Control of CFTR membrane trafficking: not just from the ER to the Golgi

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Running title: Control of CFTR membrane trafficking

Article type: Minireview

Abstract

Biogenesis of CFTR starts with its co-translational insertion in the membrane of the endoplasmic reticulum (ER) and core-glycosylation. These initial events are followed by a complex succession of steps with the main goal of checking the overall quality of CFTR conformation in order to promote its exit from the ER through the secretory pathway. Failure to pass the various checkpoints of the ER quality control targets the most frequent disease-causing mutant protein (F508del-CFTR) for premature degradation. For wt-CFTR which exits the ER, traffic through the Golgi, is major site for glycan processing, although non-conventional traffic pathways have also been described for CFTR. Once at the cell surface, CFTR stability is also controlled by multiple protein interactors including Rab proteins, Rho small GTPases and PDZ proteins. These regulate not only anterograde traffic to the cell surface, but also its endocytosis and

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/febs.12392

recycling thus achieving a fine and tight modulation of its plasma membrane levels. Exciting recent data have related autophagy and epithelial differentiation to the regulation of CFTR traffic. Herein, we review the various checkpoints of the complex quality control along the secretory traffic and the associated pathways which are starting to be explored for the benefit of CF patients.

Keywords

CFTR, chaperones, ER, ERQC, trafficking, folding, endocytosis, Rho GTPases, Rab GTPases

Abbreviations

AFT, arginine framed tripeptide; AnxA5, annexin V; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; ERQC, endoplasmic reticulum quality control; GERAD, glycoprotein endoplasmic reticulum associated degradation; MSD, membrane spanning domain; Myo5B, myosin Vb; NBD, nucleotide binding domain; PM, plasma membrane; RD, regulatory domain; SRP, signal recognition particle; TGN, trans-Golgi network; UPP, ubiquitin proteasome pathway.

Introduction

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, which when mutated causes the autosomal genetic disease cystic fibrosis (CF), is an integral membrane glycoprotein functioning as a cAMP-activated and phosphorylation-regulated chloride (Cl^-) ion channel at the apical membrane of epithelial cells. CFTR (also ABCC7) is a member of the ABC transport superfamily and its structure contains two membrane-spanning domains (MSD1 and MSD2), two nucleotide binding domains (NBD1 and NBD2) and a regulatory domain (R domain) which is a unique feature among ABC transporters (Fig.1). As happens with other multi-domain glycoproteins, CFTR is co-translationally inserted into the endoplasmic reticulum (ER) membrane and concomitantly N-linked to glycosyl groups. Generally, these posttranslational modifications, as well as interaction with chaperones and folding, occur through an iterate process until the protein acquires its fully folded native conformation [1, 2]. Being a membrane protein, CFTR follows the general route of the secretory pathway, i.e., through the Golgi, to reach the plasma membrane (PM).

The most common disease causing mutation consists in the deletion of three nucleotides that encode phenylalanine 508 in the polypeptidic chain (F508del) and this leads to failure in the protein reaching the PM. This mutation is present in 90% of CF patients in at least one of their two mutated CFTR alleles.

CFTR biogenesis and glycosylation at the ER: folding checkpoints of ER-to-Golgi traffic

Biogenesis of CFTR begins in cytosolic ribosomes that that are targeted through the signal recognition particle (SRP) to the ER membrane translocon (Sec61 complex) via the SRP receptor [1, 3]. The CFTR polypeptidic chain emerging from the ribosome is co-translationally inserted into the ER through the sequential and coordinated action of signal and stop transfer signals through the translocon [3].

Immediately after insertion into the ER membrane, the newly synthesized CFTR polypeptidic chain emerging into the ER lumen, either wild-type (wt) or mutant (F508del), is core-glycosylated at asparagine residues 894 and 900, located in the 4th extracellular loop (Fig.1). N-glycosylation occurs by the addition of a glycoconjugate with 14 osidic residues (reviewed in [4, 5]). N-glycosylation plays a pivotal role in glycoprotein intracellular processes such as folding, sorting and traffic [6]. Generally, these oligosaccharides serve multiple functions by acting mainly as stabilizers and protective shields for the glycoprotein outside the cell and as recognition targets in adhesion and immune response modulation.

However, at the ER, glycosylation plays an important role in the quality control that assesses the folding status of proteins destined for the secretory pathway. For CFTR, an integral membrane protein, glycosylation at the ER constitutes the core-glycosylated (immature) form of the protein, (also called band B) which is used to assess folding by the lectin/chaperone calnexin. For proteins undergoing secretion (being thus biosynthesized into the ER lumen) the folding status at the ER is assessed by calreticulin instead. From the ER, wt-CFTR traffics through the Golgi complex, where it is processed by multiple Golgi glycosyltransferases originating the fully mature form of CFTR (also termed band C) [7]. Usually, these bulky glycans located at the surface of secretory and cell membrane glycoprotein molecules are mostly exposed to the extracellular space. Interestingly, however, a recent study manipulated the 6-7 N-glycan sites of another human membrane glycoprotein – tyrosinase – which exposes these glycans towards the lumen of subcellular organelles. Results from this study demonstrate that removal of two close glycosylation sites arrests the post-translational productive folding process resulting in terminally misfolded mutants subjected to degradation through the mannosidase driven ER-associated degradation (ERAD) pathway [8].

