

# Misassembled mutant $\Delta F508$ CFTR in the distal secretory pathway alters cellular lipid trafficking

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## Summary

Most patients with cystic fibrosis (CF) have a single codon deletion ( $\Delta F508$ ) in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) that impairs assembly of the multidomain glycoprotein. The mutant protein escapes endoplasmic reticulum (ER) quality control at low temperature, but is rapidly cleared from the distal secretory pathway and degraded in lysosomes. CF cells accumulate free cholesterol similar to Niemann-Pick disease type C cells. We show that this lipid alteration is caused by the presence of misassembled mutant CFTR proteins, including  $\Delta F508$ , in the distal secretory pathway rather than the absence of functional CFTR. By contrast, cholesterol distribution is not changed by either D572N CFTR, which does not mature even at low temperature, or G551D, which is processed normally but is inactive. On expression of the  $\Delta F508$  mutant, cholesterol

and glycosphingolipids accumulate in punctate endosomal structures and cholesterol esters are reduced, indicating a block in the translocation of cholesterol to the ER for esterification. This is overcome by Rab9 overexpression, resulting in clearance of accumulating intracellular cholesterol. Similar but less pronounced alterations in intracellular cholesterol distribution are observed on expression of a temperature-rescued mutant variant of the related ATP-binding cassette (ABC) protein multidrug resistance-associated protein 1 (MRP1). Thus, on escape from ER quality control, misassembled mutants of CFTR and MRP1 impair lipid homeostasis in endocytic compartments.

Key words: CFTR, Cholesterol, Cystic fibrosis, Glycosphingolipids, Lipid trafficking

## Introduction

Correct intracellular cholesterol trafficking and distribution among subcellular organelles is essential for the maintenance of cholesterol homeostasis. Cholesterol is a highly hydrophobic lipid that is transported through the aqueous cytosol either by vesicles or by a nonvesicular pathway involving soluble carrier proteins (Mukherjee and Maxfield, 2004). A major source of cellular cholesterol is endocytic uptake of lipoproteins such as low-density lipoprotein (LDL). The lipoprotein-derived cholesterol is rapidly released from endosomes and lysosomes, and is transferred to the endoplasmic reticulum (ER) for esterification, where newly synthesized cholesterol is produced. Retrograde vesicular transport through the Golgi complex has been implicated in the transport to the ER, but the detailed mechanism whereby cholesterol moves to the ER is still uncertain (Chang et al., 2006).

In the inherited lysosomal storage disorder Niemann-Pick disease type C (NPC), transport to the Golgi and ER is blocked and cholesterol and other lipids accumulate in late endosomal compartments (Kruth et al., 1986). White et al. (White et al., 2004) recently observed similar accumulation of free cholesterol in cystic fibrosis (CF). CF is primarily a disease of defective epithelial salt and fluid transport resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Quinton, 1999); however,

the disease phenotype is pleiotropic and includes reports of a variety of lipid alterations (Peretti et al., 2005). More than 1400 different mutations in the gene encoding CFTR have been identified in patients with CF; of these, a deletion of phenylalanine 508 ( $\Delta F508$ ) is the most common, being present in more than 90% of patients with CF (Sferra and Collins, 1993).

The CFTR protein is a member of the ABCC subfamily of ATP-binding cassette (ABC) membrane transport proteins, which play important functional roles in many tissues including the liver, especially the biliary tree, lung, pancreas, retina and immune system (Dean, 2005). Several ABC proteins are involved in multidrug resistance of human cancer cells, but in addition an increasing number have been implicated in genetic disease and in many cases protein misfolding has been shown to be responsible. Over time, CFTR has become a prototype for studies of ABC protein misfolding in genetic disease. Deletion of phenylalanine at position 508 in CFTR results in the temperature-sensitive  $\Delta F508$  protein that cannot mature conformationally at 37°C. Growth of cells at reduced temperature or other manipulations enable the nascent mutant protein to avoid ER quality control, but it is then very rapidly cleared from the distal secretory pathway and degraded in lysosomes (Gentzsch et al., 2004; Sharma et al., 2004).

During the course of experiments to examine the endocytic

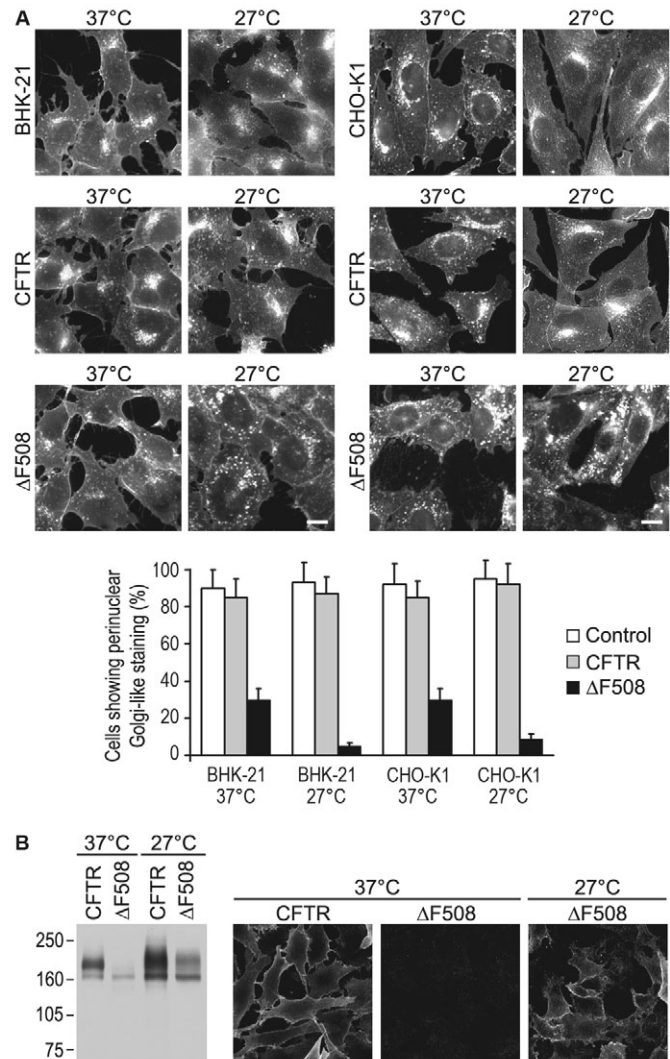
compartments visited by  $\Delta F508$  CFTR, we observed redistribution and accumulation of cholesterol similar to that seen in NPC and to that reported by Kelley and coworkers (White et al., 2004), who attributed this effect to the lack of CFTR function. We show in this study that the presence of several misassembled mutant CFTR proteins in the distal secretory pathway rather than the absence of functional CFTR is responsible for the disturbance of cholesterol trafficking. We present evidence that cholesterol and glycosphingolipids accumulate in endocytic compartments as a result of a block in translocation to the Golgi and ER. Interestingly, we observed similar alterations in intracellular cholesterol distribution on expression of a temperature-rescued variant of a related ABC protein, multidrug resistance-associated protein 1 (MRP1), raising the possibility that there might be a more generalized impact on lipid homeostasis caused by misfolded membrane proteins that avoid ER quality control.

