University of Dundee

Functional Rescue of a Misfolded Eukaryotic ATP-binding Cassette Transporter by Domain Replacement

Louie, Raymond J.; Pagant, Silvere; Youn, Ji-Young Youn; Halliday, John J.; Huyer, Gregory; Michaelis, Susan

Functional Rescue of a Misfolded Eukaryotic ATP-binding Cassette Transporter by Domain Replacement*[S]

Raymond J. Louie[‡], Silvere Pagant[‡], Ji-Young Youn[‡], John J. Halliday[‡], Gregory Huyer[§], Susan Michaelis[§], and Elizabeth A. Miller[‡1]

From the [‡]Department of Biological Sciences, Columbia University, New York, New York 10027 and the [§]Department of Cell Biology and Anatomy, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

ATP-binding cassette (ABC) transporters are integral membrane proteins that couple ATP binding/hydrolysis with the transport of hydrophilic substrates across lipid barriers. Deletion of Phe-670 in the first nucleotide-binding domain (NBD1) of the yeast ABC transporter, Yor1p, perturbs interdomain associations, reduces functionality, and hinders proper transport to the plasma membrane. Functionality of Yor1p-ΔF was restored upon co-expression of a peptide containing wild-type NBD1. To gain insight into the biogenesis of this important class of proteins, we defined the requirements for this rescue. We show that a misfolding lesion in NBD1 of the full-length protein is a prerequisite for functional rescue by exogenous NBD1, which is mediated by physical replacement of the dysfunctional domain by the soluble NBD1. This association does not restore trafficking of Yor1p-ΔF but instead confers catalytic activity to the small population of Yor1p-ΔF that escapes to the plasma membrane. An important coupling between the exogenous NBD1 and ICL4 within full-length aberrant Yor1p-ΔF is required for functional rescue but not for the physical interaction between the two polypeptides. Together, our genetic and biochemical data reveal that it is possible to modulate activity of ABC transporters by physically replacing dysfunctional domains.

ATP-binding cassette (ABC)[2] transporters are integral membrane proteins that couple ATP binding/hydrolysis with the transport of hydrophilic substrates across lipid barriers. Although the substrates of ABC transporters are extremely diverse (peptides, drugs, vitamins, and ions), the global protein architecture is highly conserved. From bacteria to humans, ABC transporters consist of two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs). The MSDs form a substrate translocation pore, and the two NBDs form ATP binding and hydrolysis pockets. Intracellular loops (ICLs), which are cytoplasmic extensions of the individual helical regions of the MSDs, contain coupling helices that form contact points between the MSDs and NBDs (1–3). Interestingly, the crystal structure of the prokaryotic ABC transporter, Sav1866, revealed that ICL1 and ICL2, originating from MSD1, contact NBD2, whereas ICLs originating from MSD2 contact NBD1. This quaternary organization, known as "domain swapping," is also present in the eukaryotic transporters P-glycoprotein, the cystic fibrosis transmembrane conductance regulator (CFTR), and yeast oligomycin resistance protein (Yor1p) (4–7). Despite the high degree of tertiary structure conservation and ubiquity of ABC transporters across kingdoms, these herculean proteins have very diverse responsibilities. The protein responsible for conferring yeast resistance to the mitochondrial poison oligomycin, Yor1p, is an exporting type ABC transporter that localizes to the plasma membrane and clears the cytosol of a variety of toxins (4, 8–12).

Like many eukaryotic ABC transporters (13–16), Yor1p contains all four of its domains on a single polypeptide. However, ABC transporters can also be expressed as half-molecules, where individual proteins contain one MSD fused to one NBD, at either the N or C terminus. TAP1 and TAP2 are mammalian proteins that heterodimerize to form a functional ABC transporter that moves peptides into the lumen of the endoplasmic reticulum (ER) during antigen presentation (17, 18). MsbA, a lipid flippase from *Escherichia coli*, also forms from homodimerization of two half-transporters, which coordinate to move essential lipids from the inner membrane to the outer membrane (19, 20). Further division of ABC transporter subunits occurs in prokaryotes where individual MSDs and NBDs can be encoded on separate genes. For ABC transporters that are composed of separate polypeptides, it is thought that the subunits physically associate to form functional transporters structurally equivalent to full transporters encoded on a single polypeptide (21). In each case, multiple critical interdomain interfaces must form to create a fully functional whole transporter.

The biogenesis of ABC transporters is of particular interest because of its relevance to human health: ABC transporters are involved in drug transport, ion homeostasis, and numerous other physiologically important functions. The predominant disease-causing allele of cystic fibrosis is a deletion of Phe-508 in the NBD1 domain of CFTR (22, 23). Biochemical studies have revealed that CFTR-ΔF508 is unable to make multiple native interdomain contacts; the ΔF mutation simultaneously disrupts the NBD1/ICL3, NBD1/ICL4, and NBD1/NBD2 inter-

---

TABLE 1
S. cerevisiae strains used in this study

Strain	Genotype	Source
BY4741	MATa *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	Open Biosystems (Huntsville, AL)
LMY094	MATa *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, yor1::KANMX*	Open Biosystems
SM5224	MATa *his3Δ1, leu2Δ0,met15Δ0, ura3Δ0, yor1::yor1ΔF670-HA-GFP*	This study
LMY901	MATa *his3Δ1, leu2Δ0, met15Δ0,ura3Δ0, yor1::yor1ΔF670-TAP::KANMX*	This study

faces (5). The inability to achieve native quaternary structure results in mislocalization, ubiquitin-dependent degradation by ER-associated degradation (ERAD), and consequently a loss of chloride ion transport across the apical membrane of epithelial cells (24, 25). A number of studies on CFTR and the yeast a-factor pheromone transporter Ste6p have explored the ability to modulate the biogenesis of ER-retained ABC transporters by co-expressing various fragments that function either to replace a defective domain or to sequester the quality control machinery that targets the defective protein for degradation (26–32). This raises the prospect of modulation of ABC transporter biogenesis through expression of extra copies of specific domains.

We have exploited the power of yeast genetics and biochemistry to further define the mechanism by which an exogenous domain is able to rescue biogenesis of an aberrant ABC transporter using a misfolded allele of the yeast oligomycin resistance protein, Yor1p. Deletion of a critical Phe residue (Phe-670) in the first NBD of Yor1p causes protein misfolding, ER retention, and degradation via the ubiquitin-proteasome pathway (8, 10, 11). The absence of functional ΔF670 at the plasma membrane causes sensitivity to the mitochondrial toxin oligomycin. We demonstrate that co-expression of wild-type NBD1 rescues functionality of Yor1p-ΔF and a second processing mutant in NBD1, whereas processing mutations located in other domains were not rescued. Exogenous NBD1 physically associates with full-length Yor1p-ΔF, likely substituting for the misfolded domain. This domain replacement restores the essential interaction between NBD1 and intracellular loop 4 (ICL4) and is dependent on the presence of a mutation that impairs interdomain contacts. Finally, we demonstrate that association with NBD1 does not dramatically rescue trafficking of Yor1p-ΔF but likely confers partial function to the small population of Yor1p-ΔF that escapes to the plasma membrane.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. To create a TAP-tagged form of Yor1p-ΔF, the chromosomal copy of *YOR1* was first replaced with *yor1-ΔF-HA-GFP* by a pop-in/pop-out strategy. Briefly, the integrating plasmid pSSM2058 was linearized with MluI and transformed into yeast, which created a strain containing the *URA3* marker sandwiched between wild-type *YOR1* and the *yor1-ΔF-HA-GFP* allele. Transformants were then transferred to 5-fluoroorotic acid plates to select for strains that had undergone recombination to "pop out" one of the *YOR1* genes along with the flanking *URA3* gene. Strain SM5224, containing integrated *yor1-ΔF-HA-GFP*, was verified by analytical PCR, oligomycin sensitivity, and ER localization. The TAP fusion (protein A-derived ZZ domain, TEV protease cleavage site, and an S tag) was introduced by generating a PCR fragment derived from the plasmid, pKW804 (a gift from David

Drubin, University of California, Berkeley), and containing homology to the 3′-UTR of *YOR1*, which was transformed into SM5224 to generate LMY901, a strain expressing Yor1p-ΔF-TAP, marked with KanMX. Correct integration of the tag was checked by PCR and immunoblotting.