The most frequent mutant protein, F508del-CFTR, is almost completely retained at the ER due to its incorrect folding, and from there it is degraded via the ubiquitin-proteasome pathway (UPP) (reviewed in [4, 5]). The machinery responsible for this retention is generally called the ER quality control (ERQC) [9] and, despite the large amount of data already gathered from multiple studies on the ERQC, it is still unclear how it distinguishes a native protein conformation from a misfolded one. However, it is generally accepted that the folded status of proteins depends both on intrinsic structural motifs ("protein autonomous") which are determined by its primary sequence and on extrinsic factors ("protein non-autonomous") present in the cellular milieu e.g., molecular chaperones [10]. Understanding factors that correct folding and/or prevent ER retention of F508del-CFTR may ultimately be of benefit for the treatment of CF. As the protein autonomous factors determining CFTR folding and in particular F508del-CFTR misfolding were recently reviewed [10] we focus here on protein-non-autonomous factors and respective folding assessment checkpoints.

Chaperone complexes containing Hsc70/Hsp70 and Hsp90 are the two major cytosolic folding machines that monitor the folding status of cytoplasmic proteins or those possessing large cytoplasmic domains. Both have been described to participate in CFTR folding [11-14] (reviewed in [4] and [10]).

Although Hsp70 has long been known to play a key role in the machinery that distinguishes between wt- and F508del-CFTR, recent data has shown that F508del-CFTR (but not wt-) is present in a stalled folding intermediate in stoichiometric association with the core Hsp's 40, 70 and 90, referred to as the "chaperone trap" [15]. In fact, a previous study had characterized such Hsp70-interactions *in vitro* to clarify the mechanism that senses misfolded F508del-CFTR *in vivo*. Interestingly, it was found that isolated F508del-NBD1 binds Hsc70 with higher affinity than wt-NBD1 and that Hsp70-NBD1 binding affinity can be reduced by either ATP or ADP. These nucleotides, however, increase the difference between the Hsp70-binding affinities for F508del- *vs* wt-NBD1, while one small molecule CFTR corrector could reduce it [16].

These and other findings are in full support of a previously proposed model that describes two major checkpoints for the early ERQC of CFTR [17]. According to this model, while wt-CFTR passes through this first Hsc70-mediated ERQC checkpoint, F508del-CFTR is kinetically trapped here (Fig.2), as Hsp/Hsc70 recognizes (and strongly binds to) the exposed hydrophobic residues of mutant protein as its newly synthesized polypetidic chain fails to acquire the native conformation. Although F508 is located at the

outer surface of NBD1, without a major impact on the structure of the isolated domain [18], it is now evident from full-length CFTR protein modelling data that it makes crucial contacts during the inter-domain folding process [19, 20]. This is also supported by earlier data showing that formation of Hsc70-Hdj-2 complexes with nascent wt-CFTR is greatly reduced after expression of the R-domain, suggesting that the intramolecular NBD1–R-domain interaction, catalyzed by Hdj-2/Hsc70, is a critical step in the CFTR folding pathway and that it is defective in the biogenesis of F508del-CFTR [21].

The second ERQC checkpoint (Fig.2) involves the core-glycosylation of CFTR (see above) and the calnexin cycle [2]. In contrast to F508del-CFTR which is mostly retained for UPP-degradation at the Hsp70 folding checkpoint, wt-CFTR proceeds in the folding pathway through this cycle, where it undergoes successive rounds of release-deglucosylation and reglucosylation-rebinding to the ER-membrane chaperone/ lectin calnexin [17]. According to this model, some wt-CFTR unable to acquire a folded conformation, and possibly a small amount of F508del-CFTR escaping degradation at the 1st ERQC checkpoint, undergo proteolytic glycan-mediated-ERAD (GERAD) at this 2nd (calnexin-dependent) checkpoint [17].

A 3rd ERQC checkpoint occurs when CFTR is transported to the Golgi via COPII (coat protein II) coated vesicles that form at the ER exit sites [22]. At this point, misfolded F508del-CFTR fails to exit the ER due to exposure of ER retention motifs - four arginine-framed (RXR) tripeptides (AFTs). Indeed, upon substitution of one Arg by Lys in each of these motifs F508del-CFTR is released to the cell surface [22, 23]. Besides the AFTs retention motifs active export of wt-CFTR from the ER also relies on presence of a di-acidic code (the "DAD" motif located in NBD1 – Fig.2) which acts as a positive cargo signal necessary for Sec24-mediated COPII packing [24] and whose disruption reduces both Sec24-CFTR association and ER exit [25].

