The cystic-fibrosis-associated ΔF508 mutation confers post-transcriptional destabilization on the *C. elegans* ABC transporter PGP-3

Liping He1, Jennifer Skirkanich1, Lorenza Moronetti1, Rosemary Lewis1,2 and Todd Lamitina1,2,*

SUMMARY

Membrane proteins make up ~30% of the proteome. During the early stages of maturation, this class of proteins can experience localized misfolding in distinct cellular compartments, such as the cytoplasm, endoplasmic reticulum (ER) lumen and ER membrane. ER quality control (ERQC) mechanisms monitor folding and determine whether a membrane protein is appropriately folded or is misfolded and warrants degradation. ERQC plays crucial roles in human diseases, such as cystic fibrosis, in which deletion of a single amino acid (F508) results in the misfolding and degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl– channel. We introduced the ΔF508 mutation into *Caenorhabditis elegans* PGP-3, a 12-transmembrane ABC transporter with 15% identity to CFTR. When expressed in intestinal epithelial cells, PGP-3wt was stable and efficiently trafficked subject to the same Creative Commons License terms.

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INTRODUCTION

Over 30% of all cellular proteins are either secreted or integral membrane proteins. Maturation and folding of this class of proteins is essential for cellular function and this process begins in the endoplasmic reticulum (ER). If proteins fold properly within the ER, they exit this compartment to undergo further post-translational modifications and sorting to their final cellular destination. Proteins that become misfolded in the ER are recognized by ER quality control (ERQC), a system that monitors and manages ER protein biosynthesis (Vembar and Brodsky, 2008). Proteins that are targeted by ERQC can be dealt with through two physiologically distinct pathways. First, they can be refolded through the unfolded protein response (UPR), a multifaceted stress response that upregulates ER chaperones, increases ER volume and adjusts ER translation to match folding demands (Marciniak and Ron, 2006). Second, they can be degraded through a process called ER-associated degradation (ERAD) (Vembar and Brodsky, 2008). At a molecular level, ERAD is a complex process, requiring chaperones, protein dislocation channel(s), ATPases, ubiquitin conjugating enzymes and proteasome subunits to appropriately recognize, remove and degrade misfolded substrates from the ER membrane and/or lumen (Brodsky and Wójcikiewicz, 2009; Hegde and Ploegh, 2010). Numerous types of misfolding events can activate ERAD, including environmental stressors or genetically encoded mutations in ERQC client proteins (Ward et al., 1995; Kelly et al., 2007). However, even in the absence of environmental or genetic perturbations to protein folding, ERAD is an active process. In some cases, as much as ~75% of newly synthesized proteins are selected for degradation by ERAD owing to errors in protein translation or inappropriate folding trajectories (Varga et al., 2004).

A well-known ERAD substrate is the cystic fibrosis transmembrane conductance regulator (CFTR) (Jensen et al., 1995). CFTR is a 12-transmembrane integral membrane protein of the ABC transporter superfamily. CFTR is only expressed in vertebrates, in which it functions as a cAMP-regulated Cl– channel (Marciniak and Ron, 2006). Efficient degradation of PGP-3ΔF508 required the function of several *C. elegans* ER-associated degradation (ERAD) homologs, suggesting that destabilization occurs through an ERAD-type mechanism. Our studies show that the ΔF508 mutation causes post-transcriptional destabilization and degradation of PGP-3 in *C. elegans* epithelial cells. This model, combined with the power of *C. elegans* genetics, provides a new opportunity to genetically dissect metazoan ERQC.

1Department of Physiology, Perelman School of Medicine, and 2Cell and Molecular Biology Graduate Group, Cell Biology and Physiology Program, University of Pennsylvania, Philadelphia, PA 19104, USA

*Author for correspondence (lamitina@mail.med.upenn.edu)

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biochemical levels, but how these mechanisms operate in an organismal setting is poorly understood.

Although the ΔF508 mutation has a significant effect on CFTR folding, it is also a determinant of protein folding in other ABC transporters. For example, introduction of the ΔF508 mutation into P-glycoprotein (PGP), an ABC transporter closely related to CFTR, disrupts PGP transmembrane packing and maturation within the ER and causes a loss of PGP activity (Loo et al., 2002; Chen et al., 2004). In yeast, ΔF508 inhibits the function of STE6, an ABC transporter required for yeast mating (Teem et al., 1993; Teem et al., 1996) and YOR1, a broad spectrum ABC transporter that mediates drug efflux (Louie et al., 2010; Pagant et al., 2010). Similar to mutant CFTR, efficient degradation of these ABC transporters also requires the function of chaperones, ubiquitin enzymes and other ER-associated molecules, suggesting that similar mechanisms govern ERAD-dependent degradation of mutant CFTR and other ABC transporters (Nakatsukasa et al., 2008). Although PGP, YOR1 and STE6 are distinct from CFTR in that they act as transporters and not ion channels, these studies suggest that the ΔF508 mutation within the context of NBD1 is a broad, evolutionarily conserved determinant of ABC transporter protein stability from yeast to humans.