Cultures were grown at 30 °C in standard rich medium (1% yeast extract, 2% peptone, and 2% glucose) or synthetic complete dextrose medium (0.67% yeast nitrogen base, 2% glucose, and essential amino acids mix). For testing growth on oligomycin, saturated overnight cultures were diluted 10-fold and spotted onto YPEG plates (1% yeast extract, 2% peptone, 3% ethanol, and 3% glycerol) that were supplemented with 0.2 μg/ml oligomycin (Sigma-Aldrich) and 0.1% galactose as required. For TAP purification and *in vitro* vesicle budding, the cells were grown in synthetic complete raffinose medium (0.67% yeast nitrogen base, 2% raffinose, and essential amino acids mix) supplemented with 0.1% galactose. For counterselection of URA^+ strains, the cells were grown on synthetic complete dextrose medium supplemented with a final concentration of 0.1% of 5-fluoroorotic acid.

Plasmids—Plasmids used in this study are listed in Table 2. pSM2058 contains the *yor1-ΔF-HA-GFP* gene, flanked by 697 base pairs of the endogenous 5′-UTR and 512 base pairs of the 3′-UTR, cloned into the XmaI and SacI sites of the integrating plasmid, pSM170. The HA-GFP epitope tag was cloned into pSM2058 via a PCR-introduced NotI site (which introduced three Ala residues) prior to the *yor1-ΔF* stop codon. pEAE83 containing Yor1p-HA in pRS316 was a gift from Scott Moye-Rowley (University of Iowa). This plasmid was the basis for site-directed mutagenesis (33) to obtain several HA-tagged mutants. Overexpression of NBD1 of *YOR1* (LMB203) was achieved by cloning the sequence corresponding to amino acids 532–820 into the BamHI/HindIII site of p425gal (34). The overexpression construct of NBD1-ΔF was created by site-directed mutagenesis to create LMB204. Myc-tagged NBD was created using *in vivo* homologous recombination; a PCR fragment containing the Myc epitope tag, amplified from pYM4, with additional flanking homology to the 3′ end of the NBD1 gene, was co-transformed with LMB203 or LMB204 to create NBD1-Myc (pRL177) and NBD1-ΔF-Myc (pRL178), respectively. To create pRL138, the *yor1-ΔF670* allele, including 500 bp of upstream and 200 bp of downstream DNA, was cloned into the BamHI site of pRS426. To create plasmid-borne Yor1p-ΔF-TAP constructs, the TAP sequence from pKW804 was cloned into pRL138 to create pRL210. Using pRL210 as template, site-directed mutagenesis was used to introduce the I1084P mutation and create the plasmid pRL211 (*yor1-ΔF/I1084P-TAP*).

Protein Purification—Tandem affinity purification of Yor1p-ΔF and its associated partners was performed with IgG-

TABLE 2
Plasmids used in this study

| Plasmid	Genotype	Source
pSM2058	5′-UTR *yor1-ΔF670*-HA-GFP 3′-UTR in pSM170	This study
pEAE83	HA-tagged *YOR1* in pRS316	Katzmann *et al.* (11)
pEAE93	GFP-tagged *YOR1* in pRS316	Katzmann *et al.* (11)
pRS315 pdr1–3	*pdr1–3* in pRS315	Carvajal *et al.* (48)
pLM309	ΔF670 mutation in pEAE83 (*yor1-ΔF*)	Pagant *et al.* (10)
p425GAL1	pRS425 vector with *GAL1* promoter and *CYC* terminator	Mumberg *et al.* (34)
LMB203	NBD1 cloned into p425GAL1	This study
LMB204	ΔF670 mutation in LMB203 (*nbd1-ΔF670*)	This study
pKW804	TAP tag (S-TEV-ZZ) KanMX	David Drubin (gift)
pYM4	3Myc Tag KanMX	Knop *et al.* (49)
pJH1	K627A mutation in pEAE83 (*yor1-K627A*)	This study
spQC035	G278R mutation in pEAE83 (*yor1-G278R*)	S. Pagant (unpublished observations)
spQC036	R387G mutation in pEAE83 (*yor1-R387G*)	Pagant *et al.* (4)
spQC039	I1084P mutation in pEAE831 (*yor1-I1084P*)	S. Pagant (unpublished observations)
pRS426	2μ URA vector	Christianson *et al.* (38)
pRL138	*yor1-ΔF670* cloned into BamHI site of pRS426	This study
pRL140	K627A mutation in LMB203 (*nbd1-K627A*)	This study
pRL177	LMB203 tagged with Myc (*NBD1-Myc*)	This study
pRL178	ΔF670 mutation in pRL177 (*nbd1-ΔF-Myc*)	This study
pRL179	D71E73A mutation in pLM309 (*yor1-ΔF D71E73A*)	This study
pRL180	K627A mutation in pRL177 (*nbd1-K627A-Myc*)	This study
pRL182	L631P mutation in pEAE83 (*yor1-L631P*)	This study
pRL203	L631P mutation in pLM309 (*yor1-ΔF L631P*)	This study
pRL204	I1084P mutation in pLM309 (*yor1-ΔF I1084P*)	This study
pRL210	TAP fusion in pRL138 (*yor1-ΔF-TAP*)	This study
pRL211	I1084P mutation in pRL210 (*Yor1-ΔF I1084P-TAP*)	This study
pLM037	ΔF670 mutation in pEAE93 (*yor1-ΔF-GFP*)	Pagant *et al.* (4)
pRL205	L631P mutation in pEAE93 (*yor1-L631P-GFP*)	This study

Sepharose affinity purification followed by S protein-agarose affinity purification (35). TAP-tagged strains were grown overnight in synthetic complete raffinose/gal medium to an A_{600} of 0.1–0.9 (mid-log phase), and equal numbers of cells were harvested (2000 $\times$ *g* for 10 min). Using standard techniques, the cells were converted to spheroplasts, resuspended to 100 A_{600} cell equivalents/ml of lysis buffer (20 mM HEPES, pH 7.0, 150 mM KCl, 250 mM sorbitol, 2% *n*-dodecyl β-D-maltoside, and 1× protease inhibitor mixture), lysed by homogenizing 15 times with a Dounce tissue homogenizer, followed by incubation on ice for 30 min. All of the subsequent steps were carried out at 4 °C. Unbroken cells were removed by gentle centrifugation (8000 $\times$ *g* for 2 min) followed by a high speed spin to remove insoluble material (17,000 $\times$ *g* for 20 min). The supernatant was precleared by incubation with CL-6B-Sepharose pre-equilibrated in lysis buffer (Sigma). 10 μl of cleared lysate was removed and set aside as the input. The cleared lysate was incubated with prewashed IgG-Sepharose (GE Healthcare; 500 μl of slurry/1000 A_{600} cell equivalents) for 3 h. IgG-Sepharose-bound proteins were washed several times with lysis buffer and incubated overnight in a small volume of lysis buffer plus 1 unit of AcTEV protease (Invitrogen)/100 A_{600} cell equivalents. 1 ml of lysis buffer was added, and the eluate was transferred to prewashed S protein-agarose beads (Novagen; 60 μl of slurry/1000 A_{600} cell equivalents) and incubated for 3 h. S protein-agarose-bound proteins and 10% of the input fractions were detergent-solubilized, separated by SDS-PAGE, and analyzed by immunoblotting. COPII proteins Sar1p, Sec23p/24p, and Sec13/31p were prepared as described previously (36).

