Peripheral Protein Quality Control as a Novel Drug Target for CFTR Stabilizer

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Conformationally defective cystic fibrosis transmembrane conductance regulator (CFTR) including rescued ΔF508-CFTR is rapidly eliminated from the plasma membrane (PM) even in the presence of a CFTR corrector and potentiator, limiting the therapeutic effort of the combination therapy. CFTR elimination from the PM is determined by the conformation-dependent ubiquitination as a part of the peripheral quality control (PQC) mechanism. Recently, the molecular machineries responsible for the CFTR PQC mechanism which includes molecular chaperones and ubiquitination enzymes have been revealed. This review summarizes the molecular mechanism of the CFTR PQC and discusses the possibility that the peripheral ubiquitination mechanism becomes a novel drug target to develop the CFTR stabilizer as a novel class of CFTR modulator.

Keywords: peripheral QC, CFTR, stabilizer, ubiquitination, RFFL

INTRODUCTION

Cystic fibrosis (CF) is one of the most lethal autosomal-recessive diseases caused by mutation in CFTR (Lopes-Pacheco, 2016). CFTR mutations are classified as I–VII according to their properties (I–protein synthesis defect, II-maturation defect, III-gating defect, IV-conductance defect, V-reduced quantity, VI-reduced PM stability, VII-no mRNA transcription). The most prevalent CF causing mutation, ΔF508, was classically categorized as class II mutation. However, rescued ΔF508 (rΔF508)-CFTR by corrector (e.g., VX-809/lumacaftor) or low temperature culture shows class III and VI phenotypes (Dalemans et al., 1991; Veit et al., 2016a). Although drug targets of the class II or III mutations are well studied, that of the class VI mutation are not because the mechanism of CFTR PM stability regulation are still veiled by numerous undefined molecules involved in CFTR PQC system. In this review, we summarize accumulated findings regarding the CFTR PQC from the molecular and environmental aspects and also discuss the potential of recently identified PQC machineries including endocytic adaptors and ubiquitination enzymes as targets for CFTR stabilizer which anchors the functional channel at the PM and reduces the degradation (Figure 1).

CFTR INSTABILITY AT THE PM

Nascent wild-type (WT) CFTR is N-glycosylated at the endoplasmic reticulum (ER) during translation and folded by the aid of chaperones such as calnexin (CNX), HSP70 and HSP90 (Amaral, 2004; Kleizen et al., 2005; Okiyoneda et al., 2008; Rosser et al., 2008; Glozman et al., 2009; Kim and Skach, 2012). Properly folded CFTR is then sorted to the Golgi apparatus and
Constitutive CFTR turnover

Channel function

PM turnover

FLN-A
NHERF1

CFTR
stabilize PM CFTR

endocytosis

LMTK2

Deb2
ARF

P. aeruginosa

PQC Ub ligases

PM turnover

CFTR peripheral QC

Decreased channel function

Decreased PM stability

some PQC factors affect CFTR PM stability

CFTR PM stability is deteriorated by infection, CS and CFTR potentiators.

CFTR PM destabilizing mutations and their locations are listed (Veit et al., 2016a, Cystic Fibrosis Genetic Consortium Database). N287Y mutation increases CFTR endocytosis without affecting maturation (Silvis et al., 2003). R347P, S492F, rF508, A561E, L1077P, and N1303K mutations induce severe maturation defect and PM instability (Van Goor et al., 2014). N287Y and L1077P are localized at intracellular loop 2 (ICL2) of MSD1 and ICL4 of MSD2, respectively. Both mutations are predicted to destabilize the MSD1-NBD2 and MSD2-NBD1 interactions that define CFTR conformational stability.
Internalized CFTR could be de-ubiquitinated at endosomes by deubiquitinase (DUB) and recycled back to the PM depending on the conformational states. The class VI mutations render the CFTR unstable at the PM. Additionally, the class I, II and some class III CFTR mutants also show PM instability (Lukacs et al., 1993; Haardt et al., 1999; Silvis et al., 2003; Wang et al., 2014; Veit et al., 2016a). N-glycosylation, especially the core-glycosylation, determines the cleavage and the PM stability likely by affecting the CFTR conformational stability (Glozman et al., 2009; Cholon et al., 2010). Protein translation kinetics is also a significant factor that modulates proper co-translational folding. Knock down (KD) of ribosomal protein L12 (RPL12) increases ΔF508-CFTR PM expression and stability (Veit et al., 2016b). RPL12 KD might affect protein translation kinetics associated with co-translational protein folding efficiency (Buhr et al., 2016) and thereby improve CFTR thermodynamic stability which also determines the CFTR PM stability (Okiyoneda et al., 2010; Rabeh et al., 2012). Thus, correcting the CFTR structural defects at the ER could improve the PM stability.