The nematode Caenorhabditis elegans is a powerful genetic model system that is used to investigate general mechanisms of protein misfolding and aggregation (Link et al., 2003; Briguglia et al., 2006; Cohen et al., 2006; Gidaletz et al., 2009; Wang et al., 2009). The availability of numerous mutant strains has led to the identification of many unexpected genetic pathways that can influence the folding and toxicity of misfolded substrates (Hsu et al., 2003; Morley and Morimoto, 2004; Garcia et al., 2007). Mechanistic insight into the roles of these pathways is facilitated by the optical transparency of C. elegans, which allows the steady-state levels of fluorescently tagged proteins to be visualized with subcellular resolution in live animals from birth until death. In addition to the expected roles for protein chaperones and other cell-autonomous mechanisms that have been previously identified through cell- or biochemistry-based studies, work in C. elegans has also identified surprising cell-non-autonomous mechanisms that contribute to cellular protein folding in an organismal context (Garcia et al., 2007; Prahlad et al., 2008; Prahlad and Morimoto, 2011). Although these experimental tools have been applied to models of soluble misfolded proteins (Nollen et al., 2004; Gidaletz et al., 2009), a C. elegans model for membrane protein misfolding in general and/or ΔF508-dependent folding mechanisms has not been developed.

In this study, we developed a misfolded, multipass transmembrane protein in C. elegans intestinal epithelial cells by introducing the ΔF508 mutation into the ABC transporter pgp-3. Whereas wild-type PGP-3 was stable and trafficked to the apical plasma membrane, PGP-3ΔF508 was unstable and most protein did not reach the plasma membrane. Using PGP-3ΔF508 as an in vivo sensor for membrane protein folding, we found that environmental conditions and genetic pathways that regulate CFTRΔF508 instability in mammals also regulate PGP-3ΔF508 instability in C. elegans. Our studies establish C. elegans as a new model system for the study of membrane protein quality control and provide novel opportunities for in vivo genetic, genomic and pharmacological analysis of this complex process in animals.

RESULTS

The ΔF508 mutation post-transcriptionally destabilizes PGP-3 in C. elegans

In mammals, the CFTR protein is an important representative model for the study of membrane protein folding and quality control systems (Peters et al., 2011). Although CFTR homologs are present throughout vertebrate genomes, they are absent from the genomes of invertebrates, such as C. elegans. However, CFTR is a member of the ABC transporter gene family, of which there are at least 60 C. elegans homologs (Sheps et al., 2004; Zhao et al., 2004; Zhao et al., 2007). In total, 14 of these ABC transporters encode PGP-3 variants, which have been used previously to model ΔF508 folding defects in mammalian cells (Loo et al., 2002; Chen et al., 2004). C. elegans PGP proteins exhibit 13-16% identity with human CFTR (supplementary material Table S1). Most of this homology is found within the highly conserved NBD1 and NBD2 domains (supplementary material Fig. S1). In human CFTR, the F508 mutation lies within the NBD1 domain (~amino acids 433-586 of human CFTR). To model ΔF508 folding defects in worms, we searched for a C. elegans PGP with qualities similar to those of mammalian CFTR, namely one that is expressed in epithelial cells and that is trafficked to the apical plasma membrane. Previous studies have shown that PGP-3 exhibits these properties (Lincke et al., 1993; Broeks et al., 1995). However, PGP-3 is functionally distinct from CFTR in that it functions as an ATP-dependent drug transporter and not as a cAMP-activated Cl- channel (Lincke et al., 1993; Broeks et al., 1995). We cloned pgp-3 and replaced a nine-amino-acid-encoding region (amino acids 476-484) within NBD1 with a homologous human CFTR sequence surrounding F508 (amino acids 501-509), as had been previously done with human PGP (pgp-3ΔF508) (Loo et al., 2002) (Fig. 1A). We also created a variant that lacked the F508 residue (pgp-3ΔF508ΔF). The expression of both constructs was driven by the vha-6 promoter, which is active only in polarized intestinal epithelial cells (Oka et al., 2001; Allman et al., 2009). The proteins were tagged at the N-terminus with a 3×FLAG epitope and at the C-terminus with mCherry. In the intestine, the tagged proteins were functional, as measured by the ability to rescue the slow growth phenotype of pgp-1(pk17); pgp-3(pk18) double-mutant animals (supplementary material Fig. S2). The transgenes were integrated into the same genomic location at single-copy level using the Mos-mediated transgene insertion method (Frokjaer-Jensen et al., 2008). As expected for single-copy insertions, quantitative PCR showed that the mRNA for both pgp-3ΔF508 and pgp-3ΔF508ΔF was expressed at identical levels (Fig. 1B). Similar results were obtained with extrachromosomal array and integrated overexpression lines (supplementary material Fig. S3).

