Inhibiting Endoplasmic Reticulum (ER)-associated Degradation of Misfolded Yor1p Does Not Permit ER Export Despite the Presence of a Diacidic Sorting Signal[□]

Silvere Pagant,* Leslie Kung,* Mariana Dorrington, Marcus C.S. Lee,[†] and Elizabeth A. Miller

Department of Biological Sciences, Columbia University, New York, NY 10027-6902

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Capture of newly synthesized proteins into endoplasmic reticulum (ER)-derived coat protomer type II (COPII) vesicles represents a critical juncture in the quality control of protein biogenesis within the secretory pathway. The yeast ATP-binding cassette transporter Yor1p is a pleiotropic drug pump that shows homology to the human cystic fibrosis transmembrane conductance regulator (CFTR). Deletion of a phenylalanine residue in Yor1p, equivalent to the major disease-causing mutation in CFTR, causes ER retention and degradation via ER-associated degradation. We have examined the relationship between protein folding, ERAD and forward transport during Yor1p biogenesis. Uptake of Yor1p into COPII vesicles is mediated by an N-terminal diacidic signal that likely interacts with the “B-site” cargo-recognition domain on the COPII subunit, Sec24p. Yor1p-ΔF is subjected to complex ER quality control involving multiple cytoplasmic chaperones and degradative pathways. Stabilization of Yor1p-ΔF by inhibiting its degradation does not permit access of Yor1p-ΔF to COPII vesicles. We propose that the ER quality control checkpoint engages misfolded Yor1p even after it has been stabilized by inhibition of the degradative pathway.

INTRODUCTION

Eukaryotic cells maintain a strict policy of protein quality control to prevent the improper deployment of aberrant proteins within the cell, which may impair cellular function and ultimately result in proteotoxicity. This quality control checkpoint is of particular importance with respect to secretory proteins, which enter the endoplasmic reticulum (ER) as linear, unfolded polypeptides and generally must attain the correct quaternary structure before gaining transport competence to be delivered to downstream compartments (Ellgaard and Helenius, 2001; Bukau *et al.*, 2006). Misfolded and aberrant proteins are disposed of by ER-associated degradation (ERAD), which targets proteins for reverse translocation out of the ER, ubiquitination, and destruction by the cytoplasmic proteasome (Ahner and Brodsky, 2004). Thus, the ER plays a fundamental role in coordinating protein

synthesis, folding, degradation and transport to ensure accurate and efficient delivery of proteins to the compartments of the secretory pathway.

With recent progress in defining many of the components that participate in these processes, the complexity of these interrelated pathways is beginning to be understood. For example, ERAD is now recognized to be a multifaceted program that uses multiple machineries to degrade misfolded proteins with differently located lesions (Ahner and Brodsky, 2004; Ismail and Ng, 2006). Substrates within the ER lumen or ER membrane each have distinct topological requirements in terms of both lesion detection and protein extraction before the common endpoint of proteasomal degradation. Thus, rather than being a simple protein detection and degradation service, ERAD is attuned to dispose of proteins from different environments. Despite the advanced state of knowledge with respect to the destructive side of protein quality control, the precise nature of the interplay between the biosynthetic and degradative pathways remains poorly understood. Specifically, the mechanisms by which a single protein is diverted according to its particular folding status are largely unknown.

One critical juncture in the process of protein quality control is the uptake of newly synthesized proteins into ER-derived transport vesicles, known as coat protomer type II (COPII) vesicles for the cytoplasmic coat proteins that sculpt vesicles from the ER membrane and populate them with the appropriate cargo (Lee *et al.*, 2004). Membrane proteins are generally captured into COPII vesicles via cytoplasmic ER export signals that bind directly to the COPII subunit Sec24p, which is responsible for efficiently recruiting cargo into nascent vesicles as they form from the ER membrane (Miller *et al.*, 2002). Similarly, soluble secretory proteins bind to receptors that in turn likely contain Sec24p-

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* These authors contributed equally to this work.

[†] Present address: Department of Microbiology, Columbia University, New York, NY 10027-6902.

Address correspondence to: Elizabeth A. Miller (em2282@columbia.edu).