In Vitro Vesicle Budding—Radiolabeled semi-intact cells were prepared as described (37). Briefly, the cells were grown to mid-log phase, and five A_{600} cells were harvested, washed, and resuspended in 1.25 ml of starvation medium (synthetic complete raffinose medium with 0.1% galactose medium lacking methionine). The cells were starved for 10 min at 30 °C before the addition of TRAN35S-Label mix (~70 μCi/A_{600} of cells; MP Biomedicals). The cells were labeled for 10 min at 30 °C and then metabolically killed and converted to spheroplasts. The cells were gently lysed by resuspension in lysis buffer (20 mM HEPES, pH 6.8, 0.7 M sorbitol, 150 mM KOAc, 2 mM Mg(OAc)$_2$, 0.5 mM EGTA, and 1× protease inhibitor mixture mix) and freezing at −80 °C overnight. The cell membranes were washed once with low acetate B88 (20 mM HEPES, pH 6.8, 0.7 M sorbitol, 50 mM KOAc, 5 mM Mg(OAc)$_2$, and 1× protease inhibitor mixture mix) and twice with B88 (20 mM HEPES, pH 6.8, 0.7 M sorbitol, 150 mM KOAc, 5 mM Mg(OAc)$_2$, and 1× protease inhibitor mixture mix). Semi-intact cells were then incubated with COPII proteins (10 μg/ml Sar1p, 10 μg/ml Sec23p/Sec24p, and 20 μg/ml Sec13p/Sec31p) in a final reaction that contained 2.5 A_{600} of cells in the presence of either 0.1 mM GTP supplemented with an ATP regeneration system (final concentration, 1 mM ATP, 50 μM GDP-mannose, 40 mM creatine phosphate, 200 μg/ml creatine phosphokinase) or 0.1 mM GDP. The reactions were incubated at room temperature for 30 min, then the newly formed vesicles were separated from the donor membranes by centrifugation (16,000 $\times$ *g* for 5 min), and proteins were solubilized in 1% SDS (final concentration) and diluted with immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, and 2 mM NaN$_3$). Cargo proteins were immunoprecipitated using monoclonal anti-HA antibodies, precoupled to protein G-Sepharose beads (GE Healthcare), or polyclonal antibodies against Sec22p or Gas1p (a gift from Randy Schekman), precoupled to protein A-Sepharose beads (GE Healthcare). Immune complexes were separated by SDS-PAGE and analyzed by phosphorimaging analysis using a Storm PhosphorImager (GE Healthcare). The proteins were quantified using ImageQuant software (GE Healthcare).
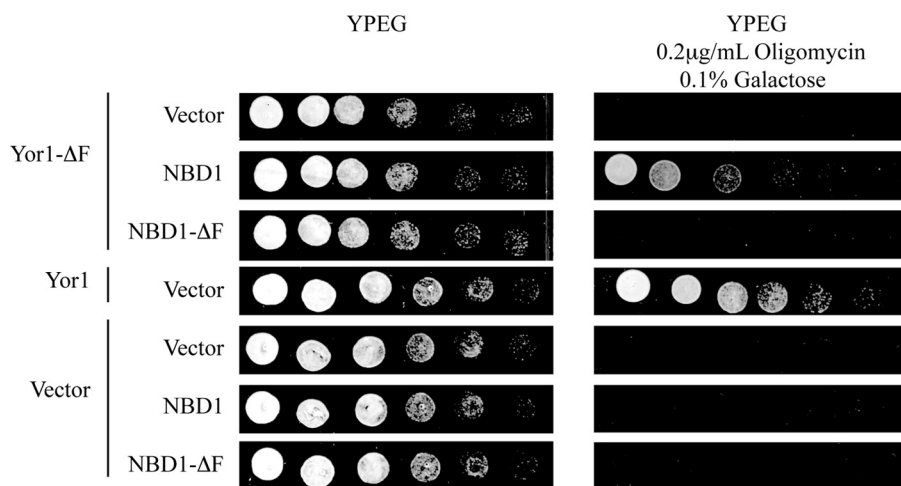
FIGURE 1. **The oligomycin sensitivity of strains expressing full-length *yor1-ΔF* is rescued by co-expression of NBD1.** 10-fold serial dilutions of *yor1Δ* yeast co-transformed with indicated plasmids were tested for viability on YPEG and YPEG supplemented with oligomycin and 0.1% galactose to induce overexpression of NBD1 constructs. Cells co-expressing wild-type *YOR1* and empty vector were resistant on oligomycin-containing medium, whereas cells containing *yor1-ΔF* and empty vector were inviable. The oligomycin-sensitive phenotype of cells expressing *yor1-ΔF* was partially rescued by the co-expression of NBD1 but not *nbd1-ΔF*. Expression of the NBD1 constructs alone did not result in oligomycin resistance.

Limited Proteolysis—Cells expressing HA-tagged forms of *YOR1* were grown to mid-log phase, and 10 A_{600} cells were harvested and converted to spheroplasts. Spheroplasts were washed twice with 20 mM HEPES, pH 7.4, resuspended in 100 μl of 20 mM HEPES, pH 7.4, and divided into four equal reactions. Each reaction was treated with a final concentration of 0, 25, 50, or 100 ng/μl trypsin (Sigma-Aldrich) for 10 min on ice. Digestion was terminated by the addition of 0.2 mg/ml (final concentration) soybean trypsin inhibitor (Sigma-Aldrich) to all reactions followed by incubation on ice for 15 min. The proteins were separated by SDS-PAGE and analyzed by immunoblotting using an anti-HA antibody (Covance).

Cross-linking—The cells expressing cysteine-substituted forms of Yor1p-HA were grown to mid-log phase, harvested, and converted to spheroplasts. Spheroplasts were washed twice with 20 mM HEPES, pH 7.4, and incubated with increasing concentrations of 3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate (Toronto Research Chemicals), prepared as 100× stock in dimethyl sulfoxide. The cells were cross-linked for 15 min at room temperature, then collected by centrifugation, and resuspended in 100 μl of 1% SDS prior to the addition of 50 μl of 3× sample buffer without reducing agent. The cells were disrupted by glass bead lysis (15 min, 4 °C) and heated to 55 °C for 5 min, and proteins were separated by nonreducing SDS-PAGE and analyzed by immunoblotting using an anti-HA antibody (Covance).

Pulse-Chase Analysis of Yor1p—The cells were grown to mid-log phase in complete synthetic medium; eight A_{600} cells were harvested, resuspended in fresh medium lacking Met/Cys, and incubated at 30 °C for 15 min while gently shaking. The proteins were labeled for 10 min by adding 30 μCi of TRAN35S-Label mix (MP Biomedicals)/A_{600} cells. A 10× chase solution (10 mM L-cysteine, 50 mM L-methionine, 4% yeast extract, and 2% glucose) was added, and two A_{600} aliquots of cells were harvested at each time point. Harvested cells were transferred to chilled tubes containing a final concentration of

20 mM sodium azide. The cells were washed once with 20 mM sodium azide and resuspended in 100 μl of 1% SDS. The cells were disrupted by glass bead lysis (15 min, 4 °C), heated to 55 °C for 5 min, diluted with 5 volumes of immunoprecipitation buffer (50 mM Tris, pH 7.5, 160 mM NaCl, 1% Triton X-100, and 2 mM sodium azide), and cleared by centrifugation. Yor1p and Gas1p were immunoprecipitated from the cleared lysate and analyzed by SDS-PAGE and phosphorimaging (as described above).

Live Cell Imaging—Strains co-expressing the constitutively active transcription factor, pdr1–3, and *yor1-ΔF-GFP*, *yor1-L631P-GFP*, or *YOR1-GFP* were grown in selective medium to mid-log phase, and images were taken on Olympus Fluoview FV500 laser-scanning confocal microscope (Olympus) with argon (488 mm), HeNe (543 nm), or HeNe-R633 beams and 100× objective with 2× zoom. The digital images were Kalman averaged from three scans. The images were analyzed using Adobe Photoshop (Adobe Systems).

