Glucose-sensitive CFTR suppresses glucagon secretion by potentiating K_{ATP} channels in pancreatic islet α cells

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DISCLOSURE STATEMENT: The authors have nothing to disclose.edu.hk

Abstract

The secretion of glucagon by islet α cells is normally suppressed by high blood glucose but this suppressibility is impaired in diabetes or patients with cystic fibrosis (CF), a disease caused by mutations in the gene encoding CFTR, a cAMP-activated Cl^- channel. However, precisely how glucose regulates glucagon release remains controversial. Here we report that elevated glucagon secretion, together with increased glucose-induced membrane depolarization and Ca^{2+} response, is found in CFTR mutant (DF508) mice/islets compared to the wildtype. Overexpression of CFTR in AlphaTC1-9 cells results in membrane hyperpolarization and reduced glucagon release, which can be reversed by CFTR inhibition. CFTR is found to potentiate K_{ATP} channel since membrane depolarization and whole-currents sensitive to K_{ATP} blockers are significantly greater in wildtype/CFTR-overexpressed α cells compared to that in DF508/non-overexpressed cells. K_{ATP} knockdown also reverses the suppressive effect of CFTR-overexpression on glucagon secretion. The results reveal that by potentiating K_{ATP} channels, CFTR acts as a glucose-sensing negative regulator of glucagon secretion in α cells, defect of which may contribute to glucose intolerance in CF and other types of diabetes.

cystic fibrosis | diabetes | glucagon | islet α cells |CFTR

Opposing the actions of β-cell-secreted insulin, glucagon secreted by pancreatic islet α cells plays an essential role in blood glucose maintenance, by promoting gluconeogenesis and glycogenolysis in response to blood glucose deprivation or hypoglycemia to increase blood glucose level (1,2). In addition to stimulating insulin secretion, high blood glucose concentrations inhibit glucagon release via incompletely understood mechanisms thought to involve both direct action on α cells and paracrine effect exerted by factors released by neighboring cells (2,3). A proper response of glucagon regulation to blood glucose level is a necessary guarantee for glucose homeostasis. Dysregulated glucagon production and/or release, apart from insulin insufficiency, also results in metabolic disorders/diseases, such as hyperglycemia, hypoglycemia and diabetes mellitus (4-6). However, precisely how glucose regulates glucagon release and whether the secretion is controlled by a mechanism intrinsic to islet α cells remain controversial (7,8).

Diabetes is the most common non-pulmonary complication in patients with cystic fibrosis (CF) (9). CF-related diabetes (CFRD), which is distinct from T1DM and T2DM, is associated with increased morbidity and mortality, with a prevalence up to 50% in CF patients aged 30 years (9-11). CFRD patients are characterized by impaired glucose tolerance, which is attributed to defects in both islet β and α cell functions, with impaired insulin secretion and decreased suppressibility of pancreatic glucagon secretion (12-15). It is generally believed that the gradual deterioration of the pancreatic islet structure due to defective exocrine pancreatic secretion and the obstruction of pancreatic duct underlies the pathophysiology of glucose intolerance in CF (11,16). However, our recent study has discovered a functional role of CFTR in pancreatic islet β cells and insulin secretion (17). We found that CFTR channel in pancreatic β cells can be

62 activated by glucose and that its Cl^- efflux contributes to the glucose-induced membrane
63 depolarization and action potentials leading to Ca^{2+} influx required for insulin secretion, defect
64 of which results in impaired and delayed glucose-induced insulin secretion, as observed in CFRD
65 patients (11,17). CFTR has been found to be expressed in glucagon-secreting human and rodent
66 α cells (18,19) and recently implicated in the regulation of glucagon secretion (19). However,
67 how exactly CFTR regulates glucagon secretion remains elusive and the effect of CFTR on
68 glucagon secretion has not been demonstrated *in vivo* at organism level. Using a CFTR mutant
69 (DF508) mouse model (20) and a CFTR-overexpressing AlphaTC1-9 cell line (21,22), we
70 undertook the present study to explore the role of CFTR in the regulation of glucagon secretion
71 by α cells. The results demonstrate that CFTR negatively regulates glucagon secretion by
72 potentiating K_{ATP} channels, defect of which results in excessive glucagon secretion found in
73 CFTR mutant (DF508) mice/ islets. These results suggest that in addition to the previously
74 reported defect in insulin secretion by the β cells (17), dysregulated glucagon secretion due to
75 CFTR mutations in α cells may also contribute to the glucose intolerance found in CF patients
76 and the pathogenesis of CFRD.

77

78 **Results**

79 **Elevated glucagon levels in CFTR mutant mice**

80 To explore the role of CFTR in regulating blood glucagon level, we made use of a CFTR mutant
81 mouse model with DF508 mutation (20), the major CFTR mutation found in over 70% of CF
82 patients (23). After fasting for over 12 hours, DF508 mice showed significantly higher blood

glucagon levels, as measured by ELISA, compared to that of the wildtype (Fig.1a). We also conducted glucose tolerance test (GTT) in these mice and measured blood glucagon levels at 10 and 30 minutes after glucose administration. As shown in Fig.1a, DF508 mice exhibited consistently higher levels of glucagon as compared to that of the wildtype. This *in vivo* result suggests that CFTR mutation may lead to dysregulated blood glucagon levels, contributing to glucose intolerance in CF.

Mutation or inhibition of CFTR increases glucagon secretion by isolated mouse islets *ex vivo*

To further investigate the role of CFTR in pancreatic islet α cells and exclude the contribution from other sources, we performed *ex vivo* experiments in isolated mouse islets. Significant suppression of glucagon release was observed in wildtype islets when treated with a high level of glucose (6 mM), compared to that with 1 mM (Fig.1b). At both glucose concentrations, treatment of the islets with a CFTR inhibitor, Inh172 (10μM), for 2 hours significantly elevated the glucagon levels (Fig.1b), which were comparable to that observed in DF508 islets (Fig.1b). These results are consistent with a suppressive role of CFTR in glucagon secretion.

Overexpression of CFTR suppresses glucagon secretion in AlphaTC1-9 cell line

Since insulin is known to inhibit glucagon release (24), the above observed glucagon increase in isolated islets, either with CFTR mutation or inhibition, might not be due to a direct effect of CFTR on α cells, but secondary to its direct effect on insulin secretion by the β cells as previously reported (17). To investigate a direct effect of CFTR on glucagon release, we overexpressed CFTR in a mouse α cell line, AlphaTC1-9, which has been used in the studies for glucagon secretion (21,22). As shown in Fig.2a and Fig.2b, CFTR was successfully overexpressed, at both protein and channel function levels. Since CFTR channel function in α cells has not been

reported, we used the patch-clamp technique to examine the whole-cell currents in CFTR-overexpressing AlphaTC1-9 cells. The K^+ in the pipette solution was replaced by Cs^+, leaving Cl^- as the major permeant ion that mediates the whole-current detected. Since CFTR can be activated by cAMP, we used forskolin (10 μM), an activator of adenylyl cyclase, to stimulate the cells and detected significant current activation, with time and voltage-independent whole-cell current profile and a linear I-V relationship typical of CFTR, in CFTR-overexpressing AlphaTC1-9 cells but not the vector-control cells (Fig.2b). The forskolin-induced currents could be blocked by CFTR inhibitor, GlyH (10μM) (Fig.2b), indicative of CFTR currents. When bathed in 1 mM glucose incubation bath, the CFTR-expressing cells showed significantly reduced glucagon release as compared to the cells transfected with empty vectors. The CFTR overexpression-induced glucagon suppression was significantly reversed by a CFTR inhibitor GlyH (10μM), shown in Fig.2c. Taken together, the results obtained from CFTR-overexpressing α cells are consistent with those from CFTR mutant mice and isolated islets, revealing a role of CFTR in suppression of glucagon secretion.

CFTR suppresses glucose-deprivation-induced Ca^{2+} increase

Physiologically, glucose induces a series of complex electrical activities in islet α cells, including membrane depolarization and Ca^{2+} influx, which are known to be required for glucagon release through exocytosis (25). However, the underlying mechanism remains elusive.

