RNF185 is a novel E3 ligase of Endoplasmic Reticulum Associated Degradation (ERAD) that targets Cystic Fibrosis Transmembrane conductance Regulator (CFTR) by 

Elma El Khouri¹, Gwenaëlle Le Pavec¹, Michel B. Toledano¹, Agnès Delaunay-Moisan¹#

¹Laboratoire Stress Oxydant et Cancers, SBIGEM, IBiTec-S, CEA-Saclay

Running Title: RNF185 is a new E3 ligase targeting CFTR

#To whom correspondence should be addressed: Agnès Delaunay-Moisan, agnes.delaunay-moisan@cea.fr, LSOC, SBIGEM, IBITECS, CEA-Saclay, 91191 Gif-sur-Yvette CEDEX France

Keywords: ERAD/CFTR/E3 ligase/quality control/ ubiquitin/degradation

Background: RNF5 is an ERAD E3 ligase targeting CFTR to co-translational degradation, and RNF185 is an RNF5 homologue.

Results: RNF185 targets CFTR and CFTRΔF508 to co-translational degradation and synergizes with RNF5 to their post-translational degradation.

Conclusion: RNF185 and RNF5 act together as major ERAD E3 ligases of CFTR.

Significance: The RNF5/RNF185 module is a new potential therapeutic target for the treatment of cystic fibrosis.

SUMMARY

In the endoplasmic reticulum (ER), misfolded or improperly assembled proteins are exported to the cytoplasm and degraded by the ubiquitin-proteasome pathway through a process called ER associated degradation (ERAD). ER-associated E3 ligases, which coordinate substrate recognition, export and proteasome targeting, are key components of ERAD. Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is one ERAD substrate targeted to co-translational degradation by the E3 ligase RNF5. RNF185 is a RING domain-containing polypeptide homologous to RNF5. We show that RNF185 controls the stability of CFTR and of the CFTRΔF508 mutant in a RING and proteasome-dependent manner, but not that of other classical ERAD model substrates. Reciprocally its silencing stabilizes CFTR proteins. Turnover analyses indicate that, as RNF5, RNF185 targets CFTR to co-translational degradation. Importantly however, simultaneous depletion of RNF5 and RNF185 profoundly blocks CFTRΔF508 degradation not only during translation, but also after synthesis is complete. Our data thus identify RNF185 and RNF5 as a novel E3 ligases module that is central to the control of CFTR degradation.

The endoplasmic reticulum (ER) hosts a machinery called ERQC for ER Quality Control that is essential to prevent the secretion of improperly folded or assembled proteins (1-3).
ERQC assists the production of functional proteins by promoting both the folding of nascent proteins through a cohort of chaperones, disulfide isomerases and glycosylation enzymes, and the degradation of terminally misfolded proteins through the so called ER Associated Degradation (ERAD) pathway (4). Both ERQC branches contribute to alleviate the folding stress imposed on the ER, thereby maintaining cell viability. When the burden of unfolded proteins overwhelms ER folding capacity, the Unfolded Protein Response (UPR) is turned on to restore ER homeostasis.

ERAD encompasses a three-step process whereby misfolded proteins or unassembled subunits are first translocated from the ER lumen or ER membrane to the cytoplasm where they are finally targeted for degradation through the ubiquitin-proteasome pathway (5). Ubiquitination is a central process in ERAD as it tightly couples ERAD substrate recognition, efficient extraction and retrotranslocation from the ER membrane to the cytoplasm and delivery to the proteasome. Ubiquitination involves the activation of the ubiquitin moiety by its loading on the E1 activating enzyme, the transfer of the ubiquitin moiety from the E1 to the E2-conjugating enzyme and finally to the substrate by the coordinated action of the E2 and E3 enzymes (6). Two ER-associated E2 enzymes, Ubc6p and Ubc7p, and two ER-membrane embedded E3 ligases, Doa10 and Hrd1, have been attributed a major function in yeast ERAD (5). In mammals, functional homologs include E2s from the Ubc6 and Ubc7 family (7,8), organized around an expanded number of E3 ligases (9,10).

One of the best-characterized E3 ligase-based complexes of ERAD is HRD. This complex is conserved from yeast to mammals, and its core components are the E3 ligase Hrd1 (HRD1 in mammals) (11,12) and the one pass ER transmembrane adaptor Hrd3 (SEL1L in mammals) (13). Although Hrd1 may directly participate to the recognition and ubiquitination of misfolded substrates (11,14), Hrd3 both increases Hrd1 specificity and broadens its range of action (15). Indeed Hrd3, by itself or through cooperation with the lectin chaperone Yos9 (OS-9 and XTP-3 in mammals), binds to and recruits substrates to the E3 ligase (13,15,16). In yeast, a functional HRD complex is also needed to support the interaction of Hrd1 with cdc48/P97/VCP (17), the core subunit of the ATPase complex required for the dislocation of the substrate from the ER membrane and its delivery to the 26S proteasome (18-20). In mammals, the interaction of E3 ligases with p97 does not require their ligase activity (21), and the tight coupling between substrate ubiquitination and dislocation observed in yeast might thus be absent or uses alternate mechanisms. In both yeast and mammals, p97 also associates with proteins of the Derlin family, which are multipass transmembrane proteins the function of which is still debated (17,21,22). Derlins interact with and are required for the degradation of select ERAD substrates (16,23-27) as part of a distinct p97-containing complex bridged to the ligase (22). It was recently proposed that Derlins facilitate substrate extraction from the ER membrane, promoting their efficient delivery to the ERAD machinery (28). In yeast, Derl1 is recruited to the HRD complex by Usa1 (Herp in mammals) (16), which otherwise regulates Hrd1 function through the regulation of its oligomerisation, turnover and activity (29-31).

In yeast, the two membrane-associated ERAD E3 ligases, Hrd1 and Doa10, encompass most of the ERAD substrate repertoire (15,16,32,33). While Hrd1 is required for the ubiquitination of substrates bearing folding lesions in ER luminal and membrane domains, Doa10 is dedicated to those containing cytoplasmic lesions.

In mammals, an expanded number of ER associated-E3 ligases have been assigned to ERAD, the substrate specificity of which, if any, is not yet elucidated. These include two homologs of yeast Hrd1, HRD1 and gp78, one homologue of Doa10, TEB4, and enzymes that do not have homologs in yeast, RNF5/RMA1, RFP2, Kf-1 and TRC8 (9,33,34). HRD1 and gp78 have multiple substrates, some of which are shared by these two ligases (35-39). Moreover, HRD1 might be preferentially in charge of ER soluble substrates (36,38).

The RING-finger containing E3 ligase RNF5/RMA1 has recently gained interest because of its function in the degradation of the ERAD substrate CFTRΔF508, a mutant form of Cystic Fibrosis Transmembrane conductance Regulator (CFTR) that is most frequently responsible for cystic fibrosis (27). CFTRΔF508 is targeted for ERAD at distinct folding stages, through the sequential action of the ligases RNF5/RMA1 and CHIP (27,40-42). CHIP is a cytoplasmic U-box protein that is recruited on Hsc70-bound CFTR and targets it to ubiquitination and degradation after translation (27,40-42). RNF5 rather senses CFTR
RNF185 is a new E3 ligase targeting CFTR folding defects during translation (27,41) and may be recruited to CFTR lesions in part by the Hsc70-associated Hsp40 DnaJ12 (43). RNF5 also associates with Derlin-1 to drive CFTR mutant degradation (27,44). Current data support a model whereby RNF5 and gp78 collaborate to trigger CFTRΔF508 degradation, with RNF5 priming CFTRΔF508 by ubiquitination during translation and gp78 subsequently elongating the ubiquitin chain to promote efficient degradation (45).

