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Defective exocytosis and processing of insulin in a cystic fibrosis mouse model

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Abstract

Cystic fibrosis-related diabetes (CFRD) is a common complication for patients with cystic fibrosis (CF), a disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). The cause of CFRD is unclear, but a commonly observed reduction in first-phase insulin secretion suggests defects at the beta cell level. Here we aimed to examine alpha and beta cell function in the *Cftr*^{tm1EUR}/F508del mouse model (C57BL/6J), which carries the most common human mutation in *CFTR*, the F508del mutation. CFTR expression, beta cell mass, insulin granule distribution, hormone secretion and single cell capacitance changes were evaluated using islets (or beta cells) from F508del mice and age-matched wild type (WT) mice aged 7–10 weeks. Granular pH was measured with DND-189 fluorescence. Serum glucose, insulin and glucagon levels were measured *in vivo*, and glucose tolerance was assessed using IPGTT. We show increased secretion of proinsulin and concomitant reduced secretion of C-peptide in islets from F508del mice compared to WT mice. Exocytosis and number of docked granules was reduced. We confirmed reduced granular pH by CFTR stimulation. We detected decreased pancreatic beta cell area, but unchanged beta cell number. Moreover, the F508del mutation caused failure to suppress glucagon secretion leading to hyperglucagonemia. In conclusion, F508del mice have beta cell defects resulting in (1) reduced number of docked insulin granules and reduced exocytosis and (2) potential defective proinsulin cleavage and secretion of immature insulin. These observations provide insight into the functional role of CFTR in pancreatic islets and contribute to increased understanding of the pathogenesis of CFRD.

Key Words

- CFTR
- F508del
- cystic fibrosis-related diabetes (CFRD)
- insulin
- glucagon

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Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in *CFTR* encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an anion channel that conducts Cl^- and HCO_3^- and is activated by ATP and cAMP (Chen *et al.* 2010). Besides being an ion channel CFTR is a regulator of other ion channels and membrane proteins (Sheppard & Welsh 1999). The F508del mutation in *CFTR* is carried by 65–80%

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of people living with CF (Koivula *et al.* 2016). In both human and mouse, F508del-CFTR is misprocessed in the endoplasmatic reticulum and the channel is subsequently degraded. However, a small portion of F508del-CFTR escapes degradation and reaches the plasma membrane where it has reduced conductivity (Wilke *et al.* 2011).

Blood glucose abnormalities are common in CF and CF-related diabetes (CFRD) is a common secondary complication. Acquiring diabetes on the background of CF leads to an increased morbidity and mortality, and depending on the age-group studied 30–50% of the adult patients have CFRD (Brennan *et al.* 2004, Dobson *et al.* 2004, de Valk & van der Graaf 2007). Deficient insulin secretion, beta cell dysfunction and various degrees of insulin resistance characterize CFRD (Hardin *et al.* 2008, Cano Megias *et al.* 2015). The pathogenesis in CFRD is still not completely understood. Patients with severe mutations in CFTR and pancreatic exocrine insufficiency are predictors of CFRD but pancreatic sufficient patients also acquire CFRD (Wooldridge *et al.* 2015). The question whether CFTR has a functional role in beta cells is not without controversy. We and others have shown that CFTR is expressed in primary human and rodent islet cells (Boom *et al.* 2007, Edlund *et al.* 2014, 2017, Guo *et al.* 2014, Huang *et al.* 2017). Moreover, single cell transcriptome analysis of human islets has revealed that CFTR mRNA is expressed in a subset of alpha and beta cells (Blodgett *et al.* 2015, Segerstolpe *et al.* 2016). However, on the basis of the single cell transcriptome analysis, others have argued that CFTR is not expressed at a significant level in the islet cells and therefore do not contribute to beta cell dysfunction in CF and CFRD (Sun *et al.* 2017, Hart *et al.* 2018). Instead, it has been suggested that CFRD is driven by a combination of beta cell loss, inflammation, islet remodeling and general morbidity (Sun *et al.* 2017, Hart *et al.* 2018). Loss of beta cell mass is implicated in CF but not to the extent seen in type 1 diabetes (Moran *et al.* 2010). Moreover, treatment with the CFTR corrector Ivacaftor restored beta cell function and insulin secretion in CF patients (Bellin *et al.* 2013, Kelly *et al.* 2019), indicating dysfunctional insulin secretion in CFRD.

Animal models resembling human CF have shed light on CFRD pathology. The newborn *Cftr*$^{-/-}$ ferrets display reduced first-phase insulin secretion and glucose intolerance (Olivier *et al.* 2012). The CF pigs are born with pancreatic inflammation similar to humans. Islet cell mass is intact but when challenged with an intravenous glucose tolerance test, glucose tolerance is impaired with increased proinsulin to insulin secretion ratio (Uc *et al.* 2015). Increased proinsulin secretion suggests impaired processing of insulin and an intrinsic beta cell defect. Also pancreatic-insufficient CF patients have increased proinsulin to C-peptide secretion both during fasting and in glucose-stimulated conditions (Sheikh *et al.* 2017, Nyirjesy *et al.* 2018). Hence, suggesting that insulin processing might be impaired in human CF. Insulin maturation within the insulin granule requires cleavage of proinsulin by endopeptidases into mature insulin and C-peptide, a process that is dependent on an acidic intragranular environment (Davidson *et al.* 1988). Acidification of the granule is achieved by simultaneous pumping of H^+ and Cl^- into the granule (Barg *et al.* 2001). Inadequate granular acidification results in an increased intragranular proinsulin to mature insulin ratio (Davidson *et al.* 1988). We have previously shown that CFTR is involved in the regulation of exocytosis and insulin secretion in human and mouse beta cells (Edlund *et al.* 2014), and we have proposed that CFTR is important for insulin granule priming by providing Cl^- to the insulin granule via granular ClC3.

The *Cftr*^{tm1EUR} mouse model (van Doorninck *et al.* 1995, French *et al.* 1996), here denoted F508del, carries the F508del mutation. The F508del mice are essentially pancreatic sufficient but have a lower body weight and intestinal disease similar to human CF (French *et al.* 1996, Wilke *et al.* 2011, Fontes *et al.* 2015). The F508del mouse model on a FVB background has reduced beta cell mass and increased insulin sensitivity at young age, but developed glucose intolerance with increased age (Fontes *et al.* 2015). Here, we have investigated intrinsic effects of CFTR in pancreatic islet hormone secretion in young F508del mice on a C57BL/6J background. We hypothesized that the F508del mutation could impair function and/or induce changes in beta cell area. We therefore investigated both changes in beta cell area and the possibility that the F508del beta cells have defective exocytosis and insulin secretion. Finally, we measured islet glucagon and somatostatin secretion. The data presented highlight the important contribution of CFTR in beta cells for functional insulin secretion.