ENVIRONMENTAL STRESSES AFFECTING THE CFTR PM STABILITY

Infection and Inflammation

The CFTR loss of function induces airway surface liquid (ASL) dysregulation which impairs clearance of infected bacteria and/or fungi, and increases the concentration of other soluble signal mediators such as cytokines, chemokines and growth factors. Pseudomonas aeruginosa (PA) is one of the most common bacteria found in CF respiratory tissue and responsible for lung injury in CF (Koch, 2002; Bhagirath et al., 2016). PA destabilizes PM CFTR by inhibiting endocytic recycling (Swiatecka-Urban et al., 2006). PA secretes CFTR inhibitory factor (Cif) that stabilizes complex formation of ubiquitin (Ub) specific peptidase 10 (USP10) and GTase activating protein (SH3 domain) binding protein 1 (G3BP1) and inhibits CFTR-USP10 interaction. Cif inhibits internalized CFTR sorting to recycling pathway by suppressing USP10 dependent CFTR de-ubiquitination at endosome, resulting in the lysosomal degradation of WT-CFTR (Bomberger et al., 2011). PA also activates transforming growth factor β1 (TGF-β1) signaling that is an important modifier of lung disease severity in CF (Harris et al., 2011). TGF-β1 inhibits functional PM expression of WT-CFTR and ΔF508-CFTR by reducing mRNA level (Snodgrass et al., 2013; Sun et al., 2014) although its role in the PQC remains unknown.

Heavy Metals

More than 10 ppb of arsenic induces the WT-CFTR ubiquitination and lysosomal degradation via c-Cbl in CF bronchial epithelial (CFBE) cells (Bomberger et al., 2012). Importantly, the phenotype of arsenic toxicity overlaps with CF patient (Bomberger et al., 2012; Mazumdar et al., 2015). Cadmium (Cd) is a major component of cigarette smoke (CS), and its inhalation is associated with decreased pulmonary function and chronic obstructive pulmonary disease. Cd reduces CFTR PM level, but it remains unknown if it reduces the PM stability (Rennolds et al., 2010).

Cigarette Smoke

Cigarette smoke is a major risk factor of chronic obstructive pulmonary disease and interferes with CFTR functionality. Ten minutes of CS exposure transiently suppresses CFTR function, induces internalization and decreases ASL height in human bronchial epithelial (HBE) cells (Clunes et al., 2012). CS promotes CFTR internalization in BHK cells and results in increased insolubility of CFTR and colocalization with vimentin, a filament protein associated with aggresome Ca2+ dependently. This observation suggesting that CS induces PM CFTR destabilization by stimulating internalization and aggregation in addition to suppressing CFTR functionality (Clunes et al., 2012; Rasmussen et al., 2014).

MOLECULAR MACHINERIES DETERMINING THE CFTR PM STABILITY

Endocytosis Adaptors and Tethering Factors

Endocytosis is a critical step of elimination of PM CFTR as a part of PQC and is regulated by several molecules. WT-CFTR is internalized slowly by CME while misfolded rΔF508-CFTR endocytosis is accelerated (Sharma et al., 2004; Swiatecka-Urban et al., 2005; Varga et al., 2008; Okiyoneda et al., 2010). KD of CME adaptor AP-2 µ2 subunit or disabled 2 (DAB2) stabilizes rΔF508-CFTR at the PM by inhibiting endocytosis (Fu et al., 2012, 2015).