We have here identified the RING-finger containing protein RNF185, a protein that is homologous by sequence to RNF5, as a novel ER-associated E3 ligase of ERAD. We show that RNF185 specifically targets CFTR and CFTRΔF508 to proteasomal degradation. As RNF5, RNF185 controls CFTR stability during translation and combined inactivation of RNF185 and RNF5 lead to a dramatic stabilization of CFTRΔF508. Our data therefore uncover a functional overlap between these two ligases which we further show takes place not only during but also after CFTR translation is complete. Overall, our data uncover a novel ERAD E3 ligase module important for the control of CFTR degradation in the ER.

EXPERIMENTAL PROCEDURE

Plasmids and antibodies

Full length human RNF185 was cloned from HEK293 cells cDNA using Transcriptor High fidelity cDNA synthesis kit (Roche). It was then PCR-amplified using the following primers: GAAGATCTGCAAGCAAGGGGCCCTCGGCC and CCGTCGAGTTAGCAATCGAGCCAGAA CATG and cloned into BamHI/XhoI sites of pcDNA3.1 Flag expression vector. RNF185 deletion mutants (RNF185ΔC: 1-176 and RNF185ΔR: 94-192) were generated by PCR-based cloning of the corresponding fragments using respectively the following primers: CCGTCGAGTTAGCGTGACAGGAACTGCTC GTC (DOWN) for RNF185ΔC GAAAGATCTAGGGGCAACAGCCAGAA CATG and cloned into BamHI/XhoI sites of pcDNA3.1 Flag expression vector. RNF185ΔC 1-176 and RNF185ΔR 94-192 (up) for RNF185ΔR. RNF185 RM (C39A, C42A) was generated by PCR-based mutagenesis using the following primers: CGAGGCCAACATCGCTGGAGACACGACAG CCC (up) and CCAAGGGCAGTGGCCTGCAGAAGTGGTCTG CCTGCC (down) and cloned along the same strategy used for the WT gene.

The pcDNA3.1 vectors expressing WT CFTR-HA and ΔF508 CFTR-HA, bearing an HA tag epitope in the C terminal end of the proteins were a generous gift from M. Benharouga. HA-CD3 construct was a kind gift from A. Weissman (46). HA-TCR was a kind gift from R. Kopito (47). α-1-antitrypsin expressing vectors (NHK, Z mutants) were kindly provided by E. Chevet (University of Bordeaux). pEGFP-C1 (Clontech) was used as a control for transfection efficiency. The following antibodies were purchased from commercial vendors: polyclonal anti-α-1-antitrypsin (DakoCytomation); monoclonal MM13-4 anti-CFTR (N-terminal tail epitope) (Millipore); monoclonal clone 24-1 anti-CFTR (R&D Systems); polyclonal anti-Derlin-1 antibody (Sigma Aldrich), monoclonal anti-Erlin2 (Sigma Aldrich), monoclonal and polyclonal anti-Flag antibodies (Sigma Aldrich); Monoclonal anti-GAPDH (Ambion), polyclonal anti-GFP (abcam); monoclonal anti-HA (Covance); monoclonal anti-ubiquitin (AssayDesign). Polyclonal anti-RNF5 was generated as described (48). Polyclonal anti-RNF185 was generated by injection of GST-RNF185 recombinant protein in rabbit and affinity purification of the resulting antibodies.

Cell culture and transfection

Human Embryonic Kidney (HEK) 293 or 293T cells were maintained in DMEM containing Glutamax (Invitrogen) supplemented with 10 % fetal bovine serum in 5 % CO2 at 37 °C. Cells were transiently transfected using TransIT-LT1 Transfection Reagent (Mirus) or using the calcium phosphate technique. HEK293 cells stably expressing CFTRΔF508 were generated by transfection with the expression vector pTracer (49) for CFTR ΔF508 and selected with zeocin (200 µg/mL).

Immunoprecipitation and immunoblotting

For immunoblotting experiments, cells were washed with PBS and lysed on ice in buffer A containing 50 mM Tris HCl pH 8, 150 mM NaCl, 1 % Triton 100X, 0.1 % SDS, 1 mM EDTA, 0.5 % DOC, protease Inhibitor Cocktail Tablets Complete (Roche) and 1 mM PMSF. Equal amount of proteins were loaded on SDS-PAGE after denaturation (5 minutes at 95°C or 10 minutes at 42°C for CFTR samples). Levels of endogenous RNF185 were monitored by immunoprecipitation using RNF185 polyclonal antibody in buffer A.
RNF185 partners were co-immunoprecipitated following lysis in IP buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 0.5 % Triton 100X, 1 mM EDTA, protease Inhibitor Cocktail Tablets Complete (Roche) and 1 mM PMSF). For CFTR/RNF185 co-immunoprecipitations, cells lysis was performed in a buffer containing 20 mM Hepes pH 7, 150 mM NaCl, 1 mM EDTA, 1 % NP40 (buffer B). Immunoprecipitations were conducted with the indicated antibodies after a 15 minutes preclear using 20 µL of Sepharose 4B beads. After extensive washing in lysis buffer, the samples were further processed for loading on SDS-PAGE.

Protein samples were separated on SDS-PAGE (6% Acrylamide:Bisacrylamide (40 % 37.5:1) for CFTR samples or 14 % for RNF185 samples) and transferred onto nitrocellulose membranes. After membrane blocking in 5 % Milk in PBS or in Odyssey blocking buffer (Li-Cor Biosciences), immunoblot analysis was performed using the indicated primary antibodies. Anti-mouse IgG or anti-rabbit IgG secondary antibodies labeled with fluorophores of different wavelengths were used to visualize specific protein signals by Infrared Imaging technology (Odyssey, LI-COR).

For cycloheximide chase analysis, HEK293T cells were transfected with the indicated plasmids and 24 hours later, cells were treated with cycloheximide (Sigma, 100 µg/mL) for the indicated times.

Proteasome inhibition

HEK293 cells were transfected with the indicated plasmids and 24 hours later treated with ALLN (N-Acetyl-L-leucinyll-L-leucinyll-L-Norleucinal-CHO; Calbiochem, 10 µg/ml) or the equivalent volume of DMSO for 12 hours. Cells were lysed and proteins extracted with buffer A. Soluble and insoluble fractions of protein lysates were analyzed by Western Blot.

Pulse labeling analysis

After a 30 minutes incubation in a methionine/cysteine-free medium (Invitrogen), cells were incubated for the indicated times with a pulse labeling medium containing EasyTag™ EXPRESS35S Protein Labeling Mix (100 µCi/ml) (PerkinElmer). After the indicated labeling times, cells were washed with cold PBS and resuspended on ice in buffer B. After pre-clearing, the extracts were centrifuged and 5X RIPA buffer (250 mM TRis-HCl pH7,5, 750 mM NaCl, 5% Triton X-100, 5% Deoxycholate, 0.5% SDS) was added to the supernatants to reach a 1X final concentration. Immunoprecipitation was then performed using anti-HA antibody (transfected CFTR) or 24-1 anti-CFTR antibody (stable cell lines). After washing, immunoprecipitated material was denatured at 42°C and loaded on a 6% SDS-PAGE. Fixed gels were then exposed on Biomax MR film (SIGMA).

When mentioned, cells were pretreated with ALLN or DMSO for one hour in complete DMEM medium prior starvation, then for 30 minutes in the starvation medium to achieve proteasome inhibition.