Materials and methods

Animals and tissues

The global *Cftr*^{tm1EUR} mouse model (C57BL/6J, F12 backcrosses), heterozygous for the F508del *Cftr* mutation, was obtained from the Erasmus Medical Center (Rotterdam, Netherlands) (Wilke *et al.* 2011, Fontes *et al.* 2015). Heterozygous mice were bred and

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held in isolator cages and fed with standard diet (R36; Lactamin AB, Stockholm, Sweden) and water *ad libitum* (Ohlsson *et al.* 2008). Offspring was genotyped by PCR analysis followed by digestion of the PCR product with SspI. The following primer pairs were used: CF-P580: 5′-GGACGCAAAGAAAGGGATAAG; and CF-P581: 5′- CACAACACTGACACAAGTAGC. Female and male wild type (WT) (weight: 23±0.5 g) and homozygous F508del (weight: 20±0.4 g) mice littermates were used in experiments. Littermates aged 7–10 weeks were included in the studies. In one experiment, we used beta cells from C57BL/6J/AKR/CH3 mice. All experiments were performed in accordance with ethical statements and regulations approved by the ethical committees in Stockholm and Lund. Animals were killed by cervical dislocation and islets isolated by collagenase (Sigma-Aldrich) digestion.

Immunocytochemistry

Islets were handpicked in Hanks buffer (Sigma-Aldrich), dispersed in Ca^{2+}-free buffer, transferred to cell culture media (RPMI-1640; SVA, Uppsala, Sweden) supplemented with 10 mmol/L glucose, 10 v/v FBS (Sigma-Aldrich), 100 IU/mL penicillin/streptomycin, 2 mmol/L L-glutamin (both from HyClone, South Logan, UT, USA) and incubated overnight at 37.5°C, 5% CO_2. The cells were fixed in paraformaldehyde, and stained as described (Vikman *et al.* 2006). Primary antibodies insulin (Millipore) and CFTR (MATG1061; RD Biotech, Besançon, France) (Carvalho-Oliveira *et al.* 2004) were used. Corresponding secondary antibodies (Jackson ImmunoResearch) were detected using confocal laser microscopy (Zeiss instruments) and analyzed using Zen software as described (De Marinis *et al.* 2010). The INS1-823/13 beta cell line lack CFTR (Ct_{CFTR}=undetermined, Ct_{HPRT}=23, Ct_{PPIA}=18, n=6 passages) and was used as a negative control for MATG-1061 specificity (Supplementary Fig. 1A, see section on supplementary data given at the end of this article).

Immunohistochemistry and morphometry

Pancreatic sections (5 µm) were deparaffinized and hydrated as described elsewhere (Wierup *et al.* 2004). Sections were incubated with primary antibodies against insulin (9003; EuroDiagnostica, Malmö, Sweden), glucagon (7811; EuroDiagnostica), somatostatin (sc-7819; Santa Cruz Biotechnology) and secondary antibodies (Jackson ImmunoResearch). Immunofluorescence was examined in an epifluorescence microscope (Olympus BX60; Olympus), see example hormone staining in Supplementary Fig. 2A. For beta cell mass quantification all islets in three different parts of each pancreas (minimum 200 µm apart) were assessed in a blinded fashion using NIS-Elements software (NIS-Elements 3.1; Nikon). Total insulin-, glucagon- or somatostatin-stained area and total section area were measured and alpha, beta or delta cell mass was calculated as the ratio between the two areas. Beta cell number per islet was calculated using DAPI as a marker of cell nuclei. All nuclei surrounded by insulin staining were regarded as beta cells. An average of 44±4 islets per animal were analyzed (n_{WT}=21 pancreatic sections from seven mice, $n_{F508del}$=21 pancreatic sections from seven mice).

Hormone secretion

Freshly isolated islets were dispersed, handpicked and stimulated in Krebs buffer supplemented with 1 mmol/L glucose, 1 mg/mL albumin for 30 min followed by 15 min stimulation in 50 mmol/L KCl or 1 h stimulation in 1 mmol/L, 2.8 mmol/L or 16.7 mmol/L glucose with or without 10 µmol/L forskolin (Sigma-Aldrich) and/or 50 µmol/L GlyH-101 (Calbiochem) as indicated. After stimulation, islets were dissolved in RIPA buffer (50 mmol/L TRIS-HCl, 150 mmol/L NaCl, 0.5 mmol/L NaDeoxycholate, 2 mmol/L EDTA, 50 mmol/L NaF, 1 v/v Triton-X, 0.1 v/v SDS (all from Merck) and sonicated. Supernatant and dissolved islets were analyzed with RIA using antibodies to detect insulin (RI-13K), C-peptide, glucagon (GL-32K; all three from Millipore) and somatostatin (Mercodia AB, Malmö, Sweden). Secreted proinsulin was analyzed with rat/mouse proinsulin ELISA (10-1232-01, Mercodia AB). Secretion experiments were performed independently on four occasions using islets pooled from 3 to 5 animals of each genotype and 3–5 technical replicates with 12 islets per replicate for each condition (n=4). Secretion experiment in high K^+ (50 mM) were performed on islets from individual male mice (N_{WT}=5 mice, $N_{F508del}$=5 mice).

Patch-clamp measurements

An EPC-10 amplifier and Patchmaster software (HEKA Elektronik, Lambrecht(Pfalz), Germany) were used to evoke and record whole-cell currents and changes in membrane capacitance in dispersed beta cells as described in Eliasson *et al.* (2003). Mouse beta cells were distinguished from alpha and delta cells by the electrophysiological properties of the Na^+ current inactivation (Gopel *et al.* 2000). The extracellular solution contained the following (in mmol/L): 118 NaCl, 20 TEA-Cl, 5.6 KCl, 2.6 $CaCl_2$,

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1.2 $MgCl_2$, 5 HEPES and 5 Glucose (pH 7.4 NaOH). The intracellular solution contained the following (in mmol/L): 125 CsOH, 125 L-glutamic acid, 10 CsCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 3 Mg-ATP, 0.05 EGTA and 0.1 cAMP (pH 7.15 CsOH). All chemicals are from Merck.

Transmission electron microscopy

Islets were fixed in 2.5% glutaraldehyde and treated with osmium (both from Sigma-Aldrich) as described in Andersson *et al.* (2011). Analysis to estimate total number of granules as volume density N_v (granules/μm^3) and number of docked granules as surface density N_s (granules/μm^2) were performed using the diameter of the insulin granules (D_{WT}=272±2.8 nm and $D_{F508del}$=272±3.3 nm) and an in-house program in MATLAB. A granule was considered docked if the center of the granule was within 150 nm (~D/2) from the plasma membrane (Olofsson *et al.* 2002).

