Supplemental Information

A Young Hispanic with c.1646G>A Mutation Exhibits Severe Cystic Fibrosis Lung Disease: Is Ivacaftor an Option for Therapy?

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METHODS

Patient Characteristics

This individual received standard care at The University of Tennessee CF Research and Care Center at LeBonheur Children's Hospital. The medical record was analyzed retrospectively after expedited IRB approval (UTHSC 11-01576-XM).

Genotyping

Genotyping was performed at Ambry genetics (Aliso Viejo, CA) that showed ΔF508 mutation on one chromosome and S549N on the other.

Sweat Testing

Diagnostic sweat testing was performed by using pilocarpine iontophoresis from duplicate samples from right to left arms. Collection was conducted by the filter paper method according to CF Foundation /NCCLS guidelines (1). The chloride concentrations were measured by using a digital chloridometer (Labconco, Kansas City, MO) with a minimal sweat weight of 75 mg.

Tissue Culture

HEK-293 cells were used to express wild type (WT) and mutant CFTRs. The cells were cultured in DMEM/F12(1:1) (Invitrogen, Grand Island, NY) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. Human cystic fibrosis bronchial epithelial cells ($CFBEo^-$) were cultured in MEM (Invitrogen) medium supplemented with 10% FBS and 1% Penicillin/Streptomycin. The cells were maintained in 5% CO_2 incubator at 37 ºC.

Site-Directed Mutagenesis

pcDNA3-WT-CFTR was used to generate mutant S549N-CFTR and G551D-CFTR by using site-directed mutagenesis (Quikchange site-directed mutagenesis kit, Stratagene, La Jolla, CA). A Flag tag was also inserted at the fourth outer loop of full length WT and mutant CFTRs (the insertion of Flag tag does not affect CFTR channel function or its localization). All sequences were confirmed at Molecular Resource Center at The University of Tennessee Health Science

Center (UTHSC). HEK-293 cells and CFBEo$^-$ cells were transiently transfected with pcDNA3 containing WT- or mutant CFTR cDNA by using Lipofectamine 2000 (Invitrogen). The stable HEK-293 cells and CFBEo$^-$ cells expressing Flag-WT-CFTR or Flag-S549N-CFTR were generated by using puromycin selection (2 μg/ml).

Short-circuit Currents (I_{sc}) Measurements

CFBEo$^-$ cells stably expressing WT-CFTR or S549N-CFTR were grown on Costar® Transwell permeable supports (Cambridge, MA; filter area: 0.33cm²) until they reached a resistance of > 1400 Ω and then mounted in an Ussing chamber. The CFTR-mediated I_{sc} were measured as previously reported (2). Briefly, Epithelia were bathed in Ringer's solution (mM) (*Basolateral*: 140 NaCl, 5 KCl, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 10 Hepes, 10 glucose, pH 7.2, [Cl^-] = 149), and low Cl^- Ringer's solution (mM) (*Apical*: 133.3 Na-gluconate, 5 K-gluconate, 2.5 NaCl, 0.36 K_2HPO_4, 0.44 KH_2PO4, 5.7 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 10 Hepes, 10 mannitol, pH 7.2, [Cl^-] = 14.8) at 37°C, and gassed with 95% O_2 and 5% CO_2. Reagents were added as described in the corresponding figure legends.

Iodide Efflux Assay

HEK-293 parental cells were grown on poly-lysine coated 60-mm culture dishes and transiently transfected with WT-CFTR, S549N-CFTR, or G551D-CFTR by using Lipofectamine 2000. After 48 h, iodide efflux assay was performed as previously described (3). Briefly, cells were loaded for 60 min at room temperature with loading buffer (136 mM NaI, 137 mM NaCl, 4.5 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5 mM HEPES, pH 7.2). Extracellular NaI was washed away thoroughly (7 times) with efflux buffer (136 mM $NaNO_3$ replacing 136

mM NaI in the loading buffer) and cells were equilibrated for 1 min in a final 1-ml aliquot. The first four aliquots were used to establish a stable base line in efflux buffer alone. Agonists were added to the efflux buffer and samples were collected every minute for 6 min in the continued presence of agonists (i.e., the efflux buffer used for subsequent replacements also contained agonists at the same concentration). The iodide concentration in each sample was measured by using an iodide selective electrode (Orion research Inc., Beverly, MA) and the efflux rates were reported as nanomoles (nmol)/min. HEK-293 parental cells were used as negative controls.