The mechanism described above for the early stages of CFTR trafficking constitutes the so-called "conventional" secretory pathway.

Non-Conventional CFTR Traffic

Another mechanistic model describes an "unconventional" traffic route for CFTR transport to the PM that is insensitive to blocking of the conventional trafficking pathway from the ER to the Golgi [26]. This route involves tubular structures migrating peripherally to the central Golgi and appears to be

dependent on syntaxin 13 [27]. According to this model, the total pool of wt-CFTR (folded and misfolded) is captured with equal efficiency by the COPII machinery. However, only CFTR protein that has undergone a degree of maturation will reach the *cis*-Golgi, while the rest is recycled back to the ER in COPI vesicles [26]. Proteins recycled back to the ER re-enter the folding process and are either sent once more to the *cis*-Golgi, or converge towards ubiquitination and are degraded via UPP [28].

More recently, ER stress was described to induce cell surface trafficking of the ER core-glycosylated wt- and F508del-CFTR via a GRASP55-dependent pathway [29]. In this study GRASPs (Golgi reassembly stacking proteins) were proposed to be among the tethering factors that *(i)* are involved in the ER stress-induced non-conventional secretion, *(ii)* specifically associate with cargo molecules through their PDZ domains, and *(iii)* are activated by specific upstream kinases [32].

The major final difference between these two "unconventional" traffic routes for CFTR to the cell surface is actually the protein glycosylation status. In the former the protein still travels back to early Golgi for oligosaccharide processing to the complex form [27]. In contrast, when trafficking through the GRASP55-dependent route, CFTR reaches the PM in its core-glycosylated form [29]. As complex glycosylation is a critical factor in the stabilization of membrane proteins, the possible therapeutic significance of this alternative route as a strategy to rescue mutant CFTR is probably limited.

Autophagy and CFTR traffic

As mutant CFTR is targeted for degradation at the proteasome, the formation of protein aggregates, termed "aggresomes", has been reported to occur [30]. Such "aggresomes" are then degraded mainly through autophagy through the lysosomal pathway. Recently, this pathway was shown for the first time to also play a role in the mechanisms responsible for discarding mutant protein. In fact, dysregulation of the autophagy machinery in F508del-CFTR expressing cells was implicated in the impairment of "aggresome" clearance. Luciani *et al* have shown that upregulation of reactive oxygen species (ROS) production (also a characteristic of cells expressing defective CFTR) leads to aggresome-sequestration of phosphatidylinositol-3-kinase (PI(3)K) complex III and accumulation of p62, which regulates aggresome formation, a mechanism mediated by aggresome-sequestration of the autophagy-related protein beclin 1 [31]. Interestingly, restoration of beclin 1 but also treatment with either cystamine or antioxidants is able to rescue F508del-CFTR trafficking to the membrane, an effect later shown to increase the

activation of F508del-CFTR activity by the potentiators genistein, VX-770 (*Kalydeco™*) and VRT-532 [32].

This autophagy defect was also reported to impair the clearance of *B.cepacia* vacuoles in F508del-CFTR expressing macrophages. Remarkably, stimulation of autophagy by rapamycin decreases both *B. cepacia* infection both *in vitro* by enhancing the clearance via induced autophagy and also *in vivo* contributing to a decrease of bacterial burden in the lungs of CF mice [33].

Regulation of CFTR at the plasma membrane: the role of the Ras superfamily of small GTPases

Regulation of CFTR trafficking, turnover and retention at the surface of epithelial cells is also a complex event in which the folding status of the protein is assessed [34]. Several members of the Rab and Rho subfamilies of the Ras superfamily of small guanosine triphosphatases (small GTPases) have been implicated in the regulation of these late stages of PM traffic. This superfamily, of which the founding members are the Ras oncoproteins, comprises over 150 human members, divided into five major branches on the basis of sequence and functional similarities: Ras, Rho, Rab, Ran and Arf [35, 36]. These low molecular weight proteins (~200 amino acids) maintain a structurally and mechanistically preserved GTP-binding core despite considerable divergence in sequence and function. These variations dictate specific subcellular locations, which in conjunction with a complex network of the proteins that serve as their regulators and effectors allow these small GTPases to modulate a remarkably complex and diverse range of cellular processes [36, 37].

Rab GTPases comprise the largest branch of the Ras superfamily, with over 60 members so far characterized [36]. Rab proteins are key regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways [38]. They allow motor proteins to interact with membranes facilitating vesicle motility and, through interaction with a multitude of other proteins, they coordinate the correct docking and fusion of vesicles with the appropriate target membranes [38, 39]. Several Rab GTPases have been implicated in the regulation of the intracellular transport and the PM delivery of CFTR (Fig.3). Mature, fully-glycosylated CFTR exits the trans-Golgi network (TNG) in vesicles that enter the exocytic pathway ultimately leading to the insertion of the channel into the PM [40]. This process is facilitated by myosin-Vb (Myo5B), a molecular motor that drives vesicle transport through actin cytoskeleton fibres, and is controlled by Rab11, which physically interacts with both CFTR and Myo5B [41].

