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Review

Emerging relationship between CFTR, actin and tight junction organization in cystic fibrosis airway epithelium

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Summary. Cystic fibrosis (CF), one of the most common genetic disorders affecting primarily Caucasians, is due to mutations in the *CF Transmembrane Conductance Regulator* (*CFTR*) gene, encoding for a chloride channel also acting as regulator of other transmembrane proteins. In healthy subjects, CFTR is maintained in its correct apical plasma membrane location via the formation of a multiprotein complex in which scaffold proteins (such as NHERF1) and signaling molecules (such as cAMP and protein kinases) guarantee its correct functioning. In CF, a disorganized and dysfunctional airway epithelium brings an altered flux of ions and water into the lumen of bronchioles, consequent bacterial infections and an enormous influx of inflammatory cells (mainly polymorphonuclear neutrophils) into the airways. Recent evidence in healthy airway cells supports the notion that CFTR protein/function is strictly correlated with the actin cytoskeleton and tight junctions status. In CF cells, the most frequent CFTR gene mutation, F508del, has been shown to be associated with a disorganized actin cytoskeleton and altered tight junction permeability. Thus, the correct localization of CFTR on the apical plasma membrane domain through the formation of the scaffolding and signaling complex is likely fundamental to determine a physiological airway epithelium. The correction of CFTR mutations by either gene or drug therapies, as well as by stem cell-based interventions, can determine the resumption of a physiological organization of actin stress fibers and TJ structure and barrier function, further indicating the close interrelationship among these processes.

Key words: Cystic fibrosis, Airway epithelium, Actin cytoskeleton, Tight junction, Barrier function, Neutrophils, Mesenchymal stem cells

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Introduction

In epithelial cells, the maintenance of the polarized phenotype is mainly due to the actin cytoskeleton (Chifflet and Hernandez, 2012) which is essential for various processes necessary to maintain epithelium integrity. Actin filaments control cell morphology, adherence to extracellular matrix and prevention of apoptosis (White et al., 2001), and opening and closing of the intercellular space, i.e. the so called paracellular pathway (Bruewer and Nusrat, 2006).

Tight junctions (TJs) are a complex of highly dynamic structures that play a vital role in establishing/maintaining epithelial polarity and barrier function. TJs can be seen as a hub on which different molecules land and interact with TJ proteins. The paracellular permeability to ions and small solutes is dependent on TJs and, consequently, fine regulation of this transport pathway is crucial for normal epithelial cell functions. Moreover, ion transporters and channels

have been identified having a function to modulate TJ structure and paracellular permeability, including Na^+,K^+-ATPase, Na^+-glucose cotransporter and also chloride channels (Rajasekaran et al., 2008). The actin cytoskeleton is an essential component of functional TJs and allows a correct polarization of epithelial cells. Moreover, the actin cytoskeleton can modulate membrane channel proteins either by direct binding or indirectly via actin binding proteins (Sasaki et al., 2014), suggesting that the epithelial function can depend on the interplay between TJs and signalling proteins linked to TJs, actin and actin-binding proteins, and channel/transporters.

Alterations in TJ organization and function are involved in the pathophysiology of chronic inflammatory diseases such as inflammatory bowel disease, multiple sclerosis, allergies and diabetes and their complications (Harhaj and Antonetti, 2004; Turner, 2006; Cereijido et al., 2007). Indeed, it is well known that inflammatory cytokines can modulate the TJ barrier function in the gut (Macdonald and Monteleone, 2005). Importantly, it has been recently reported that an altered epithelial permeability is implicated also in cancer (Sawada, 2013).

Cystic fibrosis (CF) and related diseases are due to mutations in the *CF Transmembrane Conductance Regulator* (*CFTR*) gene, the product of which is a chloride channel itself and a regulator of other ion channels expressed by epithelial cells (Vankeerberghen et al., 2002). These mutations lead to a lack/dysfunction of the CFTR protein on the epithelium of various organs, including pancreas, lung, intestine, sweat glands, vas deferens and epididymis, and thus determining tissue damage accompanied by atrophy and fibrosis. TJs regulate selective permeability of epithelial monolayers to ions and small molecules, thus possibly modulating in concert with CFTR (and other channels) the vectorial ion transport through the epithelia. Indeed, TJs are inherently coupled with ion channels. To date, it is not well known whether the TJs modulate the CFTR-linked activities or *vice versa*, CFTR protein could influence TJ functions. However, many observations - which we will discuss throughout this review - seem to point out that CF can be thought of as a disorder of the TJs and actin cytoskeleton organization.

Tight junctions and airway epithelium: physiological aspects

The airway epithelium of the human upper respiratory tract is an impermeable barrier that protects against inhaled pathogens or substances. TJs constitute one of the intercellular junctional complexes, in particular the most apically located. These junctions regulate solute and water flow through the paracellular space ("gate function") and determine cell polarity dividing the apical side from the basolateral side, also contributing significantly to the "barrier function" of conductive airway epithelium. Moreover, TJs regulate cell signalling (Gonzalez-Mariscal et al., 2008), gene expression, cell proliferation and differentiation (Balda and Matter, 2009; Koch and Nusrat, 2009). Although adherens junctions (AJs) and TJs are distinct intercellular junctions, they can interact with each other and these interactions seem to be crucial during junction assembly (Matter and Balda, 2003b).

Epithelial TJs are constituted by different transmembrane proteins, such as: 1) MARVEL protein family members (i.e., occludin, tricellulin, and MARVELD3); 2) members of claudin family; 3) the immunoglobulin-like proteins superfamily such as junctional adhesion molecules (JAMs); and 4) a complex of cytoplasmic scaffolding proteins containing PDZ (for postsynaptic density 95/disclarge/zona occludens) domains, such as zonula occludens (ZO) proteins. Transmembrane proteins connect the membranes of neighbouring cells forming a tight seal, while scaffolding proteins anchor transmembrane proteins to the cytoskeleton (Ganesan et al., 2013; Rezaee and Georas, 2014).

Both the measurements of either transepithelial electrical resistance (TER) or permeability to probes of different size (dextran and mannitol) are the current methods to study the tight junctions function ("gate function" and "barrier function" respectively) in cells grown *in vitro* onto semi-permeable membranes (Matter and Balda, 2003a; Steed et al., 2010). Regarding the airway epithelium, these methods were applied to immortalized cell lines, even though the use of primary epithelial cells differentiated at air-liquid interface (ALI) could better recapitulate the complexity of the structure of the airway epithelium *in vivo* (Lin et al., 2007).