RESULTS

Co-expression of NBD1, but Not nbd1-ΔF, Restores Function to Yor1p-ΔF—Using viability in the presence of oligomycin as a phenotypic measure of Yor1p function, we tested whether functionality could be restored to Yor1p-ΔF by co-expression of NBD1. The DNA sequence encoding the predicted NBD1 domain from *YOR1* was cloned into a 2μ plasmid under the control of the galactose-inducible *GAL1* promoter (34). This construct was co-transformed with a plasmid encoding the *yor1-ΔF* gene into a *yor1Δ* strain and tested for oligomycin resistance upon galactose-induced overexpression. Consistent with observations for CFTR (26, 27), overexpression of NBD1 rescued *yor1-ΔF* and restored partial oligomycin resistance, whereas cells co-expressing an empty vector remained inviable (Fig. 1). As a control, we tested whether the expression of NBD1 alone was sufficient to confer oligomycin resistance. In the absence of *yor1-ΔF*, the overexpression of NBD1 did not permit viability, which indicates that the partial resistance that is induced by the overexpression of NBD1 is dependent on *yor1-ΔF* (Fig. 1). To ensure that the functional rescue of Yor1p-ΔF by co-expression of NBD1 was through trans-complementation and not the result of recombination to create a wild-type allele, oligomycin resistance was tested in the presence of glucose, which represses the *GAL1* promoter. Under these conditions, cells co-expressing *yor1-ΔF* and NBD1 were no longer resistant on oligomycin (data not shown).
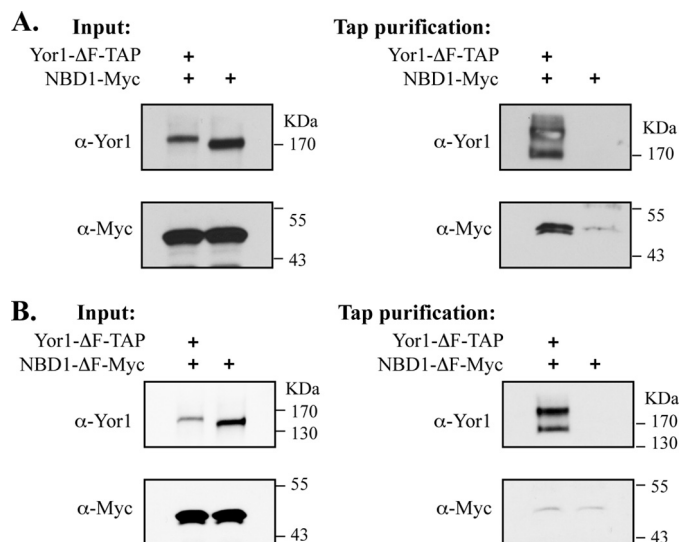
We next investigated whether rescue of the Yor1p-ΔF phenotype by co-expression of NBD1 was caused by sequestration of the quality control machinery or saturation of the ER-associated degradation (ERAD) pathway that would allow

Yor1p-ΔF to elude ER retention. This mode of rescue has been previously described for an analogous situation with misfolded CFTR truncations, whereby co-expression of misfolded CFTR fragments engaged the ER quality control and ERAD machinery and led to an increase in CFTR-ΔF processing (28, 29). If this mechanism operated in the rescue of Yor1p, then co-expression of *nbd1-ΔF* should also be proficient in, if not better at, conferring oligomycin resistance to a strain expression *yor1-ΔF*. However, co-expression of *nbd1-ΔF* was incapable of rescuing *yor1-ΔF* (Fig. 1). Together, these data indicate that the co-expression of NBD1 and not NBD1-ΔF is able to rescue

FIGURE 2. **NBD1, but not NBD1-ΔF, physically associates with Yor1p-ΔF.** Strains co-expressing *yor1-ΔF-TAP* or untagged *YOR1* in combination with the indicated mutants of NBD1-Myc were subjected to lysis and TAP affinity purification. A portion of lysate was removed for input analysis (*left panels*). Lysate and purified complexes were analyzed by SDS-PAGE, and immunoblotting with α-Yor1p and α-Myc polyclonal antibodies. Analysis of input proteins revealed comparable starting levels of NBD1-Myc, which did not co-purify with untagged Yor1p. *A*, NBD1-Myc co-purified with Yor1p-ΔF-TAP. *B*, NBD1-ΔF-Myc did not co-purify with Yor1p-ΔF-TAP.
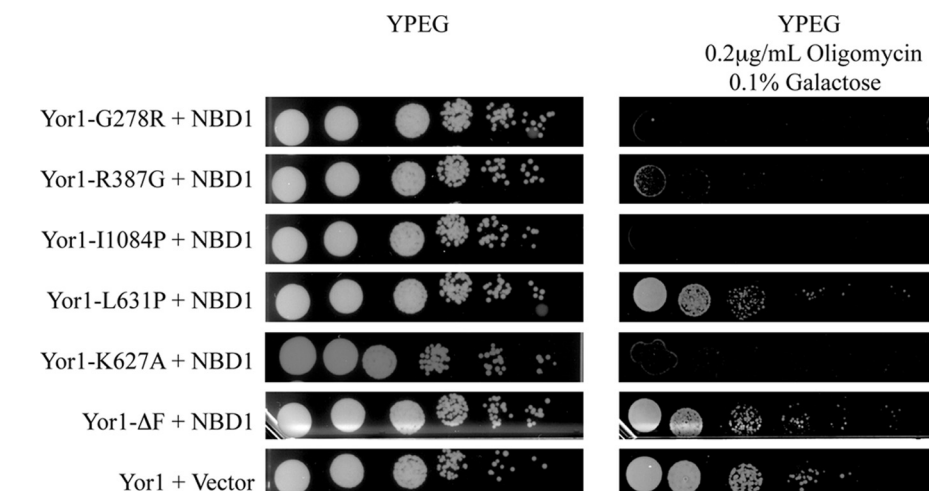
yor1-ΔF through a mechanism that is likely independent of protein quality control sequestration.

NBD1 Physically Associates with Yor1p-ΔF—Given that exogenous NBD1 did not rescue Yor1p-ΔF by saturating protein quality control, we investigated whether the extra NBD1 domain associates directly with Yor1p-ΔF. To test this hypothesis, a Myc tag was added to the NBD1 construct, which was then introduced into a strain of yeast expressing *yor1-ΔF* with a C-terminal TAP tag. Using the TAP tag, Yor1p-ΔF-TAP and its binding partners were purified, resolved by SDS-PAGE, and analyzed by immunoblotting. NBD1-Myc co-purified with Yor1p-ΔF-TAP but was not precipitated in a control reaction from an untagged strain (Fig. 2*A*). Additionally, NBD1-ΔF-Myc did not co-purify with Yor1p-ΔF-TAP, consistent with its inability to restore oligomycin resistance (Fig. 2*B*). Together these data suggest that the physical association between the exogenous NBD1 and Yor1p-ΔF is required for functional rescue.