In the intestine, PGP-3ΔF508 was trafficked to the apical plasma membrane (Fig. 1C). Trafficking of PGP-3ΔF508 occurred through a COPII-dependent pathway, because RNA interference (RNAi) of C. elegans sec-24.1, a homolog of yeast sec-24, which is required for the trafficking of proteins from the ER to the Golgi (Barlowe et al., 1994), strongly attenuated overall abundance and apical membrane localization of PGP-3ΔF508 (supplementary material Fig. S4). Compared with PGP-3ΔF508, the steady-state fluorescence of the PGP-3ΔF508 protein was significantly reduced (Fig. 1C). Quantitative analysis of mCherry fluorescence showed that there was significantly reduced total fluorescence and reduced apical membrane fluorescence for PGP-3ΔF508 compared with PGP-3ΔF508ΔF (Fig. 1D), although image
enhancement showed that some PGP-3ΔF508::mCherry protein could be detected at or near the apical membrane (supplementary material Fig. S5). Given that ΔF508 is known to cause protein misfolding, we considered the possibility that the apparent reduction in fluorescence was due to global misfolding of the PGP-3ΔF508 protein and the linked mCherry reporter. However, this was not the case: western blot analysis confirmed a reduction in PGP-3ΔF508 protein levels compared with PGP-3wt (Fig. 1E). Furthermore, in extrachromosomal array lines, which exhibit highly variable expression levels, the slope of the mRNA:fluorescence relationship was substantially reduced in PGP-3ΔF508 animals as compared with PGP-3wt animals (supplementary material Fig. S3). Together, these data show that C. elegans PGP-3 is a COPII-trafficked membrane protein and that introduction of the ΔF508 mutation into NBD1 causes a post-transcriptional reduction in both the overall steady-state levels and apical membrane abundance of PGP-3.

**Genetic or physiological activation of the osmotic stress response stabilizes PGP-3ΔF508**

In mammalian cells, CFTRVΔF508 can be stabilized following exposure of cells to either low temperature or high levels of glycerol or other organic osmolytes (Sato et al., 1996; Howard et al., 2003). We investigated whether exposure of worms to these conditions might similarly stabilize PGP-3ΔF508. Worms exposed to either low temperature (growth at 15°C or 24-hour exposure to 4°C; data not shown) or high temperature (1-hour exposure to 35°C heat shock; supplementary material Fig. S6) did not exhibit significantly altered expression levels of PGP-3ΔF508. However, exposure of worms to hyperosmotic environments, which stimulates the synthesis and accumulation of the osmolyte glycerol in the intestine (Lamitina et al., 2004; Lamitina et al., 2006; Rohlfing et al., 2010; Rohlfing et al., 2011), significantly stabilized PGP-3ΔF508 total and apical membrane fluorescence (Fig. 2). Hyperosmotic stress also increased the abundance and apical membrane localization of PGP-3wt, although, unlike PGP-3ΔF508, PGP-3wt stabilization was not strong enough to be detected by western blot analysis (Fig. 2). mRNA levels for PGP-3, as measured by qPCR, were not affected by hyperosmotic stress (supplementary material Fig. S7). These data show that physiological activation of the osmotic stress response (Osr) pathway, but not other stress response pathways, post-transcriptionally stabilizes PGP-3ΔF508 in C. elegans.

We also examined whether genetic activation of known protein folding pathways improved PGP-3ΔF508 stability. Mutations in both the daf-2–IGF (insulin-like growth factor) signaling pathway and the Osr pathway protect against related but distinct types of protein misfolding and aggregation in C. elegans (Hsu et al., 2003;...
Moronetti Mazzoe et al., 2012). Therefore, we tested whether activation of these pathways might stabilize PGP-3ΔF508. We found that daf-2(e1370) mutants had no effect on the stability or trafficking of PGP-3ΔF508 (supplementary material Fig. S8). However, Osr mutants, such as osm-7 or osm-11, significantly improved the stability of PGP-3ΔF508 (Fig. 3). Osr mutants increased both the total amount of PGP-3ΔF508 protein as well as the amount of protein that was trafficked to the apical plasma membrane (Fig. 3). As was the case during hyperosmotic adaptation, Osr mutants also stabilized PGP-3wt. Together, these data show that PGP-3ΔF508 stability can be regulated by genetic and physiological activation of the Osr pathway but not by other pathways known to regulate protein folding, such as the insulin/IGF pathway. Because IGF signaling is known to suppress the folding defects associated with other non-membrane-localized substrates (Hsu et al., 2003; Morley and Morimoto, 2004), these data suggest that the genetic mechanisms regulating the folding and stability of the PGP-3ΔF508 membrane protein are distinct from those regulating the folding of previously described cytosolic substrates in C. elegans.