## Results

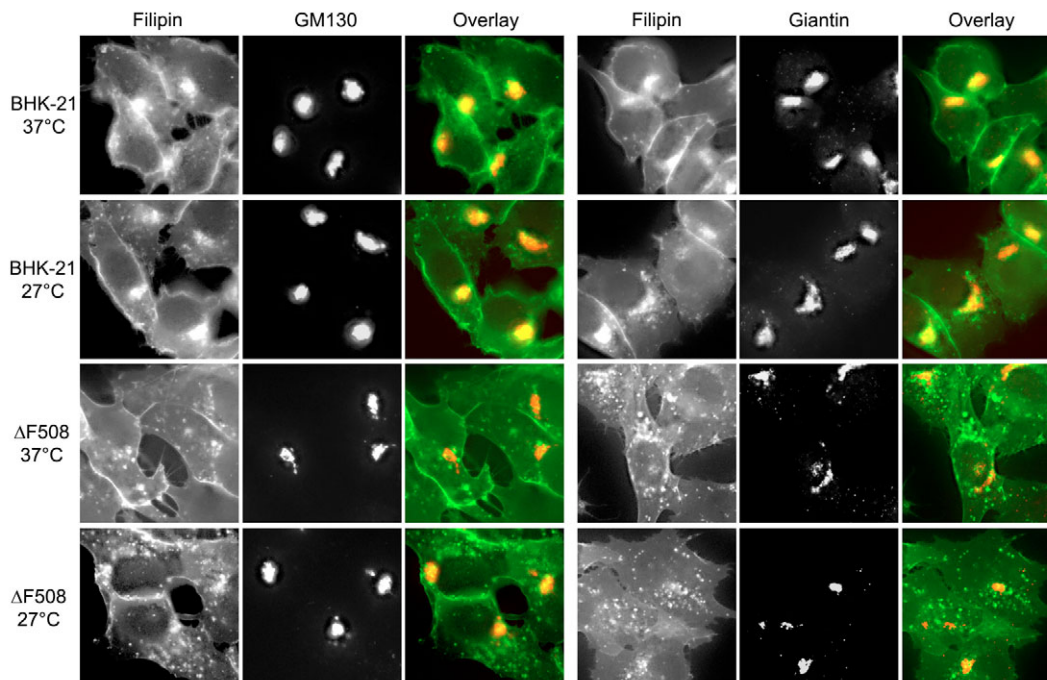
**$\Delta F508$  CFTR perturbs intracellular cholesterol trafficking**  
Following the report of White et al. (White et al., 2004), we employed filipin labeling (Sokol et al., 1988) to detect free cholesterol in baby hamster kidney cells (BHK-21) and Chinese hamster ovary cells (CHO-K1), both of which do not express CFTR endogenously, before and after stable transfection with wild-type or  $\Delta F508$  CFTR (Fig. 1A). Both cell types expressing either wild-type or no CFTR showed staining of a condensed perinuclear region. By contrast, this structure was nearly unstained in  $\Delta F508$ -expressing cells, which instead show intense labeling of punctate vesicular structures throughout the cytoplasm. Importantly, this striking change in distribution was most pronounced in cells that had been cultured at 27°C rather than 37°C, providing an initial clue to the means by which the mutant protein causes this change. To verify that  $\Delta F508$  CFTR escapes ER quality control at reduced temperature, we applied western blotting of lysates from BHK-21 cells expressing CFTR and  $\Delta F508$  CFTR at 37°C and 27°C, and detected mature  $\Delta F508$  CFTR in cells grown at low temperature (Fig. 1B). Immunofluorescence microscopy on nonpermeabilized cells detecting an external epitope confirmed cell-surface localization of  $\Delta F508$  CFTR in cells that were incubated at 27°C. At the lower temperature, the  $\Delta F508$  protein is able to transit from the ER to the Golgi, where it acquires complex oligosaccharide chains and proceeds from there to the cell surface. Thus, the change in cholesterol distribution is most dramatic under conditions where  $\Delta F508$  can enter the distal secretory pathway.

To confirm that cholesterol localizes to perinuclear Golgi-related compartments in BHK-21 cells that were not expressing wild-type or  $\Delta F508$  CFTR, we employed two commonly used Golgi markers, GM130 (Golgi matrix protein of 130 kDa) (Nakamura et al., 1995) and giantin (376 kDa Golgi complex membrane protein) (Linstedt and Hauri, 1993) and costained cholesterol with filipin (Fig. 2). Most of the intracellular cholesterol localized to a perinuclear location that was also labeled by GM130 or giantin. However, in cells expressing  $\Delta F508$  CFTR, cholesterol was distributed throughout the cells in a punctate pattern that was especially obvious at lower temperature (Fig. 1A and Fig. 2). Incubation at lower temperature did not alter the appearance of the Golgi

compartment detected by GM130 and giantin antibodies in either the untransfected host BHK-21 cells or those expressing  $\Delta F508$  CFTR (Fig. 2).



**Fig. 1.**  $\Delta F508$  CFTR perturbs intracellular cholesterol trafficking. (A) Cholesterol redistribution in cells expressing  $\Delta F508$  CFTR is enhanced at reduced temperature. BHK-21 and CHO-K1 cells expressing CFTR and  $\Delta F508$  CFTR were grown at 37°C and 27°C and stained with filipin. Bar, 10  $\mu$ m. The histogram indicates the percentage of cells showing perinuclear Golgi-like staining versus punctate late endosomal/lysosomal staining. For quantification, at least 90 cells were counted from three different experiments and standard deviations are indicated. (B)  $\Delta F508$  CFTR escapes ER quality control at reduced temperature. Western blot of lysates from BHK-21 cells expressing CFTR and  $\Delta F508$  CFTR grown at 37°C and 27°C shows that  $\Delta F508$  CFTR matures when cells are grown at low temperature. Cell lysates were separated by 6% SDS-PAGE and proteins were detected by immunoblotting after transfer to nitrocellulose using anti-CFTR antibody 596. Immunofluorescence microscopy on nonpermeabilized cells using mouse mAb HA11 to detect an external HA epitope in an expanded second extracytoplasmic loop of CFTR, followed by Alexa Fluor 488 goat anti-mouse IgG, confirms cell-surface localization of  $\Delta F508$  CFTR in cells that were incubated at 27°C. BHK-21 clones stably expressing CFTR and  $\Delta F508$  CFTR with HA epitope have been described earlier (Gentzsch et al., 2004).



**Fig. 2.** Most cholesterol colocalizes with Golgi markers GM130 and giantin in BHK cells expressing wild-type or no CFTR, but not in cells expressing  $\Delta F508$  CFTR. GM130 was detected with mouse anti-GM130 mAb followed by goat anti-mouse Alexa Fluor 568 IgG conjugate; giantin was detected by rabbit anti-giantin antibody followed by goat anti-rabbit Alexa Fluor 568 IgG conjugate; and cholesterol was stained with filipin. In the overlay panels, GM130 and giantin are shown in red and filipin staining is shown in green.

### Cholesterol redistribution correlates with CFTR misprocessing not dysfunction

To test the idea that the perturbation of cholesterol homeostasis was due to the presence of a misassembled mutant protein in the distal secretory pathway we examined CFTR variants (Fig. 3A) that are dysfunctional but processed normally (G551D), misprocessed but not rescued from ER quality control at low temperature (D572N) or, like  $\Delta F508$ , misprocessed but rescued at low temperatures (1410X). The diffuse punctate filipin staining pattern was clearly apparent at 27°C in 1410X as in  $\Delta F508$  expressing cells (Fig. 3C).

