Science Advances
AAAS

advances.sciencemag.org/cgi/content/full/6/8/eaax9914/DC1

Supplementary Materials for

IRE1α kinase–mediated unconventional protein secretion rescues misfolded CFTR and pendrin

Hak Park, Dong Hoon Shin, Ju-Ri Sim, Sowon Aum, Min Goo Lee*

*Corresponding author. Email: mlee@yuhs.ac

Published 19 February 2020, *Sci. Adv.* **6**, eaax9914 (2020)
DOI: 10.1126/sciadv.aax9914

This PDF file includes:

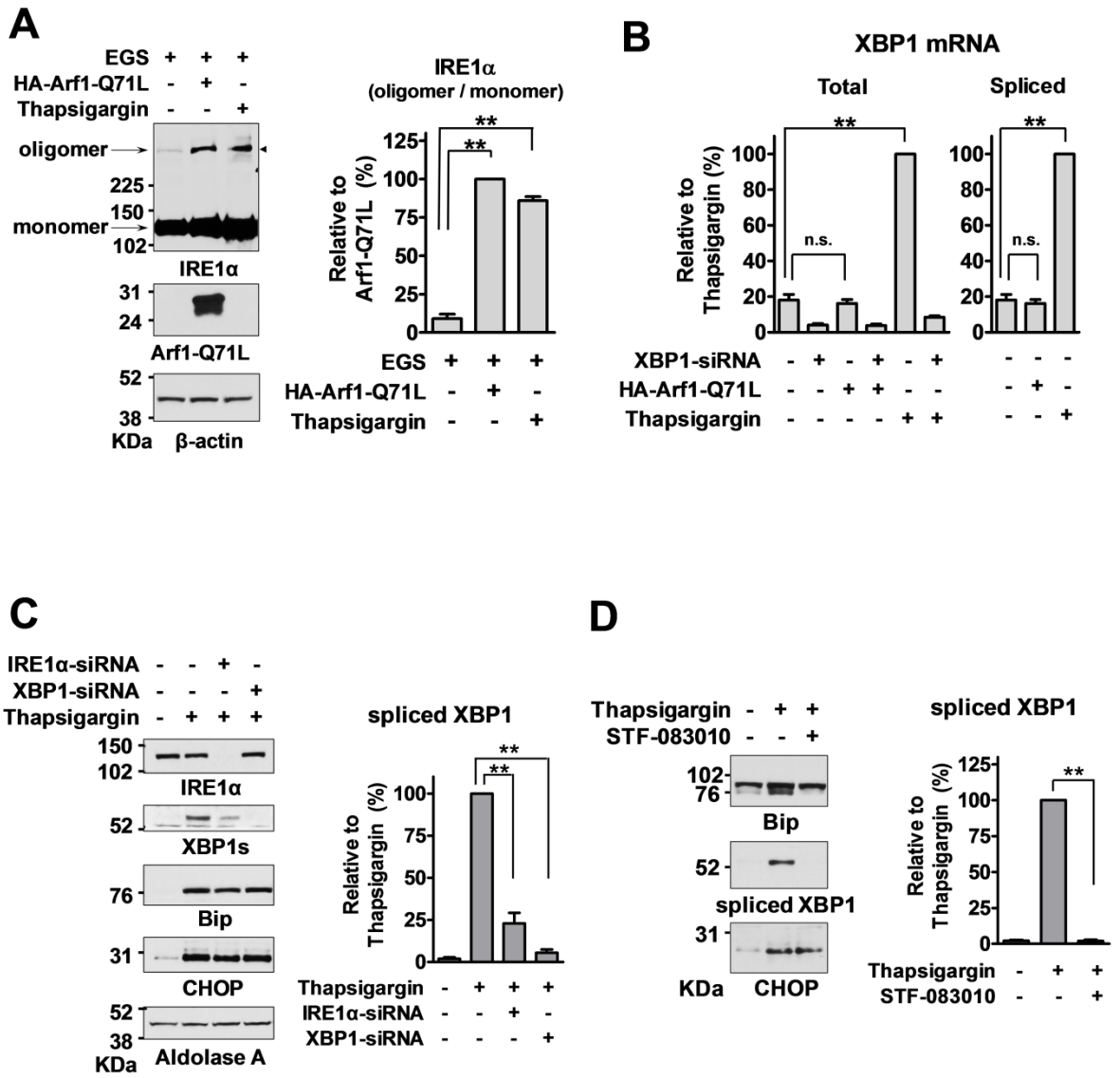

Fig. S1. XBP1 splicing and IRE1α endonuclease activity are not involved in UPS of ΔF508-CFTR (control experiments of Fig. 1). (**A**) Analysis of IRE1α oligomerization. HEK293 cells were transfected with plasmids encoding for Arf1-Q71L (48 h) or treated with thapsigargin (5 μM, 12 h) to induce ER stress, and then incubated with the cross-linking reagent EGS (500 μM, 30 min). Arrowheads indicate the cross-linked IRE1α oligomeric (upper band) and monomeric (lower band) forms of IRE1α. Representative immunoblots are shown in the left panel and the results of multiple experiments are summarized in the right panel (n = 3). (**B**) The mRNA quantification of total *XBP1* (left) and spliced *XBP1* (right) was performed by quantitative RT-PCR as detailed in the Materials and Methods. HEK293 cells were transfected with Arf1-Q71L plasmids (48 h) or treated with thapsigargin (5 μM, 12 h). Some cells were pretreated with XBP1-specific siRNA for 48 h. The results of multiple experiments are summarized (n = 3). Thapsigargin, but not Arf1-Q71L, increased both total and spliced *XBP1* mRNA levels. (**C**) Effects of IRE1α- and XBP1-specific siRNAs on XBP1 splicing. HEK293 cells were treated with thapsigargin (5 μM, 12 h) and/or transfected with the indicated siRNAs (100 nM, 48 h). Thapsigargin increased the protein level of spliced XBP1 (XBP1s), which was reduced by siRNAs against IRE1α or XBP1. Representative immunoblots are shown in the left panel and the quantification results of multiple experiments are summarized in the right panel (n = 3). (**D**) STF-083010 inhibits the RNase activity of IRE1α. HEK293 cells were treated with thapsigargin (5 μM, 12 h) and/or treated with STF-083010 (60 μM, 12 h), followed by immunoblot analysis for the XBP1s protein. Representative blots are shown in the left panel and the results of multiple experiments are summarized in the right panel (n = 3). Bar graph data are shown as the mean ± SEM. $^{**}P < 0.01$.