RNA interference analysis

HEK 293T cells were transfected at a final concentration of 40 nM with siRNA oligonucleotides directed against RNF185 (5' GAUAUUUGCCACAGCAUUU 3' or 5' CUUUCUGUUGCCGGUUAU 3') or a non-specific control (5' UACAAUGACGAUGCUU 3') using the calcium phosphate method. 24 hours later, cells were then transfected with HA-CFTR WT or HA-CFTRF508 plasmids. Trace amounts of EGFP-C1 plasmid were co-transfected with CFTR plasmids to control CFTR transfection levels. Cells were then collected 48 hours after the initial siRNA transfection. RNF185 silencing was controlled either by immunoprecipitation using RNF185 antibody followed by immunoblot or by RT-QPCR using RNF185 specific primers.

To perform RN5/RNF185 double knockdown, HEK293 cell lines stably expressing a control or a validated shRNA sequence targeting RNF5 (48) were generated. shRNA were expressed in the pSS-H1 vector (a gift from D.Billadeau, Mayo Clinic, Rochester MN) downstream of the RNA polymerase II-dependent H1 promoter.

Quantitative-PCR Analysis

Cells or tissues were collected and washed in PBS and RNAs were extracted using Macherey-Nagel RNA extraction kit according to the manufacturer instructions. 1 µg of RNAs was then used for cDNA synthesis using MMLV reverse transcriptase (Invitrogen) and hexaprimers (Roche). Quantitative PCR was then performed using Bio-Rad iCycler IQ5 PCR Thermal Cycler. The PCR reaction was performed using SYBR green PCR mastermix amplification reagent (Invitrogen) and transcript-specific primers. The house-keeping gene
RNF185 is a new E3 ligase targeting CFTR

GAPDH was used as reference for cell experiments. 18S RNA and ppiA1 genes were used as internal standards for expression analysis in tissues. The transcript-specific primers used are the following: human RNF185 5’-CTGTCACGCTTCTTCTATTTGT-3’ (forward) and 5’-GCCAGCATTAGGCAATCAG-3’ (reverse); mouse RNF185 5’-TCTTCTGTGCGGCTTACA-3’ (forward) and 5’-TTGCAGACTGGAACACTTGTC-3’ (reverse); GAPDH: 5’-ATGGGGAAGGTGAAGGTCG-3’ (forward) and 5’-GGGGTCATTGATGGCAACAATA-3’ (reverse); GRP78 5’-CACAGTGGTGCCTACCAAGA-3’ (forward) and 5’-TGTCTTTTGTCAGGGGTCTTT-3’ (reverse); RN18S 5’-TGCTTGGCTCCTTTCT-3’ (forward) 5’-TTGGCAAATGCTTTCGCTC-3’ (reverse); PPIA 5’-ATGGGAAAGGTGAAGGTCG-3’ (forward) and 5’-GCCTTCTTCCACCTGCCAAA-3’ (reverse).

For analysis of RNF185 expression upon UPR induction, HEK 293 cells were grown in a six-well plate. 24 hours later, cells were treated with tunicamycin (2 µg/mL) and harvested at the indicated times. The levels of RNF185 and GRP78, used as a control for UPR induction, were evaluated by Q-PCR and quantified using GAPDH as an internal standard.

In vitro ubiquitination assay

Bacterially expressed GST-RNF5, GST-RNF185 and GST-RNF185 RING mutants were purified on FPLC using fast-flow GST columns (GE). In vitro ubiquitination was performed according to the instructions provided with the Ubiquitin Conjugation Initiation Kit (Boston Biochem). Briefly, the assays were carried out at 37°C in a 30 µl-reaction mixture containing 0.5 M Hepes pH 8.0, 250 nM E1 Enzyme Solution, 600 µM Ubiquitin Solution, 1 mM Mg-ATP Solution, and 0.4 µM of separately provided E2 enzymes. Reactions were terminated by the addition of 20 µL of 5X SDS sample buffer and proteins were separated by 10 % SDS PAGE and visualized by immunoblot using anti-GST and anti-ubiquitin antibodies.

Immunostaining

HEK 293 cells were grown on coverslips and seeded on 24 well plates 24 hours prior to TransIT transfection. Endoplasmic reticulum was visualized by co-transfecting ER-GFP (GFP KDEL) with the indicated plasmids. To visualize mitochondrial network, cells were treated for 45 min with 100 nM MitoTracker (Invitrogen) in DMSO prior fixation. Cells were washed in PBS and fixed with 4 % formaldehyde in PBS for 30 min. After 3 washes in PBS, cells were permeabilized with 0.5 % Triton for 4 minutes and processed as described above. Cells were then incubated with 3 % BSA in PBS for 30 min. Cells were then incubated with the indicated antibodies (Flag: 1: 50000; GFP: 1: 20000 in BSA 3 % PBS). Image acquisition was done on a Zeiss LSM510 Meta confocal microscope (Plan-Apochromat 63×1.4-numerical-aperture [NA1.4] objective). Images were further analyzed using ImageJ and Adobe Photoshop CS6 software.

RESULTS

1. RNF185 is a conserved ubiquitous E3 ligase of higher eukaryotes

By performing a BLAST analysis against the human RNF5 protein, we identified human RNF185, which exhibits more than 70 % of sequence identity with RNF5. Apart from the RING domain, a high degree of sequence identity is found both in the two C-terminal transmembrane domains and in the central region (Fig 1). By searching sequence databases, we identified homologs of RNF5 and RNF185 in several species. Drawing the phylogenic tree of the RNF family showed a clear partition of RNF5 and RNF185 into distinct taxonomic units, especially in mammals (Fig S1). Such partition was less apparent in insects and plants, although several homologs were found in each organism. Interestingly, only one family member was found in nematodes and no member could be found in S. cerevisiae, although RNF5/RNF185 homologs exist in specific fungi and in amoebae. As shown by quantitative-PCR, RNF185 is widely expressed in mouse tissues with high levels of expression in heart and testis (Fig 2A).

To test the activity of RNF185 as a bona fide RING-dependent E3 ligase, we performed in vitro self-ubiquitination assays with GST-purified recombinant RNF185, in the presence of ubiquitin, ATP, E1 and different E2 enzymes. Auto-ubiquitination was monitored by western blot using an anti-ubiquitin antibody (Fig 2B). In the presence of UbcH5c, GST-RNF185 exhibited a potent self-
ubiquitination activity that was comparable to that observed for RNF5. Self-ubiquitination, although much lower, could also be detected in the presence of UbcH6, but was absent in the presence of UbcH7. We next tested the requirement of the RING domain for the ubiquitination activity of RNF185 by introducing two point mutations at cysteine 39 and 42 (RNF185 RM) or by truncating the entire RING domain (RNF185 ΔR) (see Fig 2C). These mutants were both devoid of auto-ubiquitination activity (Fig 2D), establishing that RNF185 has a RING-dependent E3 ligase activity.

2. RNF185 localizes to the ER membrane and interacts with ERAD components

Fluorescence microscopy was used to localize an N-terminal Flag-tagged version of RNF185. In the HEK293 (Fig 3A), HeLa and RPE (Fig S2A and S2B) cell lines, RNF185 largely co-localized with the GFP-KDEL ER marker. Further in HEK293, endogenous RNF185 also localized to the ER, as shown using an antibody raised against RNF185 (Fig 3B, Fig S2C). A previous report indicated that RNF185 localizes to mitochondria and not to the ER (50), in discordance with our observations. We therefore compared the imaging-fluorescence signal of Flag-tagged RNF185 with that of Mitotracker. Although we could not totally discount a mitochondrial localization (Fig 3C), the ER localization of RNF185 appeared clearly predominant in our experimental conditions.