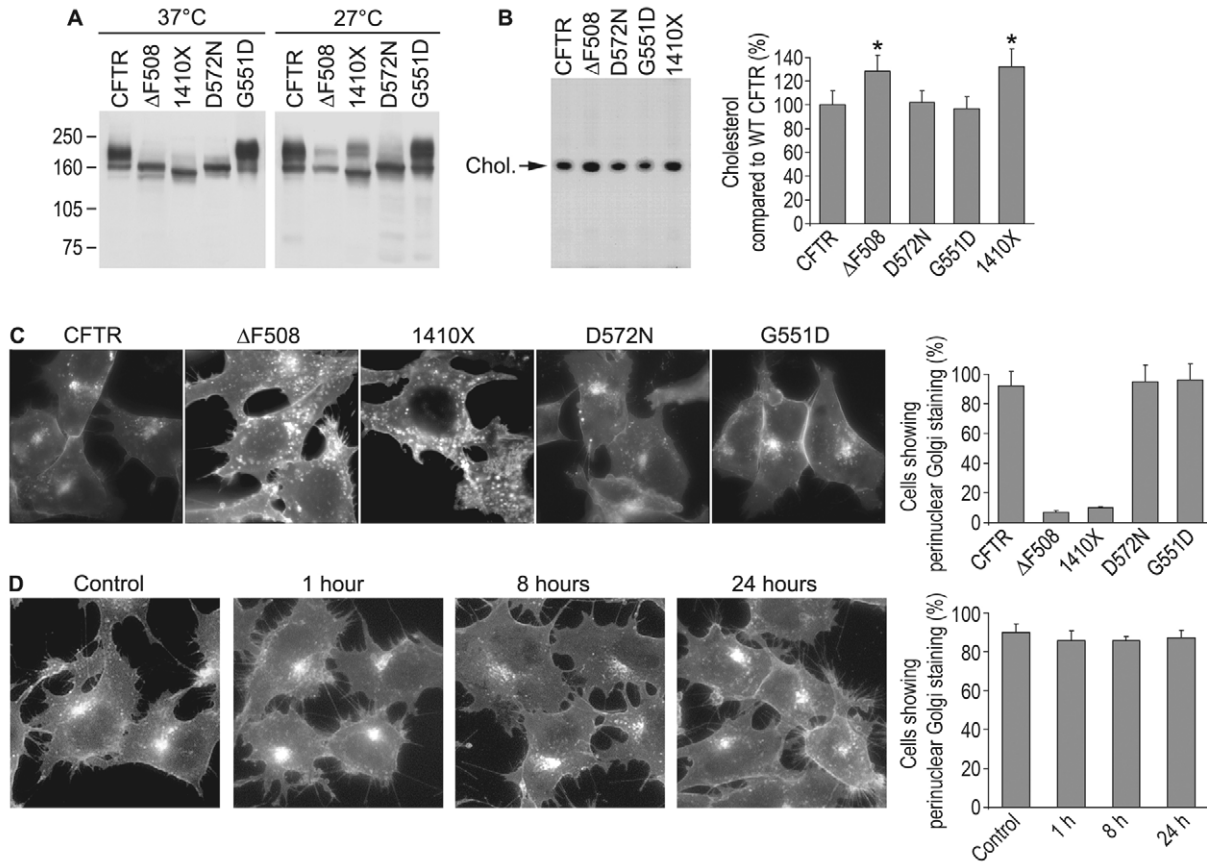
Mature, complex-glycosylated forms of C-terminal CFTR truncation mutants like 1410X were shown to have a five to sixfold faster degradation rate than wild-type CFTR (Haardt et al., 1999) and resemble in this aspect rescued  $\Delta F508$  (Lukacs et al., 1993; Sharma et al., 2001; Sharma et al., 2004). Neither the severe disease-causing mutation G551D, which prevents CFTR channel activation although it is processed normally (Cutting et al., 1990; Gregory et al., 1991), nor the D572N mutation, which is retained at the ER at high or low temperature, changed cholesterol distribution from normal.

We extracted cellular lipids from cells expressing the different CFTR variants grown at 27°C and separated cholesterol by thin-layer chromatography. There were increased amounts of free cholesterol in those variants where it appeared redistributed (Fig. 3B). As independent verification that altered cholesterol homeostasis was not caused by a lack of a functional CFTR, we treated cells with the CFTR channel inhibitor I-172 (Ma et al., 2002) (Fig. 3D). The compound was without effect on cholesterol localization in cells expressing wild-type CFTR. Combined with the fact that cells not

transfected with any CFTR have the same normal cholesterol distribution as those transfected with the wild-type CFTR, these data argue strongly that some factor other than reduced CFTR function is responsible for accumulation of the sterol.

### A related mutant ABC protein $\Delta F728$ MRP1 affects cellular cholesterol distribution

Rescued  $\Delta F508$  CFTR that escapes ER quality control at low temperature rapidly disappears from the cell surface and is degraded in lysosomal compartments (Gentzsch et al., 2004). Our hypothesis is that the presence of the misassembled CFTR glycoprotein in the distal secretory pathway might be responsible for disturbance in cholesterol trafficking and we tested whether the counterpart of the  $\Delta F508$  mutation in CFTR in another member of the same subfamily of ABC proteins, the MRP1 multidrug transporter (Buyse et al., 2004; Deeley and Cole, 2003), would have a similar effect. That this mutation does have a comparable effect on maturation and trafficking from the ER and is temperature sensitive is shown in Fig. 4A,B. Western blotting and immunofluorescence microscopy of cells expressing MRP1 or  $\Delta F728$  MRP1 grown at 37°C and 27°C confirmed that  $\Delta F728$  MRP1 is a temperature-sensitive misfolding mutant that matures at low temperature and proceeds to the cell surface.  $\Delta F728$  MRP1, when rescued at 27°C, causes a partial redistribution of filipin labeling from the normal tightly clustered perinuclear structures to diffuse pan-cytoplasmic vesicles (Fig. 4C). The effect, however, is somewhat less pronounced than with  $\Delta F508$  CFTR rescued by low temperature. The behavior of rescued  $\Delta F728$  MRP1 in the endocytic pathway has not been characterized, but it appears that a temperature-sensitive assembly variant of a second ABC



**Fig. 3.** Misassembled rather than dysfunctional CFTR mutants cause cholesterol redistribution. (A) Influence of reduced temperature on maturation of different CFTR variants. Western blots showing maturation of CFTR and CFTR variants  $\Delta F508$ , 1410X, D572N and G551D grown at 37°C and 27°C. Maturation of CFTR mutants  $\Delta F508$  and 1410X CFTR is rescued by low temperature. D572N CFTR is retained at the ER at high or low temperature and the severe-disease-causing mutation G551D, which prevents CFTR channel activation, is processed normally at 37°C and 27°C. Cell lysates were separated by 6% SDS-PAGE, transferred to nitrocellulose and proteins were detected by immunoblotting using anti-CFTR antibody 596. (B) Free cholesterol (Chol.) is elevated in CFTR-processing mutants. Free cholesterol content of cells expressing CFTR variants at 27°C was quantified after cellular lipid extraction from equal numbers of cells and thin-layer chromatographic analysis to compare amounts of cholesterol in cells expressing mutant CFTR with the amount of cholesterol detected in cells expressing wild-type CFTR. Values are representative of three independent experiments. Cells expressing  $\Delta F508$  and 1410X CFTR show a significant increase in cholesterol content ( $*P < 0.05$ ) in comparison with cells expressing wild-type CFTR. (C) Cholesterol distribution in these variants grown at 27°C. Microscopy showing filipin staining of CFTR and CFTR variants  $\Delta F508$ , 1410X, D572N and G551D grown at 27°C. The percentage of cells showing perinuclear Golgi staining is shown in a histogram. At least 75 cells were counted and values are representative of three independent experiments. (D) CFTR inhibitor I-172 is without effect on cholesterol distribution. Filipin staining of BHK-21 cells expressing CFTR untreated (control) or treated with 10  $\mu\text{M}$  CFTR inhibitor I-172 for 1, 8 or 24 hours. Neither the percentage of cells showing Golgi staining by filipin nor the relative filipin intensity changes in the presence of the inhibitor. The histogram shows the percentage of cells with perinuclear Golgi staining. At least 75 cells were counted and values are representative of three independent experiments.