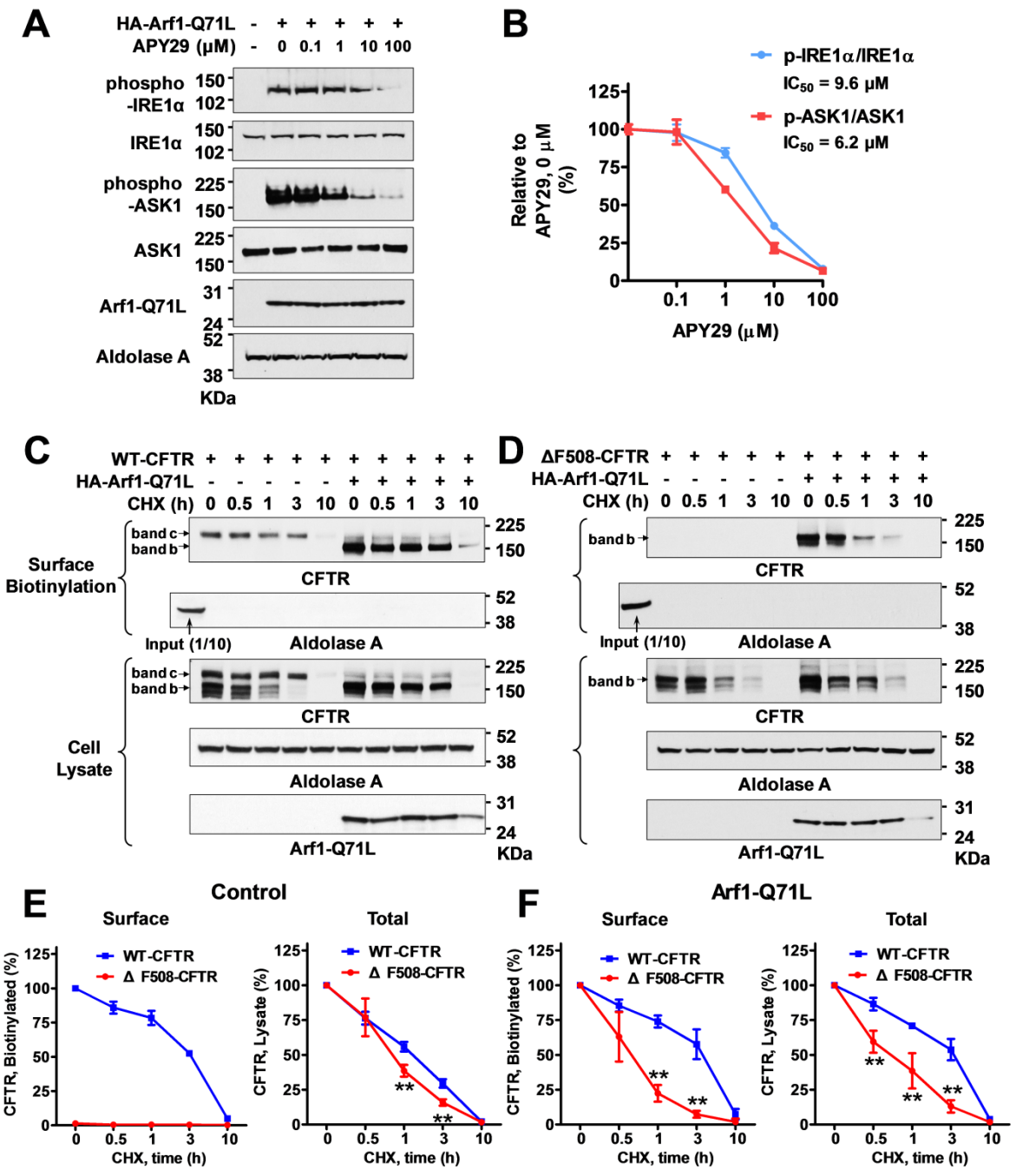

Fig. S2. IRE1α kinase–ASK1 pathway is required for UPS of ΔF508-CFTR (control experiments of Fig. 2). (**A** and **B**) APY29 inhibits phosphorylation of IRE1α and ASK1 in a dose-dependent manner. Phosphorylation of IRE1α and ASK1 was induced by transfection with Arf1-Q71L plasmids (48 h) and identified with phospho-specific antibodies. Representative immunoblots are shown in **A** and the results of multiple experiments are summarized in **B** (n = 3). The IC_{50} values of APY29 on IRE1α and ASK1 phosphorylation were 9.6 and 6.2 μM, respectively. (**C**–**F**) Protein stability of WT- and ΔF508-CFTR was analyzed in HEK293 cells treated with the protein synthesis inhibitor cycloheximide. Cells were transfected with plasmids encoding for WT- or ΔF508-CFTR (48 h). Some cells were co-transfected with Arf1-Q71L plasmids to induce UPS. Surface biotinylation assays were performed after incubation with cycloheximide (0.1 mg/mL) for the indicated time periods. Representative results of WT-CFTR and ΔF508-CFTR are shown in **C** and **D**, respectively. Quantification of the CFTR levels at the cell-surface and total lysates in control and Arf1-Q71L-transfected cells are summarized in **E** and **F**, respectively (each n = 3). Protein stability of ΔF508-CFTR is lower than WT-CFTR under all conditions. Data are shown as the mean ± SEM. $^{**}P < 0.01$, compared to WT-CFTR.