Destabilization of PGP-3ΔF508 is dependent on C. elegans ERAD homologs

Misfolded membrane proteins within the ER trigger ERAD, an evolutionarily conserved degradation pathway. Genetic studies in yeast and biochemical studies in mammalian cells have identified several evolutionarily conserved proteins that play key roles in ERAD (Vembar and Brodsky, 2008). We identified C. elegans homologs for many ERAD genes based on sequence homology (Table 1) and examined their roles in the regulation of PGP-3ΔF508 and PGP-3wt stability in vivo using RNAi (for genes whose mutant phenotype is lethal or for genes without an available mutation) or available loss-of-function mutants to knock down gene expression. Previous studies have suggested that the Derlin proteins play important roles in ERAD (Ye et al., 2004; Younger et al., 2006; Schaheen et al., 2009), as well as in the trafficking and degradation of misfolded plasma membrane proteins (Dang et al., 2011). C. elegans contains two Derlin homologs, cup-2 and R151.6 (der-2), homologous to Derlin-1 and Derlin-2, respectively. cup-2 mutants were previously shown to increase the abundance of some membrane proteins, as well as to cause the activation of the UPR (Ye et al., 2004; Schaheen et al., 2009). The function of R151.6 is unknown. We found that both R151.6(RNAi) and cup-2(2ar506) loss-of-function mutants increased total and apical membrane protein levels of PGP-3ΔF508. However, these RNAi knockdowns had no effect on the steady-state levels of PGP-3wt (Fig. 4, Table 1; supplementary material Table S2). In addition to the Derlin proteins, we also discovered a role for Cdc48 (p97) homologs in the degradation of PGP-3ΔF508. The Cdc48 genes encode AAA ATPases that are required for ERAD in both yeast and mammalian cells (Huyer et al., 2004; Goder et al., 2008; Nakatsukasa et al., 2008). C. elegans expresses three Cdc48 homologs, cdc-48.1, cdc-48.2 and cdc-48.3. We found that post-embryonic RNAi inhibition of cdc-48.1 and cdc-48.2, but not cdc-48.3, increased total and apical levels of PGP-3ΔF508 but had little effect on PGP-3wt (Fig. 4, Table 1; supplementary material Table S2). Functional inhibition of other ERAD regulators, including the ER chaperone calreticulin, and the RING finger E3 ubiquitin ligase rnf-5, also weakly stabilized PGP-3ΔF508 (Table 1; supplementary material Table S2). Together, these data show that the reduction in PGP-3ΔF508 protein requires the activity of C. elegans ERAD homologs and suggest that PGP-3ΔF508 is degraded through an ERAD-type mechanism.

**DISCUSSION**

The ΔF508 mutation is a critical regulator of protein stability in CFTR and other ABC transporters across a wide range of species. Analyses of the pathways that participate in ΔF508 substrate recognition and degradation have mostly been limited to biochemical approaches in single cells. Here, we introduce a C. elegans model that allows investigation of ΔF508-dependent recognition and degradation mechanisms in a multicellular, live-animal, epithelial tissue setting. Using genetic approaches, we show that proteins with well-established roles in ERAD-type degradation contribute to the degradation of PGP-3ΔF508 in C. elegans. Furthermore, we show that...
Fig. 3. Genetic activation of the Osr pathway stabilizes PGP-3ΔF508 and PGP-3wt. (A) DIC and wide-field fluorescence images of PGP-3ΔF508 in the indicated Osr mutant backgrounds grown on standard (50 mM NaCl) NGM plates. Arrows point to sites of PGP-3ΔF508 apical membrane localization. Scale bars: 10 μm. (B) Quantification of total and apical membrane fluorescence from animals expressing PGP-3ΔF508 or PGP-3wt in the indicated genetic background. Data are normalized relative to the wild-type control. *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA. (C) Representative western blot of PGP-3ΔF508 and PGP-3wt in the indicated genetic background. The numbers beneath each set of images represent the normalized PGP-3:actin ratio for the experiment shown. One of two representative experiments is shown.