Immunoprecipitation and Western Blotting

HEK-293 cells were transiently transfected with WT or mutant CFTR and lysed at 48 h posttransfection in lysis buffer (PBS containing 0.2% Triton-X-100 and protease inhibitors: 1mM phenylmethylsulfonylfluoride, 1μg/ml pepstatin, 1μg/ml leupeptin and 1μg/ml aprotinin). The lysate was centrifuged (16000 × *g* for 10 min at 4˚C) and the clear supernatant was used for immunoprecipitation of CFTRs by using α 24-1 IgG mab (R&D Systems, Minneapolis, MN) crosslinked to protein A/G beads (Santa Cruz, Santa Cruz, CA). The beads were washed three times with lysis buffer. The proteins were eluted from the beads in SDS-PAGE sample buffer, subjected to SDS-PAGE on 4-15% gel (Bio-Rad, Hercules, CA), transferred to PVDF membrane, and probed for CFTR with α NBD-1-R polyclonal antibody.

Surface Labeling Assay and Immunofluorescence Microscopy

HEK-293 cells expressing Flag-tagged WT-CFTR or S549N-CFTR were grown on 35-mm cell culture dishes, fixed with 3.7% formaldehyde for 10 min, blocked with 1% BSA for 30 min, and then treated with α-Flag HRP (0.2μg/ml) for 90 min. The HRP substrate (1-step Ultra TMB,

Pierce) was added to the dishes for 10 min and the reaction was stopped by adding equal amount of 2M H_2SO_4 (4). Samples were collected and the absorbances were measured at 450 nm on FLUOstar Omega plate reader (BMG labtech).

For immunofluorescence microscopy, HEK-293 cells expressing Flag-tagged WT-CFTR or S549N-CFTR were grown on poly-lysine coated 35-mm glass-bottomed dishes, fixed with 3.7% formaldehyde for 10 min, blocked with 1% BSA for 30 min, and incubated with mouse α-Flag antibody (1:200 dilution) for 1 h. After washing, the cells were incubated with Alexa-Fluor 488 rabbit α-mouse IgG (1:50 dilution) for 1 h and then subjected to confocal imaging on a Carl-Zeiss LSM5 PASCAL confocal microscope (Thornwood, NY).

Statistical Analysis

Statistical analyses were performed by using Student's *t*-test and P value < 0.05 was considered significant (*).

References

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and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proc Natl Acad Sci U S A* 2003;100:342-346.

4. Penmatsa H, Zhang W, Yarlagadda S, Li C, Conoley VG, Yue J, Bahouth SW, Buddington RK, Zhang G, Nelson DJ, Sonecha MD, Manganiello V, Wine JJ, Naren AP. Compartmentalized cyclic adenosine 3',5'-monophosphate at the plasma membrane clusters PDE3A and cystic fibrosis transmembrane conductance regulator into microdomains. *Mol Biol Cell* 2010;21:1097-1110.

Figure Legends

Supplemental Figure 1. (Axial view) chest computed tomography (CT) shows extensive bilateral upper lobe bronchiectasis (arrows, L>R).

Supplemental Figure 2. S549N-CFTR did not exhibit Cl^- channel function. (A) The ribbon diagram of NBD1 structure of CFTR. S549N mutation occurs in the signature sequence (LSGGQ). (B) S549N-CFTR did not exhibit Cl^- channel function as evidenced in iodide (I^-) efflux assay. HEK-293 cells were transiently transfected with WT- or S549N-CFTR cDNA. PKA activating agonists (10 μM forskolin, 100 μM IBMX and 200 μM cpt-cAMP) were added to activate CFTR channel. (C) The maximal iodide efflux rate at 2-min after addition of PKA activating agonists quantified from experiments shown in (B). n = 3, ns: not significant.