Claudins

Claudins are a family of proteins (at least 27 claudin family members have been identified) with short N-terminal and longer C-terminal cytoplasmic domains and two extracellular loops. All claudins (except claudin-12) possess a carboxy terminal PDZ binding motif, which interacts with one of the three PDZ domains of the scaffolding proteins such as ZO-1, contributing to the tight junction organization (Itoh et al., 1999). Different claudins are expressed throughout the tracheobronchial tree with a cell-type specific expression (Frank, 2012), while claudin-3, -4 and -18 are abundantly expressed in the alveolar epithelium (LaFemina et al., 2010). It has been demonstrated that claudin-4 expression is strongly increased in acute lung injury *in vivo*, supporting a protective role for claudin-4 in epithelial barrier function (Wray et al., 2009). By reverse transcribed-PCR, we observed that claudins-1 and -4 were expressed by human bronchial epithelial cell line 16HBE14o- expressing wild-type (wt) CFTR while only claudin-1 was expressed by the human CF bronchial epithelial cell line CFBE41o- (homozygous for F508del-CFTR mutation) upon formation of polarized monolayers

(Castellani et al., 2012).

Occludin

Occludin is a TJ protein presenting four hydrophobic segments and two extracellular loops, one enriched with tyrosine and glycine residues and a second one with several residues of tyrosine. Like claudins, the carboxy terminal of occludin binds to ZO-1, but not by a PDZ-domain (Li et al., 2005). Its physiological function still remains controversial. Several studies inducing overexpression of occludin in MDCK cells suggest that this protein actively promotes the paracellular flux of macromolecules (Balda et al., 1996; McCarthy et al., 2000). Moreover, occludin mediates barrier responses to cytokines, perhaps through an effect on caveolin-1 or other lipid microdomain signaling processes at the junction (Van Itallie et al., 2010). Two studies (Saitou et al., 2000; Schulzke et al., 2005) involving occludin-null mice showed no differences in structure and function of TJ in different tissues such as intestine and stomach if compared with wild type mice, even though lung barrier function was not described in these works. Thus, the role of occludin in regulating epithelial integrity and paracellular permeability at the lungs *in vivo* requires further studies.

JAMs

JAMs are members of the immunoglobulin superfamily comprising JAM-A, JAM-B, JAM-C, JAM-4, JAM-L and CAR (Coxsackie and Adenovirus receptor). JAMs are involved in multiple functions including TJ assembly, regulation of endothelial and epithelial paracellular permeability, leukocyte recruitment during inflammation, angiogenesis, cell migration and proliferation and platelet activation (Luissint et al., 2014). It has been reported that JAM-A failed to reconstitute TJ strand-like structures when overexpressed in mouse fibroblasts lacking TJs, while the effect of reconstitution was obtained with overexpression of claudin-1 in the same cellular system, suggesting that JAM-A was not directly involved in the formation of TJ strands (Itoh et al., 2001). Recently, it has been demonstrated that JAM-A plays a key role in the regulation of TJ dynamics in alveolar epithelial cells both *in vivo* and *in vitro* by coordinating interactions among claudins, the TJ scaffold, and the cytoskeleton. Specifically, depletion of JAM-A diminished claudin-18 localization to tight junctions and decreased ZO-1 expression, resulting in a compromised actin cytoskeleton assembly (Mitchell et al., 2015).

ZO-1

ZO-1, the first identified tight junction protein, belongs to a family of multidomain proteins (including ZO-2 and ZO-3) able to form interactions with other TJ proteins, the actin cytoskeleton and a number of actin binding proteins (Fanning et al., 2002), and other signalling molecules (Rodgers et al., 2013). Concerning the airway epithelial cells, several studies reported a direct correlation between alteration in ZO-1 expression and decreased barrier function in different models. For example, in a polarized model of airway epithelial barrier *in vitro*, obtained by growing 16HBE14o- cells onto permeable membrane filters for seven days to form a polarized monolayer, our research group (Castellani et al., 2012) detected a TER indicative of a tight barrier (~500 $\Omega \cdot cm^2$) and observed a complete organization of ZO-1 with a typical chicken-wire pattern by confocal analysis, while CFBE41o- cell monolayers showed a disorganized ZO-1 mostly localized in the nucleus with a low TER (~100 $\Omega \cdot cm^2$). Interestingly, despite the different organization, the levels of expression of ZO-1 in the two cell lines were similar.

In 16HBE14o- cells, lipoxin A4 (LXA_4) treatment significantly increased ZO-1 levels at the plasma membrane stimulating the increase of TER, and this effect was inhibited by boc-2, a LXA_4 receptor antagonist (Grumbach et al., 2009). These results are consistent with the low levels of LXA_4 found in the airway cells of CF patients (Karp et al., 2004), allowing the transmigration of inflammatory cells via disorganized TJs.

Signalling

TJs are involved in two main types of signal transduction process: 1) signals towards TJs guiding their assembly and therefore also paracellular permeability, mediated by G proteins, protein kinase A (PKA), different protein kinases C (PKC) and Rho family GTPases; and 2) signals from TJs regulating gene expression, cell proliferation and differentiation (Matter and Balda, 2003b). Different studies have linked different Rho GTPase family members, including RhoA, Rac and Cdc42 to the regulation of junctional signalling mechanisms (Samarin and Nusrat, 2009; Terry et al., 2010; McCormack et al., 2013). In particular, RhoA-dependent modification of paracellular permeability involves Rho-associated protein kinase (ROCK). Moreover, the actin cytoskeleton directly regulates TJs by interacting with occludin, cingulin and the ZO-proteins. Our research group demonstrated that RhoA is involved in both the NHERF1-induced assembling of actin stress fibers (Favia et al., 2010) (Fig. 2) and in TJ re-organization and function in airway epithelial cells *in vitro* (Castellani et al., 2012).

Actin and cytoskeleton

The importance of actin filaments in maintaining the integrity of the epithelial barrier has extensively been demonstrated. In polarized epithelial cells, the cytoskeleton displays distinct spatial organization in the apical and basolateral compartments, with the apical region rich in actin filaments assembled in several

structures. Myosin and actin constitute a ring that encircles the cells at the level of TJ and AJs (Ivanov et al., 2010). Activation of actomyosin contraction depends on myosin light chain kinase (MLCK) that phosphorylates myosin II regulatory light chain (MLC). A constitutive expression of MLCK in confluent polarized intestinal epithelial cells (Caco-2) increased MLC phosphorylation, reorganizing perijunctional F-actin and increasing tight junction permeability (Shen et al., 2006).