We next inspected the localization of Flag-tagged mutants of RNF185 in HEK293 cells (Fig 3A and 3C). RNF185 ΔC, a mutant with a truncation of the most distal transmembrane domain (amino acids 176 to 192) (Fig 2C), did not localize to the ER, but instead displayed a diffuse fluorescence pattern (Fig 3A and 3C), indicating that this domain is required for ER membrane targeting. In contrast, both RING mutants RNF185 RM and RNF185 ΔR essentially localized to the ER structure, indicating that the E3 ligase activity of RNF185 is not required for its localization.

Having shown that RNF185 is an E3 ligase localized in the ER, we next probed the interaction of its Flag-tagged version with select ERAD components by Flag-immunoprecipitation in HEK293T cells. Flag-RNF185 could efficiently pull down Derlin-1, as reported for Flag-RNF5 (Fig 4A). Flag-RNF185, as well as Flag-RNF5 also efficiently pulled down Erll2 (Fig 4A), a prohibitin-like scaffold protein of the ERAD that has been reported to target IP3R (51). We next probed the RNF185 interaction with Ubc6 and Ubc7 that are the two ER membrane-associated E2 ligases of ERAD. Flag-RNF185 efficiently pulled down both Ubc6e/UBE2J1 and UBE2J2 (Fig 4B), but not Ubc7/UBE2G1 (data not shown), indicating a preferential association of RNF185 with the Ubc6 E2 ligase family.

As ERAD components are transcriptionally induced as part of the UPR (38,52), we monitored RNF185 expression upon treatment with tunicamycin, a drug causing ER stress by blocking glycosylation. RNF185 transcripts increased in response to tunicamycin, peaking at 12 hours after the onset of treatment (Fig 4C, left panel), a time-course comparable to the one observed for the bona fide UPR target GRP78 (Fig 4C, right panel).

In summary, our data show that RNF185 is a RING domain-dependent E3 ligase of the ER that interacts with ERAD components and is transcriptionally induced during the UPR.

3. RNF185 targets CFTR and CFTRΔF508 to ERAD

Our data strongly suggest a role of RNF185 in ERAD. To further substantiate this hypothesis, we checked whether modulating RNF185 cellular levels would affect the stability of ERAD model substrates. CFTR and CFTRΔF508 are both targeted to ERAD, due to inefficient folding, which leads to the degradation of two thirds of the former and 99% of the latter (53-55). CFTR migrates as two bands; the faster one represents the immature ER-localized B form, and the slower one the plasma membrane-localized mature C form (Fig 5A, upper panels). CFTRΔF508 is only seen as the ER-retained immature B form. In cells over-expressing RNF185, the levels of both CFTR and CFTRΔF508 dramatically decreased, in proportion with the dose of RNF185 (Fig 5A). Such an effect was dependent on RNF185 ubiquitin ligase activity, as it was not seen with the RING mutants RNF185 RM or RNF185 AR (Fig 5B). At the highest dose of RNF185, intensity of the wild-type CFTR C form band was 5 fold lower (Fig 5A, lane 4, 2 μg of DNA) than that of the control sample (Fig 5A, lane 1). The RNF185-dependent decrease of the wild-type CFTR C form band reflects an increased degradation of the ER localized B form rather than a maturation defect, as suggested by the concerted change of immature and mature CFTR.

CFTRΔF508 levels also showed a 5-fold decrease upon RNF185 expression. Such a decrease was already seen at the lowest dose of RNF185 (fig
proteasomal and autophagic pathways.

NHK and Z variants are folding-defective mutants by co-immunoprecipitation. Immunoprecipitation proteasome inhibitor MG132 to prevent RNF185- of the ER luminal enzyme While the NHK mutant is a dominant negative effects by titration of their substrates. TCR transmembrane proteins recognized as abnormal T cell receptor subunits when individually expressed (8,56-59). The NHK and Z variants are type I monomers of the ER luminal enzyme α1-antitrypsin (AAT). The NHK mutant is a bona fide ERAD substrate, Z variant is cleared up by both the proteasomal and autophagic pathways (60-63). Over-expressing RNF185 did not affect the stability of any of these proteins (Fig S4A and S4B). Over expressing E3 ligase RING mutants can cause dominant negative effects by titration of their substrates away from E2-dependant ubiquitination. However, neither of the RNF185 RING mutants had an effect on the stability of the isolated TCR or mutant AATs (Fig S4C and S4D).

Overall, these data indicate that RNF185 is a novel ER E3 ligase that regulates CFTR turnover.

4. RNF185 affects CFTR and CFTRΔF508 stability through the ubiquitin-proteasome system.

To evaluate whether RNF185 affects CFTR turnover through proteasomal degradation, we monitored the effect of the proteasome inhibitor ALLN (N-Acetyl-L-leucinyl-L-leucinyl-L- Norleucinal-CHO). Consistent with previous observations by Ward and colleagues (55), proteasome inhibition strongly stabilized WT and mutant CFTR, with preferential accumulation of the immature B form in both the detergent soluble and detergent insoluble fractions (Fig 5E). ALLN also mitigated the RNF185-dependent decrease of both CFTR and CFTRΔF508 levels, an effect that was more pronounced for the latter. To detect CFTR-ubiquitin conjugates, we blocked protein deubiquitination by adding N-ethylmaleimide during the lysis of cells that were otherwise treated with proteasome inhibitors (Fig S5A). In both ALLN and MG132 treated cells, the amount of CFTR ubiquitin conjugates increased in the presence of RNF185, which indicates that RNF185 targets CFTR to ubiquitin-proteasome-dependent degradation.

As shown in Fig. 4B, RNF185 interacts with the E2 ligases Ubc6e/UBE2J1 and UBE2J2, the former of which is known to regulate CFTR turnover (7). We thus evaluated the role of these two enzymes in RNF185-dependent CFTR degradation. Co-expressing RNF185 and Ubc6e/UBE2J1 had an additive effect on CFTRΔF508 levels decrease that was not seen with the catalytically dead Ubc6eC91S (Fig S5B, compare lane 2 and 3 with lane 4). These data suggest that these two enzymes cooperate in CFTR degradation. However, we could not observe a rescue of the RNF185-dependent degradation of CFTRΔF508 following Ubc6eC91S overexpression, an effect that would be expected on the basis of a transdominant negative effect of Ubc6eC91S. Therefore, either Ubc6eC91S do not behave as a transdominant negative mutant in these conditions, or alternatively another E2 ligase cooperates with RNF185 to degrade CFTR. RNF185 also interacted with UBE2J2 (see fig. 4B), yet simultaneously co-expressing both Ubc6 dominant-negative mutants did not prevent RNF185-dependent degradation of CFTR (data not shown). UbcH5 is also known to regulate CFTR degradation (42), and knocking down expression of all three UbcH5 isotypes (a, b and c) rescued RNF185-dependent CFTR levels decrease, but only
partially (Fig S5C). This suggests that the UbcH5 family could also serve as E2 ligases for RNF185.

In summary, these data indicate that RNF185 targets CFTR to degradation by the ubiquitin-proteasome system, but do not allow to strictly conclude with regards to the preferred RNF185-partner E2 ligase in this function, whether it is Ubc6e or UbcH5 or both.