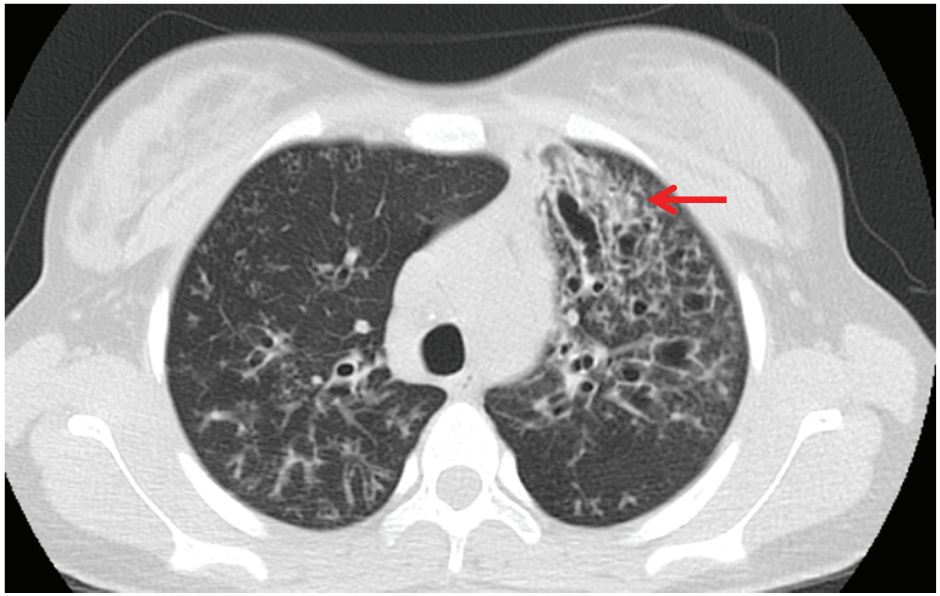
Supplemental Figure 3. S549N-CFTR is expressed at the plasma membrane of cells. (A) Immunofluorescence micrographs of stable HEK-293 cells expressing Flag-WT-CFTR (positive

control) or Flag-S549N-CFTR. The cells were grown in poly-lysine coated culture dishes, treated with mouse α-Flag antibody followed by labeling with Alexa-Fluor 488 rabbit α-mouse IgG, and subjected to imaging. HEK-293 parental cells were used as negative control. (B) The results from surface labeling assays show that S549N-CFTR is expressed at the plasma membrane of cells. HEK-293 cells transfected with Flag-WT-CFTR or Flag-S549N-CFTR were grown on poly-lysine coated culture dishes and treated with α-Flag antibody conjuagted to HRP. HRP substrate 1-step Ultra TMB was then added and the reaction was stopped after 10-min with H_2SO_4. Samples were collected and absorbance was measured at 450 nm. HEK-293 parental cells transfected with pcDNA3 empty vector were used as negative control. n =3.