The effects of exposure to cigarette smoke on actin cytoskeleton and TJs in the airway epithelial cells were intensely studied in the Calu-3 cells, derived from human bronchial submucosal glands. In particular, cigarette smoke exposure caused an increase in phosphorylation of MLC with activation of ROCK, as well as an increase of polymerized F-actin content and a redistribution of the TJ proteins from the normal apical site to a more basal location with a concomitant increase in macromolecular permeability (Olivera et al., 2010).

CFTR and cystic fibrosis

CFTR expression and function

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein is a cAMP-activated ion channel directly involved in the transport of chloride and participating in the transport of other ions, namely sodium and bicarbonate, in absorbing/secreting epithelia of many organs (Rowe et al., 2005; Chan et al., 2009). Indeed, CFTR protein is expressed in the trachea, lung, pancreas and several tissues of the reproductive system (Quinton, 1999; Rowe et al., 2005; Chan et al., 2009; O'Sullivan and Freedman, 2009).

CFTR belongs to ABC transporters that usually contain two nucleotide-binding domains (NBD) and two transmembrane domains (TM), each with six spans of alpha helices. CFTR is unique among ABC transporters because it has a regulatory (R) domain that is phosphorylated by protein kinase A (PKA) and C (PKC). The two halves of CFTR (each comprised of a NBD and a TM) homodimerize to form a functional transporter (Riordan, 2005) (Fig. 1).

CFTR protein has been implicated also in cellular processes apparently unrelated with ion transport, such as epithelial cell polarization and differentiation. Hollande and collaborators initially suggested that the targeting of CFTR to the apical membranes is directly linked to the process of cellular polarization in cancerous pancreatic cells (Hollande et al., 1998). In a following study, pancreatic CFPAC-1 cells transfected with wt CFTR, showed a distribution of both the Golgi complex and microtubules consistent with what was usually observed in polarized epithelial cells, indicating the importance of CFTR in maintaining the integrity of the biosynthetic/secretory pathway (Hollande et al., 2005). An increased proliferative activity was observed in intestinal crypt epithelial cells of CFTR-null mice (Gallagher and Gottlieb, 2001), according to what was observed by Leigh et al., who showed an increased proliferative index in the airways of CF subjects (Leigh et al., 1995). In line with these findings, a delayed differentiation and regeneration in non-infected human

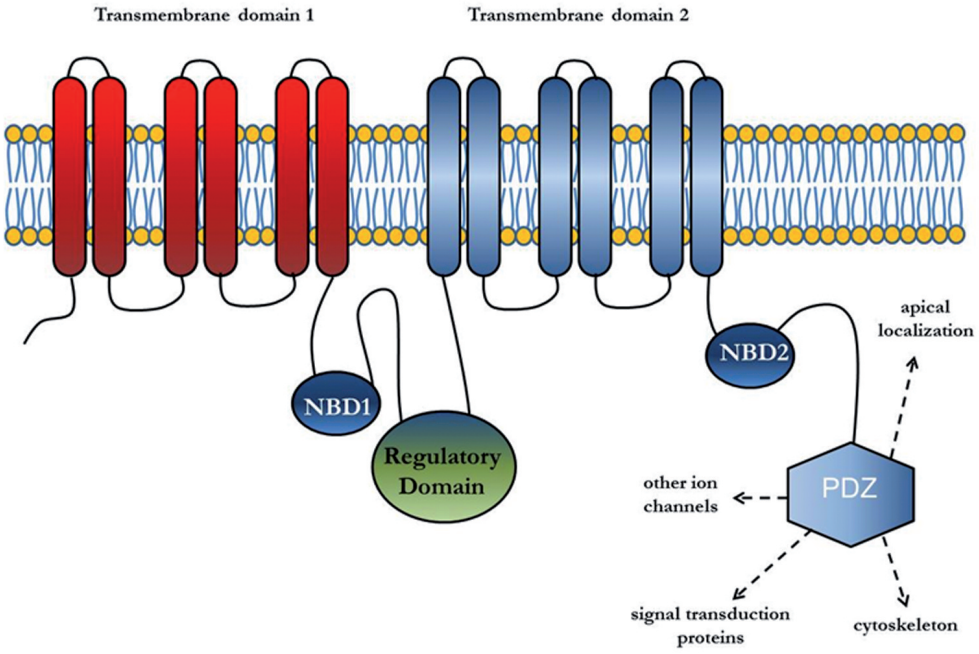

Fig. 1. CFTR structure and interactions with other intracellular proteins. The CFTR protein consists of 1480 amino-acids and is composed of different domains. The two transmembrane domains (each composed of 6 trans-membrane units) form the chloride channel. The nucleotide binding domains (NBD1, NBD2), each accommodating an ATP binding site, have an important role in the opening and closing of the channel. The regulatory domain is involved in the opening of the channel by the protein kinase A-dependent phosphorylation. The carboxy terminal part of the protein is comprised of a PDZ-interacting domain which is required for CFTR apical polarization and interaction with the PDZ domain of other proteins, allowing CFTR interaction with the actin cytoskeleton, other ion channels and signalling molecules.

CF nasal epithelia has been demonstrated (Hajj et al., 2007).

More recent observations demonstrate that: 1) CFTR interacts at the level of TJs with ZO-1 through its PDZ-binding domain; 2) CFTR deficiency impairs TJs assembly and reassembly, and prevents tubulogenesis in epididymal cell line DC2; 3) CFTR keeps in control the transcription factor ZO-1 nucleic acid binding protein (ZONAB, also known as YBX3) at the TJ level, while CFTR inhibition or knockdown induces the translocation of ZONAB into the nucleus leading to upregulation of proliferation (Ruan et al., 2014). These findings suggest that the role of CFTR in the epithelial organization seems to be unrelated with its channel activity. Developmental defects have been recently reported in the trachea of young CF patients and newborn CFTR-knockout pigs (Meyerholz et al., 2010). Altogether, these studies indicate the possible role of CFTR in the control of epithelial proliferation and differentiation in several tissues in strict relationship with TJ proteins.