Intragranular pH measurements

Dispersed single cells from C57BL/6J/AKR/CH3 mice were plated onto poly-D-lysine-coated glass bottom culture dishes and incubated overnight. Before experiments, cells were incubated with 1 μM LysoSensor green DND-189 (L-7535; Invitrogen; Thermo Fisher) in Krebs buffer for 40 min. During experiments, cells were perfused at a flow rate of 1 mL/min at 32°C with Krebs buffer supplemented with 2.8 mM or 16.7 mM glucose and with 10 μM forskolin in the presence or absence of 25 μM GlyH-101. Confocal laser microscopy (Zeiss instruments) was used to measure DND-189 fluorescence (excitation 443 nm; emission 505 nm) and data was analyzed using Zen software. To subtract rundown, fluorescence was normalized to traces measured at 2.8 mM glucose alone.

In vivo measurements

Animals were fasted overnight and 1 mg/g glucose in PBS was injected intra-peritoneally (maximum 200 μL/animal) and blood glucose levels were measured. Venous blood was collected from tail-vein of unanesthetized animals. Blood glucose levels were measured using Glucometer Elite Strips (Bayer) and serum insulin and glucagon levels were analyzed with ELISA (Insulin: 10-1247-01, Glucagon: 10-1281-01; Mercodia AB). Rate of glucose clearance was estimated by subtraction of the blood glucose value at each time point with the previous blood glucose value divided by the time.

Statistical analysis

Data are presented as mean±S.E.M. of number of animals (N) or number of experiments/cells (n). Students t-test and ANOVA were used to calculate statistical significance and a $P<0.05$ value was considered statistically significant.

Results

F508del islets have reduced beta cell mass

We studied CFTR protein expression in dispersed F508del and WT beta cells using confocal immunocytochemistry. CFTR was detected using the MATG-1061 monoclonal antibody; shown to recognize not only CFTR but also F508del-CFTR (Carvalho-Oliveira *et al.* 2004) (Fig. 1A). Plasma membrane expression of CFTR in beta cells from F508del mice was lower than in WT mice (Fig. 1B), which is in accordance with reduced surface expression of F508del-CFTR in other tissues (French *et al.* 1996). The 2D surface area size of individual beta cells was ~20% smaller in F508del compared to WT beta cells (Fig. 1C).

We next calculated beta cell area from pancreas sections immunostained for insulin (Supplementary Fig. 2A), since a previous observation in the F508del mice suggests that the main reason for reduced insulin secretion in these mice is reduced beta cell mass (Fontes *et al.* 2015). Our calculations confirmed a reduced beta cell area in F508del mice (Fig. 1D). However, when we counted the number of nuclei as a measurement of number of beta cells within the insulin stained area there was no difference between F508del and WT mice (Fig. 1E). The pancreas weight was similar between the animals (Supplementary Fig. 2B). Alpha or delta cell area was not different between F508del mice and WT mice (Fig. 1F and G, Supplementary Fig. 2A).

F508del beta cells secrete more proinsulin and less C-peptide

We asked the question if the *Cftr* mutation affects beta cell function and measured insulin secretion from isolated islets. We measured insulin release after 1 h incubation in the non-stimulatory (basal) glucose concentration 1 mmol/L and the stimulatory glucose concentration 16.7 mmol/L, respectively. CFTR was activated using the cAMP-increasing agent forskolin and CFTR function was blocked by the CFTR-antagonist GlyH-101. We found reduced basal insulin secretion in F508del (Fig. 2A).

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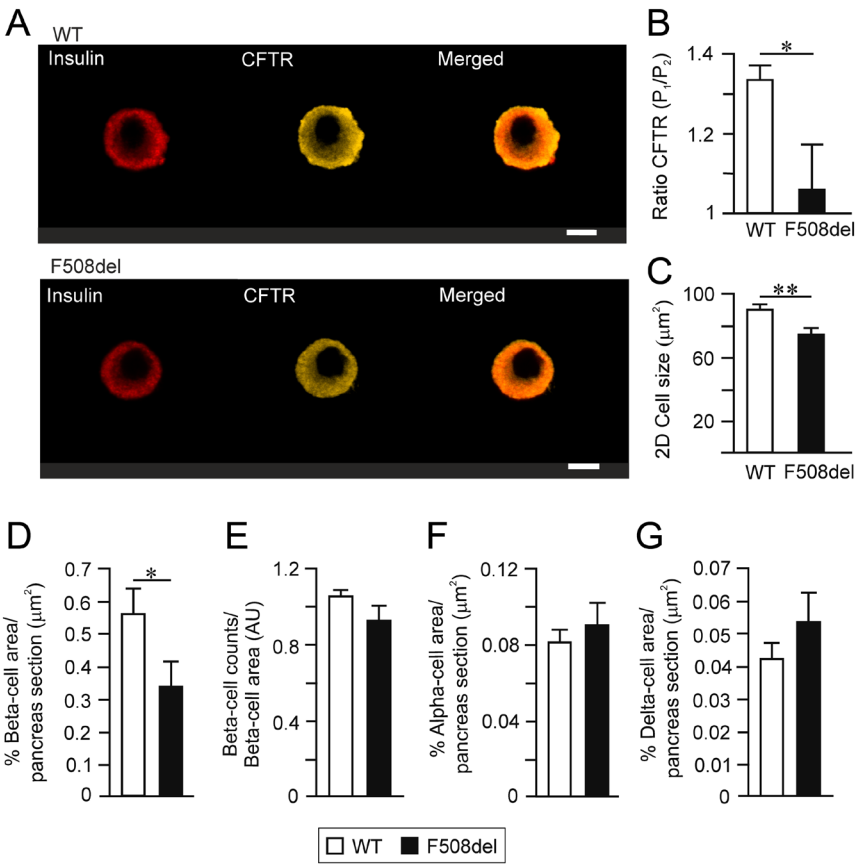

Figure 1
Morphological investigation of WT and F508del islets and beta cells. (A) Representative image of CFTR (yellow) and insulin (red) localization using confocal immunocytochemistry in fixed single beta cells from a WT and a F508del beta cell. Scale bar 5 µm. (B) The ratio of CFTR protein expression in the plasma membrane region (P_1) compared to the cytosol (P_2) in WT ($n = 14$) and F508del ($n = 11$) in insulin positive cells as in (A). (C) Single 2D area cell size of insulin positive cells measured on confocal images using Zen software. (D) Beta cell mass in WT and F508del islets using insulin immunohistochemistry. (E) Number of beta cells within the insulin stained area in the histology sections in (D). (F) Alpha cell mass and (G) delta cell mass as in (D). Data are presented as mean ± S.E.M., white bar: WT and black bar: F508del, *$P < 0.05$, **$P < 0.01$. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0570.

As expected, addition of forskolin did not affect basal insulin secretion in the WT, whereas it slightly enhanced basal release in the F508del islets.