5. RNF185 affects CFTR co-translational degradation

We next sought to quantify the change in the rate of CFTRΔF508 degradation prompted by RNF185 over-expression through a measure of CFTRΔF508 half-life, after inhibiting translation with cycloheximide (CHX) (Fig 6). Upon RNF185 over expression, the increased degradation of CFTRΔF508 was reflected by a decrease of its half-life from 44 minutes to 29 minutes in the presence of RNF185 (Fig 6A). Upon RNF185 knockdown however, despite an elevated level of CFTRΔF508 at the initial time point, the CFTRΔF508 half-life was not significantly altered (Fig 6B). The CHX-based protocol only reports on the stability of fully translated CFTR protein, ignoring any co-translational degradation. To evaluate whether RNF185 could preferentially affect CFTRΔF508 stability during translation, we monitored the accumulation of metabolically labeled CFTRΔF508 after adding [35S]-Met/[35S]-Cys for a defined period of time (Fig 7A). As expected, the amount of labeled CFTRΔF508 increased with time, but the over expression of RNF185 decreased CFTRΔF508 labeling by up to 50% compared to the control condition and this, at all time points examined. The pulse labeling experiment measures the net balance between protein translation and degradation, the latter occurring after and possibly also during translation. To exclude an impact of RNF185 on CFTR translation efficiency, we repeated the pulse-labeling experiment in the presence of the proteasome inhibitor ALLN. ALLN totally corrected the RNF185-dependent decrease of the amount of [35S]-labeled protein at all the time points tested (Fig 7A), which indicates that RNF185 over-expression only affects CFTR degradation and not translation. The rate of post-translational degradation can be calculated from a fitted curve deduced from the values obtained in the CHX experiment (Fig S6A), and this rate can then be used to predict the impact of post-translational degradation on the amount of accumulated [35S]-labeled CFTRΔF508 (see appendix and Fig S6B). Such calculation predicts that the decrease in the amount of [35S]-labeled CFTR caused by RNF185 over-expression at 20 min would be 7% at best, if degradation was exclusively post-translational, a decrease much lower than the 35-50% decrease observed in the pulse experiment (appendix and Fig 7B). We thus conclude that upon overexpression, RNF185 targets CFTR proteins for ubiquitination and degradation both during and after protein synthesis.

We next performed the same experiment in conditions upon RNF185 knockdown (Fig 7C). Strikingly, the amount of accumulated [35S]-labeled CFTRΔF508 was at least twice the control condition. As RNF185 knockdown did not affect the rate of CFTR post-translational turn-over (see Fig 6B), these data again point to a preferential effect of RNF185 on CFTR stability during synthesis.

6. RNF5 and RNF185 have a redundant function on the control of CFTR stability

The control of CFTR co-translational degradation has previously been attributed to RNF5 (27). We therefore compared the impact of RNF5 and RNF185 knockdown on CFTR turnover. Initial assays using RNF5-specific siRNA did not produce any significant stabilization of CFTRΔF508. However, HEK293 cells stably expressing an RNF5-directed shRNA sequence succeeded, as it caused a 3-fold increase in CFTRΔF508 steady-state levels (Fig 8), compared to the 2-fold increase observed upon RNF185 knockdown (see Fig 5C, Fig 8). As already shown above for RNF185, the effect of each single knockdown mainly reflected an E3 ligase dependent co-translational regulation of CFTR stability, as each did not significantly impact CFTRΔF508 turnover rate after CHX addition (Fig 6 and Fig 8). We next monitored the impact of knocking-down RNF5 and RNF185 simultaneously. Strikingly, the combined depletion of both ligases led to a drastic stabilization of CFTRΔF508. This was reflected by a 4.5-fold increase in CFTRΔF508 steady-state levels, and also by a net decrease of CFTR turnover rates after CHX addition. Importantly, the pool of stabilized CFTRΔF508 was only found in the Triton soluble fraction (data not shown), indicating that stabilized CFTR proteins do not form aggregates and should be accumulating in a foldable state.
These data strongly suggest that RNF5 and RNF185 are functionally redundant in the control of CFTR stability. Moreover, they reveal a new overlapping function for these enzymes in the post-translational control of CFTR stability.

DISCUSSION

We have herein characterized RNF185 as a novel RING E3 ligase of the mammalian ERAD machinery. We have shown that, as demonstrated for many other ERAD components, RNF185 is an ER-associated E3 ligase whose expression is induced upon ER stress. As such, RNF185 affects the stability of the ERAD substrates CFTR and CFTR\textsubscript{F508} in a RING-domain and ubiquitin-proteasome dependent fashion but not that of the other ERAD model substrates TCR\textsubscript{a68}, CD3\textsubscript{e71} and alpha1 anti-trypsin. Whether RNF185 indeed is specific for CFTR proteins remains to be tested by the use of a broader range of ERAD substrates including other multipass membrane proteins. Our data indicate that RNF185 can sense CFTR folding defects while CFTR is being synthesized, a function previously attributed to RNF5. Simultaneous depletion of RNF185 and RNF5 indeed unraveled their functional redundancy in controlling CFTR degradation. Importantly, our data now indicate that combined depletion of RNF5 and RNF185 blocked CFTR turnover not only during translation but also after CFTR synthesis has been completed. We thereby uncovered a new redundant function of these E3 ligases in controlling the stability of full-length CFTR proteins.

RNF185 functions in CFTR ERAD

Interaction data suggest that RNF185 belongs to a functional complex that comprises the general ERAD components Erlin2, Derlin1 and E2 ligase Ubc6e, of which the latter two have previously been shown to contribute to CFTR turnover. Our data also suggest that Ubc6e might cooperate with RNF185 in targeting CFTR to degradation. However our data also show that this functional interaction is not exclusive and that E2s from the UbcH5 family can also cooperate with RNF185, which is in agreement with the identification of this E2 enzymes as RNF185 interacting partners, along with other E2 enzymes (64). The effect of UbcH5 knockdown on the RNF185-stimulated degradation of CFTR is however hard to interpret as UbcH5 depletion also impact CHIP-dependent CFTR degradation (42). Thus, which of the two E2 enzymes is the bona fide RNF185 partner in CFTR degradation or whether both types of E2 contribute to this function cannot be strictly established from our data. In fact, this situation is reminiscent of the one of RNF5, for which its coexpression with Ubc6e had an additive effect on CFTR stability, while its coexpression with mutant Ubc6e failed to restore CFTR levels above the ones monitored in cells expressing RNF5 alone (27). Moreover, UbcH5a mutant expression can partially prevent RNF5-stimulated CFTR degradation, suggesting RNF5 can also team up with enzymes of the UbcH5 family. It is thus possible that both RNF185 and RNF5 use alternate E2s to stimulate CFTR degradation.

RNF185 can target CFTR and CFTR\textsubscript{F508} for degradation during translation

CFTR folding is a complex process, which involves cooperativity between protein domains and occurs both during and after translation (65). Not surprisingly, CFTR quality control is exerted both during and after translation, as indicated by studies on the turnover rates of CFTR mutant proteins (27) and CFTR ubiquitination during translation (14,55). The latter observation thus suggests the existence of E3 ligases that operate during CFTR synthesis. A role for RNF185 as one of these ligases is strongly suggested by our pulse labeling and turnover experiments. Upon \textsuperscript{35}S pulse labeling (see Fig 7A and 7B), RNF185 over-expression caused a 50% decrease in the amount of \textsuperscript{35}S incorporated into CFTR relative to the control, which was much greater than the 7% theoretical decrease in the case of exclusive post-translational degradation. The involvement of RNF185 in CFTR co-translational quality control is further supported by our knockdown experiments, indicating a lack of post-translational stabilization of CFTR\textsubscript{F508} after CHX-dependent translation arrest (Fig 6B), and a significant increase in the amount of CFTR synthesized upon RNF185 over-expression (Fig 7B). That RNF185 affects CFTR co-translational degradation is also supported by our observation that RNF185 associates with Derlin-1, a protein suggested to promote CFTR ERAD early during synthesis by virtue of its interaction with CFTR N-terminal domains (25).