Abbreviations used: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; COPII, coat protomer type II; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; MSD, membrane spanning domain; NBD, nucleotide binding domain; TMD, transmembrane domain.

binding sites (Otte and Barlowe, 2004). Misfolded proteins seem to be largely excluded from COPII vesicles, but the mechanism by which these proteins fail to enter vesicles is not clear.

Recent experiments using chimeric ERAD substrates demonstrated that degradation of a misfolded protein can be overcome when a strong ER export motif is appended to the protein (Kincaid and Cooper, 2007). This suggests that rather than using an active ER retention mechanism, quality control may result from a competitive interaction between ERAD and forward transport. Misfolded proteins may be rapidly destroyed by the ERAD machinery, leaving little opportunity to engage the COPII coat. Thus, disabling the degradative pathway might be expected to stabilize a misfolded protein sufficiently to drive forward transport. Indeed, inhibiting ERAD can allow rescue of forward transport, and, in some cases, the deployment of a functional protein (Kota *et al.*, 2007). One mechanism that may play a role in establishing a hierarchy of degradation over forward transport is the presentation of an ER export motif; misfolded proteins may simply fail to present an appropriate signal, resulting in their default degradation. This may be especially relevant to soluble secretory proteins, which contain ER export motifs that mediate interaction with their transmembrane receptors (Otte and Barlowe, 2004). Simple failure to engage the receptor would ultimately cause degradation. However, misfolded membrane proteins, which interact directly with Sec24p, present a more difficult quality control problem (Mossessova *et al.*, 2003). Where the misfolding lesion is located in a cytosolic domain, it is easy to imagine how structural changes may obscure an ER export motif. This is thought to be true of the human cystic fibrosis transmembrane receptor (CFTR); the most common disease-causing mutation, $\Delta F508$, is located in the same cytosolic domain as the ER export signal, potentially disrupting interaction with Sec24p (Wang *et al.*, 2004). Conversely, where the misfolding lesion is in a luminal or transmembrane region, or in a cytosolic domain distinct from that containing the ER export signal, interaction with Sec24p may be preserved. How these proteins are excluded from COPII vesicles remains unclear, but luminal or cytosolic chaperones that might bind the misfolded domain may play a role by actively retaining the protein or obscuring an interaction with Sec24p. Finally, the discovery that some misfolded proteins require a round-trip to the Golgi before ERAD suggests that misfolded proteins may not be entirely absent from COPII vesicles (Vashist *et al.*, 2001). As for ERAD, the mechanism that excludes a protein from COPII vesicles may be unique for each specific aberrant protein.

We study the close relationship between protein folding and forward transport through the secretory pathway in the budding yeast, *Saccharomyces cerevisiae*. We describe here the biogenesis of a plasma membrane protein, Yor1p, which is an ATP-binding cassette (ABC) transporter that functions as a pleiotropic drug pump to clear toxic substances from the yeast cytoplasm (Katzmann *et al.*, 1999). ABC transporters are a large family of proteins that contain distinct arrangements of membrane spanning domains (MSDs), which form a channel in the lipid bilayer, and nucleotide-binding domains (NBDs) that provide the driving force for substrate translocation across the membrane. The arrangement of MSDs and NBDs in Yor1p places it in the same class of ABC transporters as human CFTR (Riordan *et al.*, 1989). Deletion of a phenylalanine residue in NBD1 of Yor1p (F670), equivalent to the $\Delta F508$ mutation in CFTR, causes ER retention and proteasomal degradation of Yor1p (Katzmann *et al.*, 1999). In CFTR, this mutation results in a channel that is

capable of conducting chloride (Drumm *et al.*, 1991; Li *et al.*, 1993), but it is recognized by the ER quality control machinery and retained in the ER where it is degraded via ERAD instead of being deployed to the apical membrane (Cheng *et al.*, 1990; Denning *et al.*, 1992; Ward *et al.*, 1995). Extensive work elucidating the mechanisms of destruction of aberrant CFTR have revealed that it is a complex process involving multiple cytoplasmic chaperone complexes (Zhang *et al.*, 2001; Youker *et al.*, 2004; Younger *et al.*, 2004, 2006), but the relationship between the degradative and forward transport itineraries remains unclear.