In accordance with our previously published data (Edlund *et al.* 2014), inhibition of CFTR by GlyH-101 reduced glucose-stimulated insulin secretion in the presence of forskolin in WT islets by ~40% (Fig. 2B). We have previously demonstrated the specificity of GlyH-101 for CFTR (Edlund *et al.* 2017). The specificity was further confirmed using INS1-823/13 cells, which do not express CFTR (Supplementary Fig. 1A). GlyH-101 did not affect glucose and IBMX-stimulated insulin secretion in INS1-823/13 cells (Supplementary Fig. 1B).

Glucose-stimulated insulin secretion did not differ between F508del islets and WT islets (Fig. 2B). Moreover, forskolin elevated glucose-stimulated insulin secretion to a larger extent in the F508del islets (Fig. 2B). We believe these surprising results are partly due to reduced insulin content in F508del islets compared to WT islets (Supplementary Fig. 3A), and partly due to the insulin RIA which does not discriminate between insulin and proinsulin. We therefore measured the secretion of proinsulin and C-peptide separately from the same samples. Proinsulin secretion from F508del islets was markedly increased during cAMP-stimulation (Fig. 2C, Supplementary Fig. 3D). Moreover, GlyH-101 increased proinsulin secretion from WT islets while having no further effect on F508del islets (Fig. 2C). In line with that observation, C-peptide secretion was decreased in F508del islets (Fig. 2D, Supplementary Fig. 3E).

F508del beta cells have defective exocytosis of insulin granules

We have previously suggested that CFTR regulates exocytosis in beta cells (Edlund *et al.* 2014). We therefore measured depolarization-induced insulin secretion after incubation in 50 mmol/L K^+ during 15 min to investigate insulin secretion downstream of beta cell depolarization. Depolarization-induced insulin secretion was reduced by ~30% in F508del islets (Fig. 2E) compared to WT. In the simultaneous presence of forskolin the reduction was nearly ~40% (Fig. 2E). During this short stimulation in the absence of glucose there was no difference in proinsulin secretion between F508del and WT mouse islets (Fig. 2F).

To examine the effect of CFTR deficiency on exocytosis in detail we measured exocytosis in single F508del and

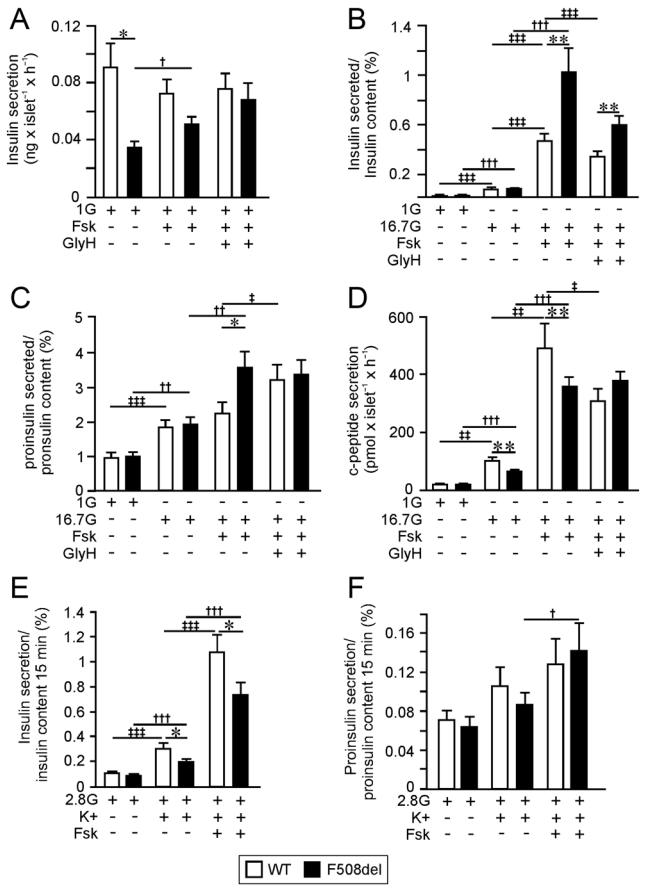

Figure 2
Insulin, proinsulin and c-peptide secretion from islets isolated from F508del and WT mice. (A) Insulin secretion at 1 mmol/L glucose (1 G) and cAMP-amplified using 10 µmol/L forskolin (Fsk). CFTR was inhibited using 50 µmol/L GlyH-101 (GlyH) in WT and F508del islets (n_{WT} = 4, $n_{F508del}$ = 4). (B) Insulin secretion at 16.7 mmol/L glucose (16.7 G) and cAMP-amplified using forskolin in WT and F508del islets expressed per insulin content. CFTR was inhibited using 50 µmol/L GlyH-101 (GlyH, n_{WT} = 4, $n_{F508del}$ = 4). (C) Proinsulin secretion per proinsulin content and (D) C-peptide secretion measured in the same sample as in (B). (E) Depolarization-induced insulin secretion per insulin content in 2.8 mmol/L glucose (2.8 G) and 50 mmol/L KCl (K^+) with or without 10 µM forskolin (Fsk) in islets from separate mice (not pooled, N_{WT} = 5, $N_{F508del}$ = 5). (F) Proinsulin secretion per proinsulin content measured in the same samples as in (A). Data are presented as mean ± S.E.M., white bar: WT and black bar: F508del, $^*P < 0.05$, $^{**}P < 0.01$ WT vs F508del, $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$ within WT as compared to another condition, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ within F508del as compared to another condition.

WT beta cells using standard whole-cell capacitance measurements. The increase in membrane capacitance, elicited by a train of ten 500-ms depolarizations from −70 mV to 0 mV, was significantly reduced in F508del beta cells compared to WT beta cells (Fig. 3A, B and C). The increase in membrane capacitance evoked by the first two depolarizations in the train, reflecting ATP-independent exocytosis of primed and pre-docked granules (Eliasson *et al.* 1997), was reduced by ~50% (Fig. 3C). ATP-dependent exocytosis, the latter eight depolarizations, was less affected (Fig. 3C). The reduced exocytosis was not due to a reduced influx of Ca^{2+} (Supplementary Fig. 4).

Docking of granules is important for functional exocytosis and we wanted to confirm that mutations in *Cftr* reduce the docked pool of granules. To this end, transmission electron microscopy was employed (Fig. 3D, E, F and G). In line with our previous findings in NMRI mouse and human beta cells treated with CFTR inhibitors (Edlund *et al.* 2014), F508del beta cells had a reduced surface density (N_s) of granules (Fig. 3F) while the total volume density (N_v) was similar between F508del and WT beta cells (Fig. 3G).