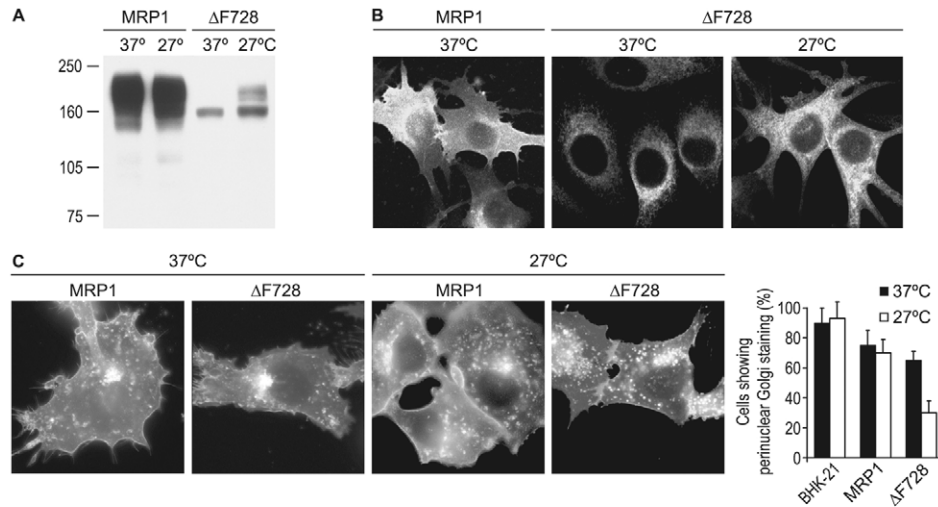
protein also affects cellular cholesterol handling. So-called folding mutations of other ABC proteins have been identified that contribute to several other human genetic diseases (Hanrahan et al., 2003) but their possible influence on the trafficking of sterols or other molecules has not been investigated.

#### Distribution of fluorescent sphingolipid analog is altered in cells expressing $\Delta F508$ CFTR

Given that cholesterol was present in punctuate endosomal locations and quantification of cholesterol revealed that the total amount was increased (Fig. 1A and Fig. 3B), we tested the hypothesis that cholesterol accumulates as a result of a block in transit from the endosomes to the Golgi and ER, the site of esterification. Pulse labeling with the fluorescent analog

NBD-cholesterol resulted primarily in the diffuse punctate pattern at early times, regardless of CFTR genotype (not shown), which persisted at later times in cells expressing  $\Delta F508$  but not in cells expressing wild-type or no CFTR (Fig. 5A). Although cholesterol accumulated in endosomal/lysosomal compartments in cells expressing  $\Delta F508$  CFTR, perinuclear Nile Red staining, which detects cholesteryl esters and other neutral lipids, was reduced (Fig. 5B). This might indicate a block in translocation of cholesterol to the Golgi and ER for esterification, similar to that observed in NPC.

Cholesterol and sphingolipids have high affinity for one another and are the two main components of lipid raft microdomains, and therefore accumulation of one can cause trapping and accumulation of the other (Simons and Gruenberg, 2000). Furthermore, cholesterol affects



**Fig. 4.** Misprocessed ABC protein  $\Delta F728$  MRP1 also affects cellular cholesterol distribution. (A)  $\Delta F728$  MRP1 is a temperature-sensitive misfolding mutant.  $\Delta F728$  MRP1 matures when cells are grown at low temperature. Western blot of lysates from BHK-21 cells expressing MRP1 or  $\Delta F728$  MRP1. BHK-21 cells stably expressing MRP1 or  $\Delta F728$  MRP1 were grown either at 37°C or shifted to 27°C. Lysates were separated by SDS-PAGE, transferred to nitrocellulose and detected using mAb 897.2. (B)  $\Delta F728$  MRP1 escapes ER quality control at low temperature and proceeds to the cell surface. Immunofluorescence microscopy showing localization of MRP1 and  $\Delta F728$  MRP1 at 37°C and 27°C. Immunostaining was performed on cells permeabilized with 0.1% saponin using mouse mAb 897.2 followed by Alexa Fluor 488 goat anti-mouse IgG. (C) Cells expressing  $\Delta F728$  MRP1 show redistribution of cholesterol similar to cells expressing  $\Delta F508$  CFTR. BHK-21 cells expressing MRP1 or  $\Delta F728$  MRP1 were grown at 37°C or 27°C and stained with filipin. The percentage of cells showing Golgi staining is shown in a histogram. At least 75 cells were counted and values are representative of three independent experiments.

sphingolipid trafficking and modulates membrane trafficking along the endocytic pathway in sphingolipid storage diseases (Choudhury et al., 2004; Puri et al., 1999). We found in pulse-chase labeling experiments with the most commonly used fluorescent sphingolipid analog BODIPY-lactosylceramide (BODIPY-LacCer) (Marks et al., 2005) that it did not transit from the punctate cholesterol-containing vesicles to the Golgi in cells expressing rescued  $\Delta F508$  CFTR, but did transit in cells expressing wild-type or no CFTR (Fig. 5C). Thus, there is a block in glycosphingolipid as well as cholesterol trafficking.

#### Rab9 overcomes the cholesterol block induced by $\Delta F508$ CFTR

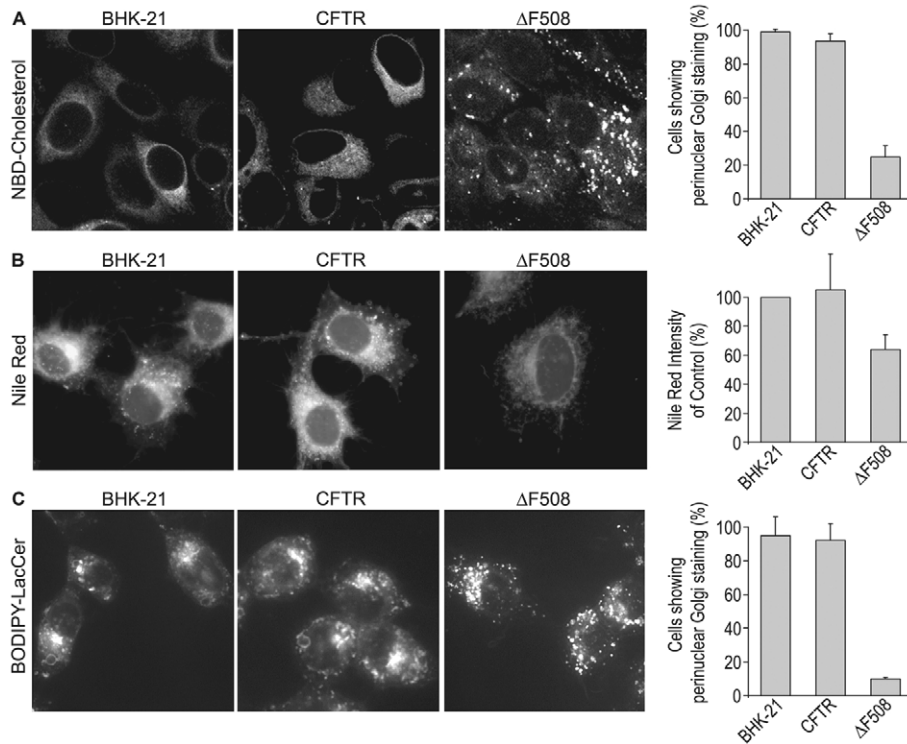
In the case of NPC, we had shown earlier that elevated intracellular cholesterol could be reduced by overexpression of the small GTPase Rab9 that promotes trafficking from late endosomes to the trans-Golgi network (Choudhury et al., 2002; Narita et al., 2005). More recently, we observed that overexpression of Rab9 drives rescued  $\Delta F508$  CFTR along this route (Gentzsch et al., 2004). We asked whether we could rescue cholesterol trafficking in cells expressing  $\Delta F508$  by overexpression of Rab9. BHK-21 cells expressing CFTR or  $\Delta F508$  CFTR were transfected with a plasmid encoding an enhanced green fluorescent protein (EGFP) fusion of Rab9 (Choudhury et al., 2002) and 24 hours later the cells were shifted to 27°C and incubated for another 24 hours. Three representative panels of cells expressing wild-type and  $\Delta F508$  CFTR that had been transfected with Rab9-EGFP are shown in Fig. 6 and cells that overexpress Rab9-EGFP are indicated by an arrow in the filipin panel. The punctate filipin staining is cleared from cells expressing Rab9-EGFP and  $\Delta F508$  CFTR, but remains unchanged in a perinuclear Golgi-like location in

cells expressing Rab9-EGFP and CFTR. The histogram in Fig. 6 shows that Rab9 overexpression is able to clear the punctate cholesterol accumulations and this might be the consequence of Rab9 overcoming an endosome-to-Golgi cholesterol trafficking block in  $\Delta F508$ -expressing cells.