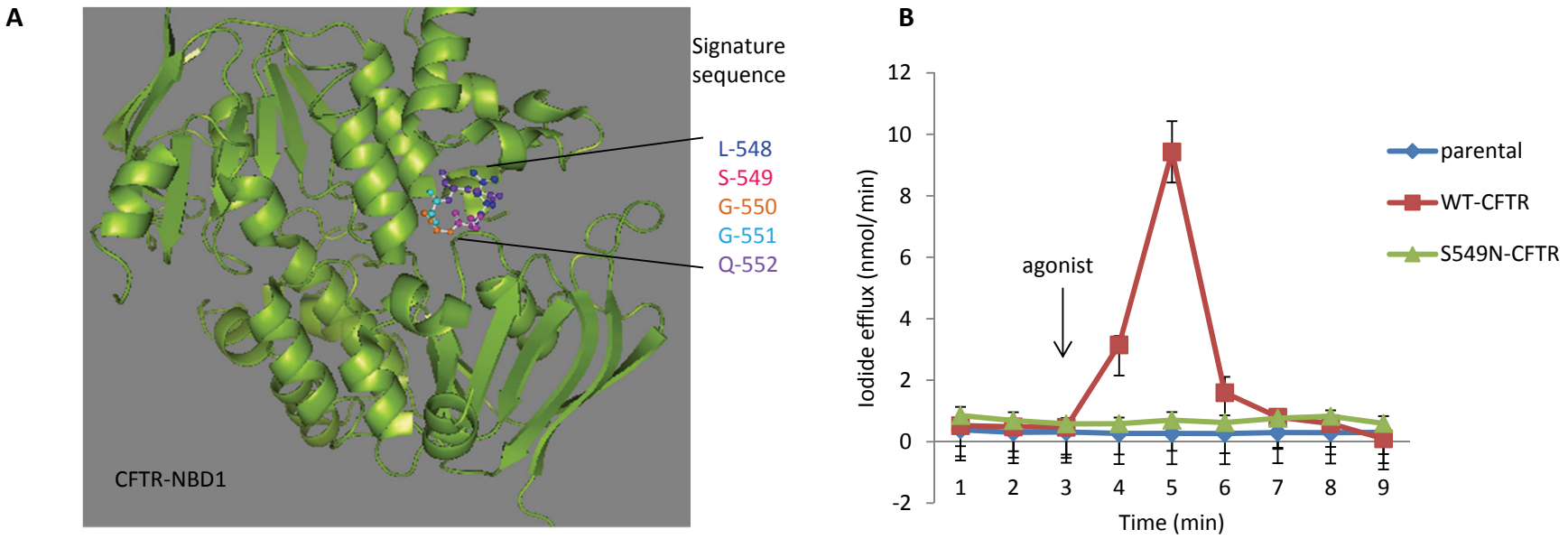
Supplemental Figure 4. CFTR potentiator P1 restored the Cl^- channel function of S549N-CFTR. (A) CFTR potentiator P1 restored the channel function of S549-CFTR and G551D-CFTR. HEK-293 cells expressing Flag-WT-CFTR, Flag-S549N-CFTR or Flag-G551D-CFTR were grown in poly-lysine coated plates with or without P1 treatment (200 μM). HEK-293 parental cells were used as negative control. n = 3. (B) The maximal iodide efflux rate at 2-min after addition of PKA activating agonists for cells expressing WT- or S549N-CFTR with or without P1 treatment. The data were quantified from experiments shown in (A). (C) P1 does not increase S549N-CFTR expression level. The cells were lysed at the end of iodide efflux assays and subjected to immunoprecipitation and Western blotting for CFTR by using α NBD-1-R polyclonal antibody.

Supplemental Figure 5. CFTR potentiator restored Cl^- channel function of S549N-CFTR expressed in CFBEo$^-$ cells. The cells were grown on Costar® Transwell permeable supports until

they reached a resistance of more than 1400 Ω and then mounted in an Ussing chamber. Forskolin (FSK) and $CFTR_{inh}$-172 were added at the apical side of the cell monolayers. Ivacaftor and P1 were added to both sides of cell monolayers. DMSO was used as a negative control. (A) FSK (10 μM) and Ivacaftor (100 μM) were used to activate WT-CFTR expressed in $CFBEo^-$ cells. (B) P1 (100 μM) potentiated the channel function of S549N-CFTR in the presence of FSK (10 μM). $CFTR_{inh}$-172 (20 μM) was used to verify the responses observed were CFTR-dependent.



Supplemental figure 1



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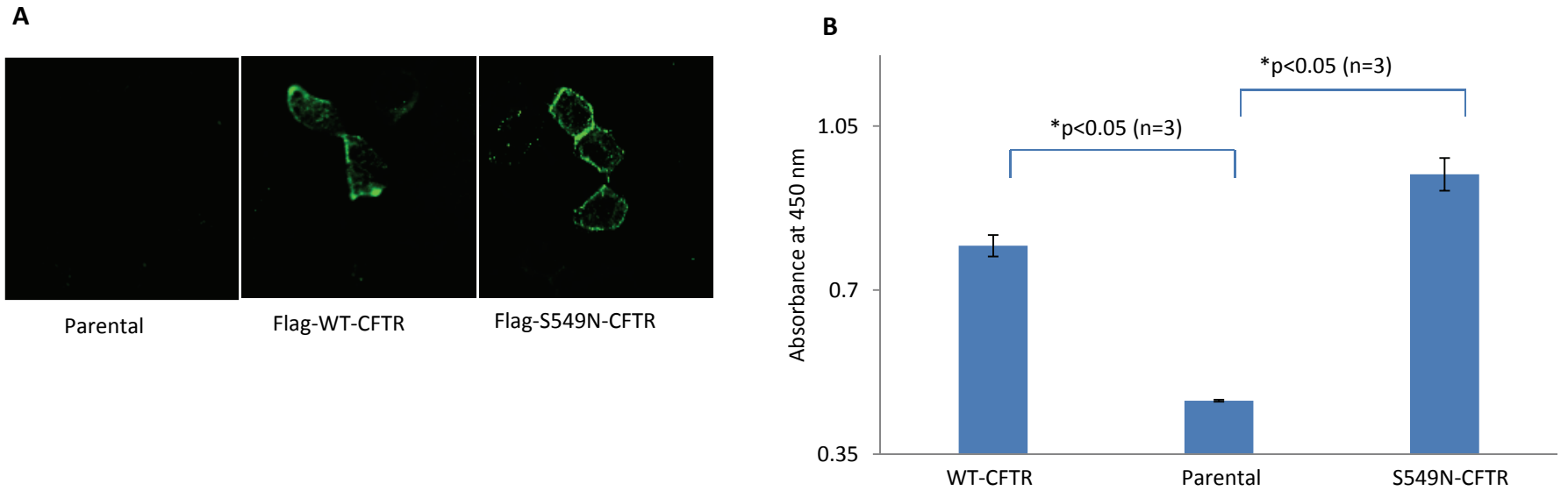