In humans, the ΔF508 mutation reduces the abundance of the CFTR protein to levels that are insufficient to support its function. Likewise, introduction of ΔF508 into the yeast ABC transporters Ste6p and Yor1p also inhibits the function of these ABC transporters (Teem et al., 1993; Pagant et al., 2007). Surprisingly, we found that, although introduction of ΔF508 into C. elegans PGP-3 leads to post-transcriptional protein destabilization, PGP-3ΔF508 is still functional, as measured by its ability to rescue pgp-1; pgp-3 double mutants (supplementary material Fig. S2). PGP-3ΔF508 also rescued pgp-1; pgp-3 mutants when expressed under the less active pgp-3 promoter at single-copy level, even though this promoter reduced total and apical expression by ~80% and preserved the post-transcriptional reductions in PGP-3ΔF508 protein levels observed with the vha-6 promoter (data not shown). Why might this PGP-3ΔF508 protein be functional in C. elegans? One possible explanation is that, although PGP-3ΔF508 protein levels are reduced, they are still at or above the levels needed to provide function. Although the ΔF508 mutation virtually eliminates all functional CFTR protein from human airway epithelial cells, it has a substantially weaker effect on CFTR in other tissues and organisms (Ostedgaard et al., 2007; Luo et al., 2009; van Barneveld et al., 2010). Moreover, data from human airway epithelia suggest that only 25% of normal CFTR function is needed to rescue epithelial defects (Zhang et al., 2009). In C. elegans, PGP-3ΔF508 levels are ~20% that of PGP-3wt (Fig. 1). By these criteria, there might still be a sufficient level of protein to rescue pgp-3 mutant phenotypes in our C. elegans model. Although these findings currently preclude us from determining whether treatments that stabilize PGP-3ΔF508 also improve function, these data do show that the PGP-3ΔF508 protein in C. elegans, like CFTRΔF508 in humans, is active. The development of PGP-3 transgenes under the control of weaker promoters, as well as the development of new ERQC substrates, should eventually provide assays that allow us to examine both substrate function and stability.

a physiologically significant stress response pathway can be leveraged to ameliorate the folding defects caused by ΔF508 in vivo. Other in vivo transgenic models of ΔF508-mediated misfolding have been developed in animal model systems, including pigs, ferrets and mice (Stoltz et al., 2010; Sun et al., 2010; Ostedgaard et al., 2011; Wilke et al., 2011), and these systems permit detailed analysis of ΔF508-associated pathophysiology. However, none of the models permit the application of high-throughput genetic screening approaches, which is the major strength of the C. elegans system. It is important to note that C. elegans PGP-3 probably exhibits different functional characteristics from human CFTR, because PGP-3 is a transporter whereas CFTR is an ion channel. Moreover, our C. elegans ΔF508 model does not recapitulate certain clinically important aspects of ΔF508-associated tissue pathophysiology, such as progressive and chronic lung infection, because worms do not have airway epithelia. However, our studies show that the ΔF508 mutation does reproduce the general molecular pathophysiology of CF. Given the power of C. elegans genetics, this model offers an opportunity to dissect the underlying genetic mechanisms that contribute to ΔF508 instability using unbiased forward and reverse genetic screens and PGP-3ΔF508:mCherry fluorescent stability as a phenotype. In support of the feasibility of this approach, we have recently carried out forward genetic screens and identified several mutants with enhanced PGP-3ΔF508 protein levels that do not significantly alter mRNA levels (data not shown). The stabilization observed in these mutants is substantially higher than that seen following inhibition of ERAD genes or activation of the Osr pathway, suggesting that they affect a distinct pathway. Such in vivo genetic screening approaches for regulators of ΔF508 protein stability are not yet technically or financially practical using other in vivo model systems. The evolutionary position of C. elegans in relation to yeast and mammals could provide new insights into membrane protein quality control that cannot be revealed through either yeast or mammalian studies on their own.
Our findings reveal an important role for the Osr pathway in regulating PGP-3F508 stability. We found that activation of this pathway, either through physiological or genetic mechanisms, stabilizes PGP-3F508 in the intestine. Activation of other proteotoxic stress pathways, including the hsf-1-dependent heat-shock response and the longevity-promoting IGF signaling pathway, did not alter PGP-3F508 stability, suggesting that the specific physiological targets of osmotic stress stabilize PGP-3F508. A primary physiological role of the osmotic stress pathway is to direct the accumulation of the organic osmolyte glycerol (Lamitina et al., 2003; Rohlfing et al., 2011) suggest that this strategy may be feasible. In this respect, an important future goal is to test whether similar mechanisms for genetic activation of the Osr pathway exist in mammals and whether activation of these pathways improves CFTR folding in vivo. Although there are many physiological arguments against this strategy, the isolation of mutant C. elegans with this phenotype and observation that these animals appear relatively normal (Wheeler and Thomas, 2006; Rohlfing et al., 2011) suggest that this strategy may be feasible. Can we test whether similar mechanisms for genetic activation of the Osr pathway exist in mammals and whether activation of these pathways improves CFTR folding in vivo? Although there are many physiological arguments against this strategy, the isolation of mutant C. elegans with this phenotype and observation that these animals appear relatively normal (Wheeler and Thomas, 2006; Rohlfing et al., 2011) suggest that this strategy may be feasible. In this respect, an important future goal is to test whether similar mechanisms for genetic activation of the Osr pathway exist in mammals and whether activation of these pathways improves CFTR folding in vivo. Although there are many physiological arguments against this strategy, the isolation of mutant C. elegans with this phenotype and observation that these animals appear relatively normal (Wheeler and Thomas, 2006; Rohlfing et al., 2011) suggest that this strategy may be feasible. In this respect, an important future goal is to test whether similar mechanisms for genetic activation of the Osr pathway exist in mammals and whether activation of these pathways improves CFTR folding in vivo. Although there are many physiological arguments against this strategy, the isolation of mutant C. elegans with this phenotype and observation that these animals appear relatively normal (Wheeler and Thomas, 2006; Rohlfing et al., 2011) suggest that this strategy may be feasible. In this respect, an important future goal is to test whether similar mechanisms for genetic activation of the Osr pathway exist in mammals and whether activation of these pathways improves CFTR folding in vivo. Although there are many physiological arguments against this strategy, the isolation of mutant C. elegans with this phenotype and observation that these animals appear relatively normal (Wheeler and Thomas, 2006; Rohlfing et al., 2011) suggest that this strategy may be feasible.