We first compared the changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) between DF508 and wildtype mouse islet α cells in response to glucose deprivation, i.e. switching the glucose concentration in the perfusion solution from high (6 mM) to low (1 mM). Since α cells are known to be located at the periphery of mouse islets (26-28), we selected islet peripheral cells

as α cells for Ca^{2+} imaging (Fig.3a), and at the end of each experiment, added adrenaline to confirm that the observed $[Ca^{2+}]_i$ activities were from α cells (Fig.3a) since adrenaline is known to induce $[Ca^{2+}]_i$ increases only in α cells, but not other cell types in the islet (29). As shown in Fig.3a, both wildtype and DF508 cells showed an increase in $[Ca^{2+}]_i$ in response to a change in extracellular glucose from 6 to 1 mM. However, the glucose-deprivation-induced $[Ca^{2+}]_i$ response was found to be significantly higher in DF508 islet α cells as compared to that in wildtype cells (Fig.3a). These results suggest that CFTR exerts an suppressive effect on the glucose-deprivation-induced Ca^{2+} increase; whereas mutation (DF508) of CFTR can remove the suppressive effect of CFTR resulting in an increased $[Ca^{2+}]_i$ response.

CFTR suppresses glucose-deprivation-induced membrane depolarization

Since membrane depolarization is known to activate voltage-sensitive Ca^{2+} channels and induce Ca^{2+} entry, the inhibitory effect of CFTR on glucose-deprivation-induce Ca^{2+} response might be due to its effect on membrane potential (Vm). We therefore also compared the changes in Vm induced by glucose deprivation in isolated islets from DF508 and wildtype mice. Again, islet peripheral cells were selected as α cells for Vm measurement. As shown in Fig.3b, deprivation of glucose (from 6 to 1 mM) gave rise to membrane depolarization (increases in Dibac signal) in both wildtype and DF508 islet peripheral cells. It should be noted that glucose reduction results in membrane hyperpolarization in β cells (17), and thus, the observed depolarization in response to glucose deprivation in these islet peripheral cells should be originated from α cells but not β cells. Similar to the Ca^{2+} response, DF508 islet α cells, as shown in Fig.3b, exhibited significantly greater changes in Dibac intensities corresponding to a more depolarizing response (30.35 ± 0.84 mV) upon glucose deprivation (6 to 1 mM) as compared with wildtype cells (7.94 ±

149 0.45 mV). The larger membrane depolarization in response to glucose deprivation observed in
150 DF508 α cells are consistent with the greater glucose deprivation-induced Ca^{2+} response
151 observed in DF508 α cells compared to wildtype (Fig.3a).

152 **CFTR is activated by glucose and conducts Cl^- efflux**

153 How does CFTR contribute to the glucose-sensitive Vm change in α cells? Since CFTR is a Cl^-
154 channel, its activation by glucose may induce Cl^- flux affecting the membrane potential (Vm),
155 i.e., Cl^- influx resulting in hyperpolarization or Cl^- efflux in depolarization. We first examined the
156 glucose sensitivity of CFTR currents in CFTR-overexpressing AlphaTC1-9 cells using the patch-
157 clamp technique. The K^+ in the pipette solution was replaced by Cs^+, leaving Cl^- as the major
158 permeant ion that mediates the whole-current detected. Addition of 20 mM glucose
159 significantly enhanced the whole-currents, similar to that induced by forskolin, which were
160 abolished by subsequent addition of GlyH (10μM, Fig.4a), indicating activation of CFTR by
161 glucose in α cells, similar to previously reported in β cells (17).

162 We then measured the intracellular Cl^- concentration ($[Cl^-]_i$) of AlphaTC1-9 cells using a Cl^-
163 sensitive dye MQAE. In the presence of 10 mM glucose, application of Inh172 (10μM) induced a
164 change in MQAE signal reflecting an increase in $[Cl^-]_i$, (data are shown as (Fmax-Fmin)/F of
165 MQAE, see methods) which was significantly enhanced by overexpression of CFTR (Fig. 4b). This
166 suggests that in the presence of 10 mM glucose, when CFTR is presumably activated as
167 demonstrated in Fig.4a, CFTR mediates Cl^- efflux, inhibition of which results in Cl^-
168 accumulation/increase in the cells. We then reasoned that glucose deprivation should render
169 CFTR channel from open to close state, which should mimic the effect of CFTR inhibitor,
170 resulting in an increase in $[Cl^-]_i$ when the perfusion solution is switched from high to low glucose

concentration. Indeed, the results showed that glucose deprivation (switching from 10 to 0 mM) induced a change in MQAE reflecting an increase in $[Cl^-]_i$, with significantly greater change observed in CFTR-overexpressing cells compared to that of the vector control cells (Fig.4c), confirming a role of CFTR in conducting Cl^- efflux. These results suggest that the CFTR channel activity in α cells, similar to that in β cells, is sensitive to extracellular glucose change, and that the turning off of CFTR upon glucose deprivation results in the accumulation of Cl^- in the cells.

Overexpression of CFTR results in hyperpolarization of membrane potential in α cells

The observed suppressive effect on glucagon release in wildtype mice/islets or CFTR-overexpressing α cells compared to mutant mice/islets or vector control/CFTR-inhibited cells prompted us to compare the resting Vm in AlphaTC1-9 cells with or without CFTR overexpression using the patch-clamp technique with KCl-containing solutions. The results showed that the cells overexpressing CFTR, in both high (10 mM) and low (0 mM) glucose conditions, exhibited a more hyperpolarized resting Vm (-54.67 ± 5.84 mV and -32.00 ± 4.32 mV, respectively) than that of the vector-control cells (-31.83 ± 4.52 mV and -21.20 ± 1.15 mV, respectively) (Fig.5a). While these results are in line with a suppressive role of CFTR in glucagon release, it is puzzling why CFTR-overexpressing α cells are more hyperpolarized in contrast to a more depolarized Vm previously observed in β cells (17), although CFTR in both cell types exhibits similar glucose sensitivity and mediates Cl^- efflux under the same conditions. In fact, the measured resting Vm by the patch-clamp experiments in α cells showed greater hyperpolarization in cells exposed to high glucose (10 mM), when CFTR is supposed to be activated, as compared to that in low glucose (0 mM), when CFTR activity is low. Opposite results should have been observed if CFTR was solely responsible for the Vm changes. There

must be other channel(s) contributing to the hyperpolarizing effect of CFTR in α cells. Since activation of $GABA_A$ receptor, also a chloride channel, has been reported to suppress glucagon secretion in islet α cells (24), we tested possible involvement of $GABA_A$ receptor in mediating the effect of CFTR in α cells. The results showed that treatment with bicuculline (10μM), a $GABA_A$ receptor antagonist, did not alter the suppressive effect of CFTR overexpression on glucagon secretion in AlphaTC1-9 cells (Suppl.Fig.S1), excluding the involvement of $GABA_A$ receptor.

CFTR suppresses glucagon secretion by potentiating K_{ATP} channel activity in α cells

K_{ATP} channels, which are inhibited by high glucose with the increase in ATP/ADP ratio but open when ATP/ADP is low at glucose deprivation, have been proposed as a key glucose-sensing mechanism in α cells with Na^+ and Ca^{2+} channels as the downstream mediators regulating glucagon release (7,8), although their precise role remains obscure. Interestingly, CFTR has been reported to interact and modulate other ion channels (30,31), including the ROMK (kir1.1), one type of ATP-sensitive potassium channels (K_{ATP}) (32). Thus, it is possible that in addition to its Cl^--transporting capacity, CFTR may regulate K_{ATP} in α cells, thereby modulating the Vm change required for glucagon release.

To test this, we first examined K_{ATP} expression and found similar expression levels of K_{ATP} genes, SUR1 and Kir6.2, in AlphaTC1-9 cells with or without CFTR expression (Suppl.Fig.S2). We next measured K_{ATP} activity using patch-clamp recording of Vm in AlphaTC1-9 cells with or without CFTR overexpression, in conjunction with the use of glibenclaminde (GLIB), a selective K_{ATP} blocker. The results showed that in the low glucose (1mM) bath, GLIB (100μM) induced Vm

depolarization in AlphaTC1-9 cells with the CFTR-overexpressing (pCFTR) cells exhibiting a significantly greater response (22.3±3.5 mV) than that of control cells (pVector, 11.6 ± 0.6 mV) (Fig.5b). Similar results were observed using Dibac measurement on isolated islets with the wildtype α cells showing greater depolarizing response to K_{ATP} inhibition by another blocker, tolbutamide (100μM, Suppl.Fig.S3), as compared to DF508 α cells. We further measured K_{ATP} currents in AlphaTC1-9 cells. A series of voltages (-80 to -20 mV) were applied to elicit K_{ATP} currents in the presence of CFTR inhibitor (Inh172) exclude CFTR currents. As shown in Fig.5c, at 10 mM glucoses, pCFTR expressed cells showed significantly larger whole-cell currents that were sensitive to GLIB (100μM), consistent with a role of CFTR in potentiating K_{ATP} activity in α cells.