The macromolecular complex of CFTR

In airway epithelial cells, apical localization of CFTR protein and its chloride channel activity strictly depend on interactions of its PDZ-interacting domain in the C-terminus with other proteins primarily localized to the apical surface of epithelial cells (Moyer et al., 1999; Li and Naren, 2005). Thus CFTR localization to the apical plasma membrane and its function are regulated by these macromolecular complexes, which contain signaling molecules, kinases, transport proteins, PDZ-domain-containing proteins, myosin motors, Rab GTPases, and soluble N-ethylmalemide sensitive factor attachment protein receptors (SNAREs) (Guggino and Stanton, 2006). The first PDZ domain protein that was identified to bind to and regulate CFTR was the Na^+/H^+ exchanger regulatory (NHERF1) that was originally discovered as an essential inhibition co-factor of the Na^+/H^+ exchanger NHE3 (Lamprecht et al., 1998). Besides NHERF1 (also known as EBP50, ezrin-binding protein, 50 kDa), NHERF2, CAP70 (CFTR-associated protein, 70 kDa, also known as NHERF3), NHERF4, and CAL (CFTR-associated ligand) can bind to CFTR through their PDZ-domains (Wang et al., 1998; Hegedus et al., 2003; Guggino, 2004; Shenolikar et al., 2004; Li and Naren, 2005; Bossard et al., 2012). NHERF1 contains two PDZ domains and a C-terminal portion through which it can bind to the N-terminal domain of the ERM proteins ezrin, radixin, or moesin (ERM binding domain). Importantly, the ERM protein ezrin has been found to promote stress fibers assembly and cortical actin polymerization in response to the activation of RhoA (Mackay et al., 1997; Favia et al., 2010). The active, open form of ezrin co-localizes with NHERF1 at or near the plasma membrane of polarized cells where they reciprocally stabilize each other and function together in organizing macromolecular complexes (Morales et al., 2004). Ezrin activity is regulated by intramolecular interactions between its own N-(FERM) and C-terminal binding sites (Morales et al., 2007) (Fig. 2). In the inactive, cytosolic state this intramolecular interaction masks its NHERF1 and actin binding sites, and two events appear to be required for ERM protein activation: the interaction of phosphatidylinositol 4,5-bisphosphate (PIP2) with the FERM domain of ezrin and the phosphorylation of T567 residue in the COOH terminal region (Bretscher et al., 2002;

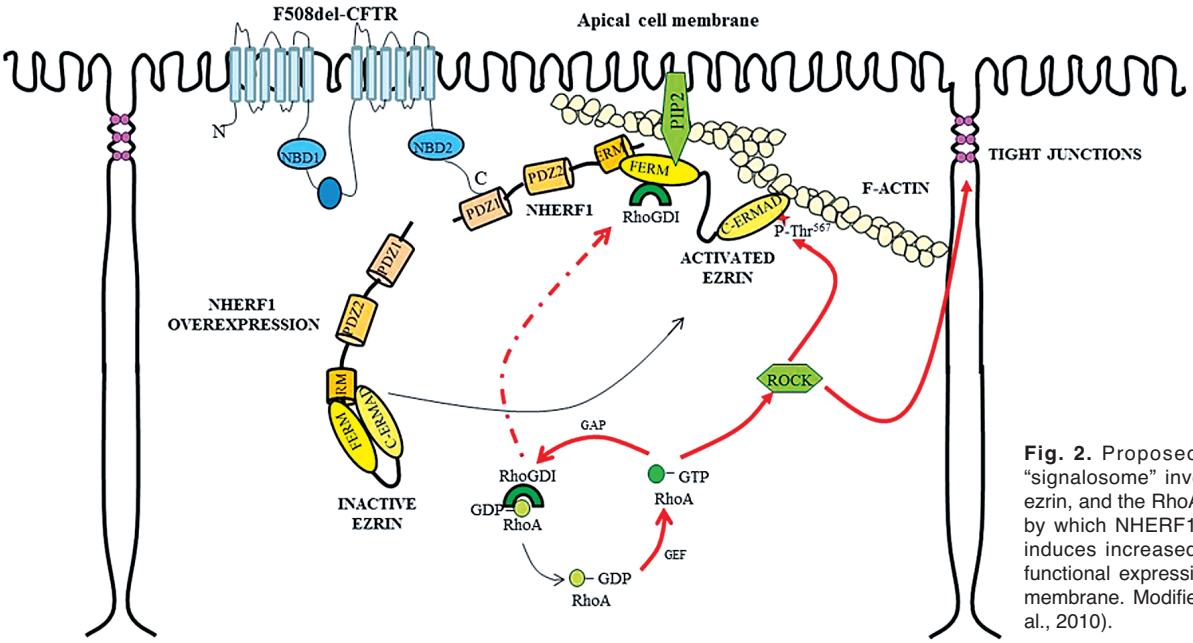

Fig. 2. Proposed model for the "signalosome" involving NHERF1, ezrin, and the RhoA/ROCK pathway by which NHERF1 overexpression induces increased F508del-CFTR functional expression on the apical membrane. Modified from (Favia et al., 2010).

Ivetic and Ridley, 2004). In airway epithelial cells, CFTR regulation has been demonstrated to depend on the organization of a multi-protein complex involving F-actin, the scaffolding protein NHERF1 and ezrin. This protein complex, besides stabilizing CFTR in highly restricted domains at the plasma membrane (Haggie et al., 2006; Jin et al., 2007), plays an important role in the control of CFTR function as ezrin, an A-kinase anchoring protein, tethers PKA in the proximity of CFTR, allowing cAMP-dependent control of chloride efflux (Short et al., 1998; Moyer et al., 2000; Sun et al., 2000). The lack of an organized subcortical cytoskeletal organization observed in CF airway epithelial cells (CFBE41o-) compared to non-CF 16HBE14o- cells has been found to cause a cytosolic accumulation and a concomitant reduced compartmentalization of cAMP in the membrane region, resulting in reduced compartmentalization of PKA activity. In line with these results, wt CFTR knockdown in 16HBE14o- cells induced a shift of cAMP and PKA distribution from apical membrane to the bulk cytoplasm together with a loss in the cytoskeletal organization (Monterisi et al., 2012). These data indicate that both an organized subcortical cytoskeleton and the CFTR expression on the plasma membrane are responsible for a correct cAMP/PKA compartmentalization in the plasma membrane region.