CFTR has a postsynaptic density 95, disks large, zonula occludens-1 (PDZ) binding motif at C-terminus and binds with Na+/H+ exchanger regulatory factor (NHERF1) PDZ domain. NHERF1 tethers CFTR with Ezrin and works as a scaffold protein that supports CFTR efficient channel activation and apical PM localization (Favia et al., 2010; Arora et al., 2014; Loureiro et al., 2015). NHERF1 also binds to misfolded ΔF508-CFTR and increases the PM stability by inhibiting carboxy terminus of HSP70-interacting protein (CHIP) Ub ligase interaction (Loureiro et al., 2015). An exchange protein directly activated by cAMP1 (EPAC1) selective activating cAMP analog 007-AM promotes WT-CFTR and NHERF1 interaction and increases CFTR PM stability in CFBE cells by suppressing endocytosis (Lobo et al., 2016). EPAC1 activation can rescue ΔF508-CFTR PM expression, and its effect is further improved with VX-809 combination (Lobo et al., 2016).

The CFTR-associated ligand (CAL) negatively regulates ΔF508-CFTR PM abundance through its PDZ domain (Wolde et al., 2007). CAL inhibition enhances the functional stability of ΔF508-CFTR at the apical PM, implying an attractive therapeutic target for CFTR PM stabilizer (Cushing et al., 2010). However, CAL also interacts with syntaxin 6 (STX6) and Golgi-localized E3-ligase membrane associated RING-CH type finger
2 (MARCH2) and regulates WT-CFTR PM expression (Wolde et al., 2007; Cheng and Guggino, 2013). Filamin-A (FLN-A) is a membrane tethered actin adaptor protein and interacts with CFTR N-terminus region. S13F mutation of CFTR compromises FLN-A binding and consequently destabilizes the PM CFTR (Thelin et al., 2007). FLN-A binds with both WT and rF508-CFTR at similar level, however, its contribution to the CFTR PQC remains unclear.

### Protein Kinases

The CFTR PM stability is regulated by phosphorylation. CFTR is predominantly phosphorylated at the R domain and also at nucleotide binding domain 1 (NBD1) and C-terminus residues by protein kinase A (PKA), protein kinase C (PKC), casein kinase II (CK2) and AMP-activated protein kinase (AMPK) for the channel function (Chappe et al., 2003; Kongsuphol et al., 2009; Luz et al., 2011). CK2 is predicted to regulate CFTR PM stability by phosphorylation at Thr-1471 where NHERF1 could interact (Venerando et al., 2013). Lemur tyrosine kinase 2 (LMTK2) phosphorylates CFTR at Ser-737 (Wang and Brautigan, 2006) and its KD or mutation at CFTR Ser-737 suppresses the endocytosis and increases CFTR PM density and stability (Luz et al., 2014). However, LMTK2 KD only modestly improves the PM function of rΔF508-CFTR (Luz et al., 2014). Spleen tyrosine kinase (SYK) phosphorylates CFTR at Tyr-512 and decreases CFTR PM levels possibly through the disordered regions. RFFL promotes K63-linked polyubiquitination to destabilize the PM CFTR (Thelin et al., 2007). Leukemia receptor tyrosine kinase (LMTK2) phosphorylates CFTR at Ser-737 in response to phorbol 12-myristate 13-acetate (PMA) (Zhu et al., 2008). c-Cbl also binds with WT-CFTR and decreases the PM stability of conformationally defective CFTR such as rF508-CFTR (Luz et al., 2014). LMKT2 KD only modestly improves the PM function of WT-CFTR (Bomberger et al., 2009). The role of USP10 in the CFTR PM stability of conformationally defective CFTR such as rF508-CFTR at the PM of polarized CFBE cells (Fu et al., 2015). E3 ligase c-Cbl may play a role in the CFTR peripheral QC, but its contribution could be modest since its KD slightly increases rΔF508-CFTR PM stability in CFBE cells (Chih et al., 2013; Fu et al., 2015). c-Cbl also binds with WT-CFTR and decreases the PM stability without affecting the ubiquitination, suggesting that c-Cbl could regulate constitutive CFTR turnover of folded CFTR by inducing endocytosis through its C-terminus adaptor function (Ye et al., 2010).