To confirm that the suppressive effect of CFTR on glucagon secretion involves K_{ATP} channels, we knocked down the functional subunit of K_{ATP}, SUR1, in the CFTR-overexpressing AlphaTC1-9 cells by SUR1-targeting siRNAs (siSUR1). The success of transfections was confirmed by RT-PCR and quantitative PCR, showing upregulation of CFTR and down regulation of SUR1 (Fig.6a). We then compared the glucagon released from CFTR-overexpressing AlphaTC1-9 cells with or without K_{ATP} knockdown. As shown in Fig.6b, knockdown of K_{ATP} subunit substantially reversed the suppressive effect of CFTR overexpression on glucagon release induced by either low (1 mM) or high (6 mM) glucose, confirming the involvement of K_{ATP} channels in the suppressive action of CFTR in α cells.

Discussion

The present results have revealed a suppressive role of CFTR in the regulation of glucagon secretion, providing a novel mechanism intrinsic to pancreatic α cells. The results obtained from the CFTR/DF508-overexpressing α cells, in addition to that obtained from wildtype and DF508 mice/ isolated islets, have clearly demonstrated that glucagon secretion can be regulated intrinsically by α cells with CFTR as a key player in suppressing glucagon release since CFTR mutation/inhibition at animal, tissue or cell levels consistently results in elevated levels of glucagon secretion compared to that observed in the wildtype mice/islets or CFTR overexpressing cells. These results are consistent with a recent report that demonstrates an inhibitory role of CFTR in regulating glucagon release in both mouse and human islet α cells (19). However, the previous study did not provide experimental evidence to explain how CFTR can exert an inhibitory role on glucagon secretion by acting as a Cl- channel. Our present finding has demonstrated that the suppressive effect of CFTR on glucagon secretion is attributed to a more hyperpolarized Vm, and a less degree of glucose-deprivation-induced depolarization and Ca^{2+} response observed in wildtype/CFTR-overexpressing α□cells (in islets or cell line) compared to that observed in DF508/CFTR inhibited cells.

Compared to what we previously reported in β cells (17), similar features of CFTR are observed in α cells. In both types of cells, CFTR is activated by glucose, which could be explained by either glucose-induced ATP production (33,34) and secondarily cAMP increase (35-37), or glucose-dependent kinases (38,39), although detailed activation mechanism remains to be elucidated. Also, CFTR appears to mediate Cl^- efflux in both cell types. Although Cl^- currents are usually thought to be inhibitory (24,40), CFTR mediated Cl^- efflux is shown to depolarize membrane potential and contributes to the electrical spikes promoting insulin secretion in β cells (17).

However, the present results suggest that CFTR plays a suppressive role in glucagon secretion in α cells. These results present a paradox in CFTR function, while with similar channel characteristics, stimulating insulin secretion in β cells but inhibiting glucagon secretion in α cells.

Interestingly, the same paradox is also observed for K_{ATP} channels. While it has been well established that glucose-induced closure of K_{ATP} channels in β cells is crucial for insulin secretion, the same condition inhibits glucagon release in α cells, making their role in the process controversial (7,41). Thus, the most important finding from the present study is that CFTR potentiates K_{ATP} channel activity in α cells, providing a perfect solution to the paradox. The greater extent of sensitivity to the K_{ATP} channel blocker in either Vm or whole-cell current measurements observed in CFTR-overexpressing or wildtype islet α cells compared to that of vector-overexpressing or DF508 cells (Fig.5b & Suppl.Fig.S3) indicates greater K_{ATP} channel activity in the wildtype/overexpression with intact CFTR, suggesting potentiation of K_{ATP} channel by CFTR, which is impaired in DF508. The involvement of K_{ATP} channel in mediating the suppressive action of CFTR on glucagon secretion is further supported by the reversal of the suppressive effect of CFTR overexpression with the knockdown of K_{ATP} channels in the α cell line. A question is remained as to how CFTR potentiates K_{ATP}. CFTR is known to interact with multiple membrane proteins (42-44) and it is reported to modulate K_{ATP} (kir1.1) activity in the kidney through protein-protein interaction (45). Thus, similar mechanism may also apply to α cells, which awaits further investigation.

Taken together, we propose a working model for regulating glucagon release intrinsic to α cells involving both CFTR and K_{ATP} channels (Fig.7). In this model, activation of CFTR by high glucose, presumably by elevated intracellular ATP/ADP as previously reported by others (46-49), leads to

activation of K_{ATP} channels overcoming the inhibitory effect of ATP/ADP on K_{ATP} channels, which results in a strong hyperpolarization that diminishes the Ca^{2+} response required for glucagon release as observed in the present study. In the case of DF508 or CFTR inhibition, the potentiating effect of CFTR on K_{ATP} channels is removed, resulting in enhanced depolarization required for Ca^{2+} response, and thus enhanced glucagon release, which is consistently observed in the present study with DF508 mice/islets/cells. This model not only explains how CFTR exerts an inhibitory effect on glucagon release in α cells but also provides an explanation to a long standing puzzle as to how K_{ATP} channels work to inhibit glucagon release under a glucose concentration known to suppress their function. The present finding suggests that it is not the glucose-induced closure of K_{ATP} channels, but rather, the potentiation of K_{ATP} channels by CFTR in response to a rise in glucose that underlies glucose-induced glucagon suppression. Given the recognized key roles of K_{ATP}, such as governing Vm and triggering Na^+ inactivation, in regulating glucagon secretion by α cells (7,8), the unexpected CFTR involvement and its potentiating effect on K_{ATP} channels in α cells provides a novel mechanism regulating α cell glucagon secretion.

What are the clinical implications of the present findings? The importance of CFTR in regulating blood glucose is highlighted by the presence of glucose intolerance in CF patients, which has been attributed to defects in both β and α cell functions, among other contributing factors (50). In addition to impaired insulin secretion (51-53), glucagon suppression following oral glucose is increasingly impaired with decreasing glucose tolerance in CF patients (14,54,55). The present study demonstrating the importance of glucose-activated CFTR in suppressing glucagon release and the inability of DF508 mice/islets to suppress glucagon release provides an explanation to the reduced glucose suppressibility found in many CF patients. Since insulin from β cells can

also exert an inhibitory effect on α cell glucagon release (24,56-58), a defect in insulin secretion due to defective CFTR (51,59,60), may also contribute to the impaired glucagon suppressibility in CF. The results from the present study, together with our previous finding in β cells (17), suggest a dual role of CFTR in regulating glucagon secretion, a direct action on α cells and an indirect effect through insulin from β cells. Therefore, both defects in insulin and glucagon secretion may contribute to glucose intolerance in CF and the development of CFRD. Of note, glucose suppressibility is also impaired or absent in patients with type 1 and type 2 diabetes mellitus (5,61,62). The presently demonstrated critical role of CFTR in suppressing glucagon secretion suggests a plausible cause of diabetic conditions due to abnormal CFTR expression/function in general population other than CF, which warrants future investigation.

Methods

Animals

cftr^{tm1Kth} mice (20) carrying CFTR mutation, DF508, were kept in a temperature-controlled room with a 12-hour light/dark cycle, and provided with food and water *ad libitum* in the Laboratory Animal Service Center, the Chinese University of Hong Kong (CUHK). For glucose tolerance assay, blood was collected from mice via tail vain 12 hours after fasting at 0, 10 and 30 min after intraperitoneal injection of glucose (1g/kg body weight). All procedures were approved by the Animal Ethical Committee of CUHK.

Isolation of mouse pancreatic islets

Mouse islets were isolated from wildtype or DF508 mice of 12-14 week-old as previously described (17,63). Purified islets were cultured in RPMI-1640 medium (Invitrogen) with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Cell culture

The mouse pancreatic α cell line, AlphaTC1 clone 9 (AlphaTC1-9), was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 16.7 mM glucose and supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C.