#### Discussion

Although CF is not primarily a disease of lipid metabolism, there is a very large literature describing lipid changes in patients with CF (Peretti et al., 2005). Most prominent is essential fatty acid deficiency (Strandvik et al., 2001), which is generally attributed to defective fat absorption and processing in the intestine, secondary to both the lack of pancreatic lipases and changes in the absorptive epithelium as a result of the absence of the CFTR ion channel. However, there are some suggestions that mutant CFTR might play a more direct role in epithelial essential fatty acid utilization (Bhura-Bandali et al., 2000; Freedman et al., 2004). Serum lipoprotein cholesterol ester depletion occurs in patients with CF, and this is more pronounced in those with greater essential fatty acid deficiency (Levy et al., 1993).

Prior to the report of White et al. (White et al., 2004), little attention had been paid to the cellular handling of cholesterol and cholesterol esters in CF. We have now confirmed and considerably extended these findings. The expression of  $\Delta F508$  and other misprocessed CFTR mutants, but not dysfunctional yet correctly processed variants, clearly causes a redistribution of free cholesterol. Several observations suggest a block in the transport of free cholesterol from a vesicular compartment, probably late endosomes, to the Golgi and ER. There is an increase in cellular free cholesterol in this vesicular compartment and a decrease in cholesterol esters that are normally formed in the ER. Overexpression of Rab9, which



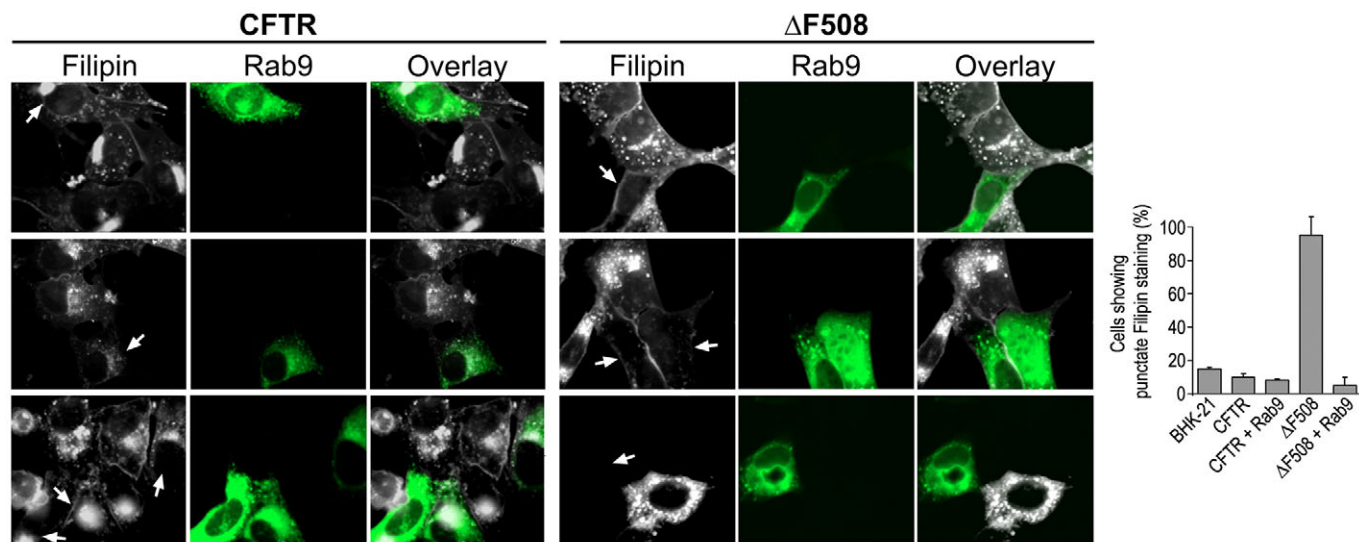
**Fig. 5.** Cells expressing  $\Delta F508$  CFTR show accumulation of glycosphingolipids as well as cholesterol, which is blocked in transfer to the Golgi and ER for esterification. (A) Transfer of NBD-cholesterol to the Golgi and ER is blocked in cells expressing  $\Delta F508$  CFTR. BHK-21 cells were grown at 27°C for 48 hours, labeled with NBD-cholesterol and chased for 4 hours with growth medium at 37°C. The histogram shows that the percentage of cells with perinuclear Golgi staining is drastically reduced in cells expressing  $\Delta F508$ . At least 75 cells were counted and values are representative of three independent experiments. (B) Nile Red staining is decreased in cells expressing  $\Delta F508$  CFTR. Nile Red staining of BHK-21 cells grown at 27°C was reduced in cells expressing  $\Delta F508$  CFTR. The average Nile Red intensity per cell is shown in a histogram. At least 90 cells were counted from three different experiments and standard deviations are indicated. (C) Distribution of fluorescent sphingolipid analog is altered in cells expressing  $\Delta F508$  CFTR. BHK-21 cells expressing CFTR or  $\Delta F508$  at 27°C for 48 hours were labeled with BODIPY-LacCer for 30 minutes at 4°C and subsequently chased for 1 hour at 37°C. The histogram shows that the percentage of cells with Golgi staining is reduced in cells expressing  $\Delta F508$  CFTR.

promotes trafficking from late endosomes to the Golgi, overcomes the cholesterol accumulation and significantly reduces intracellular free cholesterol levels. When presented to cells expressing wild-type or no CFTR, fluorescent NBD-cholesterol progressed from the dispersed punctuate pattern to the perinuclear accumulations over time but remained in the endocytic vesicular compartment in cells expressing misprocessed CFTR mutants. A blockage of the movement of glycosphingolipids between the same two locations in cells expressing misprocessed CFTR was observed using BODIPY-LacCer as also occurs in NPC (Choudhury et al., 2002).

Glycolipids are of interest in CF because asialo-GM1 ganglioside is a receptor for *Pseudomonas aeruginosa*, which is the major colonizing microbe in the CF lung. Localization of CFTR to cholesterol- and glycosphingolipid-containing lipid rafts has been reported to be required for epithelial cell signaling in response to *P. aeruginosa* infection (Kowalski and Pier, 2004).