Nedd4-2 is a member of homologous to the E6-AP carboxyl terminus (HECT) E3 which may regulate the CFTR PM expression. Nedd4-2 KD reduces ΔF508-CFTR ubiquitination at the ER, and increases the PM expression and function in CF pancreatic adenocarcinoma cell 1 (CFPAC1) and IB3-1 cells (Caohuy et al., 2009). Nedd4-2 binds both WT- and ΔF508-CFTR while its role in the WT-CFTR ubiquitination remains controversial (Koeppen et al., 2012). However, Nedd4-2 KD does not stabilize the PM rΔF508-CFTR in CFBE cells, implying its marginal contribution to the CFTR PQC (Koeppen et al., 2012; Fu et al., 2015). Nedd4-2 is unlikely a viable CF drug target because its knock out (KO) induces CF-like lung phenotype by excessive function of epithelial Na⁺ Channel (ENaC) (Kimura et al., 2011; Rotin and Staub, 2012).

A number of DUBs regulate the CFTR turnover. USP10, a DUB localized at early endosomes, interacts with WT-CFTR and reduces the CFTR poly-ubiquitination in CFBE cells. The USP10-mediated deubiquitination enhances the endocytic recycling of WT-CFTR (Bomberger et al., 2009). The role of USP10 in the PM stability of conformationally defective CFTR such as rΔF508-CFTR remains unclear.

Recently, we have discovered RING finger and FYVE like domain containing E3 Ub protein ligase (RFFL) as a novel component of the CFTR PQC machineries by a comprehensive siRNA screen in CFBE cells (Okiyoneda et al., 2018). RFFL selectively recognizes unfolded rAF508-CFTR through the disordered regions. RFFL promotes K63-linked polyubiquitination of the unfolded CFTR in post-Golgi, resulting in accelerated endocytosis and lysosomal degradation. Importantly, RFFL directly interacts with conformationally defective CFTR such as rAF508-CFTR, but not with folded WT-CFTR at the PM and endosomes. Moreover, the RFFL-mediated ubiquitination is attenuating the ubiquitination and for stabilizing the CFTR function at the PM.

### Ubiquitination Enzymes

Ubiquitination determines CFTR elimination not only at the ER, but also from the PM. Ubiquitination is mediated by a sequential action of E1, E2, and E3 enzymes and this modification could be removed by DUB. Specifically, E3 Ub ligase has been proposed to determine the substrate specificity. CHIP is the first identified E3 ligase responsible for the CFTR PQC (Okiyoneda et al., 2010). Consistent with the action at the ER (Meacham et al., 2001), CHIP selectively interacts with unfolded ΔF508-CFTR at the post-Golgi through the HSC70/HSP90 chaperones. CHIP KD reduces the ubiquitination of unfolded ΔF508-CFTR, resulting in the decelerated endocytosis and lysosomal delivery in HeLa cells (Okiyoneda et al., 2010). CHIP KD also stabilizes rΔF508-CFTR at the PM of polarized CFBE cells (Fu et al., 2015).

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conformation dependent as it selectively ubiquitinates thermally unfolded NBD1. RFFL KD enhances the functional PM expression of rΔF508-CFTR in the presence of VX-809, and this effect is further improved by inhibiting the HSC70-dependent ubiquitination machinery. Thus, RFFL plays an important role in the chaperone-independent CFTR PQC mechanism in HBE cell models.