As CFTR internalization at the cell surface is a rapid process [43] and its biosynthesis and maturation occur slowly, the recycling of internalized channel is considered a key process in maintaining a functional pool of CFTR at the PM [44]. Indeed, several studies have shown that CFTR is continuously recycled from early endosomes back to the PM via Rab11/Myo5B-driven recycling endosomes [41, 45]. Interestingly, Rab4, which has been implicated in rapid recycling of surface proteins [39], and Rab27a, a critical regulator of the exocytic pathway [46], were both shown to physically interact with CFTR (Fig.3), restraining its localization to intracellular compartments, and thus limiting channel expression at the PM [47, 48].The trafficking of CFTR from the PM to early endosomes is controlled by Rab5 [49], a critical regulator of the fusion of endocytic vesicles to these compartments [39]. Another Rab protein, Rab7, regulates the movement of CFTR away from the recycling pathway and into late endosomes [49]. Rab7 is also thought to participate in the transport of CFTR from late endosomes to lysosomes for degradation (Fig.3). Indeed, Rab7 overexpression reduced not only the surface levels but also the endosomal pools of CFTR [49]. In contrast, Rab9 can move CFTR away from lysosomal degradation by mediating its transport from late endosomes back to the *trans*-Golgi, from which CFTR may re-enter the secretory pathway towards PM insertion (Fig.3). While some *in vitro* studies have shown that F508del-CFTR cell surface expression can be increased through the manipulation of key Rab GTPases [49, 50], the mechanisms involved are still unclear. However, a better understanding of these processes may yet provide valuable targets to be used to identify new drugs to treat cystic fibrosis.

While recycling of internalized CFTR to the PM has been considered the main mechanism for sustaining a functional pool of CFTR at the cell surface, a study using multiple cell types, including airway epithelial cells, showed that up to 50% of surface CFTR exists in an immobile pool, tethered to filamentous (F) actin [51]. Tethering of CFTR to the PM involves the interaction of its C-terminal domain with the PDZ adaptor protein Na+/H+ exchanger regulatory factor isoform-1 (NHERF-1) [51-53]. NHERF-1 is important for targeting of exosome- and endosome-associated CFTR to the apical membrane of polarized epithelial cells and for anchoring of CFTR at the PM to the apical actin cytoskeleton. The latter involves the interaction of CFTR-bound NHERF-1 with the ERM (Ezrin/Radixin/Moesin) family protein Ezrin, which locks CFTR in an immobile, actin tethered complex that prevents its endocytosis [51, 53]. Interestingly, annexin5A (AnxA5) was also shown to augment CFTR whole-cell currents, independently of CFTR PDZ-binding domain, an effect consistent with the AnxA5 scaffolding role and not a result of CFTR regulated PM traffic [42].

Notwithstanding, the importance of the actin cytoskeleton in CFTR recycling and immobilization at the PM was highlighted when a dramatic decrease in the surface CFTR pool, with a corresponding increase in the amount of intracellular CFTR, was observed upon N-WASP inhibition and actin cytoskeleton disruption [54]. WASP proteins promote actin polymerization in response to signaling molecules, namely, those of the Rho family of small GTPases. Rho GTPases are found in all eukaryotic organisms and are divided into three subfamilies, grouped according to their functional and structural similarity to their three founding members RhoA, Rac1 and Cdc42. Rho proteins are key regulators of actin cytoskeleton dynamics [55, 56] but have been also implicated in the regulation of cell polarity and membrane trafficking through their modulation of F-actin remodeling [57-59]. Consistently, TC10, a member of the Cdc42 subfamily, was shown to increase CFTR levels at the cell surface by facilitating the actin-driven targeting of CFTR-containing vesicles to the PM [60] (Fig.3). Moreover, activated TC10 also reduces CFTR degradation by favouring the binding of NHERF-1 to the C-terminus of CFTR thus preventing its binding to CAL, another PDZ protein that facilitates the trafficking of CFTR to lysosomes [60, 61] (Fig.3). NHERF-1 overexpression is also sufficient to overcome CAL-induced lysosome targeting of CFTR [62], and increase polarized expression of CFTR on the apical membrane of airway cells, stimulating the vectorial transport of Cl^- [63]. Importantly, overexpression of NHERF1 was also shown to promote apical expression of the F508del-CFTR mutant channel, resulting in a significant rescue of CFTR-dependent Cl^- secretion in bronchial epithelial cell lines [64]. These findings are consistent with the report that some F508del-CFTR is able to escape the proteolytic ERQC pathway and reach the PM [65]. Furthermore, NHERF1 depletion was shown to enhance degradation of temperature rescued F508del-CFTR from the PM [66], thus suggesting that NHERF-1 overexpression contributes to the rescue of mutant CFTR by retaining it on the PM decreasing its susceptibility to degradation. These data strongly indicated an involvement of actin cytoskeleton remodelling and Ezrin-mediated actin anchoring of F508del-CFTR to the PM. Notably, the reciprocal regulation of Rho GTPases and ERM proteins during actin remodelling plays a key role in the distribution and anchorage of macromolecular protein complexes to PM microdomains which are essential to maintain cell polarity [56, 59]. Consistently, NHERF1 overexpression was shown to stimulate the activation of endogenous RhoA and of RhoA-activated kinase (ROCK), thus leading to reorganization of the actin cytoskeleton. The latter occurs with concomitant phosphorylation of Ezrin at Thr567 and stabilization of the multiprotein complex F508del-CFTR-NHERF-1-ezrin-actin at the apical PM thus rescuing CFTR-dependent Cl^- secretion [67]. Remarkably, NHERF-1-induced formation of this multiprotein complex was also shown to enhance the epithelial architecture of F508del-CFTR cell monolayers by favouring CFTR-mediated tight-junction organization,