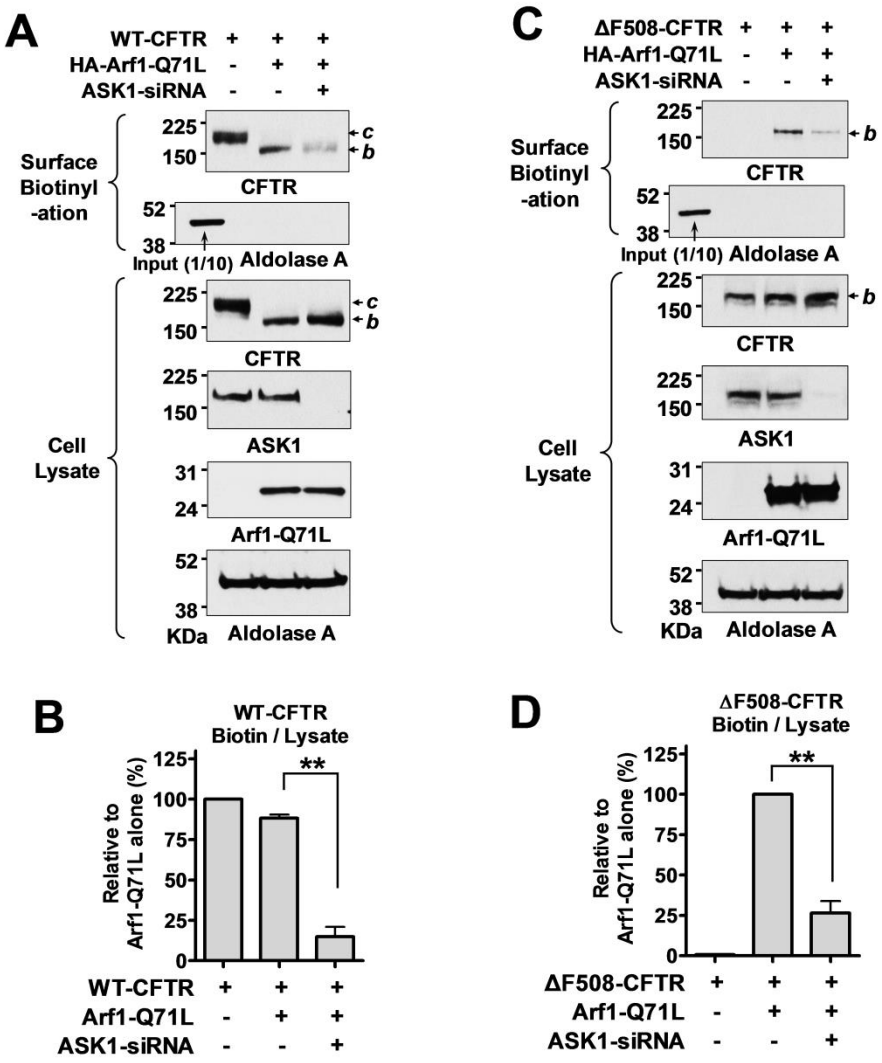

Fig. S3. Depletion of ASK1 inhibits Arf1-Q71L–induced UPS of CFTR. Surface biotinylation assays were performed with co-transfection of ASK1-specific siRNA (100 nM, 48 hours). Representative surface biotinylation results of WT-CFTR and ΔF508-CFTR are presented in **A** and **C**, respectively. The results of multiple experiments are summarized in **B** and **D** (both n = 3). *b*, core-glycosylated CFTR. *c*, complex-glycosylated CFTR. Bar graph data are shown as the mean ± SEM. $^{**}P < 0.01$.