Cleavage of proinsulin to insulin and C-peptide and the process of insulin granular priming require low intragranular pH. We therefore measured granular pH in mouse beta cells using the fluorescent dye DND-189 that specifically can be used to measure pH of acidic organelles (Fig. 3H and I). We observed a reduction in pH after stimulation with 16.7 mM glucose in presence of forskolin (in 15/32 cells) in agreement with previous measurements (Eliasson *et al.* 2003). The reduction was reverted in presence of the CFTR-inhibitor GlyH-101.

Forskolin-induced glucagon secretion in the F508del mouse is impaired

Recent data show increased glucagon secretion after CFTR inhibition in both human and mouse islets, suggesting an intrinsic function of CFTR also in alpha cells (Edlund *et al.* 2017, Huang *et al.* 2017). We asked if the CFTR mutation would have the same effect and investigated glucagon secretion properties in islets from the F508del mice. F508del and WT islets were subjected to 1 h-incubation in 1 mmol/L glucose to maximally stimulate glucagon secretion. Under this condition, F508del islets displayed higher glucagon secretion compared to WT islets (Fig. 4A). In agreement with previous findings, forskolin caused a fourfold amplification of glucagon secretion in WT islets (Fig. 4A). Interestingly, the already high level of glucagon secretion observed in F508del islets at 1 mmol/L glucose alone was not further enhanced by forskolin. At 16.7 mmol/L glucose, glucagon secretion was elevated in F508del islets as compared to WT islets. Glucagon secretion from WT islets was (as expected) reduced (Fig. 4B), whereas there was no significant difference in glucagon secretion from F508del islets as compared to 1 mmol/L glucose.

We have suggested that CFTR regulates electrical activity in the alpha cell with a small direct effect

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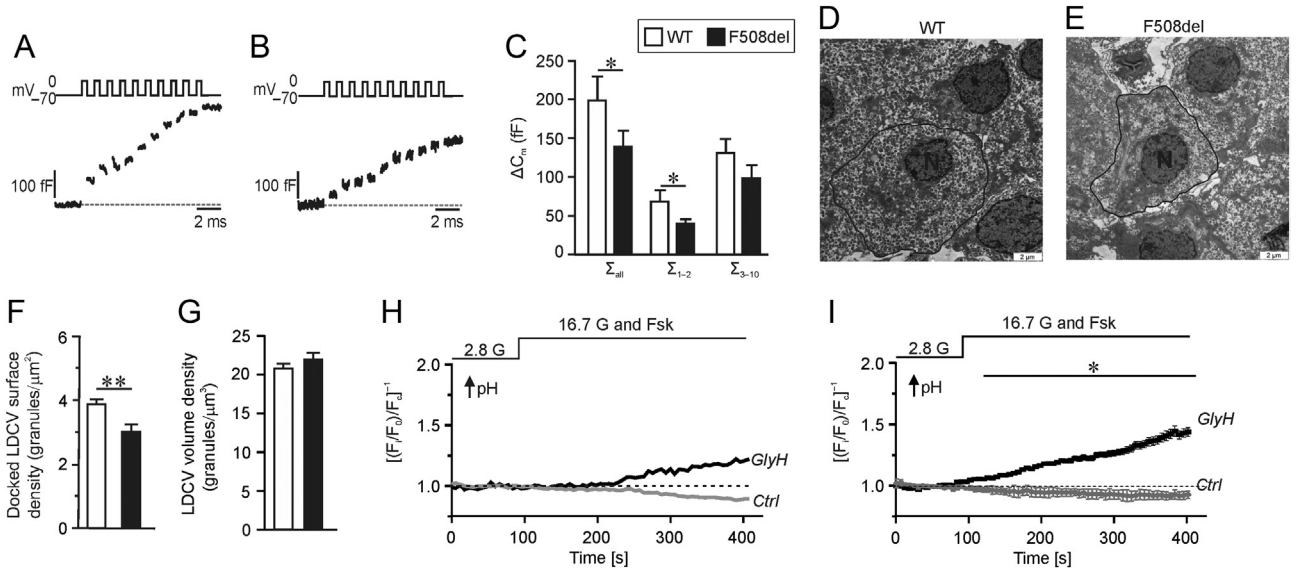

Figure 3
Exocytosis of insulin containing granules in WT and F508del beta cells. (A) Membrane capacitance increase during a train of ten 500-ms depolarizations from −70 mV to 0 mV in a single beta cell from WT mice. (B) As in (A) but on a single F508del beta cell. (C) Summary of the total increase in membrane capacitance from all experiments (Σ_{all}, n_{WT} = 16, $n_{F508del}$ = 16) as in (A) and (B), during the first two depolarizations (Σ_{1-2}) and during the latter eight depolarizations (Σ_{3-8}). (D) A transmission electron microscopic micrograph of a single beta cell in a WT islet. The plasma membrane is indicated with a solid black line and N = nucleus. (E) As in (D) but in an F508del islet. (F) An estimation of the number of large dense core vesicles (LDCV) docked at the plasma membrane measured as surface density (N_S) (n_{WT} = 28 beta cells from two mice, $n_{F508del}$ = 33 beta cells from two mice). (G) An estimation of the total amount of vesicles measured as LDCV volume density (N_v) (n_{WT} = 28 beta cells from two mice, $n_{F508del}$ = 33 beta cells from two mice). Data are presented as mean ± S.E.M., white bar: WT and black bar: F508del, *P < 0.05, **P < 0.01 WT vs F508del, ‡‡‡P < 0.001 within WT compared to another condition, †P < 0.05, †††P < 0.001 within F508del as compared to another condition. (H) Typical experiments measuring pH as the fluorescence of the probe DND-189 in the absence and presence of GlyH-101(GlyH) as indicated. DND-189 fluorescence increase with reduced pH. For clarity we present the inverse fluorescence ($[(F_i/F_0)/F_c]^{-1}$) so that traces goes below baseline for reduced pH and increases when the pH increases as indicated. Lowering of granular pH was achieved by addition of 16.7 mM glucose (G) in presence of 10 μM forskolin (Fsk). The traces were normalized to recordings performed in presence of 2.8 mM glucose alone. (I) Summary of results performed as in (H). Data are presented as S.E.M. ± S.D. (n_{Ctrl} = 15 cells, n_{GlyH} = 54 cells from N = 3 mice).

on exocytosis in rodent (Edlund *et al.* 2017). Here, depolarization-induced glucagon secretion was increased twofold in F508del islets as compared to WT islets when experiments where performed in the presence of forskolin (Fig. 4C). The dysregulated glucagon secretion in F508del islets was not explained by a difference in glucagon content (Fig. 4D).

Impaired somatostatin secretion in the F508del mouse

We measured somatostatin secretion and found that it was elevated in F508del islets compared to WT islets under all glucose conditions (Fig. 4E and F). Normally, cAMP-increasing agents amplify somatostatin secretion during glucose-stimulated conditions (Gerber *et al.* 1981, de Heer *et al.* 2008), which was also the case in the WT islets (Fig. 4F). Surprisingly, forskolin reduced somatostatin secretion in F508del islets during glucose-stimulated conditions (Fig. 4F), but increased somatostatin secretion from islets stimulated in low glucose (Fig. 4E).