A partly redundant function for RNF185 and RNF5

Among the two E3 ligases hitherto shown to affect CFTR stability in the ER (27,40,42), RNF5
RNF185 is a new E3 ligase targeting CFTR

and CHIP, RNF5 was attributed a preferential role in the co-translational sensing of CFTR folding defects. Our data now strongly indicate that RNF185 and RNF5 cooperate to target CFTRΔF508 to degradation not only during, but also after its synthesis. Indeed, we show that simultaneous inactivation of these two E3 enzymes leads to a greater increase of CFTR levels compared to the one achieved upon their single inactivation. Simultaneous inactivation of these two E3 enzymes also dramatically decreased CFTRΔF508 post-translational turnover rates, as measured upon translation inhibition. Such effect could only be uncovered once both E3 ligases have been depleted. Mechanistically, RNF5 and RNF185 redundancy could be assigned to both ligases sensing similar CFTR folding defects. Therefore, as depletion of either ligase alone stabilizes CFTR, strict redundancy would imply that the activity of each is limiting, at least in our experimental conditions. Conversely, RNF5 and RNF185 might sense different folding defects. In this case, their simultaneous depletion would be additive compared to their single knockdown. Such effect is only observed when measuring CFTR co-translational degradation rates while RNF5 and RNF185 appear fully redundant after the synthesis of the full length CFTR. We therefore favor a hypothesis where RNF5 and RNF185 might sense different folding defects during CFTR synthesis and similar ones after its synthesis has been completed. This would explain why RNF185 and RNF5 over-expression each decrease CFTR post-synthetic turnover rates while their single knockdown doesn’t. Analysis of mutated and truncated forms of CFTR was used to pinpoint the CFTR folding lesions preferentially sensed by RNF5. RNF5 was proposed to sense a defect in both the cooperative folding of the N-terminal regions occurring after synthesis of the R domain, and the association of the two MSD domains (27,41). Our preliminary data also indicate that RNF185 preferentially affects the turnover of CFTR proteins truncated after the R domain (unpublished), suggesting that as for RNF5, the synthesis of MSD2 and NBD2 C-terminal domains is not strictly required for RNF185-dependent quality control. Further experiments are needed to discriminate the mechanisms of RNF5 and RNF185-dependent checkpoints. Moreover, the relative impact of knocking down either ligase might depend on a given cell type and/or physiological condition.

The high degree of post-synthetic stabilization achieved by the double RNF5/RNF185 knockdown also questions the function of CHIP. In the ER, CHIP was proposed to preferentially act post-translationally, targeting CFTRΔF508 to degradation once the second transmembrane domain (MSD2) of CFTR has been synthetized (27). CHIP overexpression was shown to affect CFTR post-synthetic degradation rates (42). However, knockdown-based experiments supporting this model are currently lacking. Experiments are now underway to investigate the relative importance of CHIP compared to RNF5 and RNF185 in stimulating CFTR post-translational degradation in the ER.

Could other RNF185 and RNF5 functions explain the need for their functional redundancy in ERAD?

Functional redundancy between RNF5 and RNF185 could be rationalized by the need of a backup for RNF5 ERAD function, when this protein is diverted to competing processes such as the regulation of JAMP activity and the recruitment of the proteasome to the ER membranes (66), RNF5 mitochondrial recruitment and function in innate immunity signaling (67,68), or its recently described role in the regulation of mitochondrial fission (69) and autophagy (70). RNF185 also appears to carry out distinct cellular functions, including control of Wnt signaling-induced Paxillin degradation and regulation of mitophagy (see below). As such, it will be critical to assess the relative importance of the RNF module in CFTR degradation using more physiological settings including bronchial epithelial cells and CF mouse models. It is important to emphasize that both RNF5 and RNF185 does not belong to the group of the archetypal ERAD multipass Hrd1 and Doa10 E3 ligases that are conserved from yeast to mammals. As such, they maybe more likely to have versatile cellular or organismal functions in addition to the one we have described here in ERAD.

Other RNF185 functions

A homolog of mammalian RNF185 was previously characterized in Xenopus laevis as a protein affecting the stability of the cytoplasmic substrate Paxillin by direct recruitment of the proteasome (71). Wnt signaling-induced Paxillin ubiquitination in the mesoderm was shown to lead to RNF185-dependent substrate degradation. However, this process might be indirect as Paxillin ubiquitination was not shown to be operated by
RNF185 is a new E3 ligase targeting CFTR

This RNF185 function therefore differs from that we described herein in ERAD.

RNF185 was also recently described as positive regulator of mitophagy, a function that requires its ubiquitin ligase activity (50). According to this study, ubiquitination of the Bel-2 family member BNIP1 by RNF185 increases autophagosome formation by promoting p62 recruitment to mitochondria. Although we did not directly address this question, our data could not demonstrate an effect of RNF185 overexpression on the Z mutant of AAT, the degradation of which is partly autophagic (60). It thus seems unlikely that RNF185 has broad effects on autophagy. Instead, such a function of RNF185 in autophagy might be limited to its mitochondrial localization. Interestingly, the data by Tang and colleagues and ours suggest that RNF185 could provide a new link between ERAD and autophagy, as was recently shown for RNF5 (70). Identification of physiological conditions potentially regulating RNF185 localization or function in autophagy will thus be of interest to further unravel intertwined functions of RNF185 and evaluate their consequences on CFTR turnover.

Conclusion

We have identified RNF185 as a new E3 ligase that drives CFTR degradation in the ER, a function that appear redundant with RNF5. These data expand the repertoire of mammalian E3 ligases that operate in ERAD. Importantly, they provide key information for setting up strategies to efficiently block CFTR degradation in the ER and to increase the pool of foldable CFTR, potentially operational for maturation and plasma membrane targeting. Evaluating the impact of CFTR correctors, especially chemical chaperones, on the maturation of this stabilized pool will be critical to assess the therapeutic benefit that could be obtained from inactivating the RNF degradation pathways.

APPENDIX

Fitting curves and modelization

Experimental data obtained from the CHX experiment were fitted to the exponential decay function

\[ m(t) = m_0 e^{-kt} \]

where \( m_0 \) is the initial number of full length proteins, \( k \) the degradation rate and \( m(t) \) the number of full length proteins remaining at time \( t \).

Fitting curves were obtained using the R software and produced the following equations:

\[ m_{\text{vector}}(t) = 100e^{-0.016t} \]
\[ m_{\text{RNF185}}(t) = 100e^{-0.023t}, \]

identifying

\[ k_{\text{vector}} = 0.0164 \] and \[ k_{\text{RNF185}} = 0.0234. \]

The \( ^{35}S \)-labeling experiment was modeled as follows:

if one considers that \( S \), the rate of synthesis (i.e. the number of full length proteins synthetized by time unit) is constant over time, then

\[ \frac{dm}{dt} = S - km, \]

where \( m \) is the number of full length proteins, \( t \) the time and \( k \) the degradation rate that is established by the CHX experiment.

It follows that

\[ m(t) = \frac{[S/k]e^{-kt}}{1 - e^{-kt}} + C, \]

where \( C \) is a constant.

At \( t=0 \), \( m(0)=0 \), hence \( C=-S/k \) and therefore

\[ m(t) = S/k*(1-e^{-kt}) \]

is the function that predicts the accumulation of full length protein over time.

\( S \) can be decomposed as the rate of translation minus the rate of co-translational degradation.