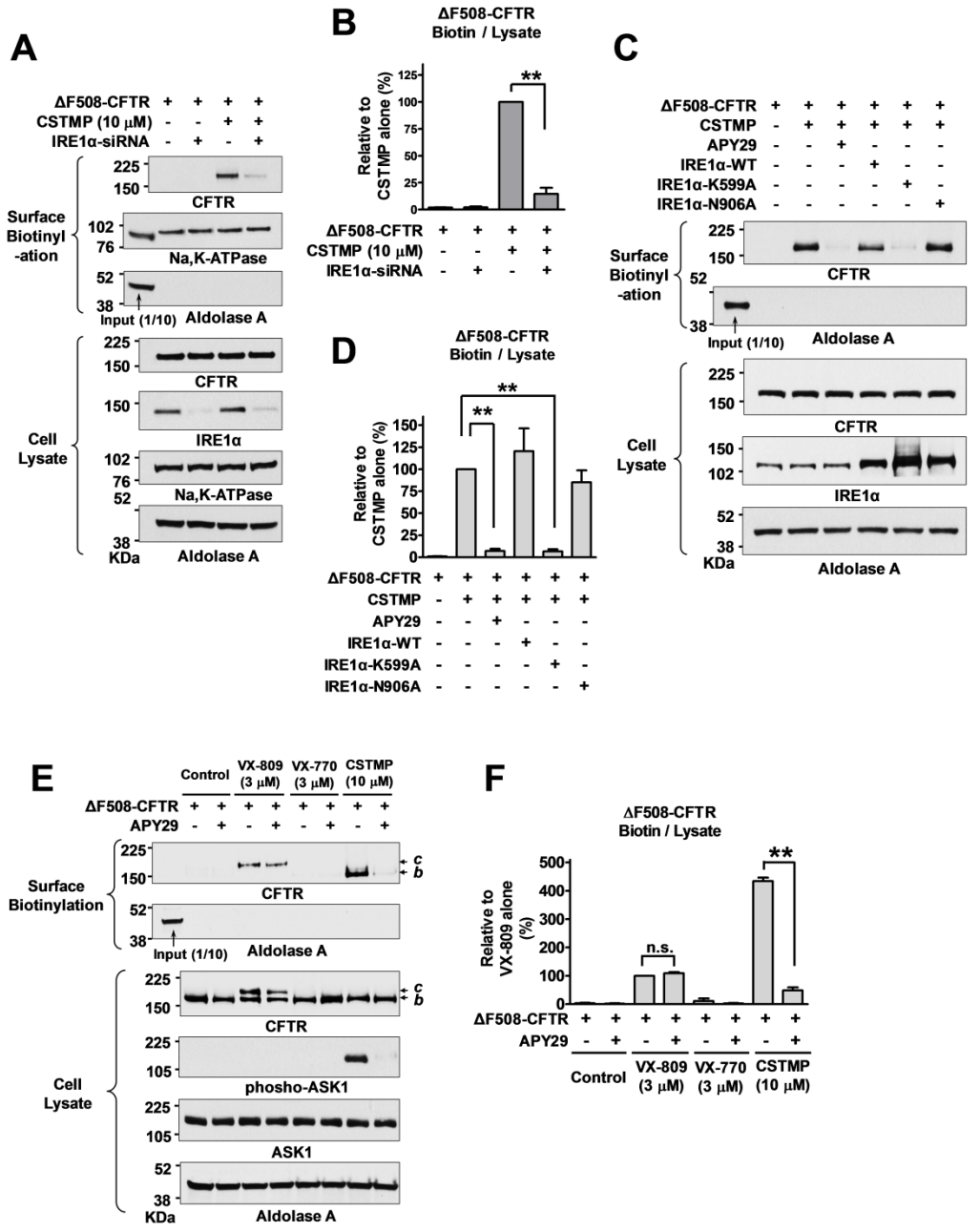

Fig. S4. CSTMP stimulates the cell surface expression of ΔF508-CFTR by activating IRE1α kinase. (**A** and **B**) Depletion of IRE1α inhibited the CSTMP-induced UPS of ΔF508-CFTR. Surface biotinylation assays were performed with co-transfection of IRE1α-specific siRNA (100 nM, 48 hours). Representative surface biotinylation results of ΔF508-CFTR are presented in **A**, and the results of multiple experiments are summarized in **B** (n = 3). Cell surface-specific labeling of proteins was confirmed by the presence of the plasma membrane protein Na,K-ATPase and the absence of the cytosolic protein aldolase A in the biotinylated fraction. (**C** and **D**) Overexpression of IRE1α kinase-dead mutant, but not its endonuclease-dead mutant, abolished the CSTMP-induced rescue of ΔF508-CFTR. Surface biotinylation assays were performed with co-expression of the IRE1α kinase-dead mutant (IRE1α-K599A) and IRE1α RNase-dead mutant (IRE1α-N906A). APY29 was used as a positive control for inhibiting the CSTMP effects. Representative surface biotinylation assays are presented in **C**, and the results of multiple experiments (n = 3) are summarized in **D**. (**E** and **F**) Comparison of the ΔF508-CFTR rescue effects induced by VX-809 and CSTMP. Surface biotinylation assays were performed in HEK293 cells treated with the CFTR corrector VX-809 (10 μM) and CSTMP (10 μM) for 24 h. Representative surface biotinylation results are shown in **E**, and the results of multiple experiments (n = 3) are summarized in **F**. VX-809 evoked the cell-surface expression of ΔF508-CFTR band C protein, which was not inhibited by APY29. $^{**}P < 0.01$, n.s., not significant. *b*, core-glycosylated CFTR. *c*, complex-glycosylated CFTR. Bar graph data are shown as the mean ± SEM.