Misfolded Alleles of yor1-nbd1 Can Be Rescued by Overexpression of NBD1—Because exogenous NBD1 physically associates with full-length Yor1p-ΔF (Fig. 2), we investigated whether the misfolded nature of Yor1p-ΔF was needed to accommodate the foreign NBD1. Similar to the effect of the ΔF lesion in NBD1, multiple mutations throughout Yor1p can perturb interdomain contacts. Specifically, mutations in ICL1 (*yor1-G278R*), ICL2 (*yor1-R387G*), and ICL4 (*yor1-I1084P*) have been shown to cause protein misfolding and oligomycin sensitivity (10). Co-expression of NBD1 did not rescue *yor1-G278R*, *yor1-R387G*, or *yor1-I1084P* (Fig. 3), suggesting that transcomplementation by the exogenous NBD1 is specific for lesions in NBD1. The Cystic Fibrosis Mutation Database was queried to identify another misfolding mutation located in NBD1. The disease-causing point mutation L468P was selected because of the severity of the substitution and its location remote from the defined ATP-binding sites. The homologous mutation in *YOR1* (*yor1-L631P*) was unable to complement a *yor1Δ* strain, and biochemical analysis revealed that Yor1p-L631P was misfolded, ER-retained, and rapidly degraded (supplemental Fig. S1). Like *yor1-ΔF*, co-expression of NBD1 rescued the oligomycin sensitivity of cells expressing *yor1-L631P* (Fig. 3). These data indicate that only alleles that induce a misfolding event in NBD1 of Yor1p permit transcomplementation by a second copy of NBD1. Consistent with this model, a properly folded but nonfunctional *yor1-NBD1* mutant was not functionally rescued by NBD1 co-expression (Fig. 3). A point mutation in the walker A motif of NBD1, K627A, does not cause protein misfolding, as indicated by protease resistance and native interdomain associations (supplemental Fig. S2), but renders cells oligomycin-sensitive through a
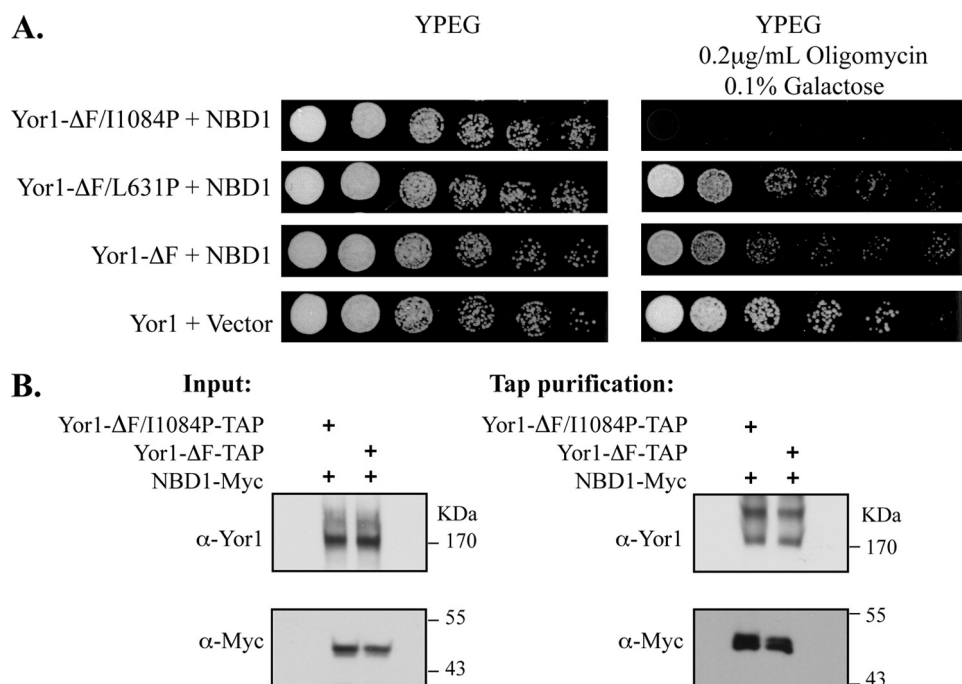

FIGURE 3. **Exogenous NBD1 can only transcomplement misfolded *yor1-nbd1* alleles.** 10-fold serial dilutions of *yor1Δ* yeast co-transformed with the indicated plasmids were tested for viability on YPEG and YPEG supplemented with oligomycin and 0.1% galactose. The co-expression of NBD1 was unable to rescue the oligomycin-sensitive phenotype of the *yor1-icl* mutants, *yor1-G278R*, *yor1-R387G*, or *yor1-I1084P*. Conversely, co-expression of NBD1 transcomplemented the misfolded *yor1-nbd1* allele, *yor1-L631P*, but not the properly folded *yor1-nbd1* allele, *yor1-K627A*.

A.

B.

FIGURE 4. **A functional NBD1/ICL4 interface is required for transcomplementation.** *A*, 10-fold serial dilutions of *yor1Δ* yeast co-transformed with indicated plasmids were tested for viability on YPEG and YPEG supplemented with oligomycin and 0.1% galactose. The cells expressing *yor1-ΔF/I1084P* were not rescued by co-expression of NBD1. The cells that expressed *yor1-ΔF/L631P* were rescued by co-expression of NBD1. *B*, cells co-transformed with plasmids containing NBD1-Myc and *yor1-ΔF-TAP* or *yor1-ΔF/I1084P-TAP* were affinity-purified using the TAP epitope tag, as described in the legend to Fig. 2. Comparable amounts of NBD1-Myc co-purified with Yor1p-ΔF/I1084P-TAP and Yor1p-ΔF-TAP.

loss of catalytic function (39). That cells expressing *yor1-K627A* were not rescued by NBD1 co-expression (Fig. 3) indicates that exogenous NBD1 can only transcomplement misfolded *yor1-nbd1* mutants.

A Functional NBD1/ICL4 Interface Is Required for Transcomplementation—If NBD1 functionally replaces a misfolded NBD1, then critical interdomain interfaces should be required for transcomplementation. We tested whether the interface between exogenous NBD1 and ICL4 of Yor1p-ΔF was required for transcomplementation. Like CFTR (5, 7) and P-glycoprotein (6), NBD1 of wild-type Yor1p interacts with ICL4, forming an interface that is disrupted by the point mutation I1084P.[3] If exogenous NBD1 uses the same interface as the native protein, then the I1084P mutation should prevent complementation. Indeed, the transcomplementation phenotype was lost when the I1084P mutation was combined with the ΔF mutation (Fig. 4A). Conversely, when the ΔF mutation was combined with a second mutation in NBD1 (L631P described above), the double mutant was still rescued by exogenous NBD1 (Fig. 4A), which suggests that additional destabilizing mutations within NBD1 of Yor1p-ΔF do not inhibit complementation by exogenous NBD1.

We anticipated that the mutation in ICL4 would abolish the physical association between exogenous NBD1 and the double mutant, Yor1p-ΔF/I1084P. We purified TAP-tagged forms of Yor1p expressed from episomal plasmids in strains also expressing NBD1-Myc. Surprisingly, we observed that NBD1-Myc still co-purified with Yor1p-ΔF/I1084P-TAP (Fig. 4B). It is

likely that disruption of the NBD1/ICL4 interface is not sufficient to abolish this association because other domain interactions are still preserved, including NBD1 with ICL3 and NBD2 (4). Instead, these data suggest that the NBD1/ICL4 interface is responsible primarily for proper functionality, likely through the coupling of ATP hydrolysis to transmembrane transport (1–3).

Rescue of Yor1p-ΔF by Exogenous NBD1 Likely Occurs at the Plasma Membrane—As a result of its aberrant quaternary structure, Yor1p-ΔF does not efficiently localize to the plasma membrane but instead is retained in the ER (8, 10, 11). Therefore, we investigated whether NBD1 association with Yor1p-ΔF restored intracellular trafficking. An *in vitro* vesicle formation assay, which recapitulates the biogenesis of ER-derived vesicles from radiolabeled permeabilized cells (37), was used to examine uptake of Yor1p-ΔF into transport vesicles in strains where NBD1 was co-expressed. Cellular membranes from [^{35}S]Met/Cys-labeled cells were incubated with purified ER vesicle coat proteins (COPII) in the presence of either GTP or GDP. The vesicles were subsequently isolated by differential centrifugation, and incorporation of Yor1p into the vesicle fraction was quantified by immunoprecipitation. Wild-type Yor1p was detected in the vesicle population in a GTP-dependant manner, whereas Yor1p-ΔF was not efficiently packaged, even when NBD1 was co-expressed (Fig. 5A), which indicates that the co-expression of NBD1 does not rescue oligomycin resistance by restoring trafficking of Yor1p-ΔF to the plasma membrane. Fig. 5B shows the quantification of the *in vitro* budding assay results.

Because co-expression of NBD1 did not reinstate trafficking of Yor1p-ΔF, we reasoned that the transcomplementation event must act on the small population of Yor1p-ΔF that escaped the ER to localize to the plasma membrane. A di-acidic ER-export signal is contained within the N-terminal cytoplasmic domain of Yor1p, which when substituted with Ala residues (D71A/E73A) abolishes the ability of Yor1p to engage the COPII cargo-binding protein, Sec24p, rendering Yor1p incapable of ER exit (10). We reasoned that this mutation would further enhance retention of Yor1p-ΔF in the ER, preventing any escape. Therefore, the di-acidic signal was mutated in the context of *yor1-ΔF*, co-expressed with NBD1, and tested for viability on oligomycin medium (Fig. 5C). The exclusively ER-retained double mutant, Yor1p-ΔF/D71A/E73A, was not rescued by NBD1 co-expression, which indicates that the transcomplementation phenotype is dependent on the fugitive population of Yor1p-ΔF that escapes ER quality control.