suggesting a role for RhoA in restoring barrier function in CF-epithelia [68]. The role of adhesion-related proteins in CF pathophysiology was also evidenced in recent studies showing a preferential association of cytokeratins (in particular, cytokeratin-8) with F508del-CFTR and that disruption of this interaction contributes to the rescue of mutant protein [69].

More recently, we have gathered evidence that the effect of NHERF-1 overexpression on CFTR surface levels relies on the activation of both RhoA and Rac1 endogenous signalling [70]. We found however, that that NHERF-1 relies on Rac1 signalling to promote Ezrin-mediated PM anchoring of CFTR, whereas its stimulation of RhoA favours CFTR recycling to the PM via ROCK and possibly actin fibre-associated vesicle transport. Indeed, Rac1 acts by stimulating PIP5K-mediated production of phosphatidyl-inositol-4,5-bisphosphate (PIP2) at the PM. PIP2 binding activates Ezrin by disrupting a head-to-tail inhibitory conformation that prevents its interaction with actin and ERM-binding proteins such as NHERF-1 [71]. PIP2 binding is also required for, and precedes, Thr567-phosphorylation by kinases such as ROCK, which further stabilizes the open conformation of Ezrin [72]. Moreover, CFTR PM-anchoring also requires Rac1-mediated *de novo* polymerization of filamentous actin (F-actin), since interference with this process prevents CFTR surface anchoring [70]. We further demonstrated that NHERF-1 coaxing of F508del-CFTR to the cell surface relies primarily on Rac1-mediated anchoring and retention. Treatment of CF airway cells with the Rac1 selective inhibitor NCS23766 completely prevents F508del-CFTR rescue by NHERF1 overexpression and, in contrast to Rac1, expression of constitutively active RhoA produced no significant increase of F508del-CFTR PM levels [70]. Data also show that activation of endogenous Rac1 signalling through treatment with hepatocyte growth factor (HGF) is sufficient to induce the cell surface anchoring and retention of F508del-CFTR. Most importantly, HGF treatment dramatically enhances the modest efficacy of small-molecule CFTR corrector C4a and investigational drug VX-809, restoring apical expression and function of the mutant channel to near 30% of wt-CFTR in primary human bronchial epithelial cells [70]. These findings reveal surface anchoring and retention as a major target pathway to be considered in CF pharmacotherapy, namely to achieve maximal restoration of F508del-CFTR in patients in combination with correctors and potentiator drugs.

CFTR traffic in proliferating and in differentiated cells

Besides the specific mechanisms controlling ERQC and membrane stability described above, CFTR trafficking is also controlled by the highly specific and tight regulation of the epithelial tissue. Firstly, the

protein is only expressed at the apical membrane of well-differentiated epithelial cells [73]. In fact, intracellular expression of CFTR and exclusion from the PM has long been reported not only for trafficking mutants, such as F508del, but also for wt-CFTR in basal cells of non-CF tissue during remodelling or non-differentiated epithelium [74]. The targeting of CFTR to the apical PM is thus directly and tightly linked to the process of epithelial differentiation/ polarization [75].

More recent studies have re-examined the causal effects of airway damage and remodelling in progression of CF lung pathology, through the usage of wound healing experiments. Interestingly, wound healing airway cells from CF patients were found to repair more slowly than non-CF cells and CFTR inhibition or silencing in non-CF primary airway cells significantly inhibited wound closure. However, these experiments were performed with cells grown on plastic, so that whether this also occurs in well-differentiated and polarized airway epithelia remains to be demonstrated [76]. Notwithstanding, transduction of wt-CFTR into CF airway cell lines or correction of CFBE-F508del and primary CF bronchial monolayers with VRT-325 significantly improved wound healing. Altogether, these observations demonstrate not only that functional CFTR plays a critical role in wound repair but also that differentiation/polarization is somehow related to CFTR trafficking [76].