Wild-type CFTR is normally rapidly endocytosed in clathrin-coated vesicles, with most recycled to the plasma membrane and a small proportion routed to late endosomes and lysosomes for degradation (Bradbury et al., 1999; Lukacs et al., 1997). However, when  $\Delta F508$  CFTR is able to escape ER quality control and reach the cell surface, it proceeds through the

endosomal compartment to lysosomes for degradation without significant recycling to the plasma membrane (Gentzsch et al., 2004; Sharma et al., 2004). Therefore, the presence of the mutant protein in late endosomes could contribute to retention of cholesterol and sphingolipids. How this might occur is largely a matter of speculation at this time. Nothing is known of the interactions of sterols or lipids with either wild-type or mutant CFTR, but it is a multi-spanning integral membrane protein fully integrated into the lipid bilayer. The  $\Delta F508$  mutation deletes a residue from a hydrophilic cytoplasmic domain of CFTR and this perturbs the association and conformation of the two large membrane-spanning domains of the protein that interact with lipid. Although the amount of the mutant polypeptide in endosomal membranes may be insufficient to retain lipid by direct binding it may have an impact on sterol-binding proteins involved in endosomal trafficking such as MLN64 (Alpy and Tomasetto, 2006), ORP1 (Olkonen et al., 2006) or caveolin-1 (Ikonen et al., 2004; Pelkmans et al., 2004). We recently found a small amount of caveolin-1 co-immunoprecipitated with CFTR (A.M. and J.R.R., unpublished observation). There may be as-yet-unknown additional CFTR-interacting cholesterol-binding proteins, possibly even including NPC1 or NPC2, which when mutated cause NPC (Carstea et al., 1997; Naureckiene et al., 2000).



**Fig. 6.** Rab9 overexpression overcomes intracellular cholesterol accumulation. BHK-21 cells expressing CFTR or  $\Delta F508$  CFTR were transfected with a plasmid encoding Rab9-EGFP (Choudhury et al., 2002). After 24 hours, the cells were shifted to 27°C and incubated for another 24 hours, before staining for intracellular cholesterol with filipin. Three representative panels of cells expressing wild-type or  $\Delta F508$  CFTR are shown. Cells that overexpress Rab9-EGFP are indicated by an arrow in the filipin panel. The punctate filipin staining is cleared from cells expressing Rab9-EGFP and  $\Delta F508$  CFTR, but remains unchanged in a perinuclear Golgi-like location in cells expressing Rab9-EGFP and CFTR. Approximately 90 cells of three different experiments were quantified and data are shown in a histogram.

The molecular details of cholesterol trafficking in several lipid storage diseases such as NPC that have been investigated for many years are still not fully understood (Maxfield and Tabas, 2005). In NPC, it has become apparent that the sterol and lipid perturbations are directly caused by mutations in genes encoding NPC1 and NPC2 (Cheruku et al., 2006; Ohsaki et al., 2006; Sleat et al., 2004). By contrast, the superficially similar changes caused by mutant CFTR are obviously secondary events in the CF cells that express mutants that cause misprocessing and not just a loss of function. This raises the possibility that there could be such secondary consequences resulting from misprocessing mutations in the genes encoding other membrane proteins and might emphasize the utility of a stringent ER quality control system to avoid or minimize these effects. We found that the counterpart of the  $\Delta F508$  mutation in a second ABC protein,  $\Delta F728$  MRP1, caused a similar cholesterol redistribution and it will be of interest to examine other ABC proteins in which misprocessing mutations are responsible for several other genetic diseases (Hanrahan et al., 2003).

It is also possible that the lipid localization changes observed are reflective of a more general perturbation of vesicular trafficking, a possibility we are currently investigating. Other alterations of the endocytic pathway, caused for example by the deficiency in lysosomal-associated membrane protein 1 (LAMP-1) and LAMP-2 (Eskelinen et al., 2004) or dynamin inactivation (Robinet et al., 2006), have been reported to disturb cholesterol traffic and, similar to what we have observed, result in accumulation of unesterified cholesterol in endolysosomal compartments.

Overall, our findings have significant implications not only in CF but possibly also more generally in providing an initial indication of the impact on cells of misfolded proteins that gain access to the distal secretory pathway.

## Materials and Methods

### Antibodies and reagents

Anti-MRP1 and anti-CFTR mouse monoclonal antibodies (mAbs) 897.2 and 596 have been described earlier (Aleksandrov et al., 2002; Hou et al., 2000), mouse monoclonal anti-hemagglutinin (HA; 16B12) and rabbit anti-giantin antibodies were from Covance, mouse monoclonal anti-GMA130 antibody was from BD Transduction Laboratories, goat anti-mouse Alexa Fluor 568 and 488 IgG conjugate and goat anti-rabbit Alexa Fluor 568 IgG conjugate were from Molecular Probes (Invitrogen). BODIPY-LacCer was synthesized as described earlier (Martin and Pagano, 1994), filipin was from Polysciences Inc. or Sigma, and Nile Red was from Eastman Kodak. CFTR inhibitor I-172 was synthesized by A. Fauq (Chemical Synthesis Core Facility, Mayo Clinic Jacksonville, Jacksonville, FL, USA) and functionality was confirmed by inhibition of CFTR single channel currents in lipid bilayer experiments (Aleksandrov et al., 2003). Anti-mouse IgG horseradish peroxidase conjugate was from Amersham Bioscience and chemiluminescent substrate was from Pierce. NBD-cholesterol (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesterol-3 $\beta$ -ol) was from Molecular Probes.

### Cells and cell culture

BHK-21 and CHO-K1 cells were obtained from the American Type Culture Collection and grown in DMEM/F12 supplemented with 5% fetal bovine serum (FBS) or MEM Alpha Medium supplemented with 8% FBS, respectively, in humidified incubators with 5% CO<sub>2</sub> at 37°C, or 24–48 hours at 27°C if indicated. BHK-21 and CHO-K1 cells stably expressing CFTR or  $\Delta F508$  CFTR have been described in our earlier publications (Chang et al., 1993; Seibert et al., 1995). BHK-21 clones stably expressing CFTR and  $\Delta F508$  CFTR with HA epitope have been described earlier (Gentzsch et al., 2004). Stable BHK-21 cell lines expressing G551D and D572N variants of CFTR or the C-terminal truncation 1410X CFTR were established as described previously (Chang et al., 1993; Gentzsch and Riordan, 2001; Loo et al., 1998). BHK-21 clones stably expressing MRP1 or  $\Delta F728$  MRP1 were constructed by transfection of cells with the corresponding cDNAs in the pNUT expression vector. To create  $\Delta F728$  MRP1 cDNA, MRP1 cDNA cloned into pNUT (pNUT/MRP1/His) (Chang et al., 1997) was used as a template for in vitro mutagenesis. The phenylalanine residue at position of 728 was deleted by using the following forward and reverse primers and the QuikChange site-directed mutagenesis kit (Hou et al., 2000). The forward and reverse primers for  $\Delta F728$  are:  $\Delta F728$  forward, 5'-CTC CGA GAA AAC ATC CTT  $\Delta\Delta\Delta$  GGA TGT CAG CTG GAG GAA-3'; and  $\Delta F728$  reverse, 5'-TTC CTC CAG CTG ACA TCC  $\Delta\Delta\Delta$  AAG GAT GTT TTC TCG GAG-3'. The  $\Delta\Delta\Delta$  signs indicate that the three nucleotides TTT, the codon for F728, were deleted in the forward and reverse primers. pNUT/ $\Delta F728$ /MRP1/His was confirmed by sequencing.

### Fluorescence microscopy

For fluorescence microscopy, cells were fixed with 4% paraformaldehyde for 10 minutes. Cholesterol was stained with 50  $\mu\text{g/ml}$  filipin as described previously (Choudhury et al., 2004). To monitor internalization of the fluorescent glycosphingolipid analog, cells were incubated 24 to 48 hours at 27°C and labeled with BODIPY-LacCer for 30 minutes at 4°C and subsequently chased for 1 hour at 37°C as described earlier (Choudhury et al., 2002). For Nile Red staining, cells were fixed for 30 minutes with 3% paraformaldehyde and were then treated for 5 seconds with ice-cold phosphate-buffered saline (PBS) containing 0.05% Triton X-100, washed and further incubated with 100 nM Nile Red in PBS for 10 minutes. Nile Red fluorescence was detected at red wavelengths and quantified by image analysis. GM130 and giantin were detected after permeabilization with 0.1% saponin in PBS using mouse anti-GM130 mAb (BD Transduction Laboratories) followed by goat anti-mouse Alexa Fluor 568 IgG conjugate or rabbit anti-giantin (Covance Research Products) followed by goat anti-rabbit Alexa Fluor 568 IgG conjugate. Fluorescence microscopy was carried out using an Olympus IX70 or a Leica DMRB fluorescence microscope and a Zeiss LSM510 confocal laser scanning microscope. Image analysis was performed using MetaMorph V4.6.9 (Universal Imaging Corp.) and ImageJ 1.34s (ImageJ; <http://rsb.info.nih.gov/ij/>) image-processing programs. In any given experiment, all photomicrographs were exposed and processed identically for a given fluorophore.