---

[3] S. Pagant, unpublished observations.
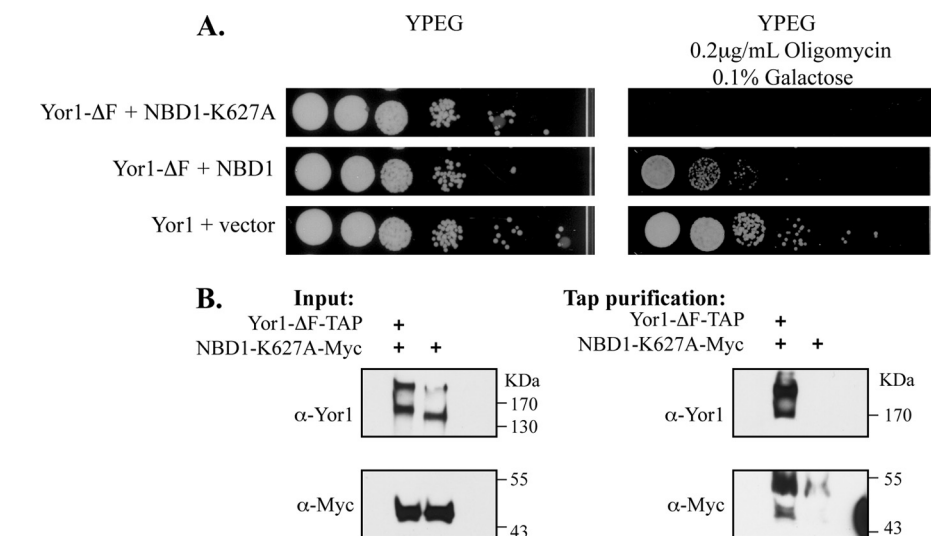
A

Yor1-HA   Yor1-ΔF-HA

B

C
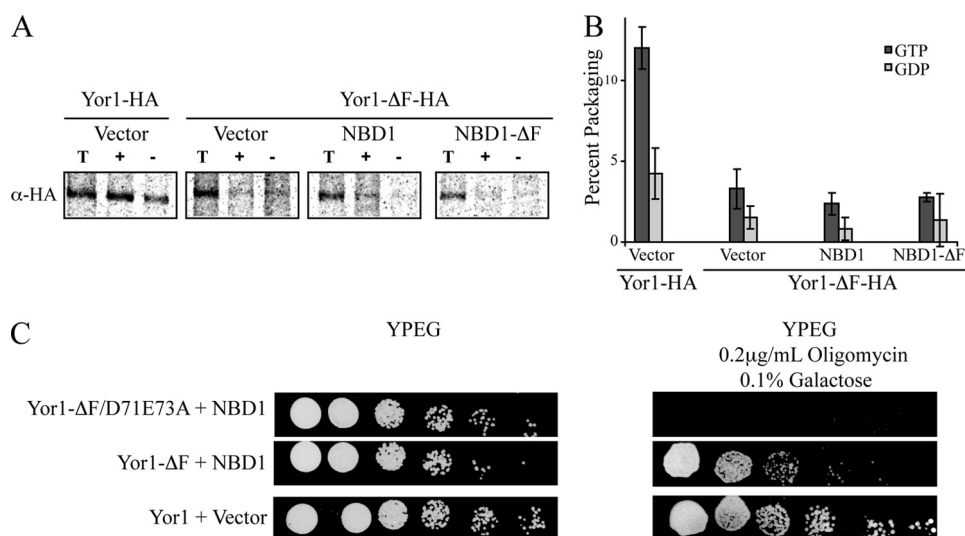
FIGURE 5. **Exogenous NBD1 does not affect trafficking of Yor1p-ΔF.** *A*, packaging of Yor1p-HA and Yor1p-ΔF-HA, co-expressed with the NBD1 constructs indicated, into COPII vesicles was examined by an *in vitro* vesicle budding assay. The cells were radiolabeled with [³⁵S]Met/Cys, permeabilized, and incubated with COPII proteins in the presence of either GTP(+) or GDP(−). Total membranes (*T*) and vesicle fractions were subjected to immunoprecipitation with HA antibodies, separated on SDS-PAGE, and analyzed by phosphorimaging. *B*, quantification of three independent *in vitro* budding experiments. The *error bars* represent standard deviations. *C*, 10-fold serial dilutions of *yor1Δ* yeast co-transformed with indicated plasmids were tested for viability on YPEG and YPEG supplemented with oligomycin and 0.1% galactose. The cells expressing *yor1-ΔF/D71E73A* were not rescued by co-expression of NBD1.

FIGURE 6. **Catalytic activity of exogenous NBD1 is required for transcomplementation.** *A*, 10-fold serial dilutions of *yor1Δ* yeast co-transformed with indicated plasmids were tested for viability on YPEG and YPEG supplemented with oligomycin and 0.1% galactose. Co-expression of *nbd1K627A* was unable to rescue *yor1-ΔF*. *B*, cells co-expressing *yor1-ΔF-TAP* or untagged wild-type *YOR1* and NBD1-K627A-Myc were affinity-purified against the TAP epitope tag, as described in the legend to Fig. 2. NBD1-K627A-Myc co-purified with Yor1p-ΔF-TAP.

Finally, if domain replacement of NBD1 restores functionality to Yor1p-ΔF, then the exogenous NBD1 should contribute to catalytic activity. We tested this by mutating the catalytic Lys residue in the Walker A site of the co-expressed NBD1. The cells were co-transformed with plasmids containing *yor1-ΔF* and *nbd1-K627A* and tested for growth on oligomycin medium. These cells were inviable on oligomycin medium (Fig. 6*A*), despite the ability of NBD1-K627A-Myc to associate with Yor1p-ΔF-TAP (Fig. 6*B*). Taken together, these data show that in addition to physically replacing the aberrant NBD1 domain of the full-length

Yor1p-ΔF, exogenous NBD1 functionally contributes essential catalytic activity to confer increased oligomycin resistance.

DISCUSSION

From bacteria to humans, the core domain architecture of ABC transporters is conserved: two MSDs and two NBDs, which can be encoded on one to four peptides, which assemble to form a functional translocation machine. In *S. cerevisiae*, a single polypeptide encodes for the ABC transporter responsible for yeast oligomycin resistance, Yor1p. Homologous to the common disease-causing allele of cystic fibrosis, deletion of Phe-670 in Yor1p causes protein misfolding, ER retention, and degradation via the ubiquitin-proteasome pathway (8, 10, 11). The present findings indicate that it is possible to restore functionality to Yor1p-ΔF through a domain replacement strategy. Fig. 7 illustrates our model of Yor1p/NBD1 transcomplementation. Full-length WT Yor1p forms numerous native interdomain contacts, traffics to the plasma membrane, and is a competent drug efflux transporter. The native quaternary structure of the catalytically nonfunctional Yor1p-K627A mutant, where interdomain contacts are formed correctly, does not permit incorporation of NBD1 and therefore does not afford rescue by co-expression of NBD1 (Fig. 7*A*). Misfolding mutations within NBD1 perturb these interdomain contacts, resulting in an aberrant assembly that permits some of the exogenous NBD1 to engage appropriate interdomain interactions. This domain substitution does not rescue trafficking but instead restores functionality to the small amount of protein that escapes ER quality control (Fig. 7*B*). The replacing NBD requires catalytic function because intact ATP binding pockets are also required for rescue (Fig. 6). In contrast, the co-expression of NBD1-ΔF does not rescue Yor1p-ΔF because it is unable to physically associate with the full-length protein (Fig. 7*C*), likely suffering from the same impaired surfaces that preclude interdomain interactions in the context of the full-length protein.