CFTR MODULATORS AFFECTING THE CFTR PM STABILITY

Pharmacological Chaperones and Chemical Chaperones
Pharmacological chaperones affect the CFTR PM stability by direct stabilization. CFTR corrector VX-809 is the first food and drug administration (FDA) approved CFTR corrector in combination with VX-770/ivacaftor (known as Orkambi). VX-809 selectively improves the processing of misfolded CFTR by stabilizing NBD1-membrane spanning domain (MSD) interface but not other misfolded proteins such as human ether-à-go-related gene (hERG) mutants (Van Goor et al., 2011; Okiyoneda et al., 2013; Ren et al., 2013; Farinha et al., 2015). VX-809 repairs not only the CFTR folding defect at the ER but also the CFTR PM instability. VX-809 washout prolongs ΔF508-CFTR functional sustainability (Van Goor et al., 2011), suggesting that improvement of the CFTR folding at the ER could increase the thermal stability and proper co- and/or post-translational modifications that renders CFTR more energetic robust conformations even at the PM. VX-809 also promotes ΔF508-CFTR and NHERF1 interaction, that may increase the PM stability (Arora et al., 2014). C3 (CFcor-325/VRT-325) and C4 (Corr-4a) also extend rΔF508-CFTR PM stability in CFBE cells probably by directing binding (Wang et al., 2007; Varga et al., 2008) although their effect could be not specific to the conformationally defective CFTR (Van Goor et al., 2011). Chemical chaperones such as glycerol also increases the rΔF508-CFTR PM stability probably by non-specifically improving the conformational stability (Okiyoneda et al., 2013).

CFTR Potentiators
The first FDA approved CFTR potentiator VX-770 improves the gating defect of some CFTR mutants. However, chronic VX-770 treatment destabilizes the PM rΔF508-CFTR in CFBE and ΔF508 homozygous CF patient HBE (CF-HBE) cells (Cholon et al., 2014; Veit et al., 2014). Importantly, chronic VX-770 treatment diminishes the VX-809 therapeutic efficacy by stimulating the elimination of PM rΔF508-CFTR (Cholon et al., 2014; Veit et al., 2014). In addition to VX-770, several CFTR potentiators including P1 (VRT-532) and P2 (PG-01) also decrease the rΔF508-CFTR PM stability (Veit et al., 2014). VX-770 and other potentiators could destabilize a variety of CFTR rare mutants referred to as CFTR2 mutants including E92K and L1077P at the PM (Avramescu et al., 2017). Thus, several CFTR potentiators may decrease the thermal stability of metastable mutant CFTR at the PM by inducing conformational change that positively affects for channel gating but negatively affects stability. High-throughput screening has identified several novel CFTR potentiators such as class A analog 4 (A04) and class P analog 12 (P12) that could not destabilize the PM rΔF508-CFTR (Phuan et al., 2015).

Proteostasis Regulating Drugs
Proteostasis regulating drugs that affect array of proteins regulating CFTR folding and QC also affect the CFTR PM stability. Histone deacteylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) alters expression of a subset of CF-interacting gene products (e.g., chaperones and DAB2) and sustains PM expression of ΔF508-CFTR in CFBE cells (Hutt et al., 2010). Tissue transglutaminase (TG2) inhibitor cystamine also stabilizes ΔF508-CFTR at the PM of airway epithelial cells by restoring BECN1 interactome which is sequestered by CFTR dysfunction (Luciani et al., 2012; Villella et al., 2013). MLK3 pathway inhibitor oxozeaenol has been reported to be effective in correcting the ΔF508-CFTR proteostasis defect in the primary HBE cells (Trzcinska-Danelutì et al., 2012). Oxozaenol could stabilize ΔF508-CFTR at the PM as MLK3 KD reduces mature ΔF508-CFTR elimination by PQC (Hegde et al., 2015).