### Western blotting

Cells were lysed and subjected to SDS gel electrophoresis on 6% polyacrylamide gels. Proteins were blotted to nitrocellulose membranes and probed with anti-CFTR mAb 596 or monoclonal anti-MRP1 antibody 897.2 and visualized by enhanced chemiluminescence detection.

### Quantitative cholesterol analysis

Cellular lipids were extracted with chloroform/methanol (2/1) from  $5 \times 10^6$  cells of each clone and cholesterol was separated by thin-layer chromatography, stained and quantified as described previously (Puri et al., 2003).

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### References

- Aleksandrov, A. A., Aleksandrov, L. and Riordan, J. R. (2002). Nucleoside triphosphate pentose ring impact on CFTR gating and hydrolysis. *FEBS Lett.* **518**, 183-188.
- Aleksandrov, A. A., Fauq, A., Gentsch, M., Aleksandrov, L. and Riordan, J. R. (2003). Influence of a thiazolidine inhibitor on CFTR single channel activity. *Pediatr. Pulmonol. Suppl.* **25**, 202.
- Alpy, F. and Tomasetto, C. (2006). MLN64 and MENTHO, two mediators of endosomal cholesterol transport. *Biochem. Soc. Trans.* **34**, 343-345.
- Bhura-Bandali, F. N., Suh, M., Man, S. F. and Clandinin, M. T. (2000). The deltaF508 mutation in the cystic fibrosis transmembrane conductance regulator alters control of essential fatty acid utilization in epithelial cells. *J. Nutr.* **130**, 2870-2875.
- Bradbury, N. A., Clark, J. A., Watkins, S. C., Widnell, C. C., Smith, H. S., 4th and Bridges, R. J. (1999). Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol.* **276**, L659-L668.
- Buyse, F., Vandendriessche, M. and Ruyschaert, J. M. (2004). Mistargeted MRPdeltaF728 mutant is rescued by intracellular GSH. *FEBS Lett.* **578**, 145-151.
- Carstea, E. D., Morris, J. A., Coleman, K. G., Loftus, S. K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M. A., Pavan, W. J., Krizman, D. B. et al. (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* **277**, 228-231.
- Chang, T. Y., Chang, C. C., Ohgami, N. and Yamauchi, Y. (2006). Cholesterol sensing, trafficking, and esterification. *Annu. Rev. Cell Dev. Biol.* **22**, 129-157.
- Chang, X. B., Tabcharani, J. A., Hou, Y. X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W. and Riordan, J. R. (1993). Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* **268**, 11304-11311.
- Chang, X. B., Hou, Y. X. and Riordan, J. R. (1997). ATPase activity of purified multidrug resistance-associated protein. *J. Biol. Chem.* **272**, 30962-30968.
- Cheruku, S. R., Xu, Z., Dutia, R., Lobel, P. and Storch, J. (2006). Mechanism of cholesterol transfer from the Niemann-Pick type C2 protein to model membranes supports a role in lysosomal cholesterol transport. *J. Biol. Chem.* **281**, 31594-31604.
- Choudhury, A., Dominguez, M., Puri, V., Sharma, D. K., Narita, K., Wheatley, C. L., Marks, D. L. and Pagano, R. E. (2002). Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J. Clin. Invest.* **109**, 1541-1550.
- Choudhury, A., Sharma, D. K., Marks, D. L. and Pagano, R. E. (2004). Elevated endosomal cholesterol levels in Niemann-Pick cells inhibit rab4 and perturb membrane recycling. *Mol. Biol. Cell* **15**, 4500-4511.
- Cutting, G. R., Kasch, L. M., Rosenfeld, B. J., Zielenski, J., Tsui, L. C., Antonarakis, S. E. and Kazazian, H. H., Jr (1990). A cluster of cystic fibrosis mutations in the first nucleotide-binding fold of the cystic fibrosis conductance regulator protein. *Nature* **346**, 366-369.
- Dean, M. (2005). The genetics of ATP-binding cassette transporters. *Meth. Enzymol.* **400**, 409-429.
- Deeley, R. G. and Cole, S. P. C. (2003). Multidrug resistance protein 1 (ABCC1). In *ABC Proteins* (ed. I. B. Holland, S. P. C. Cole, K. Kuchler and C. F. Higgins), pp. 393-422. New York: Academic Press.
- Eskelinen, E. L., Schmidt, C. K., Neu, S., Willenborg, M., Fuertes, G., Salvador, N., Tanaka, Y., Lullmann-Rauch, R., Hartmann, D., Heeren, J. et al. (2004). Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. *Mol. Biol. Cell* **15**, 3132-3145.
- Freedman, S. D., Blanco, P. G., Zaman, M. M., Shea, J. C., Ollero, M., Hopper, I. K., Weed, D. A., Gelrud, A., Regan, M. M., Laposata, M. et al. (2004). Association of cystic fibrosis with abnormalities in fatty acid metabolism. *N. Engl. J. Med.* **350**, 560-569.
- Gentsch, M. and Riordan, J. R. (2001). Localization of sequences within the C-terminal domain of the cystic fibrosis transmembrane conductance regulator which impact maturation and stability. *J. Biol. Chem.* **276**, 1291-1298.
- Gentsch, M., Aleksandrov, A., Aleksandrov, L. and Riordan, J. R. (2002). Functional analysis of the C-terminal boundary of the second nucleotide binding domain of the cystic fibrosis transmembrane conductance regulator and structural implications. *Biochem. J.* **366**, 541-548.
- Gentsch, M., Chang, X. B., Cui, L., Wu, Y., Ozols, V. V., Choudhury, A., Pagano, R. E. and Riordan, J. R. (2004). Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. *Mol. Biol. Cell* **15**, 2684-2696.
- Gregory, R. J., Rich, D. P., Cheng, S. H., Souza, D. W., Paul, S., Manavalan, P., Anderson, M. P., Welsh, M. J. and Smith, A. E. (1991). Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. *Mol. Cell. Biol.* **11**, 3886-3893.
- Haardt, M., Benharouga, M., Lechardeur, D., Kartner, N. and Lukacs, G. L. (1999). C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis. A novel class of mutation. *J. Biol. Chem.* **274**, 21873-21877.
- Hanrahan, J. W., Gentsch, M. and Riordan, J. R. (2003). The cystic fibrosis transmembrane conductance regulator (ABCC7). In *ABC Proteins* (ed. I. B. Holland, S. P. C. Cole, K. Kuchler and C. F. Higgins), pp. 589-618. New York: Academic Press.
- Hou, Y., Cui, L., Riordan, J. R. and Chang, X. (2000). Allosteric interactions between the two non-equivalent nucleotide binding domains of multidrug resistance protein MRP1. *J. Biol. Chem.* **275**, 20280-20287.
- Ikonen, E., Heino, S. and Lusa, S. (2004). Caveolins and membrane cholesterol. *Biochem. Soc. Trans.* **32**, 121-123.
- Kowalski, M. P. and Pier, G. B. (2004). Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for Pseudomonas aeruginosa-induced cellular activation. *J. Immunol.* **172**, 418-425.
- Kruth, H. S., Comly, M. E., Butler, J. D., Vanier, M. T., Fink, J. K., Wenger, D. A., Patel, S. and Pentchev, P. G. (1986). Type C Niemann-Pick disease. Abnormal metabolism of low density lipoprotein in homozygous and heterozygous fibroblasts. *J. Biol. Chem.* **261**, 16769-16774.
- Levy, E., Roy, C., Lacaille, F., Lambert, M., Messier, M., Gavino, V., Lepage, G. and Thibault, L. (1993). Lipoprotein abnormalities associated with cholesterol ester transfer activity in cystic fibrosis patients: the role of essential fatty acid deficiency. *Am. J. Clin. Nutr.* **57**, 573-579.
- Linstedt, A. D. and Hauri, H. P. (1993). Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell* **4**, 679-693.
- Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B. and Riordan, J. R. (1998). Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. *EMBO J.* **17**, 6879-6887.
- Lukacs, G. L., Chang, X. B., Bear, C., Kartner, N., Mohamed, A., Riordan, J. R. and Grinstein, S. (1993). The delta F508 mutation decreases the stability of cystic fibrosis transmembrane conductance regulator in the plasma membrane. Determination of functional half-lives on transfected cells. *J. Biol. Chem.* **268**, 21592-21598.
- Lukacs, G. L., Segal, G., Kartner, N., Grinstein, S. and Zhang, F. (1997). Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation. *Biochem. J.* **328**, 353-361.
- Ma, T., Thiagarajah, J. R., Yang, H., Sonawane, N. D., Folli, C., Galletta, L. J. and Verkman, A. S. (2002). Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J. Clin. Invest.* **110**, 1651-1658.
- Marks, D. L., Singh, R. D., Choudhury, A., Wheatley, C. L. and Pagano, R. E. (2005). Use of fluorescent sphingolipid analogs to study lipid transport along the endocytic pathway. *Methods* **36**, 186-195.
- Martin, O. C. and Pagano, R. E. (1994). Internalization and sorting of a fluorescent analogue of glucosylceramide to the Golgi apparatus of human skin fibroblasts: utilization of endocytic and nonendocytic transport mechanisms. *J. Cell Biol.* **125**, 769-781.
- Maxfield, F. R. and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-621.
- Mukherjee, S. and Maxfield, F. R. (2004). Lipid and cholesterol trafficking in NPC. *Biochim. Biophys. Acta* **1685**, 28-37.
- Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis,