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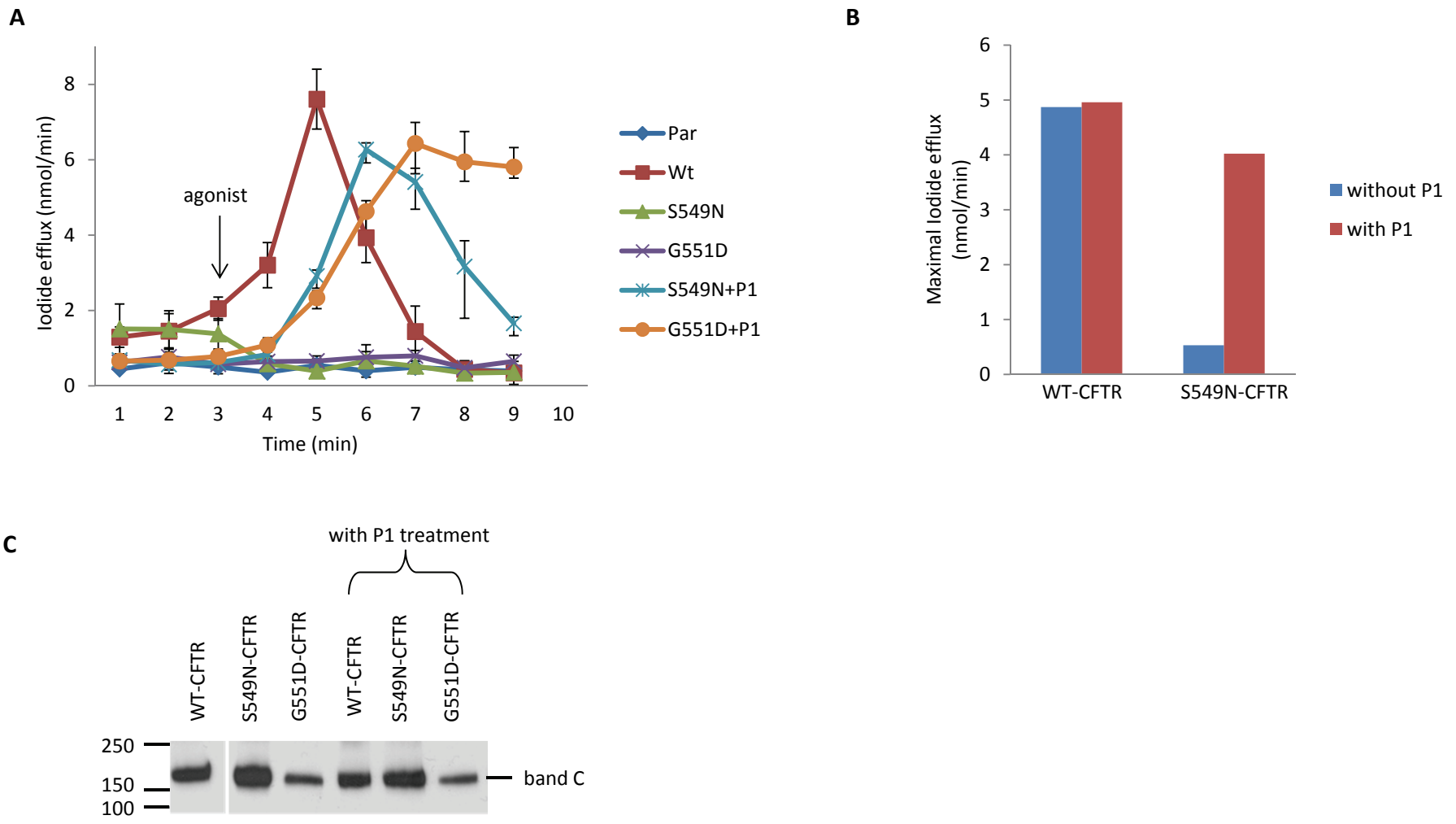