Overexpression and knockdown

AlphaTC1-9 cells were grown till 70% to 80% confluence for transfection. Various DNA plasmids (3μg per 35mm^2 dish) or interfering siRNAs (100nmole per 35mm^2 dish) mixed with Lipofectamine 2000 (6μl per 35mm^2 dish, Invitrogen) were used for transfection following the manufacturer's instruction. Cells were collected 48 hours after transfection for other experiments. Plasmids EGFP (pEGFP)or pcDNA3.1 conjugated with full-length human CFTR or DF508 mutant CFTR were kindly provided by Professor Tzyh-Chang Hwang (University of Missouri-Columbia) and Professor Yoshiro Sohma (Keio University) (64-66). pEGFP and pcDNA3.1 plasmids were used for patch-clamp and imaging experiments, respectively. To enhance fluorescence signal for cell identification in patch-clamp experiments, additional pEGFP (0.5μg per 35mm^2 dish) were transfected. Stealth siRNAs targeting mouse SUR1 gene (Assay ID. MSS277480) and the scrambled non-silencing siRNA (Medium GC content) (Cat. 12935-300) were purchased from Invitrogen.

Procedure for islet/cell handling and measurement of glucagon by ELISA

Glucagon concentrations in mouse serum or culture media were determined by ELISA (Raybiotech, Cat. EIA-GLU-1) following the manufacturer's instruction. Cells or islets were pre-incubated in Krebs-Ringer buffer (KRB) containing (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, HEPES 10 (pH 7.4) with 0.1% BSA for 15 min at 37°C, followed by 2-hour test incubation in KRB supplemented with glucose (1-6mM) and 0.1% BSA. Islets isolated from 2-3 mice of same genotype were pooled together and 10-20 size-matched islets were selected under a dissecting microscope and grouped as one replicate for ELISA assay. Adrenaline (10 µM, David Bull Laboratories) and L-arginine (10 mM, Sigma) were also added during the 2-hour test incubation with islets.

Ca^{2+}, membrane potential (Vm) and Cl^- imaging

Isolated islets were seeded on cover-slips coated with poly-L-lysine hydrobromide (25 µg/ml) and cultured in RPMI-1640 medium with 10% FBS at 37°C for 3 days. AlphaTC1-9 cells were grown on cover-slips till 50-60% confluence. For Ca^{2+} imaging, islets or cells were incubated at 37°C for 40 min with Fura-2 (3 µM, Invitrogen, California, USA) and Plutonic F-127 (1.5 µM, Invitrogen) in Margo-Ringer solution containing (in mM): NaCl 130, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2.5, HEPES 20, Glucose 10 (pH 7.4). Fura-2-loaded cells or islets were transferred to a mini chamber and mounted onto the fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan) equipped with a CCD camera (Spot Xplorer, USA). The Xenon lamp (Hamamatsu, Japan) was used to provide a dual excitation at 340 and 380 nm and emission was collected at 510 nm every 3 seconds. The ratio of 340/380 fluorescent signal intensity indicated the intracellular Ca^{2+} changes. Cells in the periphery of the mouse islets (67) were selected as α cells for Ca^{2+} imaging and further

364 confirmed by adrenaline (10 μM) stimulation (25,29,68). For Vm imaging, cells or islets were
365 bathed in Margo-Ringer solution containing a voltage-sensitive dye, DiBAC4(3) (Dibac, 2.5 μM,
366 Invitrogen). Dibac was excited by 490 nm and the emission was collected at 520nm. For
367 calibration of Dibac intensity with Vm changes, a series of incremental concentrations of
368 potassium gluconate (5, 10, 20, 40 and 60 mM) were added to the bath solution in the presence
369 of valinomycin (2μM) as previously described (69). For Cl^- imaging, cells were loaded with N-(6-
370 methoxyquinolyl)-acetoethyl ester (MQAE, 10 mM, Invitrogen) in Margo-Ringer solution at 37°C
371 for 30 min. MQAE was excited at 340 nm every 5 seconds and emission signals were collected
372 at 460 nm. An increase in MQAE intensity indicates reduction in Cl^- concentration, whereas
373 decrease in MQAE indicates Cl^- elevation. At the end of each experiment, maximal MQAE signal
374 (Fmax) was recorded in a Cl^--free solution containing (in mM): Na gluconate 130, K gluconate 5,
375 Ca gluconate 20, MgSO4 1, HEPES 20, Glucose 10 (pH 7.4) with presence of nigericin (10 μM)
376 and tributyltin (10 μM); and minimal signal (Fmin) in a buffer containing (in mM): potassium
377 thiocyanate (KSCN) 150, HEPES 10 (pH 7.2) with presence of valinpmycin (5 μM). The
378 fluorescence intensity of MQAE (F) was normalized with the difference between Fmax and Fmin,
379 and the value of (Fmax-Fmin)/F was used to directly reflect Cl^- concentration changes. In some
380 experiments where glucose deprivation was applied, the glucose in Margo-Ringer solution was
381 replaced by D-mannitol.

382 **Patch-clamp**

383 Transfected AlphaTC1-9 cells with GFP fluorescence were selected under microscope for patch-
384 clamp with an amplifier (Axopatch-200B, Axon Instruments, Foster City, CA, USA) and data
385 acquisition system (DigiData 1322A, Axon Instruments). Borosilicate glass-made patch pipettes

(Vitrex, Modulohm A/S, Herlev, Denmark) were pulled on a micropipette puller (P-97, Sutter Instrument Co., USA) to a resistance of 4–7MΩ after filled with pipette solution. When the whole-cell Giga seal was formed, the capacitance of cell was measured. Cells were bathed in Margo-Ringer with 0,1 or 10mM glucose and the pipettes were filled with solution (in mM): KCl 138, NaCl 10, MgCl2 1 and HEPES 10 with D-manitol compensated for 290 mOsm/L. For CFTR currents, cells were bathed in glucose free Margo-Ringer; pipettes were filled with a solution (in mM): CsCl 101, EGTA 10, HEPES 10, TEACl 20, MgATP 2, $MgCl_2$ 2, glucose 5.8, with osmolality adjusted to 290 mOsm/L by D-mannitol. To record the membrane voltage, current was clamped to 0.

RNA extraction, conventional and quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. 1-2 µg of Total RNA was performed reverse transcripts to obtain complimentary DNA (cDNA) using Molony murine leukemia virus reverse transcriptase (M-MLV) (Promega). The target genes were generated by PCR or qPCR with primers (SUR1: forward, 5'-GCCTTCGTGAGAAAGACCAG-3' and reverse 5'-GAAGCTTCTCCGGTTTGTCA-3'; CFTR: forward, 5'-AAAACTTGGATCCCTATGAAC-3', and reverse, 5'- GTGGGGAAAGAGCTTCAC-3'; Kir6.2: forward, 5'-GACATCCCCATGGAGAATGG-3' and reverse, 5'-TCGATGACGTGGTAGATGATGAG-3'; GAPDH: forward, 5'-AACGACCCCTTCATTGAC-3' and reverse, 5'-TCCACGACATACTCAGCAC-3'). The PCR products were run electrophoresis in 2% agarose gel with gel-red. The results were visualized under an ultraviolet transilluminator (Alpha Innotech). qRT-PCR was performed using SYBR Green Supermix (Promega) on Applied Biosystem 7500 fast real-time PCR system. A final

melting curve verified the specificity of the RT-PCR. The relative mRNA expression level was calculated by ΔΔCt method.

Western blotting

Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, 1% NP-40, 0.5% DOC, 0.1% SDS) containing protease inhibitor cocktail (Roche, cat.11697498001) for 30 min on ice. Supernatant was collected as total protein after centrifugation at 14,000 rpm for 30min. Protein were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Rabbit anti-CFTR antibody (Alomone, cat. ACL-006, 1:500, RRID: AB_2039804); Rabbit anti β-tubulin (Santa Cruz, cat. sc-9104, 1:2000, RRID: AB_2241191) were used. The protein bands were visualized with ECL western blotting detection reagent (GE Healthcare) following manufacturer's instructions and scanned by densitometer.

Statistical analysis

Data are presented as mean ± s.e.m.. Two-tail unpaired Student's t tests were used for two groups of comparison. For three or more groups, data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Interactions involving two groups were analyzed by two-way ANOVA. $P<0.05$ was considered to be statistically significant.

Acknowledgements

This work was supported in part by the National Major basic Research Program of China (2013CB967404, 2013CB967401, and 2013CB967403), the Focused Investment Scheme of the Chinese University of Hong Kong and Faculty of Medicine. We thank Xiao Sun, Chun Yuan, Xiao Feng Li, Wei Yin Lin, Ying Liu and Bin Wang for their help in animal experiments.