We also found that PGP-3F508 degradation depends on the activity of C. elegans ERAD homologs. RNAi knockdown of C. elegans homologs of Derlins, cdc-48, Calreticulin and rnf-5 all reproducibly stabilized PGP-3F508 but not PGP-3wt, suggesting that these ERAD homologs exhibit specific interactions with the destabilized substrate. We also tested whether PGP-3F508 stability was dependent on the proteasome, because all ERAD substrates in yeast are degraded to varying extents by the proteasome (Vembar
and Brodsky, 2008). However, neither pharmacological (MG-132 inhibition) nor reverse genetic approaches (RNAi against proteasome genes) to inhibit proteasome activity significantly stabilized PGP-3\(^ {AF508}\) protein levels (data not shown). This could be because PGP-3\(^ {AF508}\) degradation can occur through proteasome-independent mechanisms (Donoso et al., 2005) or because our manipulations failed to sufficiently inhibit proteasome activity or selected for animals with minimally affected activity. Yet another possibility is that proteasome inhibition does in fact stabilize PGP-3\(^ {AF508}\), but also simultaneously inhibits PGP-3\(^ {AF508}\) protein synthesis through activation of the PERK branch of the UPR, resulting in no net increase in steady-state PGP-3\(^ {AF508}\) protein levels. Notably, the PERK branch of the UPR is not found in yeast, in which, in contrast to animals, protein synthesis continues during ER stress and proteasome inhibition strongly stabilizes ERAD substrates (Mori, 2009). In this respect, \textit{C. elegans}, which contains all major branches of the UPR, could provide an important animal-specific platform to investigate in vivo integration of ER stress responses and ERAD mechanisms with single-cell resolution in a way that is not possible in yeast.

In conclusion, we have developed a \textit{C. elegans} model that recapitulates post-transcriptional \(\Delta F508\)-dependent membrane protein instability. In addition to the expected roles for ERAD homologs, our system also identified an important and specific function for the Osr pathway in stabilizing PGP-3\(^ {AF508}\). Given the simple fluorescent phenotype caused by PGP-3\(^ {AF508}\) and the amenability of \textit{C. elegans} to forward genetic screening, this system offers a new opportunity to dissect the genetics and genomics of \(\Delta F508\)-dependent membrane protein misfolding in a way that was not previously possible. Such studies could provide unique insight into the mechanistic underpinnings of \(\Delta F508\)-dependent membrane protein misfolding and instability, as well as suggest novel in vivo approaches for the treatment of protein misfolding diseases.

**METHODS**

**\textit{C. elegans} strains**

The following strains were utilized; EG4322--unc-119(ed3); ttTi5605 (Mos1), GS2555--cup-2(ar506); arl373; dpy-20(e1282), RB922--raf-5(ok793), RB1104--hsp-3(ok1083), VC1801--cpx-1(ok2234), CB1370--daf-2(e1370), MT3564--osm-7(n1515), MT3643--osm-11(n1604), drSi2[cbunc-119+, vha-6p::3\times\text{FLAG}-pgp-3(CFTR\(^ {wt}\))--mCherry::unc-54], drSi5 [cbunc-119+, vha-6p::3\times\text{FLAG}-ppg-3(CFTR\(^ {AF508}\))--mCherry::unc-54]. Standard genetic crossing methods (Brenner, 1974) were used to place mutants into the drSi2 or drSi5 backgrounds. Mutant genotypes were confirmed by PCR (for deletion alleles), DNA sequencing or phenotypic analysis.