We aimed to better define the protein folding, degradation, and forward transport pathways accessed by both the wild-type and ΔF forms of Yor1p to gain insight into how the intracellular fate of aberrant ABC transporters is controlled. Specifically, we have defined the ER export motif used by Yor1p in gaining access to ER-derived COPII vesicles and determined the site on Sec24p that likely recognizes this signal. Furthermore, we have used yeast mutants in several chaperones and the ERAD machinery to test how perturbation of these key processes directly impacts the intracellular itineraries of newly synthesized Yor1p. Because many components of the ER folding, degradation and export pathways are remarkably conserved among all eukaryotes, increasing our basic understanding of the regulation of these processes in yeast is likely to be directly applicable to protein folding problems in mammalian systems.

MATERIALS AND METHODS

Yeast Strains and Media

S. cerevisiae strains used in this study are listed in Table 1. Strains bearing mutant forms of *SEC24* as the sole copy of this essential gene were created by transforming YTB1 (*sec24 Δ ::LEU2* with wild-type *SEC24* borne on a *URA*-containing plasmid) with either wild-type or mutant forms of *SEC24* contained on a *HIS*-marked plasmid. Cells were cured of the wild-type *SEC24::URA* plasmid by growth on 5-fluoroorotic acid (0.1% final concentration), leaving the plasmid-borne copy as the sole copy of *SEC24*. *UBC7* was deleted in various strain backgrounds by transformation with a polymerase chain reaction (PCR) product composed of the *ubc7 Δ ::KANMX* disruption cassette amplified from the EUROSCARF (Frankfurt, Germany) deletion strain collection. The allelic replacement of *UBC7* was confirmed by PCR. LMY314 (*sel1 Δ ::KanMX*), LMY285 (*hrr1 Δ ::KanMX*), and LMY115 (*doa10 Δ ::KanMX*) were constructed by integration of the respective disruption cassettes into *HLJ1/YDJ1*. Disruption cassettes were previously generated by PCR amplification from the pUG6 plasmid (Guldener *et al.*, 1996). Retrieval of the *KanMX* gene in LMY285 (*hrr1 Δ ::KanMX*) was performed by *cre/lox*-mediated excision (Guldener *et al.*, 1996), and the *doa10 Δ ::KanMX* disruption cassette was introduced to create LMY313 (*hrr1 Δ doa10 Δ ::KanMX*). All allelic replacements were confirmed by PCR. Cultures were grown at 30°C in standard rich media (YPD: 1% yeast extract, 2% peptone, and 2% glucose) or synthetic complete media (SC: 0.67% yeast nitrogen base and 2% glucose, supplemented with amino acids appropriate for auxotrophic growth). For testing sensitivity to oligomycin, strains were grown to saturation, and then they were diluted to an OD₆₀₀ of 0.5 and fourfold serial dilutions were applied to YPEG plates (1% yeast extract, 2% peptone, 3% ethanol, and 3% glycerol), that were supplemented with oligomycin (Sigma-Aldrich, St. Louis, MO) from a 1 mg/ml stock in ethanol. Drug sensitivities of cells expressing mutant forms of Sec24p were assayed by streaking the strains indicated onto YPEG plates supplemented with 0.1 μ g/ml oligomycin, YPD plates supplemented with 400 μ g/ml rhodamine B (Sigma-Aldrich) or onto YPD plates supplemented with 10 μ g/ml methyl 1-(butylcarbamoyl)-2-benzimidazole-carbamate (benomyl, 95%; Sigma-Aldrich).

Plasmids

Plasmids used in this study are listed in Table 2. pEAE83 bearing *YOR1-HA* in pRS316 was a gift from Scott Moyer-Rowley (University of Iowa). This plasmid was the basis for site-directed mutagenesis by using QuikChange mutagenesis (Stratagene, La Jolla, CA) to obtain various hemagglutinin (HA)-tagged Yor1p mutants (Table 2). pRL001 represents a marker-swapped version of pEAE83, where the *URA3* marker was replaced with *TRP1* (Cross, 1997). This plasmid was used as the template for site-directed mutagenesis to introduce unpaired cysteine substitutions to test whether cross-links form from intra- or intermolecular associations. pEAE93 was a gift from Scott Moyer-Rowley and contains an in-frame fusion of green fluorescent protein