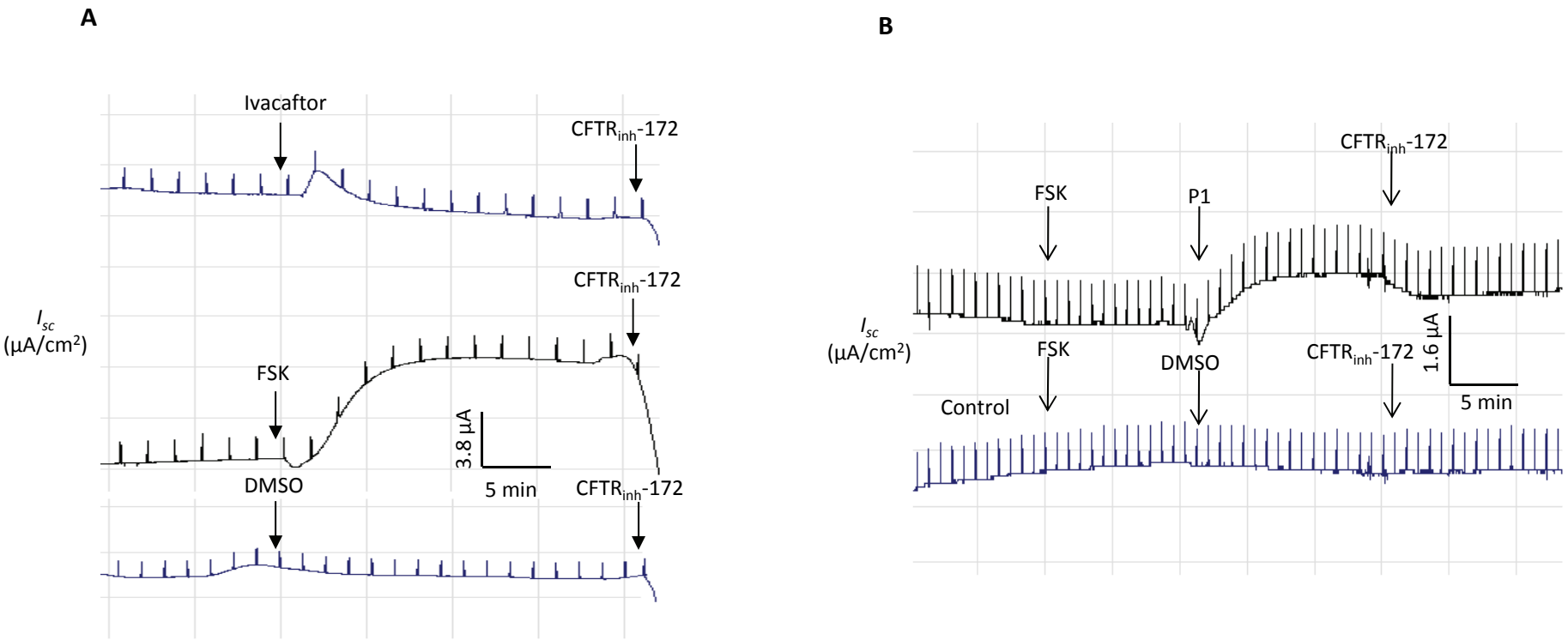


Supplemental figure 4



Supplemental figure 5