**Molecular biology and transgenics**

To generate the \textit{pgp-3} expression clones, we first amplified (Expand High-Fidelity Polymerase, Roche) the \textit{pgp-3} genomic interval (lacking start and stop codons and containing introns) from wild-type genomic DNA with primers containing attB1 and attB2 Gateway recombination sites. The resulting PCR product was recombined with pDONR221 to generate pENTRY-\textit{pgp-3}. pENTRY-\textit{pgp-3} and \textit{-ppg-3\(^ {AF508}\)} were created using a PCR fusion strategy using pENTRY-\textit{ppg-3} as the template. Briefly, a 5’ fragment, in which the 3’ primer (OG496 or OG501) replaced amino acids 476-484 of \textit{ppg-3} with amino acids 501-509 of human CFTR (with or without F508) was amplified from pENTRY-\textit{ppg-3}. A second 3’ overlapping fragment was amplified from pENTRY-\textit{ppg-3}. The 5’ and 3’ PCR products were then fused using primers flanking \textit{Agel} and \textit{KpnI} sites in \textit{ppg-3}. The fusion PCR product was digested with \textit{Agel} and \textit{KpnI} and used to replace the \textit{Agel-KpnI} fragment in pENTRY-\textit{ppg-3}. The N-terminal \textit{3\times\text{FLAG}} tag was added to pENTRY-\textit{ppg-3\(^ {wt}\)} and pENTRY-\textit{ppg-3\(^ {AF508}\)}. The full-length transgene was then inserted into the \textit{pMY26-2} backbone to generate pENTRY-\textit{ppg-3\(^ {wt}\)} or pENTRY-\textit{ppg-3\(^ {AF508}\)}.
pgp-3ΔF508 using a PCR fusion approach. A 5’ fragment that flanked the ApaI site and that contained the 3’ FLAG sequence (DYKDDDDKDYKDDDDK) was fused with a 3’ fragment that flanked an AscI site. The fusion product was digested with ApaI and AscI and ligated into either pENTRY-pgp-3wt or pENTRY-pgp-3ΔF508 to generate pENTRY-3×FLAG-pgp-3wt or pENTRY-3×FLAG-pgp-3ΔF508, respectively. The mCherry-unc-54 3’ UTR was amplified from MB14-mCherry with primers containing attB2r and attB3, then recombined with pDONOR P2r2. MB14-mCherry was created by replacing the GFP region of MB14 (kind gift of Denis Dupuy, Institut Européen de Chimie et Biologie, France) with mCherry cDNA. The 3×FLAG sequence and the human CFTR sequences were codon-optimized for expression in C. elegans (Duret and Mouchiroud, 1999). PCR-induced mutations were validated using site-directed mutagenesis and the sequence of all final entry clones was verified by DNA sequencing.

Expression clones for transgene construction (see below) were generated using Multisite Gateway technology. The promoter clone pENTRY-pha-6p, the relevant pENTRY-pgp-3 clone, and the pENTRY-mcherry-unc-54 3’ UTR were recombined in a Gateway LR reaction with pCFJ150 (Frokjaer-Jensen et al., 2008). The resulting expression clones were validated by restriction digest analysis and DNA sequencing.

Mos-mediated single-copy transgenes were created as previously described, utilizing the ttTi5605 Mos site on chromosome II and the direct-insertion screening method (Frokjaer-Jensen et al., 2008). Approximately 40 P0 young adults from the strain EG4322 unc-119(ed3); ttTi5605 were injected with each pcpp-3 expression construct and the standard concentrations of co-injection markers (Frokjaer-Jensen et al., 2008). After 12-14 days, animals that exhibited normal motility but lacked the mCherry co-injection markers were isolated and used for the insertion and long-range PCR (Long-Amp system, New England Biolabs) was performed across the genomic interval using purified genomic DNA and primers outside of the recombination interval. Products of the correct size were further analyzed by restriction digest analysis.

Quantitative PCR
Quantitative PCR was carried out as described previously (Rohlfing et al., 2010; Rohlfing et al., 2011). Primers spanning the 3’ end of the pcpp-3 gene and the 5’ end of the mCherry gene were utilized to specifically amplify the pcpp-3-mCherry transgene mRNA. Samples were normalized against expression levels for the actin gene act-2. The sequences of qPCR primers are available upon request.

RNAi
RNAi feeding clones were obtained either from the Ahringer library or the ORFeome RNAi collection (Open Biosystems). RNAi bacteria were cultured as previously described (Lamitina, 2006) and seeded onto NGM plates containing 1 mM IPTG. Bacteria were allowed to induce dsRNA for at least 24 hours. For gene knockdowns that were not lethal, hypochlorite-synchronized L1 stage animals were placed onto the RNAi bacteria and were selected for imaging ~24 hours post-L4 stage. For gene knockdowns that produced larval lethality (sec-23, sec-24.1, cdc-48.1, cdc-48.2 and rpt-5), animals were grown on control plates until the L4 stage then transferred to plates containing RNAi bacteria, where they were cultured for 24 hours before imaging.