The F508del mouse has improved glucose tolerance but reduced glucose clearance

Finally, we investigated glucose homeostasis in the F508del mouse *in vivo*. Fasting blood glucose levels were lower in F508del mice as compared to WT mice (Fig. 5A). Serum insulin was similar in WT and F508del mice (Fig. 5B), while average serum glucagon was three times higher in the F508del mice (Fig. 5C). F508del mice subjected to an intraperitoneal glucose tolerance test (IPGTT) for 120 min demonstrated overall lower increases in blood glucose levels than the WT mice, when calculated as the incremental area under the curve (iAUC) during the sampling period (Fig. 5D and E). In the WT mice, an initial glucose peak (after ~10 min) was followed by a continuous and significant lowering of blood glucose levels reaching the levels of the fasting state after 120 min, whereas in the F508del mice blood glucose remained similarly elevated during the first 30 min of the glucose challenge (Fig. 5D). We used the data measured during the IPGTT to calculate glucose clearance (Fig. 5F). Glucose clearance was delayed

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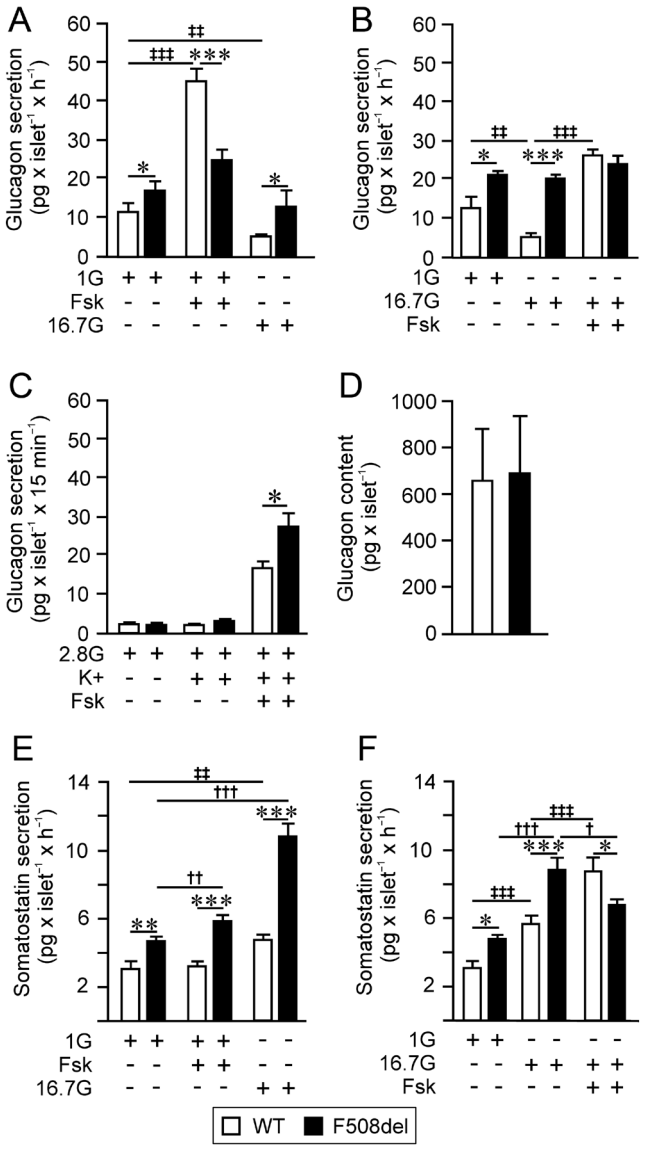

Figure 4
Glucagon and somatostatin secretion from isolated F508del and WT islets. (A) Glucagon secretion at 1 mmol/L glucose (1G) and cAMP-amplified using 10 µmol/L forskolin (Fsk) in WT islets and F508del islets (n_{WT} = 4, $n_{F508del}$ = 4). (B) Glucagon secretion at 16.7 mmol/L glucose (16.7G) amplified using forskolin in WT and F508del islets (n_{WT} = 4, $n_{F508del}$ = 4). (C) Depolarization-induced glucagon secretion in 2.8 mmol/L glucose (2.8 G) and 50 mmol/L KCl (K^+) with or without 10 µmol/L forskolin in islets from separate mice (not pooled, N_{WT} = 5, $N_{F508del}$ = 5). (D) Glucagon content in islets from (A) and (B). (E) Somatostatin secretion measured at 1 mmol/L glucose and amplified using forskolin in WT islets and F508del islets (n_{WT} = 4, $n_{F508del}$ = 4). (F) Somatostatin secretion in 16.7 mmol/L glucose and in presence or absence of forskolin in WT and F508del islets (n_{WT} = 4, $n_{F508del}$ = 4). Data are presented as mean ± S.E.M., white bar WT and black bar F508del *P < 0.05, **P < 0.01, ***P < 0.001 WT vs F508del, ‡‡P < 0.01, ‡‡‡P < 0.001 within WT as compared to another condition, †P < 0.05, ††P < 0.01, †††P < 0.001 within F508del as compared to another condition.

in F508del mice and blood glucose did not return to the fasted state in the F508del animals within the sampling period (Fig. 5D).

Discussion

We show that exocytosis is defective in F508del beta cells together with reduced number of docked insulin granules at the plasma membrane. This is in agreement with our previously published data (Edlund *et al.* 2014), which showed involvement of CFTR in human insulin secretion and beta cell exocytosis. Moreover, we present evidence suggesting that F508del mice have a defective processing and maturation of insulin, manifested as increased proinsulin secretion and decreased C-peptide secretion in forskolin- and glucose-stimulated conditions. Our data suggest that the F508del mutation results in an intrinsic beta cell defect.

During a glucose challenge, the beta cell release insulin in two phases. One rapid first-phase lasting 10–15 min followed by a slower sustained second-phase lasting for hours. Patients with CF have reduced first-phase insulin secretion (Moran *et al.* 1991, Bellin *et al.* 2013). During the IPGTT F508del mice had a lower glucose increase in response to the glucose challenge compared to WT (Fig. 5D). The F508del is a global transgenic mouse with CFTR affected in all cells of the body. The mouse most likely have adapted to its genotype, for example a leaner body (WT 23±0.5 g N_{WT}=45 vs F508del 20±0.4 g $N_{F508del}$=48, P<0.001; Supplementary Fig. 2B) and lower fasting glucose levels compared to WT (Fig. 5A). In the IPGTT, the glucose curve is shifted below the WT. Despite that, the shape of the curve indicates that F508del have a defective glucose handling. It takes 30 min before glucose elimination starts, which may reflect a reduced first-phase insulin secretion. But as we have not measured insulin secretion *in vivo* we cannot exclude that the defective glucose handling is due to peripheral insulin resistance. However, *in vivo* measurements in the *Cftr*^{tm1Eur}/F508del mice on FVB background indicate that these mice have increased insulin sensitivity at 11 week and increased insulin resistance at week 24 (Fontes *et al.* 2015) favoring that the defective glucose handling is coupled to impaired insulin secretion in our study (with 8-week-old mice). Indeed, we observe reduced insulin secretion *in vitro* in F508del mice. It is possible that the defective insulin secretion observed when studying F508del islets on C57BL/6J background *in vitro* are not detected *in vivo* due to the leaner phenotype causing increased peripheral insulin sensitivity (Backhed *et al.* 2004, Fontes *et al.* 2015).