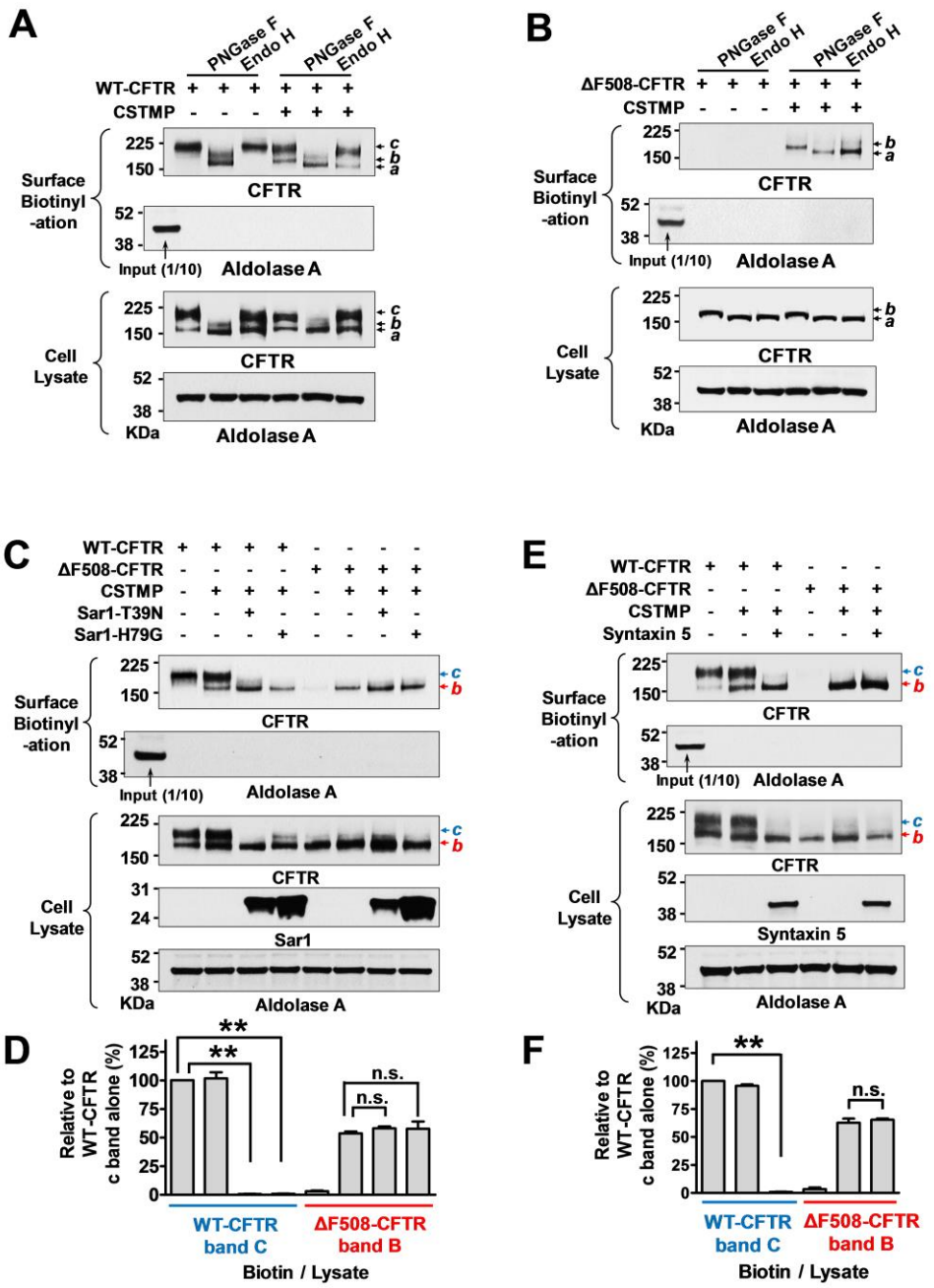

Fig. S5. CSTMP induces the cell surface expression of ΔF508-CFTR via UPS. (A and **B)** The N-glycosylation status of CFTR was analyzed. Both the total cell lysates and biotinylated fractions were digested with N-glycosidase F (PNGase F), which removes all N-linked carbohydrates, or with Endoglycosidase H (Endo H), which removes the ER-mediated N-linked carbohydrates. The cell-surface expressed band B (*b*) forms of WT- and ΔF508-CFTRs were Endo H-sensitive Three independent experiments showed similar results. (**C** and **D**) Dominant-inhibitory mutants of Sar1-GTPase (Sar1-T39N and Sar1-H79G), which inhibit conventional COPII-mediated ER-to-Golgi trafficking, were co-transfected and surface biotinylation assays were performed. Representative surface biotinylation results are presented in **C** and the results of multiple experiments (n = 3) are summarized in **D**. Sar1 mutants did not block the CSTMP-induced, cell-surface expression of band B forms of WT- and ΔF508-CFTRs. (**E** and **F**) Syntaxin 5 was overexpressed to induce Golgi acceptor blockade and surface biotinylation assays were performed. Representative surface biotinylation results are presented in **E** and the results of multiple experiments (n = 3) are summarized in **F**. Syntaxin 5 overexpression did not block the CSTMP-induced, cell-surface expression of band B forms of WT- and ΔF508-CFTRs. Data are shown as the mean ± SEM. *a*, deglycosylated CFTR, *b*, core-glycosylated CFTR. *c*, complex-glycosylated CFTR. $^{**}P < 0.01$, n.s., not significant.

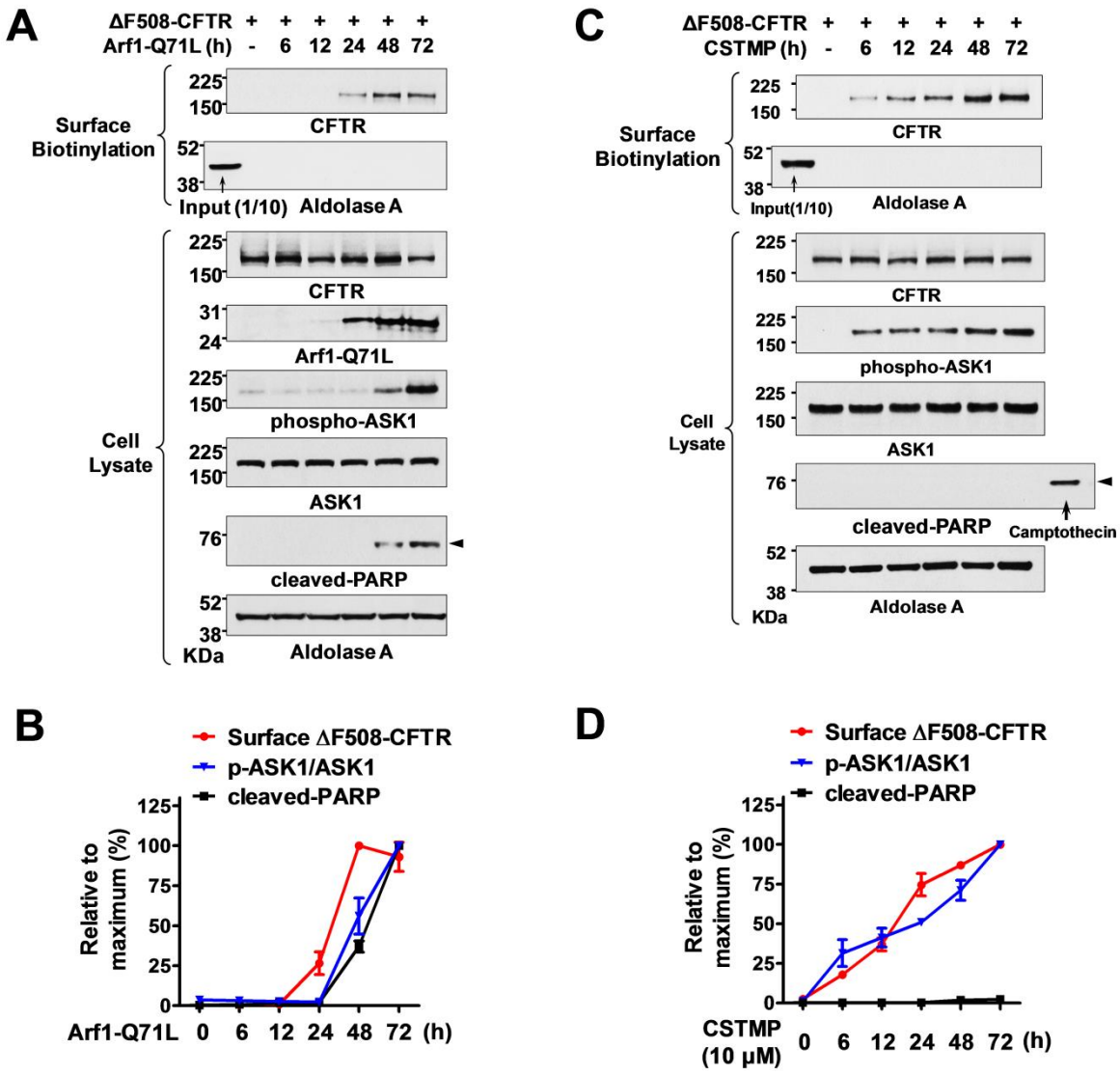

Fig. S6. CSTMP (10 μM) does not evoke apoptotic signal. (**A** and **B**) Time-series effects of ectopic Arf1-Q71L on the UPS of ΔF508-CFTR, activation of ASK1, and PARP cleavage were analyzed. The results of multiple experiments are summarized in **B** (n = 6). Transfection with plasmids encoding Arf1-Q71L for >48 h evoked PARP cleavage (arrowhead). (**C** and **D**) Time-series effects of CSTMP (10 μM) on the UPS of ΔF508-CFTR, activation of ASK1, and PARP cleavage were analyzed. The results of multiple experiments are summarized in **D** (n = 6). CSTMP (10 μM) did not evoke PARP cleavage up to 72 h. Camptothecin (2 μM, 12 h) was used as a positive control to induce the cleavage of PARP (arrowhead). ASK1 phosphorylation correlated with the CSTMP-induced UPS of ΔF508-CFTR (**B** and **D**). Line graph data are shown as the mean ± SEM.