Author contributions

H.C.C. and J.H.G conceptualized. H.C.C., Y.C.R., J.H.G and W.Q.H. designed. W.Q.H, J.H.G, Y.C.R., M.K.Y, X.H.Z and Y.W.C. performed the experiments and data analysis. H.C.C., Y.C.R, W.Q.H. and J.H.G. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Fig.1. Impaired glucose-induced suppression of glucagon secretion by CFTR mutant mice/islets or CFTR-inhibited islet α cells.

a) Plasma glucagon levels, measured by ELISA, in wildtype (WT) or CFTR mutant (DF508) mice 12 hours after fasting (0 min) and 10, 30 min after intraperitoneal administration of glucose (1g/kg body weight). n=4. *p<0.05, Two-way ANOVA. **b)** ELISA measurement of glucagon release from isolated WT or DF508 mouse islets after incubated for 2 hours in low (1mM) or high (6mM) glucose solution in the presence or absence of a CFTR inhibitor, Inh172 (10μM). n is shown in each column. ***p<0.001, *p<0.05, t-test.

Fig.2. CFTR channel activity and its suppressive effect on glucagon secretion in CFTR overexpressing-AlphaTC1-9 cells.

a) Western blotting for CFTR in AlphaTC1-9 cells transfected with empty vector plasmids (pVector) or plasmids cloned with full-length human CFTR (pCFTR). Tubulin was blotted as the loading control. **b)** Whole-cell currents recorded with CsCl pipette solution by patch-clamp in AlphaTC1-9 cells transfected with pCFTR or pVector in glucose free Margo-Ringer. Currents were elicited by a series of voltages (-80 to +80 mV) from a holding voltage of -70mV, before (Ctrl), 5-10 min after addition of Forskolin (FSK, 10μM) or subsequently GlyH101 (GlyH, 10μM), another CFTR inhibitor. Corresponding current-voltage curves are shown on the right. **c)** ELISA measurement of glucagon levels secreted from AlphaTC1-9 cells transfected with pVector or

pCFTR after 2 hour-incubation in low glucose (1mM) in the presence or absence of GlyH (10μM). n is shown in each column. ***p<0.001, *p<0.05, One-way ANOVA. Data are means ± s.e.m..

Fig.3. Suppression of glucose-deprivation-induced Ca^{2+} influx and membrane depolarization by CFTR in islet α cells.

a) Intracellular Ca^{2+} measurement by Fura-2 in isolated WT or DF508 mouse islets challenged with glucose deprivation from 6 to 1mM with representative responses over time shown. Fluorescence image shows a representative Fura-2-loaded mouse islet with selected peripheral cells (circled) for Fura-2 340/380 ratio detection. Adrenalin (ADR, 10μM) was added at the end of each experiment with responsive cells considered α cells, with statistical summary of their responses to glucose-deprivation in WT and DF508 shown on the right. n is shown in each column. ***p<0.001, t-test. **b)** Membrane depolarization assessed by Dibac, a voltage-sensitive fluorometric, in isolated WT or DF508 mouse islets in response to glucose deprivation from 6 to 1mM with representative responses over time shown. Fluorescence image shows an isolated mouse islet loaded with Dibac with circled cells selected for the detection. Dibac fluorescence intensity change was calibrated into membrane voltage change (ΔVm) and compared between WT and DF508. n is shown in each column. ***p<0.001, t-test. Data are means ± s.e.m.. Bars in fluorescence images =100μm.

Fig.4. Activation of CFTR by glucose and CFTR-mediated Cl^- efflux in islet α cells.

a) Representative whole-cell currents elicited by a series of voltages (-80 to +80 mV) from a holding voltage of -70mV in AlphaTC1-9 cells transfected with pCFTR, before (Ctrl), 10-20 min after addition of glucose (20mM) or subsequently GlyH (10μM). Corresponding current (I)-voltage (V) curves are shown on the right. n=7 (Ctrl and Glucose) and 4 (Glucose + GlyH). Error bars are smaller than symbol size except where shown. ***p<0.001, **p<0.01, Two-way ANOVA. **b)** MQAE measurement of intracellular Cl^- concentration ($[Cl^-]_i$) in 10 mM glucose with increases in response to Inh172 (10μM) in AlphaTC1-9 cells transfected with pVector or pCFTR. The fluorescent intensity of MQAE (F) was normalized with the difference between maximal (Fmax) and minimal (Fmin) values (see methods). n is shown in each column. ***p<0.001, t-test. **c)** MQAE measurement of $[Cl^-]_i$ increases in response to glucose deprivation from 10 to 0 mM in AlphaTC1-9 cells transfected pVector or pCFTR. n is shown in each column. ***p<0.001, t-test. Data are means ± s.e.m.

Figure.5. Effect of CFTR on resting membrane potential and K_{ATP} inhibition-induced membrane depolarization in AlphaTC1-9 cells.

a) Resting membrane potentials (Vm) measured within 1 min after whole-cell configuration was made with KCl-containing pipette solution by patch-clamp in AlphaTC1-9 cells transfected with pVector or pCFTR in 0 and 10 mM glucose. n=3-9. *p<0.05, One-way ANOVA. **b)** Representative time course recording of patch-clamp measurements of Vm in AlphaTC1-9 cells transfected with pCFTR or pVector in 1mM glucose bath before and after addition of a K_{ATP} blocker, glibenclaminde (GLIB, 100μM). Pipettes were filled with KCl-containing pipette solution.

678 Statistic summary of GILB induced Vm changes (ΔVm) is shown on the right. n is shown in each
679 column. *p<0.05, t-test. **c)** Representative patch-clamped whole cell currents and
680 corresponding I-V curves elicited by a series of voltages (-80 to -20 mV) from a holding voltage
681 of -70mV in AlphaTC1-9 cells transfected with pCFTR or pVector in 10 mM glucose, before (Ctrl),
682 after the addition of Inh172 (10μM), or subsequently GLIB (100μM). n = 3-6. **d)** Summary of
683 GLIB-sensitive whole-cell current density, which was obtained by subtracting the current after
684 treatment with GLIB from the current in the presence of Inh172, at -80mV, -60mV and -40mV in
685 AlphaTC1-9 cells with pCFTR or pVector. n = 3-4. *p<0.05, t-test.

686

687 **Fig.6. Involvement of K_{ATP} in mediating the suppressive effect of CFTR on glucagon secretion**
688 **in AlphaTC1-9 cells.**

689 **a)** RT- *(left)* and real-time quantitative *(right)* PCR analysis of the expression of SUR1 or CFTR in
690 AlphaTC1-9 cells after transfection with pVector, pCFTR, siRNAs targeting SUR1 (siSUR1) or non-
691 silencing control siRNAs (siNC). n=3. ***p<0.001, ns: no significance, One-way ANOVA. **b)** Levels
692 of glucagon release from AlphaTC1-9 cells transfected with pVector, pCFTR, siSUR1 or siNC, in
693 low (1mM) or high (6mM) glucose solution. n= 3-9. ***p<0.001, **p<0.01, *p<0.05. ns: no
694 significance, t-test. Data are means ± s.e.m..

695

696 **Fig.7. Working model for glucose-induced glucagon suppression in pancreatic islet α-cell**
697 **involving potentiation of K_{ATP} channel by CFTR.**

a) In α cell, CFTR is activated by glucose, possibly through the changes in its intracellular metabolites (i.e. ATP/ADP, cAMP, glucose-dependent kinases), with ATP/ADP being shown as one of the possibilities. Despite the membrane potential depolarization (Vm↑) caused by the CFTR-mediated Cl^- efflux, activated CFTR potentiates K_{ATP} channel activity, which overcomes the direct inhibitory effect of glucose on K_{ATP} channel, resulting in Vm hyperpolarization (Vm↓) and hence inhibits Ca^{2+} influx through voltage-dependent Ca^{2+} channel (VDCC) and suppresses glucagon secretion. **b)** When CFTR is defective (i.e. DF508 mutation), the potentiation effect of CFTR on K_{ATP} is removed, leading to a more depolarized Vm (Vm↑) as a result of glucose-induced K_{ATP} closure, and thus VDCC activation and excessive glucagon secretion.

Supplementary Figure legends

Suppl.Fig.S1 Effect of $GABA_A$ receptor-inhibition on glucagon secretion in AlphaTC1-9 cells.