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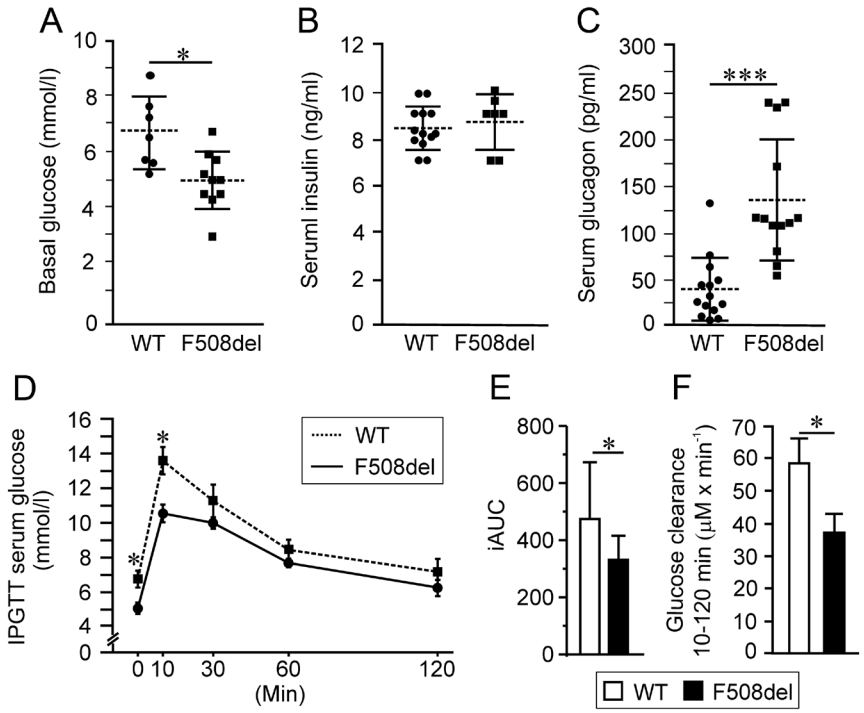

Figure 5
In vivo insulin, glucagon and glucose measurements in WT and F508del mice. (A) Fasting glucose level in WT and F508del mice (n_{WT} = 10, $n_{F508del}$ = 7). (B) Serum insulin (n_{WT} = 13, $n_{F508del}$ = 7) and (C) serum glucagon (n_{WT} = 14, $n_{F508del}$ = 13). (D) Glucose response to IPGTT (n_{WT} = 10, $n_{F508del}$ = 7). (E) Glucose response during IPGTT measured as the incremental AUC (iAUC). (F) Glucose clearance (µM/min) during IPGTT measured as the linear reduction in blood glucose level from the glucose peak at 10 min to the final time point at 120 min. Data are presented as mean ± S.E.M., WT (black filled dots, white bar, dashed line) and F508del (black filled squares, black bar, solid line); *P < 0.05, **P < 0.01 and ***P < 0.001.

In vitro, first-phase insulin secretion is suggested to be manifested by the release of primed granules measured after (1) a short (15 min) depolarizing stimulation of the islets using, for example, high concentration of K^+ or (2) stimulation of depolarization-induced exocytosis of already primed granules using single-cell patch-clamp measurements (Daniel *et al.* 1999, Olofsson *et al.* 2002). Here, we used these two techniques and found that depolarization-induced insulin secretion (Fig. 2E) and rapid exocytosis of docked insulin granules were reduced in F508del islets (Fig. 3A, B and C), suggesting defective first-phase insulin secretion in F508del beta cells. The electron microscopy analysis further confirmed a role for CFTR in insulin granule docking (Fig. 3F). It has been suggested that CFTR regulates the K_{ATP} channel at least in ferret islets (Sun *et al.* 2017). Stimulation of the islets with high K^+ or measurements of exocytosis using patch-clamp bypass the K_{ATP} channel, that is why the results presented here cannot be explained by direct involvement of the K_{ATP} channel.

The functional importance of CFTR in beta cells has been questioned (Sun *et al.* 2017, Hart *et al.* 2018). Single cell transcriptome analysis of human islet cells show that CFTR is expressed in a subset of alpha and beta cells (Blodgett *et al.* 2015, Baron *et al.* 2016, Segerstolpe *et al.* 2016). The single cell transcriptome analyses have revealed a transcriptome heterogeneity in the beta cell population suggesting that there are different pools of beta cells (Baron *et al.* 2016). Indeed, the presence of specific pacemaker beta cells dictating the beta cell response have been suggested (Johnston *et al.* 2016). Hence, in light of the transcriptomic analysis and our recent human data (Edlund *et al.* 2014), an important intrinsic functional role of CFTR in islet cells, despite relatively low expression, cannot be excluded. Our data from young F508del mice support an intrinsic beta cell defect by the observed defects in docking of granules and reduced exocytosis.

The conversion of proinsulin to insulin and C-peptide requires a low pH. H^+ are vital for optimal conversion (Davidson *et al.* 1988), and Cl^- counteract the electrostatic gradient across the granule membrane formed by the entry of H^+ (Barg *et al.* 2001). We found that both F508del beta cells and inhibition of CFTR in WT islets caused an increase in secreted proinsulin (Fig. 2C) during 1 h glucose stimulation, whereas the secretion of proinsulin was not as affected during short depolarization-induced beta cell secretion, where instead the exocytotic response was clearly reduced (Fig. 2E and 3A, B and C). Moreover, we could measure reduced pH in beta cells when priming was stimulated with glucose and cAMP: an effect that was counteracted with GlyH-101 (Fig. 3H and I). Thus, these data indicate that CFTR is involved in the pH-dependent acidification needed for proinsulin conversion and priming of granules. Taken together these results suggests

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that dysfunctional CFTR impair insulin secretion through (1) impaired docking and priming of insulin granules leading to reduced exocytosis measured by short time beta cell stimulation and (2) defective insulin maturation resulting in reduced release of mature insulin and increased release of proinsulin in the later phase of insulin secretion. It is tempting to speculate that the defective exocytosis stresses the F508del beta cell and contributes to the progressive failure seen with age reported by Fontes *et al.* (2015). Moreover, CF patients have been reported to have a skewed proinsulin to insulin ratio (Hartling *et al.* 1988, Hamdi *et al.* 1993, Sheikh *et al.* 2017, Nyirjesy *et al.* 2018), which has also been shown in F508del pig (Uc *et al.* 2015). It can be argued that loss of beta cell mass or endoplasmatic reticulum stress due to misprocessed F508del-CFTR increase proinsulin secretion (Seaquist *et al.* 1996). However, we found that the reduced beta cell area in 7–9-week-old F508del mice was mainly due to reduced cell size and not number. Moreover, proinsulin content was unchanged between F508del and WT islets (Supplementary Fig. 3B), suggesting defects mainly in the last step of insulin maturation.