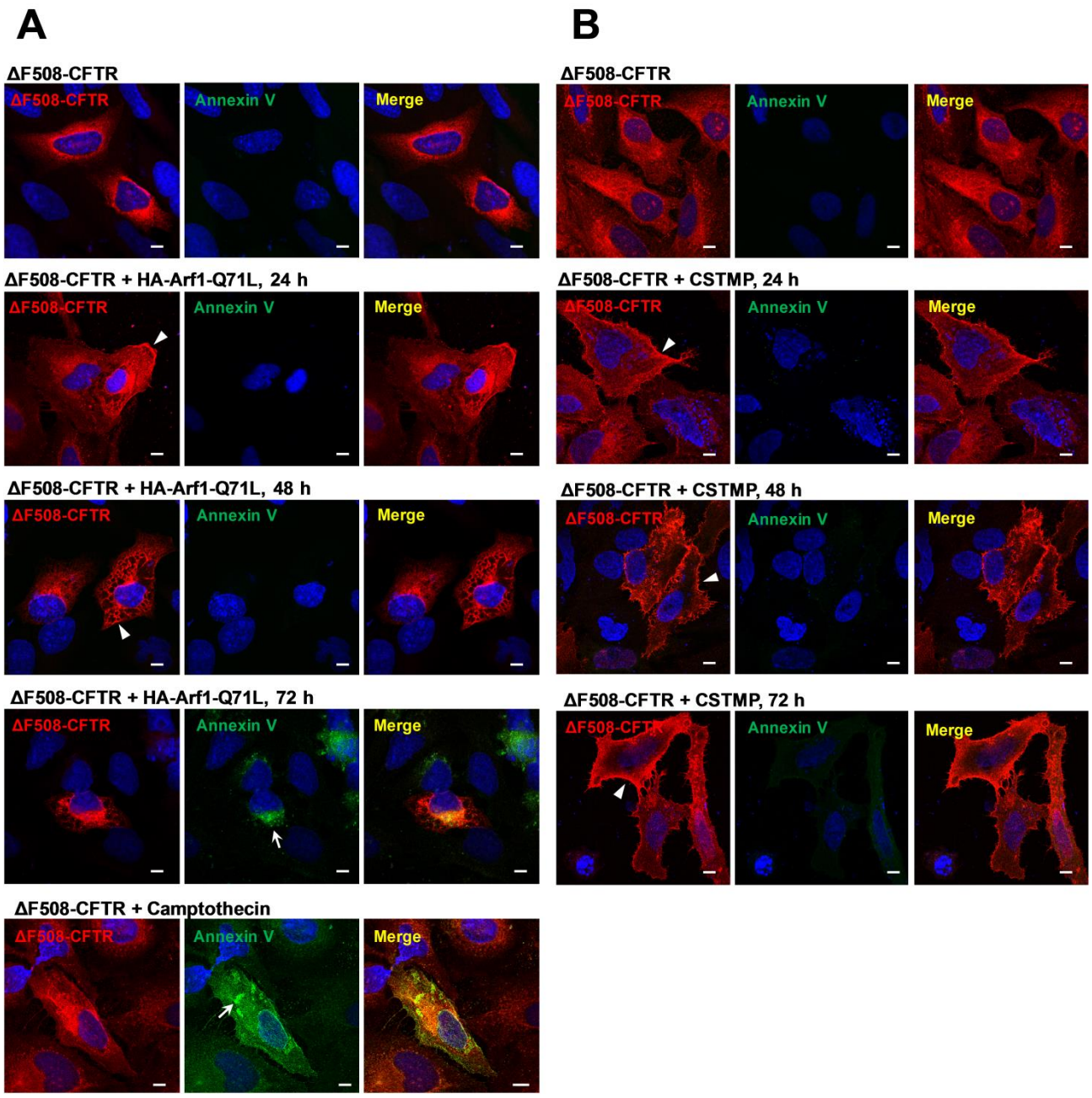

Fig. S7. CSTMP induces the cell surface expression of ΔF508-CFTR without evoking cellular apoptosis. (**A**) Prolonged overexpression of Arf1-Q71L evokes cellular apoptosis. HeLa cells were transfected with ΔF508-CFTR plasmid alone or co-transfected with Arf1-Q71L plasmid for 24, 48, or 72 h. Live cells were labeled with the apoptosis marker annexin V (green) before fixation. Anti-CFTR (M3A7) antibodies (red) were used for immunostaining after fixation and permeabilization. Arrowheads indicate the cell-surface expressed CFTR. Transfection with the Arf1-Q71L plasmid for 72 h evoked expression of annexin V (arrow). Cells treated with camptothecin (2 μM, 12 h) were used as a positive control for apoptosis. (**B**) HeLa cells were transfected with ΔF508-CFTR and incubated with 10 μM CSTMP for 0, 24, 48, or 72 h. CSTMP treatment initiated UPS of ΔF508-CFTR at 24 h (arrowhead) but did not evoke apoptosis up to 72 h. Three independent sets of experiments showed similar results. Scale bar: 10 μm.