Concluding remarks

Among CFTR's vast interactome, quality control mechanisms play a key role in assessing its folding and conformation, sorting and processing its glycan moieties, promoting its traffic through the secretory pathway, regulating its delivery to the cell surface, and controlling its endocytosis and recycling. These QC mechanisms discriminate between folded/functional and misfolded/ dysfunctional CFTR, with the latter being sent for degradation. CFTR has been shown to reach the cell surface by non-conventional secretory pathways and exciting recent data have related autophagy and epithelial differentiation to the regulation of CFTR traffic. These complex pathways are starting to be explored and novel proteins identified as potential targets for the handling of the disease to the benefit of CF patients.

Acknowledgements

Work in authors' lab has been supported by strategic grants PEst-OE/BIA/UI4046/2011 (BioFIG) from FCT, Portugal; FCT/MCTES PTDC/SAU-GMG/122299/2010 (to MDA) and FCT/MCTES PTDC/BIA-

BCM/112635/2009 grants (to CMF) and CFF-Cystic Fibrosis Foundation, USA, Ref. 7207534 (to MDA) and also ERS Romain Pauwels Research Award (to CMF). The authors are grateful to Dr. Luka Clarke for critically reading the manuscript.

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Fig.1

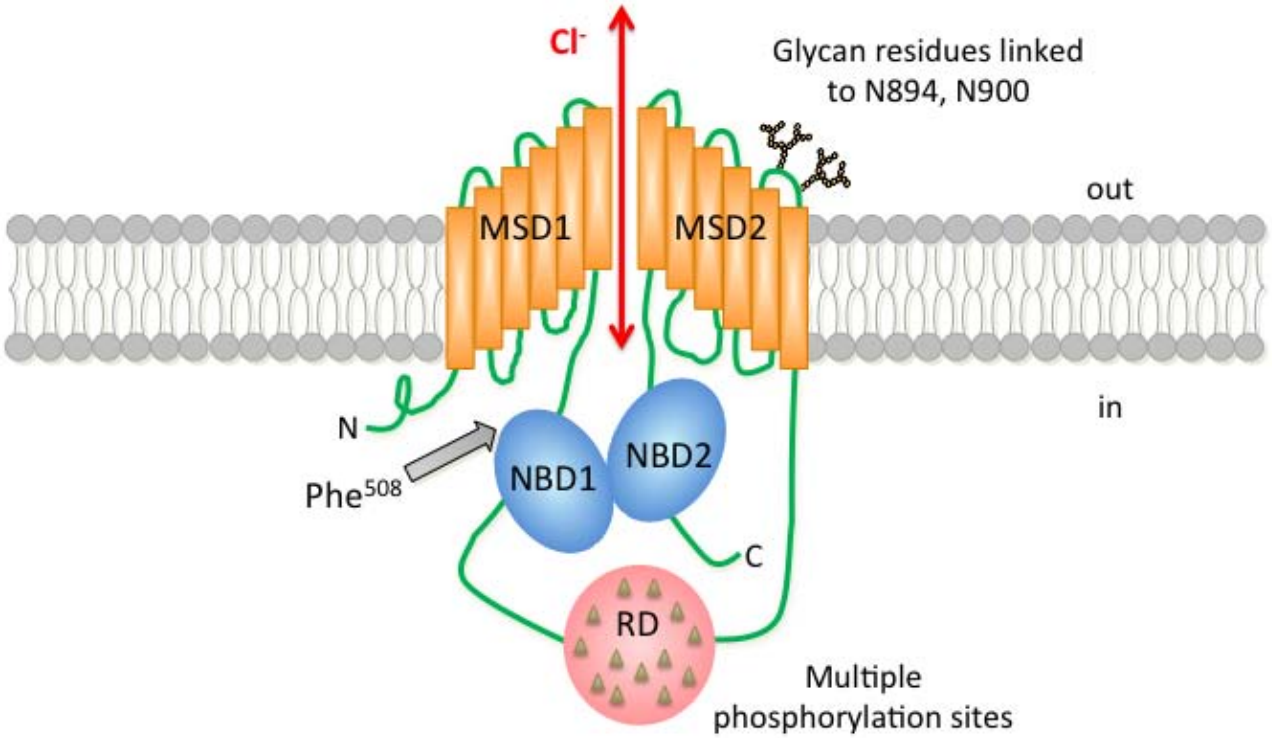

Fig. 1 – Representation of CFTR structure inserted into cell membrane. CFTR domains are indicated, i.e., membrane spanning domains (MSD1 and MSD2); regulatory (R) domain; and two nucleotide-binding domains (NBD1 and NBD2). N, amino terminus; C, carboxy terminus. Position of most frequent mutation (F508del) in NBD1 is also indicated, as well as two Asn-linked glycans, and the presence of multiple phosphorylation sites at the R domain.