Cavosonstat and CAL Inhibitor
HSP70/HSP90 Organizing Protein is adaptor protein which coordinates HSP70 and HSP90 function in protein folding and regulates CFTR maturation and PM stability (Oduro et al., 2004; Okiyoneda et al., 2010). HOP S-nitrosylation by S-nitrosogluthathione (GSNO) induces HOP degradation and increases ΔF508-CFTR PM expression (Marozkina et al., 2010). Levels of S-nitrosothiols such as GSNO are low in CF airway (Grasemann et al., 1999) and S-nitrosothiol decreases the internalization rate of rΔF508-CFTR in HBE cells (Grasemann et al., 1999, 2000; Zaman et al., 2014). Cavosonstat (SN91115) is an orally bioavailable inhibitor of GSNO reductase and restores GSNO levels (Donaldson et al., 2017). Cavosonstat is the first CFTR stabilizer in phase II trials, but it was not beneficial for improvement of lung function in combination with ivacaftor.

CFTR-associated ligand binds CFTR via a PDZ interaction domain and targets CFTR for lysosomal degradation (Cheng et al., 2004). CAL inhibition increases the PM stability of ΔF508-CFTR (Cushing et al., 2010) and cell penetrating CAL inhibiting peptide is established (Qian et al., 2015). CAL inhibitor has been developed as a cell surface CFTR stabilizer in pre-clinical level while its therapeutic efficacy and conformational selectivity remain unclear.

Ub Ligase Inhibitors
RING finger protein 5 (RNF5/RMA1) is an ER associated E3 Ub ligase that regulates early stage CFTR proteostasis at the ER (Younger et al., 2006). A RNF5 inhibitor Inh-2 identified by homology modeling and virtual ligand screening causes
significant rescue of ΔF508-CFTR in immortalized and primary HBE cells from CF patients (Sondo et al., 2018). Intriguingly, Inh-2 modestly increases mature ΔF508-CFTR half-life and this stabilization effect is further improved by VX-809. While the contribution of RNf5 in the CFTR peripheral QC remains unclear, RNf5 inhibitor may be useful to overcome the CFTR instability.

Currently, CHIP and RFFL are the only Ub ligases responsible for the CFTR peripheral QC (Okiyoneda et al., 2010, 2018). Thus, inhibiting their activity could selectively reduce the ubiquitination and elimination of unfolded CFTR from the PM, improving the limited efficacy of CF combination therapy. CHIP binds and regulates a number of substrates via chaperones (Connell et al., 2001). Moreover, inhibiting the CHIP activity induces deleterious effect as the CHIP KO mice result in the abnormal phenotypes including ataxia and pre-mature death1. In contrast, RFFL could bind and regulate a limited number of substrates because of its nature of direct binding to the CFTR through the disordered regions (Okiyoneda et al., 2018). More importantly, inhibiting the RFFL activity seems to have no venomousness since the RFFL KO mice exhibit no abnormal phenotype (Ahmed et al., 2009). Therefore, counteracting RFFL activity may provide a preferable therapeutic approach as a CFTR stabilizer that is a class of drugs that extends the PM resident time of CFTR class VI mutants. Although future studies are needed to validate the impact on ΔF508-CFTR in CF-HBE cells, developing agents selectively inhibiting RFFL-mediated CFTR ubiquitination may help improve the efficacy of CF pharmacological therapy.

**CONCLUSION AND PERSPECTIVE**

Besides the progresses of CF pharmacological therapy, stabilizing the cell surface CFTR remains challenging and is necessary to improve the limited therapeutic efficacy. Recent studies have revealed some of the CFTR PQC mechanism eliminating the functional but conformationally defective CFTR from the PM. Understanding the CFTR PQC mechanism help the development of the CFTR stabilizer, a novel class of CFTR modulator necessary to establish the robust CF pharmacological therapy.

**AUTHOR CONTRIBUTIONS**

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: TO has a patent pending in Japan for methodology to identify inhibitors of RFFL-mediated CFTR ubiquitination (2017-047626).

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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