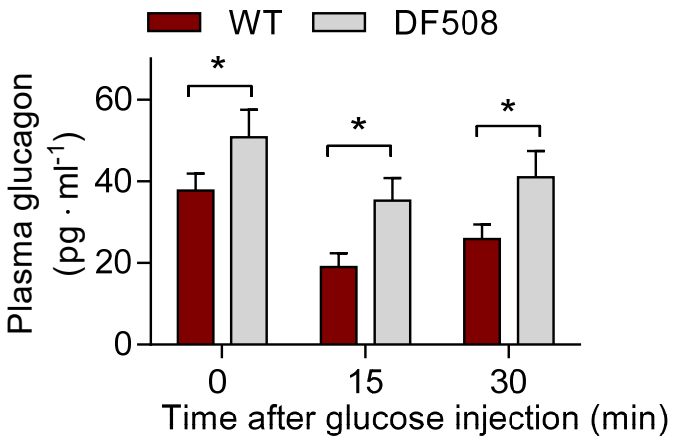
ELISA measurement of glucagon levels secreted from AlphaTC1-9 cells transfected with full-length CFTR (pCFTR), DF508 mutant CFTR (pDF508) or empty vector (pVector) after 2 hour-incubation in low glucose (1mM) in the presence or absence of a $GABA_A$ receptor antagonist, bicuculline (BIC, 10μM). n is shown in each column. ***p<0.001, One-way ANOVA. n=3.

Suppl.Fig.S2. RT-PCR analysis of the expression of CFTR, SUR1 and Kir6.2 in AlphaTC1-9 cells after transfection with pVector or pCFTR.

Suppl.Fig.S3. K_{ATP} inhibition induced membrane depolarization in islet α cells.

Membrane potential (Vm) measurement by Dibac in selected peripheral α cells in WT or DF508 mouse islets in response to a K_{ATP} blocker, tolbutamide (TOL, 100μM) in 1mM glucose. n is shown in each column. Dibac fluorescence intensity change was calibrated into membrane voltage change (ΔVm) and compared between WT and DF508. n is shown in each column. ***p<0.001, t-test.

a



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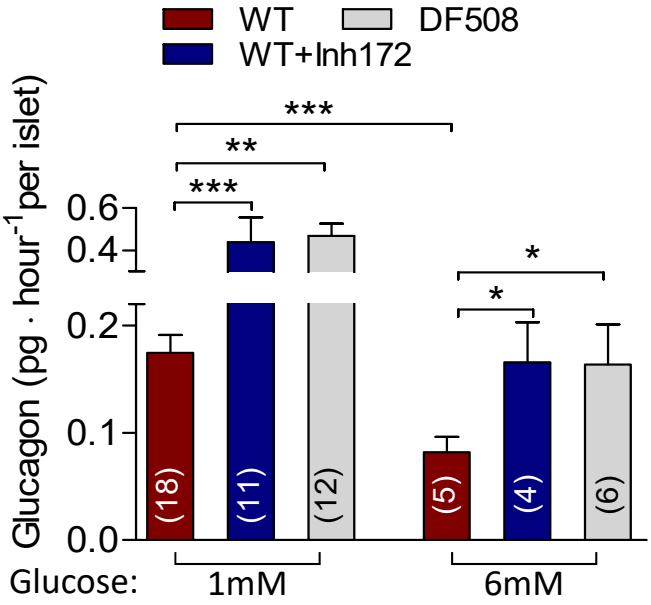


Fig.1. Huang et al

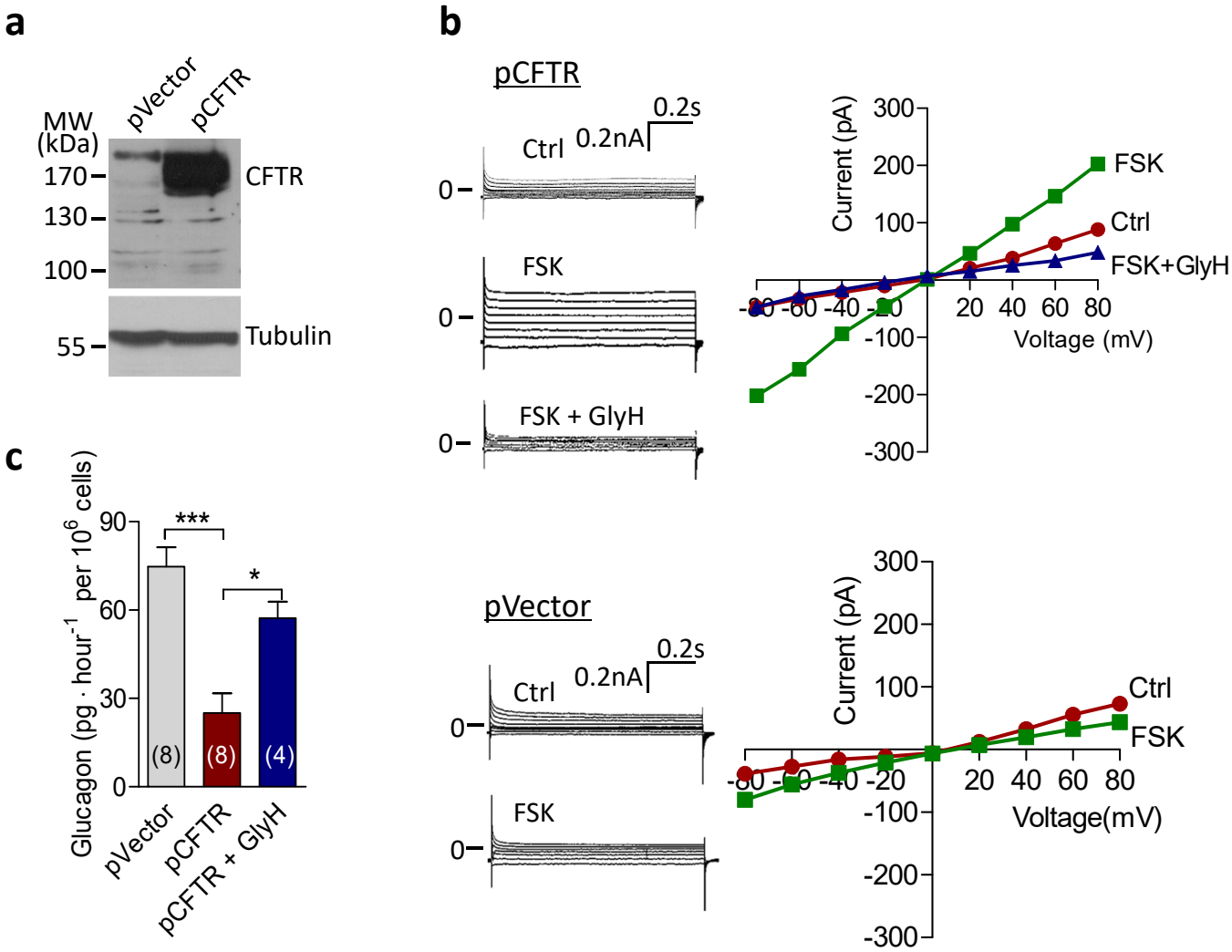
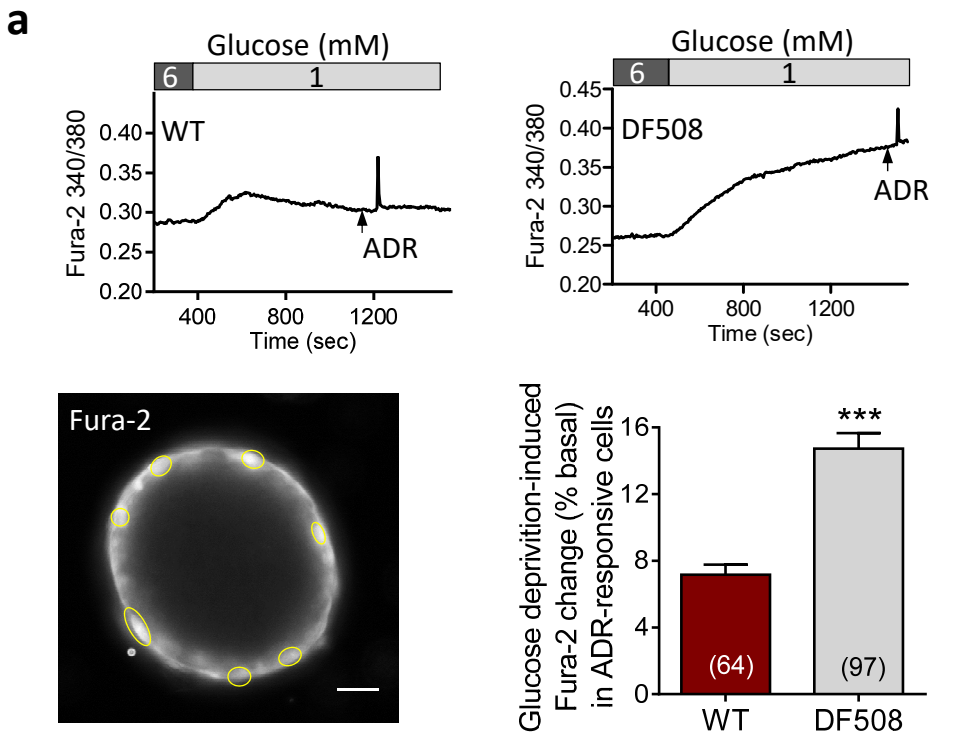