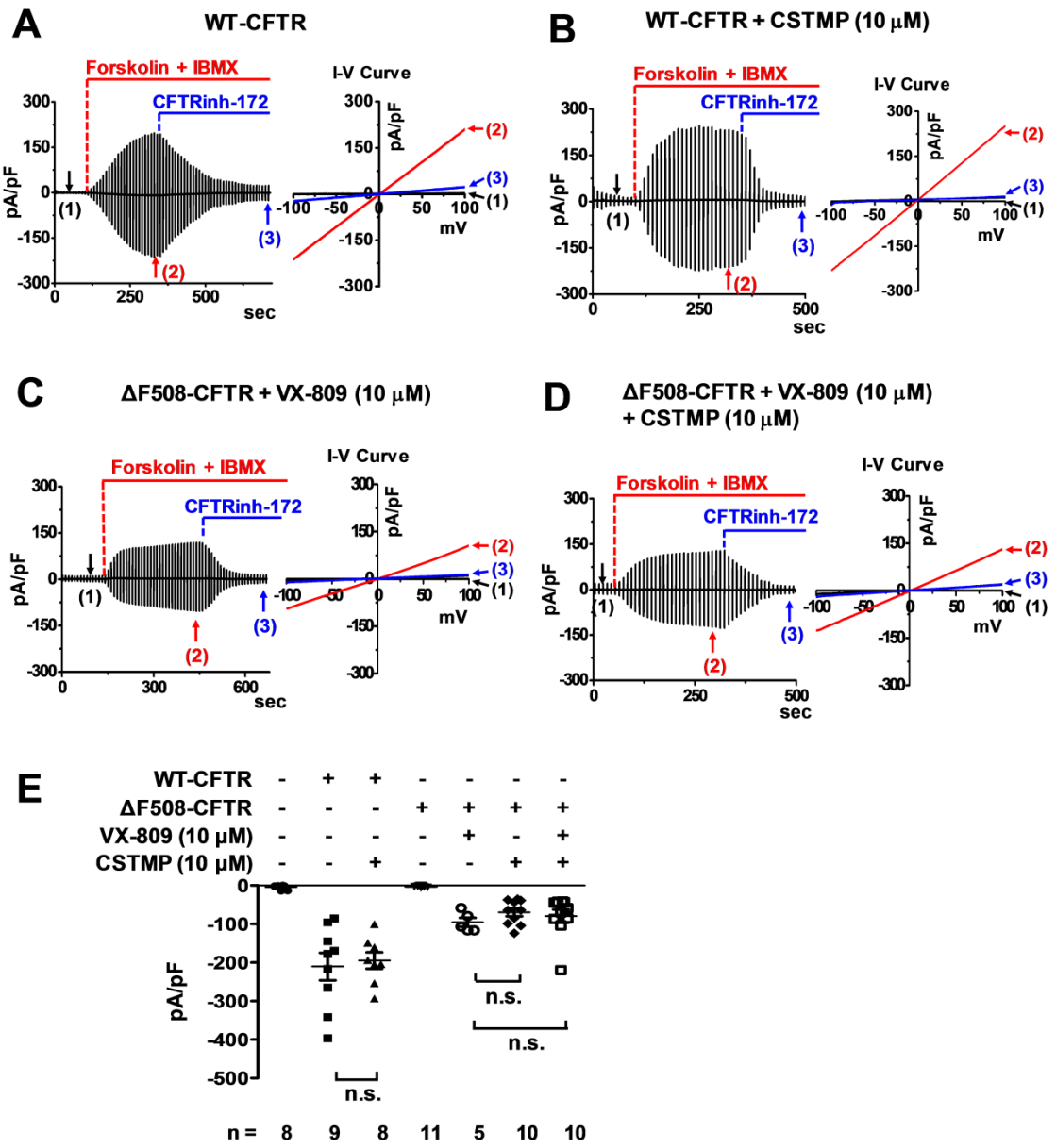

Fig. S8. Measurements of CFTR Cl^- channel activity (control experiments of Fig. 4, C to F). Whole-cell currents were measured in cells expressing WT- or ΔF508-CFTR after treatment with CSTMP and/or VX809 using the protocols described in Fig. 4. CFTR Cl^- currents were activated by cAMP (forskolin 5 μM + IBMX 100 μM) and inhibited by $CFTR_{inh}$-172 (5 μM). Representative whole-cell currents are shown in **A**–**D**. A summary of current densities measured at -80 mV is shown in **E**. Treatment with CSTMP (10 μM) did not significantly affect WT-CFTR Cl^- currents (**A**, **B**, and **E)**. Treatment with VX-809 (10 μM) evoked cAMP-induced Cl^- currents in cells expressing ΔF508-CFTR similar to CSTMP (10 μM) treatment. Combination with VX-809 (10 μM) and CSTMP (10 μM) did not show additive effects. (**C**-**E**). n, number of replicates, n.s. not significant.