Cystic fibrosis

Cystic Fibrosis (CF) is the most lethal autosomal recessive disease in Caucasians; it is caused by mutations in the *CFTR* gene, which encodes a cAMP-dependent Cl- channel expressed by epithelial cells (Sheppard and Welsh, 1999). The CFTR protein, after its synthesis in the endoplasmic reticulum (ER), is glycosylated at the level of the Golgi and transported to the apical plasma membrane. Up to date more than 2000 mutations have been identified in the CFTR gene (http://www.genet.sikkids.on.ca/cftr/app, accessed September 25, 2016) and are usually classified in six different classes according to their molecular pathology (Amaral, 2015). The most frequent CFTR mutation, known as p.Phe508del (F508del), accounting for around 66% of the mutations worldwide, is a class 2 mutation caused by a 3-bp deletion (Bobadilla et al., 2002). Although many organs are affected by CF, the chief cause of morbidity and mortality of CF individuals is due to lung disease, whose hallmarks are recurrent opportunistic bacterial infections and a deregulated neutrophil-dominated chronic inflammation. However, mutations in the *CFTR* gene have been associated with CF-related conditions, such as congenital bilateral absence of the vas deferens, idiopathic pancreatitis, rhinosinusitis, bronchiectasis, and allergic bronchopulmonary aspergillosis (Noone and Knowles, 2001).

The lack/dysfunction of the CFTR protein is reflected by the accumulation of sticky dehydrated mucus in organs lined by epithelia, such as lungs, epididymis and pancreatic ducts. It is presently believed that in the airways there is an abnormal ion and water flux consequent to decreased chloride secretion and hyperabsorption of sodium (Boucher, 2007). CFTR protein is not only a chloride channel but also seems to be involved in the tonic negative regulation of the epithelial sodium channels (ENaC) (Mall et al., 1998). In CF, the lack/dysfunction of CFTR allows an increased ENaC activity. Other pathological processes have been described in CF airways, for example a reduced fluid secretion by airway submucosal glands together with an altered secretion of mucous glycoproteins (Verkman et al., 2003), increased bacterial colonization (Pier et al., 1996), and reduced antimicrobial properties due to pH alterations (Pezzulo et al., 2012).

Chronic lung inflammation in CF is characterized by high levels of pro-inflammatory cytokines in the bronchoalveolar lavage fluid (such as IL-1β, TNF-α, and IL-8) and by a massive influx of polymorphonuclear neutrophils which serve as the immune cells in acute responses (Conese et al., 2003). This inflammatory response, however, fails in the airway bacterial clearance (Cohen and Prince, 2012).

In the intestine, meconium ileus, considered to be almost pathognomonic of the disease, is due to dehydration of the mucus and leads to gut obstruction (Quinton, 1999). In the reproductive tract, a defective purinergic signalling has been described (Ruan et al., 2012). All these alterations would contribute to worsening the phenotype with progressive atrophy and dysfunction of several organs.

Pathophysiology of tight junctions and actin cytoskeleton in CF

All the abnormalities above described might be due to an incorrect localization of the CFTR protein. The most common mutation of the CFTR gene associated with CF, the F508del, results in the synthesis of an improperly folded CFTR protein that, although being partially functional and responsive to cAMP/PKA regulation, is unable to reach the cell membrane due to retention and/or accelerated degradation in the ER. Once rescued to the cell surface, F508del-CFTR still shows a partial gating defect. Furthermore, the F508del-mutation reduces its apical membrane half-life (Lukacs et al., 1993; Sharma et al., 2001) by accelerating its endocytic retrieval from the plasma membrane and its consequent degradation (Swiatecka-Urban et al., 2005). In the polarized human CF airway epithelial cell line CFBE41o-, it has been shown that NHERF1 depletion causes F508del-CFTR which has been rescued to cell membrane, to be more unstable at the cell surface and, therefore, more susceptible to degradation (Kwon et al., 2007). In line with these observations, we have demonstrated that NHERF1 overexpression increases the wt CFTR expression on the apical membrane in human airway cells, 16HBE14o-, and rescues F508del-CFTR functional expression in CFBE41o- cells by inducing the redistribution of F508del-CFTR from the cytoplasm to

the plasma membrane and increasing the PKA-dependent activation of CFTR-dependent chloride secretion (Guerra et al., 2005). In subsequent studies (Favia et al., 2010), we found that CFBE41o- cells show a disorganization of actin cytoskeleton (lack of stress fibers); NHERF1 overexpression in these cells leads to an increase of F-actin and its redistribution at the apical membrane level. These effects are mediated by NHERF1 interactions with the phosphorylated form of ezrin. Indeed, NHERF1 overexpression leads to an increase of RhoA activity. The up-regulation of RhoA activity, in turn, leads to ROCK-mediated ezrin phosphorylation and stabilization in its open, active conformation, which further i) redistributes phospho-ezrin on the apical membrane, ii) increases the F-actin content and organization and stabilizes F508del-CFTR on the apical membrane by tethering it to the actin cytoskeleton, iii) rescues the CFTR-dependent chloride efflux. In addition, we demonstrated that NHERF1 or CFTR overexpression in CFBE41o- cell monolayers induced the reorganization of TJ proteins at the level of intercellular junctions and reduced the paracellular permeability to small solutes (Castellani et al., 2012). Wild type CFTR transient transfection partially recovered correct ZO-1 localization at TJ level, while either CFTR-NH2 (1-633aa) or CFTR S1455X transfection led to a less pronounced effect, indicating a role of C-terminus domain of CFTR in TJ re-organization (Castellani et al., 2012). In addition, we showed that the dominant-negative form of RhoA (RhoA-N19) disrupted ZO-1 localization at the TJ level and increased the permeability to dextrans restoring the CF phenotype.

More recently, we have shown that CFBE41o- cells are more rounded and wider than 16HBE14o- cells; this altered morphology could be due to the lack of stress fibers and higher membrane stiffness and lower roughness, as assessed by atomic force microscopy (Lasalvia et al., 2016). Interestingly, the overexpression of wt CFTR in CFBE41o- cells made the cells more elongated and determined the appearance of actin stress fibers (Lasalvia et al., 2016) (Table 1) suggesting that, somehow, the CFTR protein either directly or indirectly may govern the cell shape. Our hypothesis is supported by previous studies (LeSimple et al., 2010) demonstrating that GFP-CFTR expression in the CFBE41o- cells induced an increase in TER (see Table 1 for comparison of TER in different cell lines) implying that an improved CFTR trafficking is correlated with an increase in the transepithelial resistance. Overall, these findings also suggest that CFTR modulates paracellular permeability, likely at the TJ level.