Fig.2. Huang et al



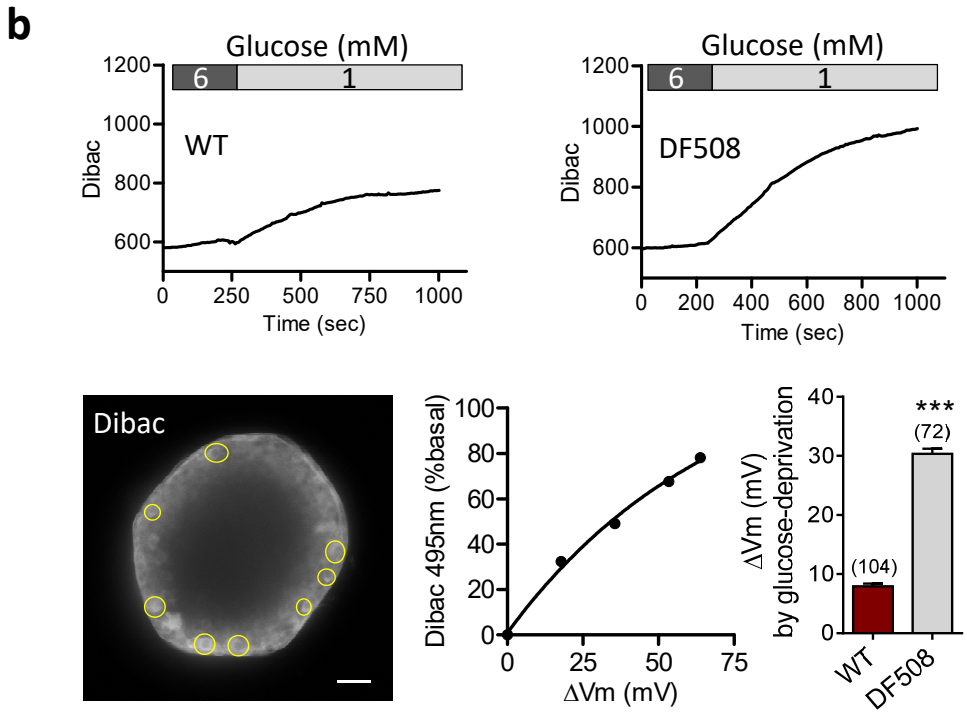


Fig.3. Huang et al

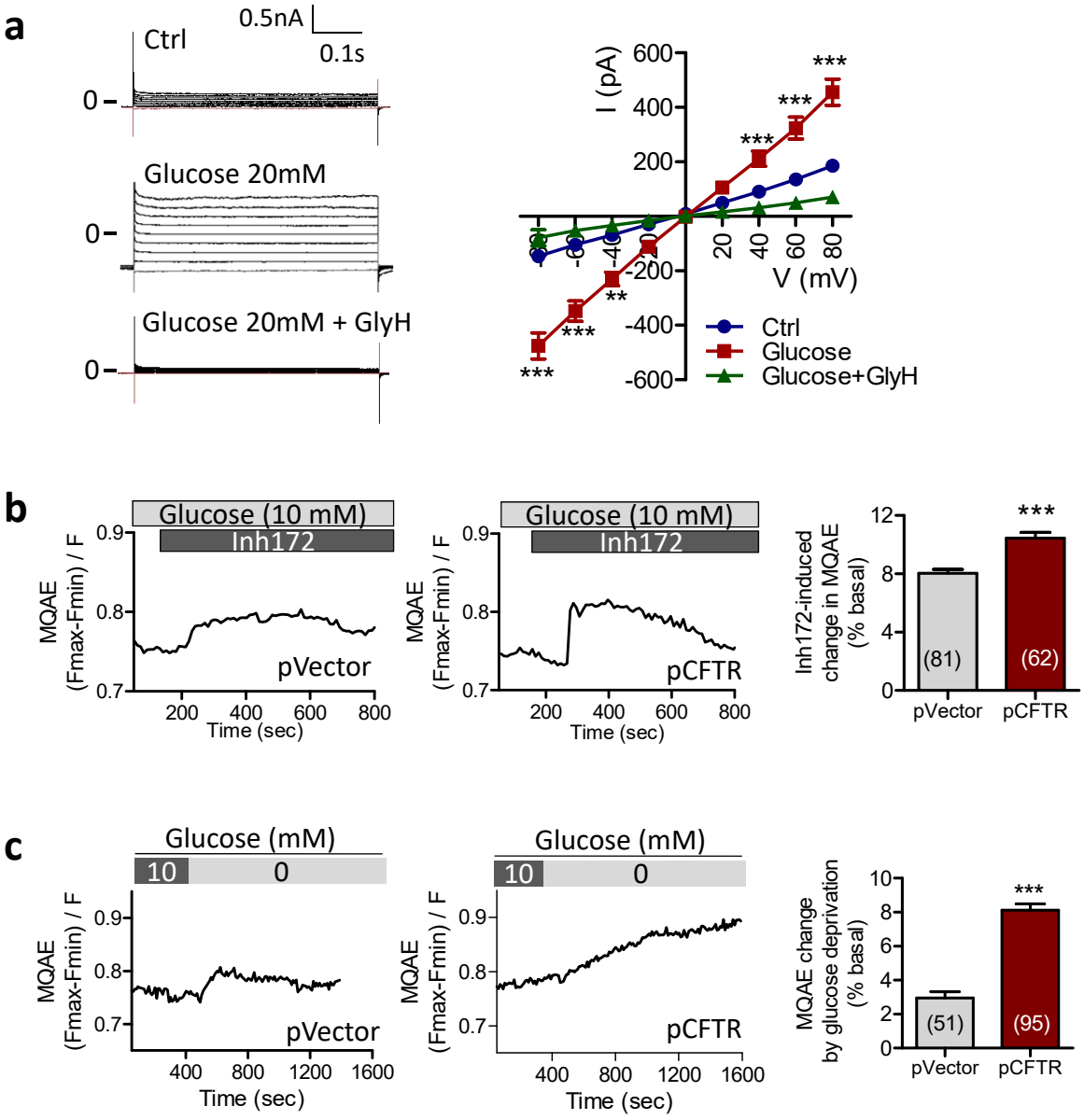


Fig.4. Huang et al

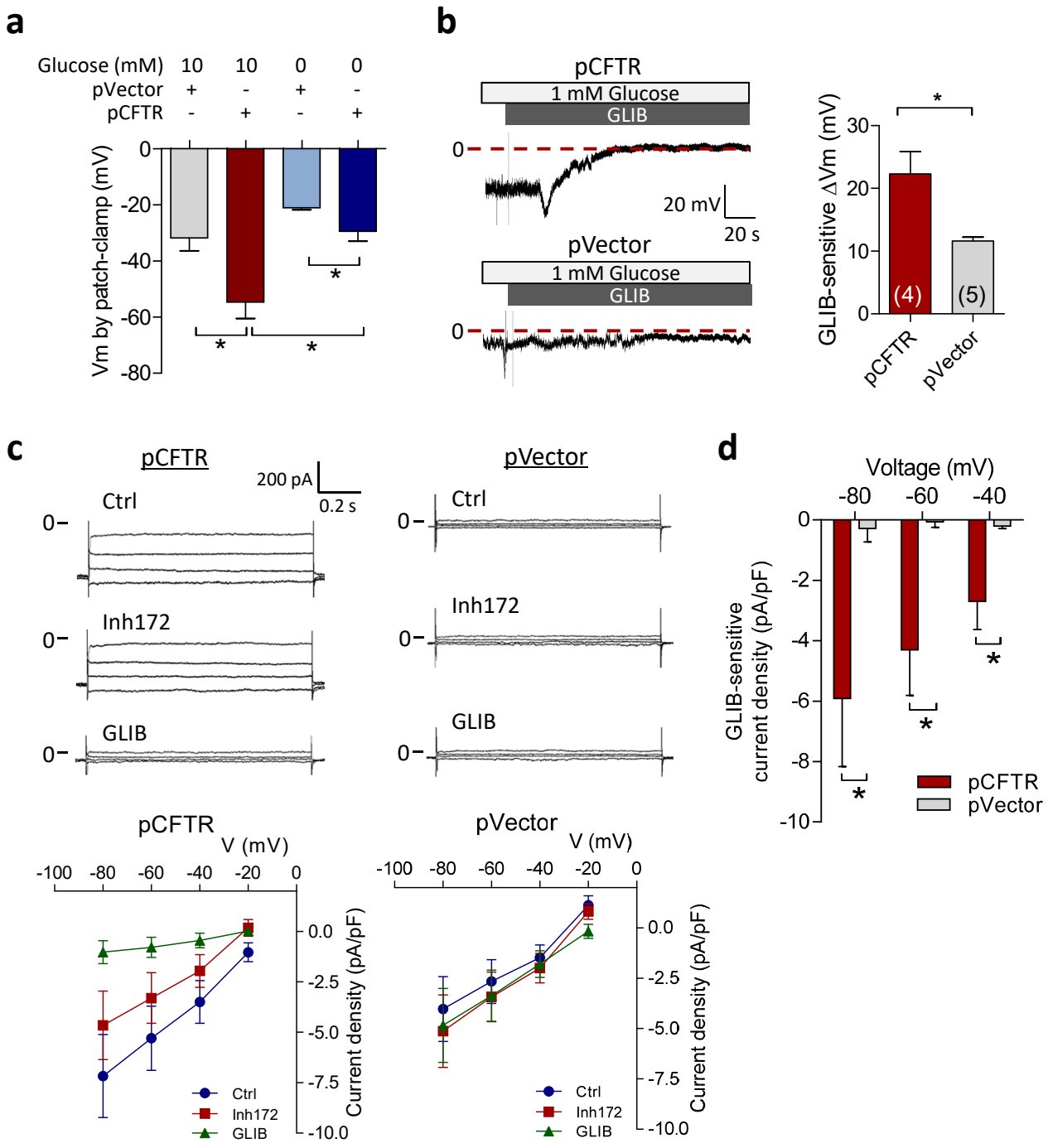
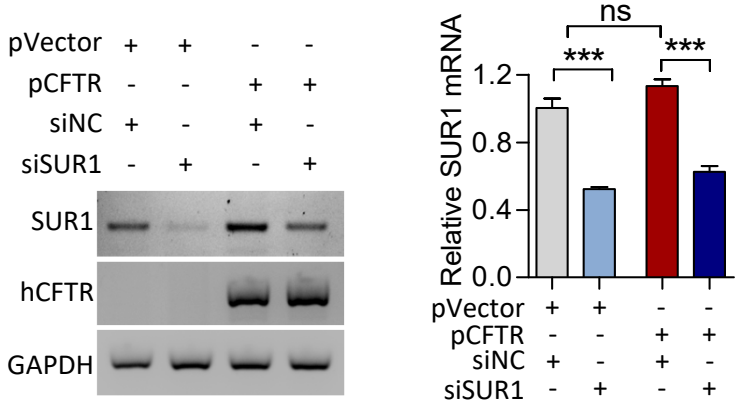


Fig.5. Huang et al

a



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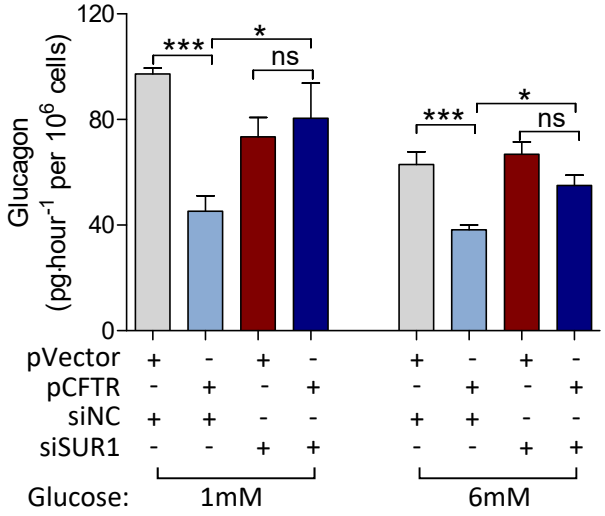
