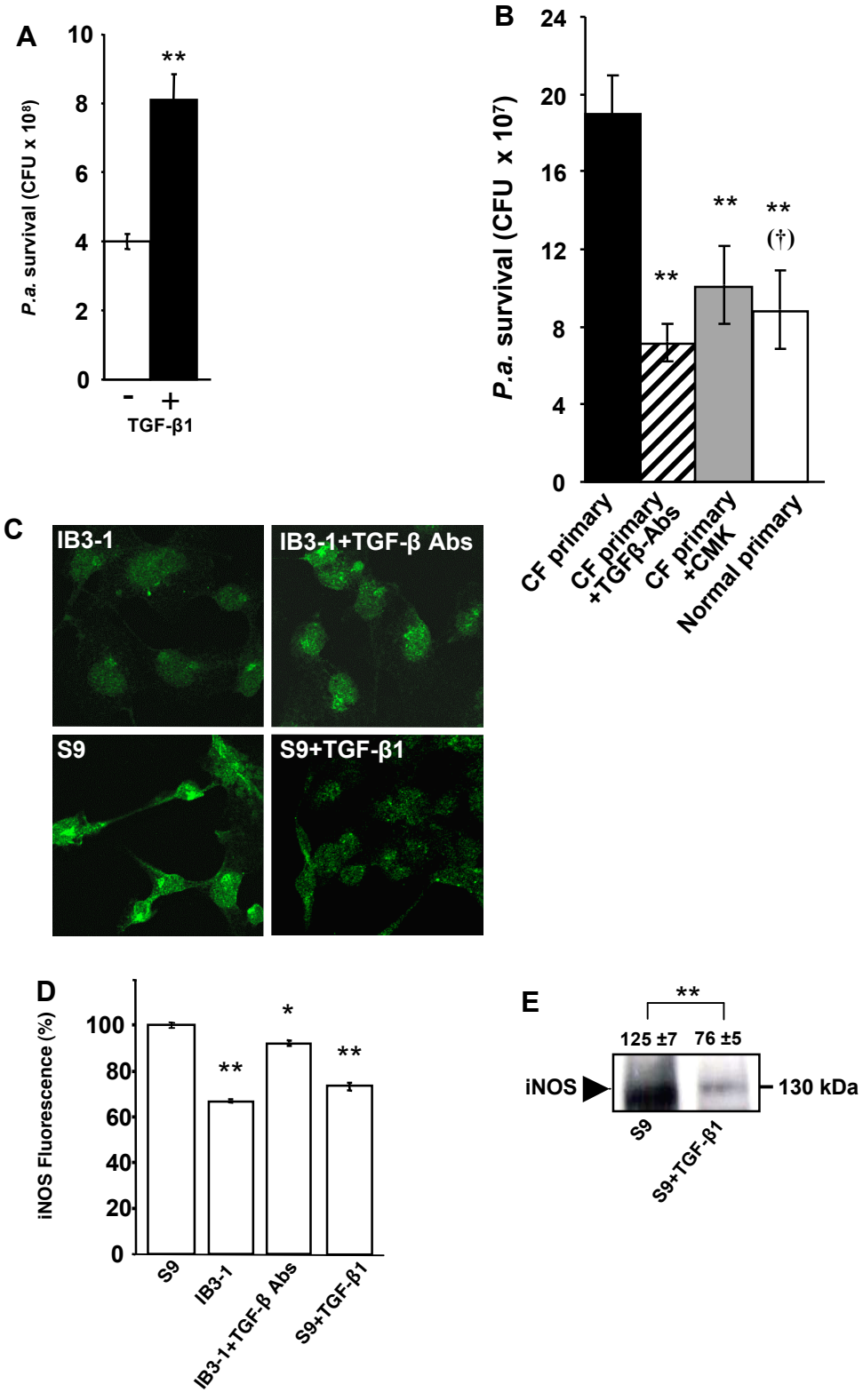
A
P.a. survival (CFU x 10⁸)
10
8
6
4
2
0
**
-
+
TGF-β1
B
P.a. survival (CFU x 10⁷)
24
20
16
12
8
4
0
**
**
**
(†)
CF primary
CF primary +TGFβ-Abs
CF primary +CMK
Normal primary
C
IB3-1
IB3-1+TGF-β Abs
S9
S9+TGF-β1
D
iNOS Fluorescence (%)
100
80
60
40
20
0
*
**
**
S9
IB3-1
IB3-1+TGF-β Abs
S9+TGF-β1
E
**
125 ±7
76 ±5
iNOS
130 kDa
S9
S9+TGF-β1