Protein preparation and western blot analysis
Synchronized young adult worms grown at 20°C on NGM OP50 plates were washed three times with M9 and immediately frozen in liquid nitrogen. Protein extraction was performed by douncing in lysis buffer [100 mM Tris-Cl, pH 8.0; 50 mM NaCl; 100 mM sucrose; 1 mM EDTA and Protease inhibitor cocktail (Roche)]. The resulting worm lysate was centrifuged at 5800 g for 10 minutes at 4°C to generate a low speed pellet and low speed supernatant. The low speed supernatant was then centrifuged at 100,000 g for 1 hour at 4°C to generate a high speed supernatant and high speed pellet. The high speed supernatant (cytoplasm) was collected and the high speed pellet (membranes) was resuspended with Tris buffer containing 0.1% Triton X-100. The protein concentration of each sample was determined using the BCA method (Pierce) and identical amounts of protein were loaded for western assays. The difference between PGP-3wt and PGP-3ΔF508 could be observed in crude worm lysates, low-speed supernatants and high-speed pellets (data not shown). Data in all figures show the high speed pellet fraction, because this fraction gave the most consistently even gel loading based on total protein levels and loading controls. The 3×FLAG epitope was detected using anti-3×FLAG (Sigma Chemical Co., M2 clone). The mCherry epitope was detected using an anti-mCherry (Clontech) antibody. Anti-VHA-5 (Vacuolar H ATPase) and anti-HSP-90 were used as loading controls for membranes and cytosol, respectively. Secondary antibodies used were anti-mouse HRP (as above) and anti-rabbit HRP (1:5882 dilution; Amersham). Bands were visualized with a Thermo Scientific chemiluminescent substrate detection system (Prod. #34080).

Microscopy
Young adult animals (24 hours post-L4 stage, unless otherwise indicated) were anesthetized with 10 mM levamisole, mounted on a 2% agarose pad and fluorescently imaged with an inverted wide-field microscope (Leica DMi4000). For deconvolved images, a Z-stack through the intestine, centered on the intestinal lumen, was acquired in 0.2 μm steps using a 1.40 NA Plan Apo 63× lens. Deconvolution was implemented through Leica AF6000 software and utilized the nearest-neighbor method and five to ten iterations. For image quantification, only raw, non-deconvolved images were utilized. In order to reduce the effects of sample photobleaching, the focal plane was first centered on the lumen of the intestine containing the apical membrane using DIC. Samples were then immediately imaged in the mCherry channel at the same single focal plane. For all images within an experiment, the exposure settings were identical between pcpp-3wt and pcpp-3ΔF508 animals.

Quantification
Leica AF6000 software was used to measure mCherry fluorescence of images. Apical membrane fluorescence was determined by measuring the average pixel intensity along the length of the apical membrane of worms imaged in the mCherry channel, using the linear quantification feature of the software. For each image, a line
Modeling membrane protein misfolding in *C. elegans*

### Clinical issue
Cystic fibrosis (CF) is a genetically inherited disease that disrupts epithelial cell function. Most cases of CF are caused by the deletion of a single amino acid (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is a CAMP-regulated Cl- channel and member of the ABC transporter gene family. The ΔF508 mutation disrupts the ability of CFTR to fold in the endoplasmic reticulum (ER). As a result, CFTRΔF508 fails to traffic to the apical membrane of epithelial cells, which impairs transcellular salt and water movement in absorptive and secretory epithelial tissues. Certain manipulations (such as osmotic adaptation, inhibition of ER quality control pathways) allow CFTRΔF508 to reach the membrane, where it can function as a Cl- channel and promote salt and water movement. Although several animal models of CFTRΔF508 misfolding (including mice, pigs and ferrets) exist, these systems do not permit rapid genetic dissection of the pathways contributing to ΔF508-dependent pathophysiology.

### Results
To complement studies of mammalian CFTRΔF508, the authors developed a fluorescence-based *C. elegans* model of ΔF508-dependent protein destabilization. Although worms do not express CFTR, they do express closely related ABC transporters called P-glycoproteins. Introduction of the ΔF508 mutation into the *C. elegans* P-glycoprotein PGP-3 post-transcriptionally destabilized the protein and inhibited its trafficking to the apical membrane of epithelial cells. Similar to mammalian cells, activation of the osmotic stress response or inhibition of *C. elegans* ER quality control proteins stabilized PGP-3ΔF508 and increased the levels of protein at the apical plasma membrane.

### Implications and future directions
This model will allow, for the first time, the use of unbiased forward and reverse genetic approaches in a live-animal setting to identify regulators of ΔF508-dependent protein destabilization. Such regulators can be easily identified in live animals as mutants that increase the abundance and/or apical membrane localization of the PGP-3ΔF508 protein, as measured by fluorescence. Homologs of these genes in humans could represent new drug targets for the treatment of CF. Additionally, polymorphisms in these genes could influence the timing and/or severity of CF disease in humans.

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