Weiser and colleagues (Weiser et al., 2011) showed that CFBE41o- cell monolayers exhibit a higher paracellular permeability and lower TER as compared with 16HBE14o- monolayers. In the same paper the authors demonstrated that stimulation of CFTR by cAMP induced an increase of paracellular permeability in 16HBE14o- cells, while in CFBE41o- cells there was a decrease of paracellular permeability together with a slight increase of TER. However, recently the same group was able to link only transepithelial resistance and not paracellular permeability to CFTR expression (Molenda et al., 2014). They showed that under non-stimulated conditions, 16HBE14o-, parental CFBE41o-, and CFBE41o- transfected with either wt CFTR or mutant F508del-CFTR had similar paracellular fluorescein flux, while CFTR activation increased the paracellular permeability only in the 16HBE14o- cell monolayers.

Altogether these observations indicate that there is a link between CFTR dysfunction and improper regulation of paracellular permeability, but these findings need to be broadly confirmed, for example in primary airway epithelial cells grown in ALI conditions.

Recently, Molina et al. (2015) analyzed the immortalised non-cancerous airway epithelial cells, derived from a patient homozygous for the F508del mutation (CuFi) and a non-CF subject (NuLi), growing in ALI cultures. CuFi presented a consistently lower TER than NuLi cells during all the period of the culture (Table 1). Interestingly, no difference between cell lines was noted in TJ protein expression (ZO-1, claudin-1 and claudin-4), although an increase in gene expression was noted in NuLi and not in CuFi cells.

In contrast to previously described findings, Nilsson and colleagues (Nilsson et al., 2010) noted that CFBE41o- monolayers exhibited a higher TER and lower permeability as compared with CFBE41o- cells corrected with CFTR stably expressed by an Epstein-Barr virus-based expression vector (CFBE pCep4) (Table 1). However, in accordance with our previous findings (Favia et al., 2010), they show that CFBE41o- cells display disorganized actin stress fibres and a blurred network at the cell boundaries, while 16HBE14o- cells are characterised by organized actin fibres at the cell borders. Interestingly, CFBE pCep4 cells displayed a condensation of the filamentous elements at the cell borders and fewer stress fibers than parental CFBE41o- cells (Nilsson et al., 2010).To reconcile these findings with the results presented by other studies, it could be that the use of different methods to recover the CF defect (adenoviral vs plasmid-derived overexpression) as well as the transgene (CFTR vs NHERF1) could be responsible for the difference in the results.

Altogether, these data highlight the close relationship among CFTR, NHERF1, actin cytoskeleton and TJ organization and function, laying down further studies aimed at identifying novel gene and drug-based approaches for the correction of the basic defect in CF and inflammatory responses.

TJ, actin, and inflammation

TJs between the polarized airway epithelial cells restrict the paracellular movement of solutes and ions, and prevent pathogens from gaining access to the submucosal compartment. In addition to their role as a

physical barrier between environmental factors and internal milieu, airway epithelial cells also play a critical role in innate and adaptive immunity by elaboration of antimicrobial molecules, proinflammatory cytokines and chemokines that recruit and activate other mucosal innate immune cells.

Neutrophil influx into the airway lumen is a pathological hallmark of CF lung disease. We assayed the neutrophil transmigration through the epithelial monolayers in a physiological way, i.e. from the abluminal to the luminal side and we found that overexpression of NHERF1 not only caused a re-organization of TJs and actin cytoskeleton but also mitigated the elevated neutrophil transmigration across the epithelium (Castellani et al., 2012).

The lumen of CF airways is replenished by pro-inflammatory cytokines (Bonfield et al., 1999), known to regulate the TJ barrier (Capaldo and Nusrat, 2008) and alter paracellular permeability in disease (Forster, 2008). The cytokines interleukin (IL)-1, IL-4, IL-10, IL-13, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ have all been shown to regulate the TJs of bronchial epithelial cells (Losa Garcia et al., 1999; Ahdieh et al., 2001; Coyne et al., 2002; Hermanns et al., 2004; Relova et al., 2005; Humlicek et al., 2007; Olson et al., 2009; Pohl et al., 2009). In Calu-3 cells, originated from submucosal glands, IFN-γ exposure had an anti-inflammatory effect and promoted epithelial barrier function (Ahdieh et al., 2001). On the other hand, IL-13 treatment caused an increase in mannitol flux and a decrease of ZO-1 protein levels and TER. Also, IL-4 treatment determined a TER decrease, indicating that IL-4 and IL-13 could act synergistically. More recently, it has been documented that exposure of Calu-3 cells to

Table 1. TER, actin cytoskeleton, and tight junctions in different airway epithelial cell lines.

Reference	Cell lines	TER ($\Omega \cdot cm^2$)	Actin cytoskeleton (F-actin)	Tight junctions
LeSimple et al., 2010	CFBE41o- (37°C)	494	ND	Permeability to mannitol: CFBE41o- (29°C)<CFBE41o- (37°C); CFBE-ΔF (29°C)<CFBE-ΔF (37°C).
	CFBE41o- (29°C)*	1005	ND	
	CFBE transfected with control Ad	380	ND	
	CFBE transfected with Ad-GFP-CFTR	638	ND	ZO-1 immunostaining: in CFBE41o- (29°C), higher ZO-1 staining at the cell-cell junctions than in CFBE41o-(37°C).
	CFBE-ΔF (37°C)	320	ND	
	CFBE-ΔF (29°C)	489	ND	
Nilsson et al., 2010	CFBE41o-	363	Disorganized stress fibers, blurred network at the cell boundaries	Permeability to lanthanium: CFBE41o- <CFBE41o- pCep4; no difference between CFBE41o- and 16HBE14o-.
	CFBE41o- pCep4	169	Few stress fibers, condensation of filamentous elements at the cell borders	
	16HBE14o-	578	Stress fibers, fiber network at the cells boundaries	
Favia et al., 2010	CFBE41o-	ND	Disorganization of actin filaments in punctate structures.	ND
	16HBE14o-	ND	Bundled F-actin filaments distributed in the basolateral and apical areas.	
Weiser et al., 2011	CFBE41o-	594	ND	Permeability to mannitol: CFBE41o- > 16HBE14o-
	16HBE14o-	875		
Castellani et al., 2012	CFBE41o-	101	ND	Immunostaining for TJ proteins: ZO-1, occludin, claudin 1, and JAM-1 expressed at the cell-cell borders in 16HBE14o- but not in CFBE41o-.
	16HBE14o-	489	ND	Permeability to dextrans: 16HBE14o- < CFBE41o-.
Molenda et al., 2014	CFBE41o-	528	ND	Immunostaining for TJ proteins: Claudin 3 expression is stronger in 16HBE than in all the CFBE cell line and its clones
	CFBE41o-/wt CFTR	431	ND	
	CFBE41o-/F508del-CFTR	470	ND	
	16HBE14o-	703	ND	
Molina et al., 2015	CuFi	250	ND	Immunostaining for TJ proteins: Similar expression of ZO-1, claudins 1 and 4 at week 7
	NuLi	650	ND	
Lasalvia et al., 2016	16HBE14o-	ND	Stress fibers	ND
	CFBE41o-	ND	No stress fibers	
	CFBE41o-/wt CFTR	ND	Appearance of some stress fibers	