If one consider the ratio

\[ \frac{m_{\text{RNF185}}(t)}{m_{\text{vector}}(t)} = \frac{[S_{\text{RNF185}}/k_{\text{RNF185}}*(1-e^{-k_{\text{RNF185}}t})]}{[S_{\text{vector}}/k_{\text{vector}}*(1-e^{-k_{\text{vector}}t})]} \];

if co-translational degradation is absent, \( S \) only reflects the rate of translation, which appears similar in the control sample and upon overexpressing RNF185 and

\[ S_{\text{RNF185}} = S_{\text{vector}}. \]

Therefore,
RNF185 is a new E3 ligase targeting CFTR

\[
\frac{m_{\text{RNF185}}(t)}{m_{\text{vector}}(t)} = \frac{k_{\text{vector}}/k_{RNF185}*(1-e^{-k_{RNF185}t})}{(1-e^{-k_{\text{vector}}t})}.
\]

Using \(k_{\text{vector}}=0.016\) and \(k_{RNF185}=0.023\) from the CHX experiment, it follows that at 20 minutes the expected ratio in the absence of co-translational degradation is

\[
\frac{m_{\text{RNF185}}(t)}{m_{\text{vector}}(t)}=0.937,
\]

which corresponds to a 6.3% decrease in \(^{35}\text{S}\)-labeled CFTR in the presence of RNF185 compared to the control condition.

Acknowledgement

We thank M. Benharouga, P. Fanen, A. Hinzpeter, A. Edelman and F. Brouillard for advice and reagents on CFTR, E. Chevet for the alpha-1 anti-trypsin expressing plasmids. We thank J.C. Aude and J.Y. Thuret for their help in phylogenetic analysis and modelisation, respectively. We thank all members of M. Toledano’s lab for fruitful discussions. We are very grateful to Ze’ev Ronai for his help in setting up this project, by providing reagents, advice and useful comments along the way. This work was supported by the Ligue contre le Cancer, Vaincre la Mucoviscidose, the Fondation pour la Recherche Medicale and ANR ERRed to MBT and ADM.

Abbreviations


References


RNF185 is a new E3 ligase targeting CFTR


RNF185 is a new E3 ligase targeting CFTR endoplasmic reticulum membrane. *Proc Natl Acad Sci U S A* 102, 14132-14138/10.1073/pnas.0505006102


RNF185 is a new E3 ligase targeting CFTR


Figure legends

Figure 1. RNF185 is a RNF5 homolog conserved in higher eukaryotes. Amino acid sequence alignment of human (GI: 45708382) and mouse (GI: 15928691) RNF185 with their human (GI:
RNF185 is a new E3 ligase targeting CFTR

5902054), mouse (GI: 9507059) and C. elegans RNF5 (GI: 3874385) homologs. The two C-terminal membrane domains are underlined. The seven cysteine residues and the histidine residue constitutive of the RING domain are circled.

Figure 2. RNF185 is a novel ubiquitously expressed E3 ligase A. Expression of RNF185 in mouse tissues. Total RNAs were purified from WT mouse tissues and were retrotranscribed for quantitative-PCR analysis using RNF185 specific primers. Ppia1 and RN18S were used as references. Analysis was carried out on RNA samples extracted from tissues of three different mice. B. RNF185 can auto-ubiquitinate. Purified GST, GST-RNF185 and GST-RNF5 were incubated at 37°C in the presence of ATP, ubiquitin, E1 and three different E2 enzymes. The reaction was next subjected to immunoblotting (IB) with anti-GST or anti-Ubiquitin antibodies. C. Schematic representation of the RNF185 constructs used in this study. RNF185 WT: Wild Type RNF185; RNF185 ΔC: RNF185 with truncation of the most distal transmembrane domain; RNF185 RM: RNF185 with two punctual mutations in the RING domain; RNF185 ΔR: RNF185 mutant with total deletion of the RING domain. D. RNF185 ubiquitin ligase activity is dependent on the RING domain. GST-RNF185 WT and its RING mutant counterparts were processed as in B.

Figure 3. RNF185 is an ER localized E3 ligase. A. Co-immunolocalization of RNF185 WT and RNF185 mutants with ER marker. HEK293 cells were co-transfected with the different RNF185 constructs and a plasmid expressing an ER localized GFP. Very low doses (0.1 μg DNA per well of a 24 well plate) of plasmid were used to prevent overexpression bias. Cells were fixed 24 hours after transfection and processed for immunostaining using Flag antibody. B. Immunolocalization of endogenous RNF185. 24 hours after transfection with ER-GFP plasmids, cells were fixed and processed for immunostaining using home-made RNF185 polyclonal antibody. C. RNF185 co-localization with a mitochondrial marker. RNF185 constructs were transfected in HEK293 cells. Mitochondria were
visualized by the addition of mitotracker to the culture medium 24 hours after transfection before processing the cells for immunostaining using Flag antibody.

**Figure 4. RNF185 interacts with ERAD components and is induced by UPR.** A. RNF185 interacts with Derlin-1 and Erlix2. HEK293T cells were transfected with the control vector, Flag-RNF185 or Flag RNF5 (0.5 µg of plasmid per well of a 6 well plate). 24 hours post-transfection, cells were lysed and co-immunoprecipitation was performed using Flag antibody. Immunoprecipitated proteins were loaded on reducing 14% SDS-PAGE and immunoblotted with antibodies against endogenous Derlin-1, endogenous Erlix2 or Flag. B. RNF185 interacts with both enzymes of the Ubc6 family. HEK293T cells were transfected with the control vector or Flag-RNF185 together with a plasmid expressing HA-UBE2J1 or myc-UBE2J2. Cells were then processed as in A. In this experiment, Flag-RNF185 co-migrates with the antibody light chain as seen in the control lane (*). C. RNF185 expression is increased after tunicamycin treatment. HEK293 cells were treated with 2 µg/ml of tunicamycin during the indicated times. Total RNAs were extracted and retrotranscribed. Quantitative-PCR analysis was performed using RNF185 specific primers and its expression levels were normalized to GAPDH levels (left panel). Results are shown as the mean of three independent experiments. Change in GRP78 expression was used as a control for UPR induction by tunicamycin (right panel).

**Figure 5. RNF185 induces the ubiquitin-proteasome-dependent degradation of CFTR proteins.** A. RNF185 overexpression decreases the steady state levels of WT CFTR and CFTRΔF508. Cells were co-transfected with control vector or increasing amounts of Flag-RNF185 (0.5, 1 or 2 µg per well) and CFTR-HA or CFTRΔF508-HA. Low amount (0.1 µg per well) of a GFP-expressing plasmid was co-transfected in each condition and the monitoring of GFP expression was used as a control for transfection efficiency. 24 hours post-transfection, cells were lysed and equal amounts of protein extracts were loaded on reducing SDS-PAGE for immunoblot (IB) with the indicated antibodies. GAPDH was used as a loading control. The core glycosylated immature form and the mature glycosylated form of CFTR are noted B and C respectively. Steady state levels of CFTR and CFTRΔF508 were quantified using ImageJ.
software and were normalized to GAPDH and GFP levels. Results from three independent experiments have been plotted and are expressed as a percentage of the control (vector) condition. B. Decrease in CFTR levels is dependent on RNF185 E3 ligase activity. Cells were co-transfected with control vector or vector expressing RNF185 WT, RNF185 RM or RNF185 AR together with CFTR-HA or CFTRΔF508-HA. As in A, co-transfection with a GFP-expressing plasmid was used to monitor transfection efficiency. Cells were next processed as in A. C. RNF185 knockdown increases CFTR levels. Cells were co-transfected with CFTR-HA or CFTRΔF508-HA together with a control or a RNF185-specific siRNA. 48 hours later the cells were processed as described in A. RNF185 extinction was monitored after immunoprecipitation of the cellular extracts with anti-RNF185 antibody. Relative steady state levels of CFTR proteins were quantified using ImageJ software. Results are expressed as a percentage of the control condition. D. RNF185 interacts with CFTR and CFTRΔF508. HEK293T cells were co-transfected with the indicated plasmids. HA-H3, a plasmid expressing HA-tagged histone H3, was used as a negative control for the immunoprecipitation (upper panel). Cells expressing WT RNF185 were treated with MG132 during 5 hours before processing with the lysis. Co-immunoprecipitations were carried out with equal amounts of cell lysates using anti-HA antibody (upper panel) or anti-Flag antibody (lower panel). The immunoprecipitates were next immunoblotted with the indicated antibodies. E. Proteasome inhibition rescues RNF185-induced decrease in CFTR levels. HEK293 cells were co-transfected with the indicated plasmids and treated with ALLN or DMSO for 12 hours, 24 hours post-transfection. After cell lysis, the detergent insoluble and soluble fractions were subjected to immunoblot analysis with the indicated antibodies.