Fig.2

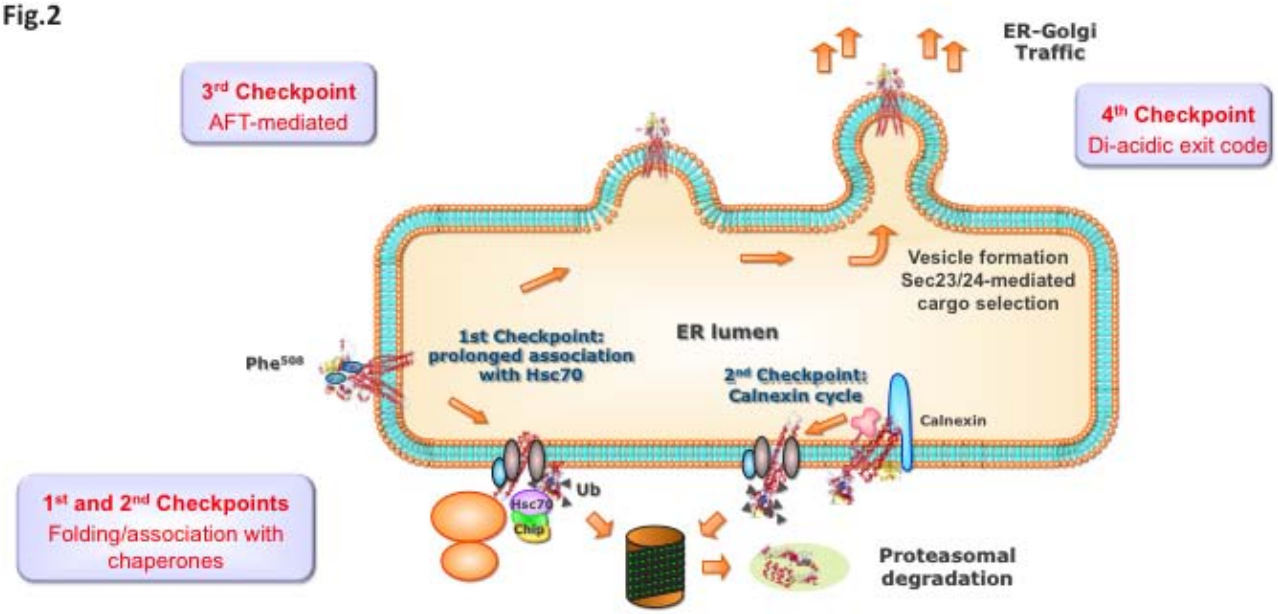

Fig.2 – Checkpoints for the ER quality control of CFTR. (**1st** checkpoint) Hsc70/Hsp70 interact with the cytosolic domains of nascent CFTR to assess its conformation - major mechanism to retain and discard F508del-CFTR. (**2nd** checkpoint) wt-CFTR proceeds in the folding pathway through interaction of its N-glycosyl residues with calnexin. Upon successful folding CFTR exits the ER, proceeding through the secretory pathway – a step that involves both (**3rd** checkpoint) dominant retention (such as the arginine-framed tripeptides) and (**4th** checkpoint) positive export signals (such as the diacidic exit code DAD).