CFBE-ΔF: CFBE41o- cells overexpressing F508del; CFBE41o- pCep4: CFBE41o- cells stably expressing wt CFTR from a plasmid. *At 29°C, the mutated F508del-CFTR is transported to the plasma membrane.

TNF-α, IL-4 or INF-γ for 48 h induced a noticeable downregulation of TJs, with a stronger suppression of ZO-1 than of occludin and a consequent significant enhancement in paracellular permeability (Petecchia et al., 2012). Exposure to these cytokines induced a EGFR-dependent MAPK/ERK1/2 pathway activation. In line with these results, Saatian and colleagues (Saatian et al., 2013) observed that IL-4 and IL-13 disrupted the airway epithelial barrier by altering the normal structure of epithelial AJs and TJs in 16HBE14o- monolayers.

IL-13 down-regulated the expression of several proteins involved in cell polarization in human nasal epithelial cells. Notably, IL-13 inhibited both mRNA and protein expression levels of ezrin and interfered with its apical localization during ciliated cell differentiation (Laoukili et al., 2001). Finally, this cytokine altered the ion transport phenotype of the human bronchial epithelium, by converting it from its normal absorptive state to a secretory phenotype (Danahay et al., 2002).

Conversely, it has been demonstrated that cytokine secretion is dependent on cytoskeleton organisation; this phenomenon primarily studied in lymphocytes was later demonstrated also in airway epithelial cells (van den Berg et al., 2006; Dudez et al., 2008).

A model of the possible relationship between the actin cytoskeleton organization, TJs and airway inflammation is depicted in Fig. 3.

Therapy of CF dysfunction in tight junctions and actin cytoskeleton

As outlined above, either NHERF1 or CFTR overexpression can recover the altered organisation of actin cytoskeleton (Favia et al., 2010; Lasalvia et al., 2016), or TJ altered barrier function (Castellani et al., 2012), recovering CFTR activity as a chloride channel (Fig. 2). However, gene delivery of such molecules as plasmids to the airway epithelium *in vivo* is not a trivial task, as demonstrated by more than two decades in this kind of effort (Griesenbach and Alton, 2009), the major hurdles being represented by pathophysiologic features (mucus plugging) and inherent problems with gene delivery vectors (immunological reactions).

More recently, a drug-based approach to CF has moved forward in the clinical arena. Moving from the detailed knowledge of the defect at the molecular level, and based on more than ten years of research, small-molecule disease-modifying drugs have been used with success in those CF patients bearing specific CFTR mutations. Correctors are termed drugs, which can allow the F508del-CFTR protein to mature and be transported on the apical membrane of treated epithelia, while potentiators are those drugs which enhance the conductance of dysfunctional CFTR proteins inserted in the apical membrane (Amaral, 2015). One of the most studied correctors is VX-809 (Van Goor et al., 2011), which enhances the stability and function on the cell surface of F508del-CFTR (Eckford et al., 2014) and improves its interaction with NHERF1 (Arora et al., 2014; Loureiro et al., 2015). Another potentially interesting compound is trimethylangelicin (TMA), a molecule that both corrects and potentiates the F508del-CFTR-dependent chloride secretion (Tamanini et al., 2011; Favia et al., 2014). We have recently demonstrated that both TMA and VX-809 correctors rescue F508del CFTR dependent chloride secretion, involving both ezrin activation and actin cytoskeletal re-organization in their effect in both primary and secondary CF airway cells (Abbattiscianni et al., 2016). An example of the re-organization of actin organisation is given in Fig. 4. Either treatment with latrunculin B, an agent determining disruption of actin stress fibers, or expression of the inactive ezrin mutant T567A reversed TMA and VX-809-induced effects (Monterisi et al., 2012; Jolly et al., 2015; Abbattiscianni et al., 2016), suggesting that clinical efficacy of correctors may be based on the appropriate conditions to activate the F508del CFTR-dependent chloride secretion, i.e. a well-

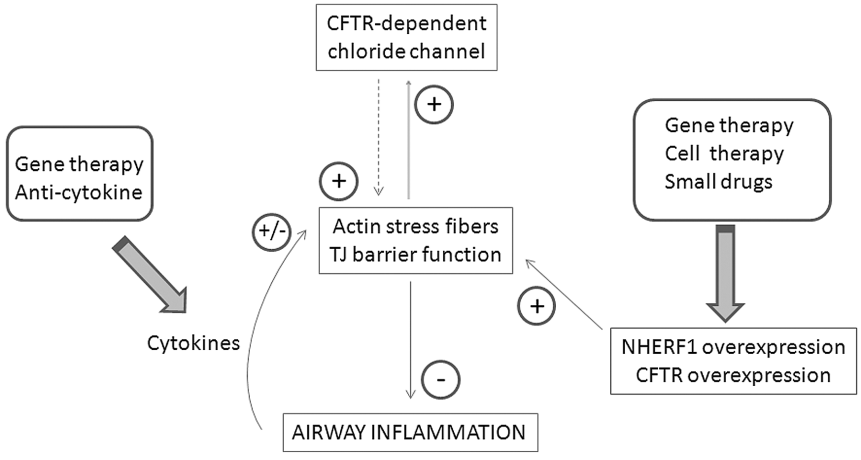

Fig. 3. Relationship of actin organisation and TJ barrier function with airway inflammation. A correct organization of stress fibers and TJs in the epithelium can arrest immune cell influx in the airways, whereas soluble mediators of inflammation, such as cytokines, can have complex effects on the structure of epithelial cells. While it is certain that a correct organization of actin stress fibers can position CFTR on the apical membrane and allows its physiological function, it is under investigation whether the organization of actin cytoskeleton is determined by the correct position and function of CFTR (denoted by the dashed arrow). NHERF1 or CFTR overexpression by gene delivery can enhance these structural features, an effect also obtained by small drug modifying-disease and cell therapy. On the other hand, gene therapy or cytokine inhibitors could block the effect of inflammation on actin cytoskeleton and TJ organisation.

organised actin cytoskeleton (Fig. 3).