**Figure 6. Analysis of CFTRΔF508 degradation by cycloheximide chase.** A. Cycloheximide (CHX) chase analysis of CFTRΔF508 upon RNF185 expression. HEK293T cells were co-transfected with CFTRΔF508-HA together with a control vector or vector expressing RNF185 WT. GFP-expressing plasmid was co-transfected as a marker for transfection efficiency. 24 hours later, protein extracts were prepared at the indicated time points after cycloheximide treatment (100 µg/mL) and loaded onto reducing SDS-PAGE. Immunoblotting was performed with the indicated antibodies. Relative changes in
the half-life of CFTRΔF508 were quantified from three different experiments using ImageJ software and normalized to GAPDH and GFP levels. The obtained values were plotted against time. B. Cycloheximide (CHX) chase analysis of CFTRΔF508 upon RNF185 knockdown. Cells stably expressing CFTRΔF508-HA were transfected with a control or an RNF185-directed siRNA. 48 hours later, cells were treated with CHX and processed as in A. Downregulation of RNF185 expression was controlled by Q-PCR (inset right panel).

Figure 7. RNF185 targets CFTRΔF508 to co-translational degradation. A. Measure of CFTRΔF508 labeling rates upon RNF185 over-expression. Cells were co-transfected with CFTRΔF508-HA together with RNF185 or the corresponding control vector. 24 hours later, the cells were labeled with 35S Met/Cys radiolabeling mix and the synthesis of 35S-labeled CFTRΔF508 protein was monitored over time by immune-precipitating equal amounts of the labeled extracts with anti-HA antibody. ALLN or DMSO was added in the medium 1h30 before labeling. Consistency of 35S-labeling between samples was controlled by loading the supernatants of the corresponding immunoprecipitation (depicted as lysates 35S). Quantification of the experiment was performed using ImageJ software and the intensity of labeled CFTR was normalized to the total amount of radioactivity initially present in the corresponding lysate. Results are expressed as a percentage of the vector condition quantified at 10 minutes in DMSO. RNF185 expression was confirmed by SDS-PAGE analysis (lower panel). B. Comparison of the experimental fitted curves (solid lines), accounting for the observed accumulation of 35S-labeled CFTRΔF508 over time in the absence (blue line) and presence (red line) of RNF185, with the theoretical curve (dashed red line), predicting the accumulation of 35S-labeled CFTRΔF508 if RNF185 only impacted CFTR post-translational degradation rate. The experimental fitted curves were obtained as described in the appendix and in Fig S6. The theoretical RNF185 curve was obtained by setting equal the rate of synthesis in the presence or absence of RNF185. C. Measure of CFTRΔF508 labeling rates upon RNF185 knockdown. Cells stably expressing CFTRΔF508 were transfected using control or RNF185-directed siRNA. 48 hours after transfection, the cells were labeled and processed as in A. Efficiency of RNF185 knockdown was controlled by Q-PCR analysis (right panel).
Figure 8. Combined depletion of RNF185 and RNF5 synergistically blocks CFTRΔF508 degradation. A. Analysis of CFTRΔF508 turnover upon combined RNF185 and RNF5 knock down. HEK 293 cells stably expressing a control shRNA or a shRNA sequence targeting RNF5 were co-transfected with CFTRΔF508-HA together with a control siRNA or a siRNA sequence targeting RNF185. 48 hours later, the cells were treated with CHX for the indicated times and processed as in Figure 6A. Immunoblotting following SDS-PAGE was performed using the indicated antibodies. Downregulation of RNF185 expression was controlled by Q-PCR (right panel). B. Relative changes in the half-life of CFTRΔF508 were quantified from three independent experiments using ImageJ software and normalized to GAPDH and GFP levels. The obtained values were plotted against time. Left panel is depicting relative values normalized to the control condition (control shRNA, control siRNA), where initial CFTR ΔF508 levels in this condition have been artificially normalized to 1. The right panel is depicting CFTRΔF508 intrinsic half-life after translation block, the initial time point for each condition being set at 100%.
Figure 2. El Khouri et al.
Figure 3. El Khouri et al.
Figure 4. El Khouri et al.
Figure 5. El Khouri et al.

A

B

C

D

E
Figure 6. El Khouri et al.
Figure 7. El Khouri et al.

A

<table>
<thead>
<tr>
<th>CFTRΔF508-HA</th>
<th>Vector</th>
<th>Flag RNF185</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse time (min)</td>
<td>10 15 20</td>
<td>10 15 20</td>
</tr>
<tr>
<td>IP HA/35S</td>
<td>DMSO % of control</td>
<td>ALLN % of control</td>
</tr>
<tr>
<td>100 133 164</td>
<td>49 64 106</td>
<td></td>
</tr>
<tr>
<td>91 160 294</td>
<td>89 161 302</td>
<td></td>
</tr>
<tr>
<td>Lysates 35S</td>
<td>DMSO</td>
<td>ALLN</td>
</tr>
</tbody>
</table>

IB: Flag
IB: GFP

B

Co-translational degradation
Post-translational degradation

Arbitrary units

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Protein levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 10 20 30</td>
<td>0 100 200 300</td>
</tr>
</tbody>
</table>

Control siRNA RNF185 siRNA

10 15 20 30

% of control

Control siRNA RNF185 siRNA

10 15 20 30

% of control

Vector, experimental fit
RNF185, experimental fit
RNF185, theoretical curve deduced from the calculation of RNF185 dependent post-translational degradation rates

C

<table>
<thead>
<tr>
<th>CFTRΔF508-HA</th>
<th>Control siRNA</th>
<th>RNF185 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse time (min)</td>
<td>10 15 20 30</td>
<td>10 15 20 30</td>
</tr>
<tr>
<td>IP HA/35S</td>
<td>Relative intensity (% of control)</td>
<td></td>
</tr>
<tr>
<td>100 155 184 417</td>
<td>65 223 605 1169</td>
<td></td>
</tr>
<tr>
<td>Lysates 35S</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. El Khouri et al.

A

<table>
<thead>
<tr>
<th>CFTR ΔF508-HA</th>
<th>Control shRNA</th>
<th>RNF5 shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after CHX (min)</td>
<td>Control siRNA</td>
<td>RNF5 siRNA</td>
</tr>
</tbody>
</table>

IB: HA

IB: RNF5

IB: GFP

IB: GAPDH

B

- Relative intensity
- Time after CHX (min)
- % of CFTR ΔF508 remaining
- Time after CHX (min)
RNF185 is a novel E3 ligase of Endoplasmic Reticulum Associated Degradation (ERAD) that targets Cystic Fibrosis Transmembrane conductance Regulator (CFTR)

Elma El Khouri, Gwenaelle Le Pavec, Michel B. Toledano and Agnes Delaunay-Moisan

*J. Biol. Chem.* published online September 9, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.470500

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2013/09/09/M113.470500.DC1