This mode of rescue is distinct from one mechanism described for rescue of CFTR-ΔF in mammalian cells, where
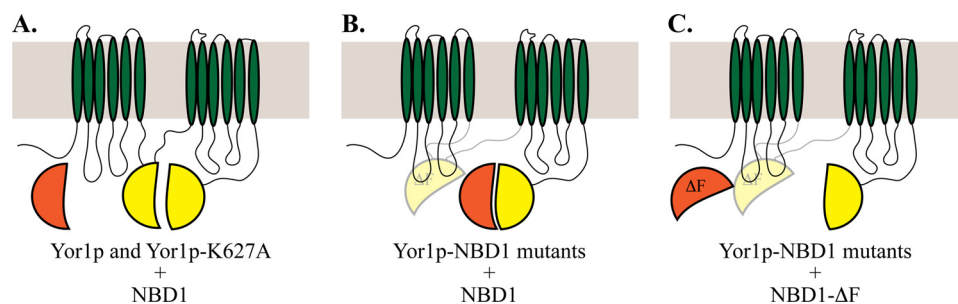
FIGURE 7. **Model of rescue by exogenous NBD1.** *A*, properly folded Yor1p and Yor1p-K627A are unable to incorporate exogenous NBD1. *B*, Yor1p-NBD1 mutants accommodate a second copy of NBD1 to restore functionality. The rescue event requires the catalytic activity of the exogenous NBD1. Communication between the second copy of NBD1 and ICL4 is required for phenotypic rescue but not physical association. *C*, co-expression of *nbd1-ΔF* does not physically associate with Yor1p-NBD1 mutants and is incapable of transcomplementation.

co-expression of aberrant or truncated CFTR fragments saturates ERAD or sequesters chaperones, restoring ion conductance through enhanced trafficking of CFTR-ΔF (28, 29). Conversely, expression of wild-type domains of CFTR can also afford rescue, likely through a mechanism of domain replacement similar to that of Yor1p-ΔF. The distinction between the two modes of rescue is important because the cellular consequences of inhibiting ER quality control are unknown, although prolonged accumulation of misfolded proteins can ultimately lead to cell death (40). Surprisingly, unlike CFTR-ΔF in mammalian cells, the inhibition of ERAD in yeast does not restore trafficking of Yor1p-ΔF (10). Consistent with this distinction, the co-expression of misfolded NBD1-ΔF, which should saturate protein quality control, failed to transcomplement Yor1p-ΔF (Fig. 1). There are two potential reasons for this. First, there may be additional layers of quality control in the yeast ER that are not present in the mammalian system. This higher stringency in yeast could be responsible for the retention of Yor1p-ΔF despite inhibition of ERAD. More likely is the second possibility: yeast and mammalian cells contain the same quality control components, but the difference lies in the severity of the ΔF mutation; the ΔF lesion in CFTR may be less detrimental than that of Yor1p. If the quaternary structure of CFTR-ΔF was sufficiently stable, then it could bypass additional layers of quality control and permit CFTR-ΔF to exit the ER upon inhibition of ERAD. The ability to increase the folding efficiency of CFTR-ΔF through reduced temperature and incubation with nonspecific osmolytes indicates that ΔF mutation in CFTR does not irreversibly impair the global protein structure (41–43). Conversely, the oligomycin-sensitive phenotype of cells expressing *yor1-ΔF* is not reproducibly rescued by reduced temperature and incubation with glycerol (data not shown), which indicates that the ΔF mutation may cause greater perturbations in the folding of Yor1p. Therefore, it is logical that the mechanism of rescue by exogenous NBD1 is not by enhanced trafficking but instead through increased functionality to the fugitive plasma membrane population of Yor1p-ΔF.

Although our data demonstrated that the second copy of NBD1 did not enhance trafficking of Yor1-ΔF, instead we infer that a small population of Yor1-ΔF is able to use its ER exit signal to traffic to the plasma membrane (Fig. 5). It is this fugitive population of Yor1p-ΔF that is rescued by NBD1. Wild-

type Yor1p employs a di-acidic ER export signal contained within the N-terminal cytoplasmic domain to bind the COPII cargo-binding protein, Sec24p, thereby promoting efficient trafficking out of the ER. In contrast, *in vitro* ER vesicle formation assays only detected marginal amounts of misfolded Yor1p-ΔF in COPII vesicles (10). It was previously unknown whether Yor1p-ΔF was capable of associating with Sec24p to traffic to the plasma membrane or whether Yor1p-ΔF exclusively exited the ER through a nonselective mechanism known as bulk flow, whereby stochastic sampling of the ER membrane during vesicle formation allows for nonspecific forward traffic of proteins. Our finding that mutation of the di-acidic motif abrogated the rescue of Yor1p-ΔF suggests that the minor population of Yor1p-ΔF that escapes ER quality control still associates with Sec24p, using the same export motif as wild-type Yor1p, to enter COPII vesicles.

This small population of Yor1p-ΔF, which managed to engage Sec24p and traffic to the plasma membrane, is competent for domain replacement because of its misfolded nature. The amount of protein that comprises this population is difficult to determine. Our attempts to measure the plasma membrane pool of Yor1p-ΔF either by subcellular fractionation or by surface biotinylation did not permit accurate quantification of this population. Therefore, we can only speculate on the precise subcellular location of the NBD1-mediated rescue of Yor1p-ΔF. The aberrant protein may be rescued during biogenesis, when initial interdomain contacts are established, or once the protein has reached the plasma membrane. The ability of ABC transporters to fold co-translationally has been demonstrated for CFTR (44) and inferred for P-glycoprotein (45). Achieving proper folding and assembly early in the biosynthetic pathway would likely preclude opportunities for domain associations with exogenous peptides. This is the probable reason that the properly folded but catalytically inactive *yor1-nbd1* allele, Yor1p-K627A (supplemental Fig. S2), is not rescued by co-expression of NBD1 (Fig. 3). Like CFTR and P-glycoprotein, Yor1p-K627A folding and assembly likely occurs co-translationally, therefore precluding the possibility for domain replacement by foreign NBD1, despite an obvious selective advantage. Thus, it is not surprising that this study identified a misfolding in Yor1p-NBD1 as an essential prerequisite for domain replacement by a second copy of NBD1 (Fig. 3).

It is not unexpected that exogenous NBD1 can associate with full-length Yor1p-ΔF, because a physical association between separately encoded domains of ABC transporters is a common feature of many prokaryotic ABC transporters. The two halves of the mammalian TAP transporter physically associate (46). More dramatically, all four domains of the heterotetrameric OppA transporter co-purify following immunoprecipitation of one MSD domain (47). Furthermore, N-terminal fragments (containing MSD1 and NBD1) of Ste6 and CFTR are able to physically associate with their full-length counterparts (30–

32), which indicates that despite encoding all domains on a single polypeptide, misfolded full-length ABC transporters are flexible enough to exclude dysfunctional domains in the context of relatively large polypeptides. Additionally, the ability of exogenous NBD1 to associate with Yor1p-ΔF at the plasma membrane is reminiscent of the functional rescue of misfolded alleles of *ste6-nbd1* that are localized to the plasma membrane and rescued by co-expression of a wild-type *STE6* fragment (30).