Another innovative approach is to analyze the possible therapeutic role that stem cells may have in the context of epithelial pathology associated with CF lung disease. Human mesenchymal stromal (stem) cells (MSCs) are pluripotent stem cells that have been identified in numerous tissues, including lung, umbilical cord, cord blood, adipose tissue, and placenta (Crisan et al., 2008). MSCs have the capacity to give rise to differentiated cell types belonging to mesodermic, ectodermic and endodermic lineages, including airway epithelial cells (Wang et al., 2005). Moreover, MSCs might provide the tissue microenvironment with paracrine mediators which act on the different cellular structural components of the lung, and which can have cytoprotective effects, and inflammatory and immunomodulatory capacities (Hayes et al., 2012).

MSCs derived from the amniotic membrane (AMSCs) are considered to be a novel cell source for cell transplantation and regenerative medicine (Miki and Strom, 2006). Human AMSCs (hAMSCs) have gained attention in this context since they are obtained from discarded material after delivery (i.e. the placenta) and have been shown to have beneficial effects when administered in animal models for a large number of diseases, including lung injury (Silini et al., 2013). Thus, hAMSCs hold great promise for the treatment of an inherited inflammatory lung disease such as cystic fibrosis. Our previously published data indicate that hAMSCs do not express CFTR when cultured alone on plastic, while they express CFTR upon co-culture on permeable filters with CFBE41o- cells (Paracchini et al., 2012), suggesting that in these conditions hAMSCs have acquired an epithelial phenotype. In further studies, we have demonstrated that hAMSC:CFBE co-cultures show a corrected phenotype concerning not only resumption of a normal chloride efflux and decreased fluid hyperabsoprtion, the two major stygmates of basic defects associated with the CF airway epithelium, but also re-organization of tight junction and actin stress fibers (Carbone et al., 2014). As the mature form of the CFTR protein was increased in co-cultures as compared with CFBE41o- monocultures, these data are in line with the hypothesis of a link between CFTR expression and activity and other epithelial cell characteristics.

Conclusions

The pathophysiology of CF disease has been focused primarily on the ion fluxes through the epithelium of the respiratory tract and of other organs such as pancreas and liver. Recent findings point to a role for epithelial structure and morphology in the dysfunction of the CF epithelia. However, at the moment, we do not know whether the lack of TJ organization and of the actin cytoskeleton can be traced back to CFTR alterations or it is exactly the contrary, i.e. the CFTR dysfunction could be due to an altered cell shape and disorganized actin cytoskeleton. It might be also the case that these are concurrent phenomena, occurring independently. We must stress here that an inflammatory milieu, such as that of CF airways, can alter both CFTR function and

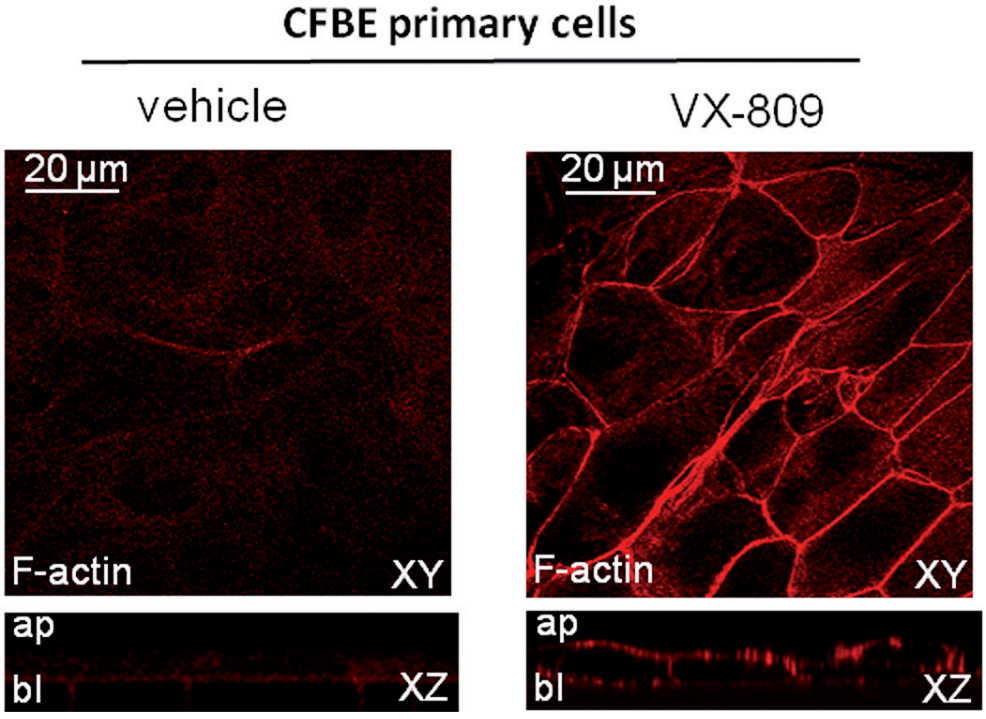

Fig. 4. Confocal analysis of cytoskeleton organization in CF primary bronchial epithelial cells and rescue by a F508del-CFTR corrector. CFBE primary cell monolayers grown at ALI (Air liquid interface) and incubated with either DMSO (vehicle) or 5 μM VX-809 for 24 h, were stained with Phalloidin-TRITC (1:5000) that detects actin filaments in red. Images show the cells by (XY) and vertical cross section (XZ) planes. ap, apical region; bl, basolateral region. Scale bar: 20 µm.

TJ/actin organization, thereby causing a complicated picture. To know in detail the relative role of these different processes may allow the design of more specific and efficacious treatments for CF.

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