Fig.3

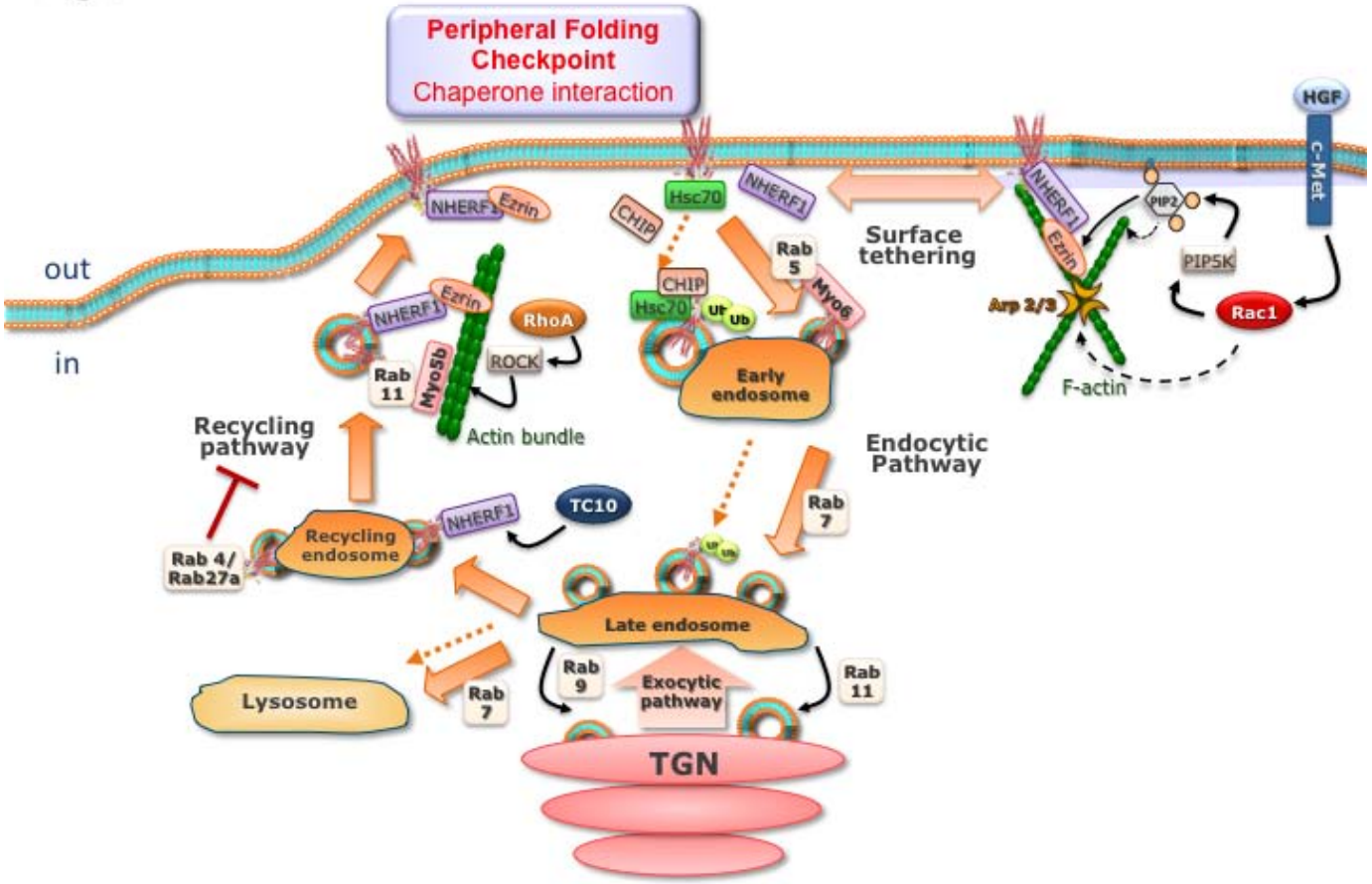

Fig.3 - Regulation of CFTR trafficking and retention at the cell surface by small GTPases. Mature CFTR exits the trans-Golgi network (TGN) in vesicles that enter the exocytic pathway, ultimately leading to the channel's insertion into the plasma membrane (PM). This process is facilitated by myosin-Vb (myo5b) Rab11 and the PDZ adaptor NHERF1. Once at the PM CFTR undergoes a final (the 5th) conformational checkpoint. Here, a prolonged interaction with chaperones leads to the ubiquitination of misfolded CFTR via CHIP-UbcH5 recruitment. This triggers the channel's rapid endocytosis, possibly involving specific Ub-binding endocytic adaptors, leading either to the deubiquitination, refolding and recycling of the protein to the PM, or to its degradation when reprocessing is not possible (e.g. F508del-CFTR). If CFTR passes this last checkpoint it can then be tethered to the the PM via the actin cytoskeleton. Additional extracellular stimuli, such as HGF signaling (see text for details), may be necessary for PM tethering of

CFTR. These stimuli trigger Rac1 GTPase activation at the PM, which leads to the branching and extension of new F-actin beneath the cell membrane. This actin meshwork is produced by the Arp2/3 actin nucleation complex and facilitated by Rac1 induction of PIP2 synthesis through PIP5K activation. PIP2 is required for the uncapping and extension of new actin filaments but also mediates Rac1 activation of Ezrin. Active Ezrin, in turn, interacts with CFTR-bound NHERF-1 and tethers the complex to submembranous F-actin, leading to the anchoring of CFTR at the PM. CFTR dissociation from NHERF-1 leads to adaptor protein-2 (AP2) binding and clathrin-mediated endocytosis of the channel, a process facilitated by myosin-VI (Myo6) and Rab5. Arriving at sorting endosomes, internalized CFTR is either sent for lysosomal degradation via Rab7 or enters the recycling pathway back to the PM, a process facilitated by RhoA signaling, possibly through ROCK activation and the formation of bundles of actin fibers. Interestingly, the CFTR recycling facilitator Rab11 can also promote the channel trafficking back to the TGN, as does the GTPase Rab9. The TC10 GTPase, on the contrary, favors CFTR delivery to the PM by enhancing NHERF1 recruitment to CFTR in sorting endosomes. Finally, Rab 4 and Rab 27a bind CFTR and antagonize its PM trafficking by retaining the channel at the intracellular endosomal compartment.