- T. E. and Warren, G.** (1995). Characterization of a cis-Golgi matrix protein, GM130. *J. Cell Biol.* **131**, 1715-1726.
- Narita, K., Choudhury, A., Dobrenis, K., Sharma, D. K., Holicky, E. L., Marks, D. L., Walkley, S. U. and Pagano, R. E.** (2005). Protein transduction of Rab9 in Niemann-Pick C cells reduces cholesterol storage. *FASEB J.* **19**, 1558-1560.
- Naureckiene, S., Sleat, D. E., Lackland, H., Fensom, A., Vanier, M. T., Wattiaux, R., Jadot, M. and Lobel, P.** (2000). Identification of HE1 as the second gene of Niemann-Pick C disease. *Science* **290**, 2298-2301.
- Ohsaki, Y., Sugimoto, Y., Suzuki, M., Hosokawa, H., Yoshimori, T., Davies, J. P., Ioannou, Y. A., Vanier, M. T., Ohno, K. and Ninomiya, H.** (2006). Cholesterol depletion facilitates ubiquitylation of NPC1 and its association with SKD1/Vps4. *J. Cell Sci.* **119**, 2643-2653.
- Oikkonen, V. M., Johansson, M., Suchanek, M., Yan, D., Hynynen, R., Ehnholm, C., Jauhainen, M., Thiele, C. and Lehto, M.** (2006). The OSBP-related proteins (ORPs): global sterol sensors for co-ordination of cellular lipid metabolism, membrane trafficking and signalling processes? *Biochem. Soc. Trans.* **34**, 389-391.
- Pelkmans, L., Burli, T., Zerial, M. and Helenius, A.** (2004). Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell* **118**, 767-780.
- Peretti, N., Marcil, V., Drouin, E. and Levy, E.** (2005). Mechanisms of lipid malabsorption in Cystic Fibrosis: the impact of essential fatty acids deficiency. *Nutr. Metab. Lond.* **2**, 11.
- Puri, V., Watanabe, R., Dominguez, M., Sun, X., Wheatley, C. L., Marks, D. L. and Pagano, R. E.** (1999). Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid-storage diseases. *Nat. Cell Biol.* **1**, 386-388.
- Puri, V., Jefferson, J. R., Singh, R. D., Wheatley, C. L., Marks, D. L. and Pagano, R. E.** (2003). Sphingolipid storage induces accumulation of intracellular cholesterol by stimulating SREBP-1 cleavage. *J. Biol. Chem.* **278**, 20961-20970.
- Quinton, P. M.** (1999). Physiological basis of cystic fibrosis: a historical perspective. *Physiol. Rev.* **79**, S3-S22.
- Robinet, P., Fradagrada, A., Monier, M. N., Marchetti, M., Cogny, A., Moatti, N., Paul, J. L., Védie, B. and Lamaze, C.** (2006). Dynamin is involved in endolysosomal cholesterol delivery to the endoplasmic reticulum: role in cholesterol homeostasis. *Traffic* **7**, 811-823.
- Seibert, F. S., Tabcharani, J. A., Chang, X. B., Dulhanty, A. M., Mathews, C., Hanrahan, J. W. and Riordan, J. R.** (1995). cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and its role in channel activation. *J. Biol. Chem.* **270**, 2158-2162.
- Sferra, T. J. and Collins, F. S.** (1993). The molecular biology of cystic fibrosis. *Annu. Rev. Med.* **44**, 133-144.
- Sharma, M., Benharouga, M., Hu, W. and Lukacs, G. L.** (2001). Conformational and temperature-sensitive stability defects of the delta F508 cystic fibrosis transmembrane conductance regulator in post-endoplasmic reticulum compartments. *J. Biol. Chem.* **276**, 8942-8950.
- Sharma, M., Pampinella, F., Nemes, C., Benharouga, M., So, J., Du, K., Bache, K. G., Papsin, B., Zerangue, N., Stenmark, H. et al.** (2004). Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J. Cell Biol.* **164**, 923-933.
- Simons, K. and Gruenberg, J.** (2000). Jamming the endosomal system: lipid rafts and lysosomal storage diseases. *Trends Cell Biol.* **10**, 459-462.
- Sleat, D. E., Wiseman, J. A., El-Banna, M., Price, S. M., Verot, L., Shen, M. M., Tint, G. S., Vanier, M. T., Walkley, S. U. and Lobel, P.** (2004). Genetic evidence for nonredundant functional cooperativity between NPC1 and NPC2 in lipid transport. *Proc. Natl. Acad. Sci. USA* **101**, 5886-5891.
- Sokol, J., Blanchette-Mackie, J., Kruth, H. S., Dwyer, N. K., Amende, L. M., Butler, J. D., Robinson, E., Patel, S., Brady, R. O., Comly, M. E. et al.** (1988). Type C Niemann-Pick disease. Lysosomal accumulation and defective intracellular mobilization of low density lipoprotein cholesterol. *J. Biol. Chem.* **263**, 3411-3417.
- Strandvik, B., Gronowitz, E., Enlund, F., Martinsson, T. and Wahlstrom, J.** (2001). Essential fatty acid deficiency in relation to genotype in patients with cystic fibrosis. *J. Pediatr.* **139**, 650-655.
- White, N. M., Corey, D. A. and Kelley, T. J.** (2004). Mechanistic similarities between cultured cell models of cystic fibrosis and niemann-pick type C. *Am. J. Respir. Cell Mol. Biol.* **31**, 538-543.