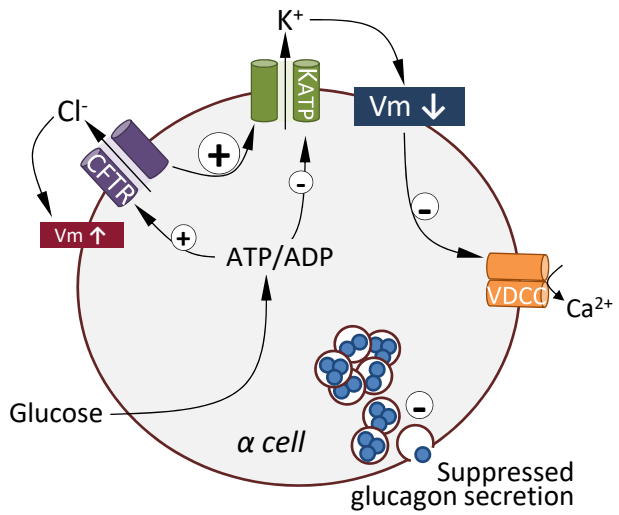


Fig.6. Huang et al

a



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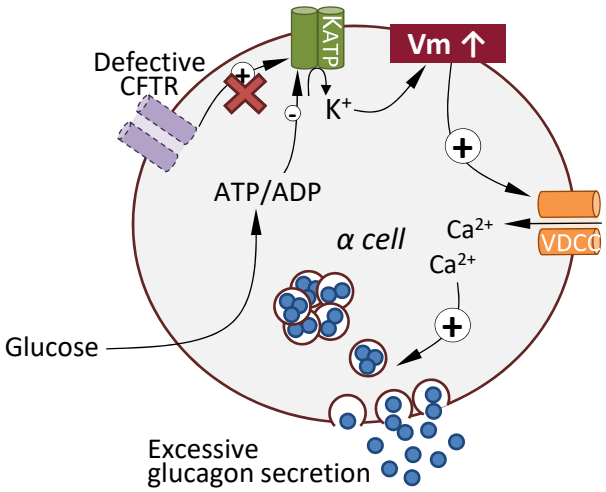
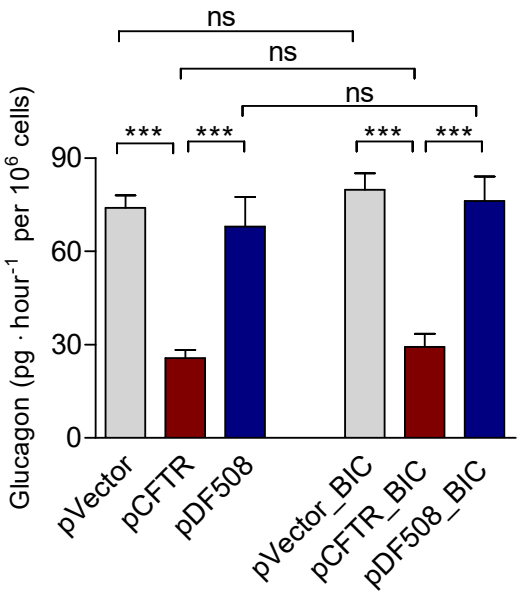
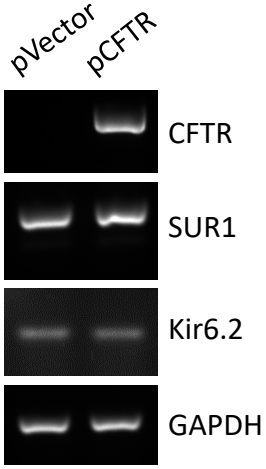


Fig.7. Huang et al

Supplementary Fig.S1



Supplementary Fig.S2



Supplementary Fig.S3