The physical association between exogenous NBD1 and Yor1p-ΔF is only one of several requirements for transcomplementation identified in this study. Critical interdomain associations, specifically between exogenous NBD1 and ICL4 of Yor1p-ΔF, are needed to restore functionality. Recent structural and biochemical studies have characterized various interdomain interactions that likely drive proper protein assembly and/or function. Work modeled on the crystal structure Sav1866 and interdomain cross-linking studies on CFTR (5) and P-glycoprotein (6) found that NBD1 of Yor1p contacts NBD2, ICL3, and ICL4 (4). The NBD/ICL interfaces are predicted to be involved in relaying confirmation changes in the NBDs to MSDs, thereby coupling ATP binding/hydrolysis with transmembrane transport. However, it is possible that some of the NBD/ICL interactions do not transmit conformational changes but rather act as scaffolds to promote proper interdomain associations. For example, the ICLs may aid in the formation of the critical ATP-binding sites by promoting proper assembly of NBD1 and NBD2. In this study we found that the addition of a misfolding mutation in ICL4 (I1084P) to Yor1p-ΔF suppressed the ability of exogenous NBD1 to transcomplement, despite retaining the physical association between the two polypeptides (Fig. 4*B*). It is likely that the functional NBD1 is held in place by interactions with ICL3 and NBD2, suggesting that the NBD1/ICL4 interface is most critical for functionality. It will be instructive to test the limits of domain replacement by co-expressing additional domains, including wild-type ICL4, and testing for rescue of oligomycin sensitivity. Such probes that could dissect the specific roles of the ICLs would facilitate dissection of the domains that drive quaternary assembly from those that are responsible for functional activity of ABC transporters.

The continued application of the genetic and biochemical approaches described here will increase our understanding of the biogenesis of this important class of proteins. Testing the limits of domain replacement will be necessary to fully elucidate how this approach can be used in modulating the functionality of clinically relevant ABC transporters.

REFERENCES

1. Biemans-Oldehinkel, E., Doeven, M. K., and Poolman, B. (2006) *FEBS Lett.* **580,** 1023–1035
2. Paumi, C. M., Chuk, M., Snider, J., Stagljar, I., and Michaelis, S. (2009) *Microbiol. Mol. Biol. Rev* **73,** 577–593
3. Seeger, M. A., and van Veen, H. W. (2009) *Biochim. Biophys. Acta* **1794,** 725–737
4. Pagant, S., Brovman, E. Y., Halliday, J. J., and Miller, E. A. (2008) *J. Biol. Chem.* **283,** 26444–26451
5. He, L., Aleksandrov, A. A., Serohijos, A. W., Hegedus, T., Aleksandrov, L. A., Cui, L., Dokholyan, N. V., and Riordan, J. R. (2008) *J. Biol. Chem.* **283,** 26383–26390
6. Zolnerciks, J. K., Wooding, C., and Linton, K. J. (2007) *FASEB J.* **21,** 3937–3948
7. Serohijos, A. W., Hegedus, T., Aleksandrov, A. A., He, L., Cui, L., Dokholyan, N. V., and Riordan, J. R. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 3256–3261
8. Katzmann, D. J., Hallstrom, T. C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Moye-Rowley, W. S. (1995) *Mol. Cell Biol.* **15,** 6875–6883
9. Epping, E. A., and Moye-Rowley, W. S. (2002) *J. Biol. Chem.* **277,** 34860–34869
10. Pagant, S., Kung, L., Dorrington, M., Lee, M. C., and Miller, E. A. (2007) *Mol. Biol. Cell* **18,** 3398–3413
11. Katzmann, D. J., Epping, E. A., and Moye-Rowley, W. S. (1999) *Mol. Cell Biol.* **19,** 2998–3009
12. Grigoras, I., Lazard, M., Plateau, P., and Blanquet, S. (2008) *Biochim. Biophys. Acta* **1778,** 68–78
13. Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M., and Tsui, L. C. (1989) *Science* **245,** 1073–1080
14. Bear, C. E., Li, C. H., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M., and Riordan, J. R. (1992) *Cell* **68,** 809–818
15. Shen, D. W., Fojo, A., Roninson, I. B., Chin, J. E., Soffir, R., Pastan, I., and Gottesman, M. M. (1986) *Mol. Cell Biol.* **6,** 4039–4045
16. Dano, K. (1973) *Biochim. Biophys. Acta* **323,** 466–483
17. Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D., and DeMars, R. (1990) *Nature* **348,** 744–747
18. Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., and Kelly, A. (1990) *Nature* **348,** 741–744
19. Karow, M., and Georgopoulos, C. (1993) *Mol. Microbiol* **7,** 69–79
20. Reyes, C. L., Ward, A., Yu, J., and Chang, G. (2006) *FEBS Lett.* **580,** 1042–1048
21. Panagiotidis, C. H., Reyes, M., Sievertsen, A., Boos, W., and Shuman, H. A. (1993) *J. Biol. Chem.* **268,** 23685–23696
22. Tsui, L. C. (1992) *Hum. Mutat.* **1,** 197–203
23. Amaral, M. D. (2004) *J. Mol. Neurosci* **23,** 41–48
24. Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R., and Smith, A. E. (1990) *Cell* **63,** 827–834
25. Zhang, F., Kartner, N., and Lukacs, G. L. (1998) *Nat. Struct. Biol* **5,** 180–183
26. Owsianik, G., Cao, L., and Nilius, B. (2003) *FEBS Lett.* **554,** 173–178
27. Clarke, L. L., Gawenis, L. R., Hwang, T. C., Walker, N. M., Gruis, D. B., and Price, E. M. (2004) *Am. J. Physiol. Cell Physiol* **287,** C192–C199
28. Cebotaru, L., Vij, N., Ciobanu, I., Wright, J., Flotte, T., and Guggino, W. B. (2008) *J. Biol. Chem.* **283,** 21926–21933
29. Sun, F., Mi, Z., Condliffe, S. B., Bertrand, C. A., Gong, X., Lu, X., Zhang, R., Latoche, J. D., Pilewski, J. M., Robbins, P. D., and Frizzell, R. A. (2008) *FASEB J.* **22,** 3255–3263
30. Berkower, C., Taglicht, D., and Michaelis, S. (1996) *J. Biol. Chem.* **271,** 22983–22989
31. Cormet-Boyaka, E., Jablonsky, M., Naren, A. P., Jackson, P. L., Muccio, D. D., and Kirk, K. L. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 8221–8226
32. Cormet-Boyaka, E., Hong, J. S., Berdiev, B. K., Fortenberry, J. A., Rennolds, J., Clancy, J. P., Benos, D. J., Boyaka, P. N., and Sorscher, E. J. (2009) *FASEB J.* **23,** 3743–3751
33. Zheng, L., Baumann, U., and Reymond, J. L. (2004) *Nucleic Acids Res.* **32,** e115
34. Mumberg, D., Müller, R., and Funk, M. (1994) *Nucleic Acids Res.* **22,** 5767–5768
35. Cheeseman, I. M., Brew, C., Wolyniak, M., Desai, A., Anderson, S., Muster, N., Yates, J. R., Huffaker, T. C., Drubin, D. G., and Barnes, G. (2001) *J. Cell Biol.* **155,** 1137–1145
36. Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994) *Cell* **77,** 895–907

37. Kuehn, M. J., Schekman, R., and Ljungdahl, P. O. (1996) *J. Cell Biol.* **135,** 585–595
38. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) *Gene* **110,** 119–122
39. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) *Trends Biochem. Sci.* **15,** 430–434
40. Wu, J., and Kaufman, R. J. (2006) *Cell Death Differ.* **13,** 374–384
41. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., and Chou, J. L. (1989) *Science* **245,** 1066–1073
42. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992) *Nature* **358,** 761–764
43. Wang, Y., Bartlett, M. C., Loo, T. W., and Clarke, D. M. (2006) *Mol. Pharmacol.* **70,** 297–302
44. Kleizen, B., van Vlijmen, T., de Jonge, H. R., and Braakman, I. (2005) *Mol. Cell* **20,** 277–287
45. Fung, K. L., and Gottesman, M. M. (2009) *Biochim. Biophys. Acta* **1794,** 860–871
46. Russ, G., Esquivel, F., Yewdell, J. W., Cresswell, P., Spies, T., and Bennink, J. R. (1995) *J. Biol. Chem.* **270,** 21312–21318
47. Doeven, M. K., Abele, R., Tampé, R., and Poolman, B. (2004) *J. Biol. Chem.* **279,** 32301–32307
48. Carvajal, E., van den Hazel, H. B., Cybularz-Kolaczkowska, A., Balzi, E., and Goffeau, A. (1997) *Mol. Gen. Genet.* **256,** 406–415
49. Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999) *Yeast* **15,** 963–972