In addition to the reduced beta cell function, loss of beta cell mass is implicated in CFRD where the continuous inflammation of the exocrine pancreas has been proposed to eventually affect the islets (Gibson-Corley *et al.* 2015). A previously reported study shows that the *Cftr*^{tm1EUR}/F508del mouse model on a FVB background has reduced beta cell mass (Fontes *et al.* 2015). We confirmed these data using the *Cftr*^{tm1eur}/F508del mouse model on a C57BL/6J background (Fig. 1D). These congruent data suggest that the phenotype links to the mutation in *Cftr* and not to the genetic background of the strain to which the *Cftr*^{tm1eur}/F508del was crossed. In addition to the previous observation we found that, the number of beta cells within each islet was similar between WT and F508del mice; suggesting that the reduced beta cell area is mainly due to smaller beta cells. Indeed, estimation of the beta cell size confirms this notion, and the beta cell area was on average ~20% smaller compared to the area in WT beta cells (Fig. 1C). From the area, we can estimate the beta cell radius (r) to be 5.3 and 4.8 µm in WT and F508del beta cells, respectively. Although the volume density of granules (N_v) was similar, an estimate of the total number of granules (N_v × beta cell volume) suggest that each beta cell contains approximately ~13,300 granules and ~10,500 granules in WT and F508del mice, respectively. Thus, F508del beta cells have ~20% less granules than the WT beta cells. Indeed, we measured a reduced insulin content in F508del islets (Supplementary Fig. 3A). The reduced beta cell size might be congenital with obstructed beta cell differentiation (Zertal-Zidani *et al.* 2013) and reduction in insulin granules or changes in cellular osmosis due to aberrant Cl^- conductance (Miley *et al.* 1998).

Individuals with CF have dysregulated glucagon secretion displayed as postprandial hyperglycemia and contradictory failure to suppress glucagon secretion. The severity of the latter increases along with worsened glucose intolerance (Moran *et al.* 1991, Lanng *et al.* 1993, Lanng 2001). Also, glucagon secretion stimulated by insulin induced hypoglycemia was decreased in both CFRD and CF patients with normal glucose tolerance (Moran *et al.* 1991). Serum glucagon was threefold higher in F508del mice, suggesting signs of defective mechanisms to suppress glucagon secretion. Indeed, high glucose concentrations failed to suppress *in vitro* glucagon secretion in F508del islets. Interestingly, the amount of glucagon secreted (in presence of cAMP) from F508del islets during 15 min and 1 h was similar (Fig. 4A, B and C), suggesting a lower maximal capacity in F508del alpha cells. For longer stimulation, the F508del alpha cells reach their full activity already in the presence of low glucose alone and addition of forskolin has a minor effect on amplification. We recently published that CFTR is important for glucagon secretion in human and mouse alpha cells (Edlund *et al.* 2017). The elevated glucagon secretion in F508del islets measured here is in agreement with our current hypothesis that CFTR is involved in the regulation of alpha cell action potential firing (Edlund *et al.* 2017), recently also supported by others (Huang *et al.* 2017). Hence, in addition to the defective insulin secretion caused by the F508del mutation, our data provide insights to why CF patients have defective glucagon counter-regulation.

Somatostatin secretion by delta cells was abnormal in the F508del islets (Fig. 4E and F). Delta cells release somatostatin with increased glucose concentration. Somatostatin receptors are present on both alpha and beta cells and binding of somatostatin inhibits adenylate cyclase, activates inwardly rectifying K^+ channels and/or inhibits Ca^{2+} channels (Kailey *et al.* 2012, Brereton *et al.* 2015). Somatostatin is an important paracrine regulator of insulin and is believed to prevent over-secretion of insulin (Brereton *et al.* 2015). Glucagon secretion is also inhibited by somatostatin (Briant *et al.* 2018). Presence of CFTR in rodent delta cells is unclear. Paracrine effects are likely to contribute to the increased somatostatin secretion. Glucagon has been shown to stimulate somatostatin secretion (Brunicardi *et al.* 2001). In F508del mice, both glucagon and somatostatin secretion is elevated so it is

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possible that the elevated glucagon secretion stimulates somatostatin secretion. Another option is that the increased somatostatin secretion in F508del is explained by altered function of the beta cell. Recently it was shown that depolarization of the beta cells spreads via gap junctions to delta cells causing release of somatostatin and inhibition of glucagon secretion (Briant *et al.* 2018). Moreover, hormone release in the islets is pulsatile and driven by Ca^{2+} oscillations (Gylfe & Tengholm 2014). The oscillations in Ca^{2+} have been shown to be disrupted by inhibition of Cl^- influx using DIDS, which instead causes a steady increase in Ca^{2+} (Eberhardson *et al.* 2000). Considering that CFTR is a Cl^- channel it is likely that mutations in *Cftr* can cause disruption in Cl^- homeostasis and disturbances in the intricate paracrine network. The exact mechanism of how the paracrine network is regulated in F508del mice is not fully elucidated and needs further investigation.

Conclusion

Taken together, the F508del mutation in *Cftr* has consequences for alpha and beta cell function *in vitro* and *in vivo*. The observed defects in the regulation of insulin secretion associated with the F508del mutation become most apparent during cAMP-dependent stimulation. In conclusion, we suggest that presence of F508del mutation will cause (1) reduced exocytosis in beta cells, pointing toward reduced first-phase insulin secretion, (2) elevated release of proinsulin, suggesting defects in the insulin maturation process and (3) alpha cell hypersecretion of glucagon. Our results provide new insight in CFRD and suggest dysfunctional hormone secretion due to alpha and beta cell defects as part of the disease mechanism.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0570.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
A E, M F-T and L E designed the study. A E, M B, M H, I G M, M A, J S E E, E S, A W, E R, E Z and N W participated in acquisition and analysis of data. A E, M F-T and L E participated in interpretation of data. B J S contributed with animal model. A E and L E drafted the manuscript. All authors revised the manuscript for important intellectual content and approved the final version. L E is the guarantor of this work.

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