Fig. S3

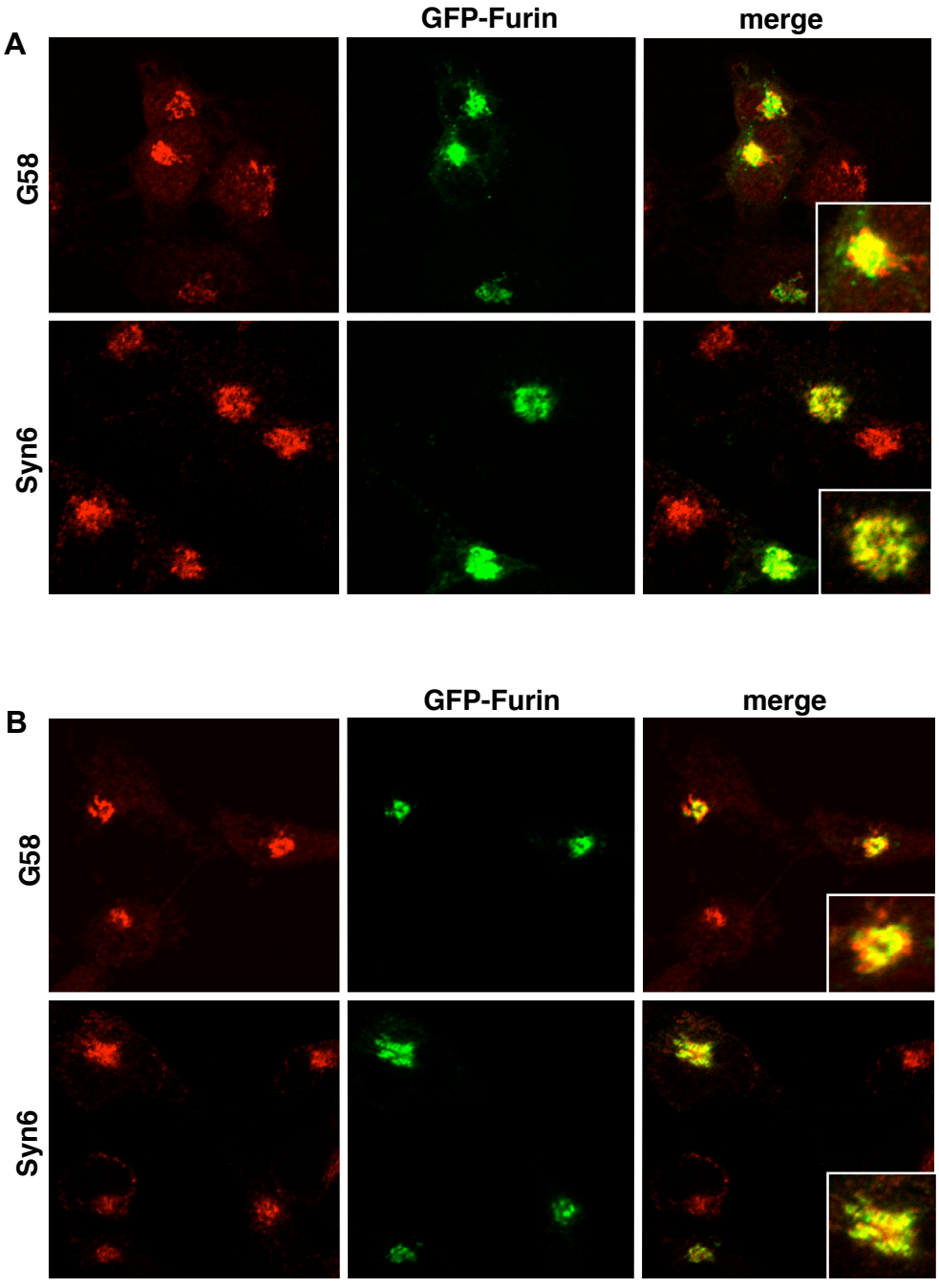

Fig. S4

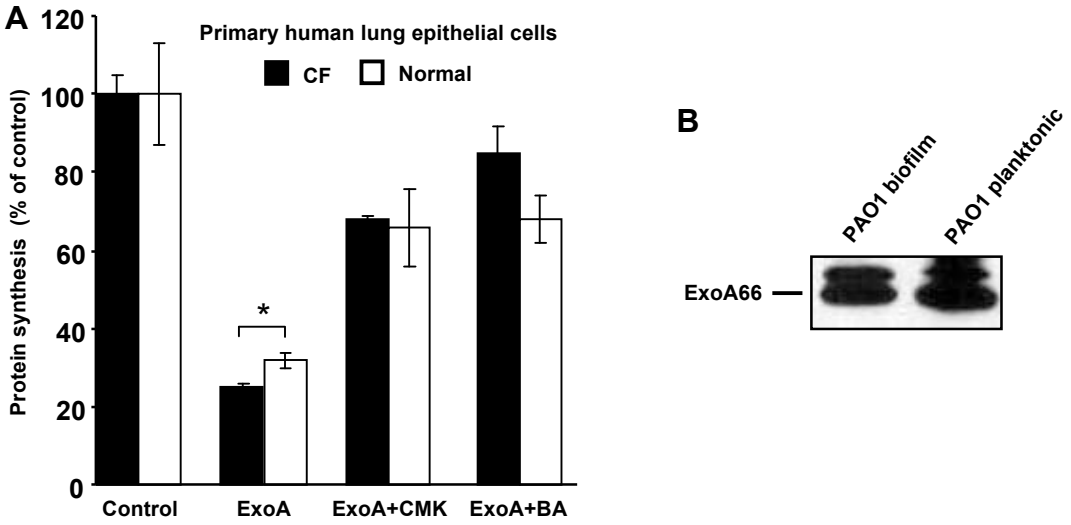
A
Primary human lung epithelial cells
CF
Normal
Protein synthesis (% of control)
120
100
80
60
40
20
0
*
Control
ExoA
ExoA+CMK
ExoA+BA
B
PAO1 biofilm
PAO1 planktonic
ExoA66

Fig. S5