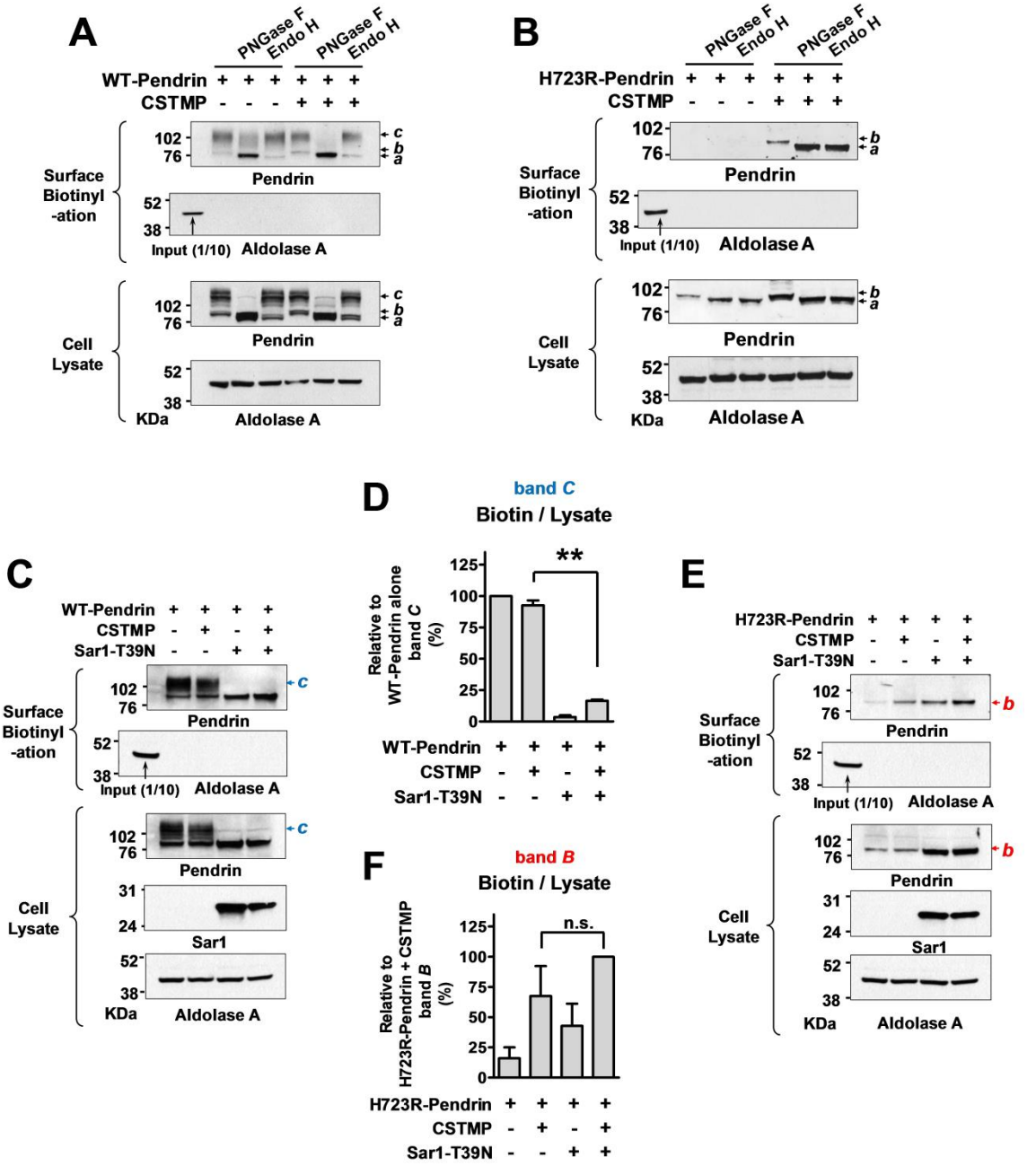

Fig. S9. CSTMP induces the cell surface expression of p.H723R-pendrin via UPS. (**A**, **B**) The N-glycosylation status of pendrin was analyzed. PANC-1 cells were transfected with plasmids encoding WT- or p.H723R-pendrin and further incubated with CSTMP (30 μM) for 48 h. Both the total cell lysates and biotinylated fractions were digested with N-glycosidase F (PNGase F) and Endoglycosidase H (Endo H). The cell-surface expressed band B (*b*) form of p.H723R-pendrin was Endo H-sensitive, while the band C (*c*) form of WT-pendrin was Endo H-resistant. Three independent experiments showed similar results. (**C**–**F**) Surface biotinylation assays were performed in PANC-1 cells with co-transfecting plasmids expressing Sar1-T39N, which inhibit COPII-mediated ER-to-Golgi transport. Representative surface biotinylation assay results are presented in **C** (WT-pendrin) and **E** (p.H723R-pendrin). Results of multiple experiments (each n = 3~4) are summarized in **D** (WT-pendrin) and **F** (p.H723R-pendrin). Sar1-T39N did not block the CSTMP-induced, cell-surface rescue of p.H723R-pendrin, but it blocked the cell surface expression of band C form of WT-pendrin. The ER-to-Golgi blockade by Sar1-T39N also evoked UPS of WT- and p.H723R-pendrins. *a*, deglycosylated pendrin, *b*, core-glycosylated pendrin. *c*, complex-glycosylated pendrin. Data are shown as the mean ± SEM. $^{**}P < 0.01$, n.s., not significant.

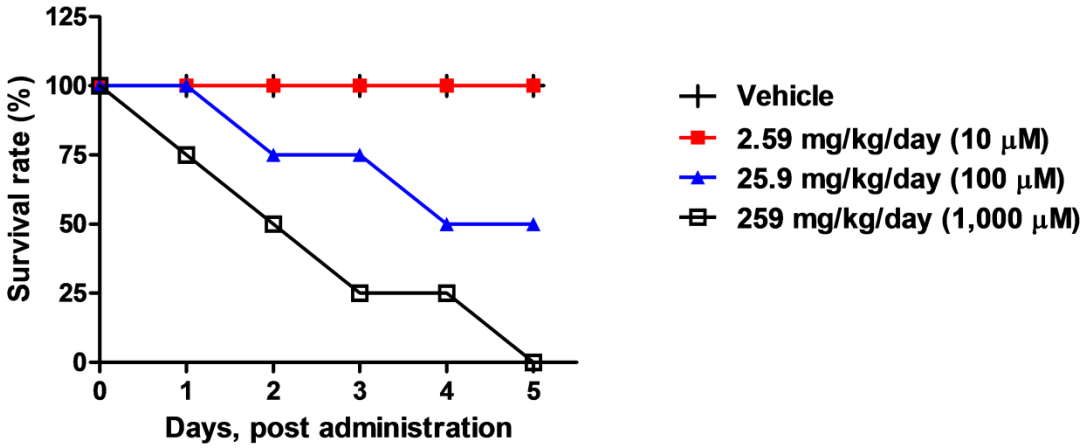

Fig. S10. The LD_{50} value of CSTMP in mice (per os) is 25.9 mg/kg per day. Four groups of four wild-type ($Cftr^{WT}$) mice (12 weeks old) received oral administration of vehicle (saline) or CSTMP at a dose of 2.59 mg/kg (equivalent to 10 μM, assuming CSTMP is evenly distributed throughout the body), 25.9 mg/kg (equivalent to 100 μM), or 259 mg/kg (equivalent to 1,000 μM) once daily for 5 days. The survival curves were plotted. The LD_{50} value was estimated to be 25.9 mg/kg/day. No mice died on the 2.59 mg/kg/day dosing schedule.