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
BY4742	<i>MATα his leu2-Δ1 lys2-801am ura3-52</i>	Open Biosystems (Huntsville, AL)
RSY620	<i>MATα leu2-3,112 ura3-52 ade2-1 trp1-1 his3-11,15 pep4::TRP1</i>	Schekman laboratory strain
YTB1	<i>MATα can1-100 leu2-2,112 ura3-1 ade2 trp1-1 his3-11,15 sec24::LEU2</i> carrying pLM22 (CEN SEC24-URA3)	Miller <i>et al.</i> (2003)
LMY006	<i>MATα his leu2-Δ1 lys2-801am ura3-52, yor1::KANMX</i>	Open Biosystems
p82a	<i>MATα, ade2-1, leu2-3, 112, his3-11, 15, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPDP-HSP82</i>	Nathan and Lindquist (1995)
G313N	<i>MATα, ade2-1, leu2-3, 112, his3-11, 15, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPDP-HSP82-G313N</i>	Nathan and Lindquist (1995)
HLJ1/YDJ1	<i>MATα, ade2, his3, leu2, ura3, trp1</i>	Youker <i>et al.</i> (2004)
hlj1/ydj1-151	<i>MATα, ade2, his3, leu2, ura3, trp1, can1-100 ydj1-2::HIS3, ydj1-151::LEU2, hlj1::TRP1</i>	Youker <i>et al.</i> (2004)
LMY109	<i>MATα, ade2, his3, leu2, ura3, trp1, ubc7Δ::KANMX</i>	This study
LMY113	<i>MATα, ade2-1, leu2-3, 112, his3-11, 15, trp1-1, ura3-1, can1-100, hsc82Δ::LEU2, hsp82Δ::LEU2, pTGPDP-HSP82-G313N, ubc7Δ::KANMX</i>	This study
LMY114	<i>MATα, ade2, his3, leu2, ura3, trp1, ubc7Δ::KANMX, yor1Δ::HIS5</i>	This study
LMY314	<i>MATα, ade2, his3, leu2, ura3, trp1, sel1Δ::KANMX</i>	This study
LMY285	<i>MATα, ade2, his3, leu2, ura3, trp1, hdr1Δ::KANMX</i>	This study
LMY115	<i>MATα, ade2, his3, leu2, ura3, trp1, doa10Δ::KANMX</i>	This study
LMY313	<i>MATα, ade2, his3, leu2, ura3, trp1, hdr1Δ, doa10Δ::KANMX</i>	This study

(GFP) to the C terminus of Yor1p; this plasmid was the basis for site-directed mutagenesis to introduce N- and C-terminal diacidic mutations. pRS315-PDR1-3 bearing a dominant active allele of the transcription factor Pdr3p was a gift from Scott Moye-Rowley, and it was used to overexpress Yor1p-GFP fusions (Epping and Moye-Rowley, 2002).

Live Cell Imaging

The subcellular localization of Yor1p and various mutants was visualized by fluorescence microscopy of Yor1p-GFP fusion proteins. Strains expressing the appropriate gene fusion were grown in selective medium to mid-log phase and examined with an IX 81 inverted microscope (Olympus, Melville, NY) equipped with a 60 $\times$ numerical aperture 1.4 PlanApo optics and a Cooke Sensicam QE air-cooled charge-coupled device camera. Images were collected with IPLab 4.0 and analyzed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

To visualize Yor1p and the N- and C-terminal diacidic mutants, HLJ1/YDJ1 cells were cotransformed with Yor1p-GFP plasmids and pRS315-PDR1-3 containing the dominant-active transcription factor *pdr1-3*, which causes up-regulation of several ABC transporters (Carvajal *et al.*, 1997).

Protein Purification

COPII proteins Sar1p, Sec23p/24p, and Sec13/31p were prepared as described previously (Barlowe *et al.*, 1994).

In Vitro Vesicle Budding

Microsomal membranes were purified from RSY620 cells expressing Yor1p-HA or Yor1p Δ F-HA as described previously (Wuestehube and Schekman, 1992). In vitro vesicle budding was performed essentially as described pre-

Table 2. Plasmids used in this study

Plasmid	Genotype	Source
pEAE83	HA-tagged YOR1 in pRS316	Katzmann <i>et al.</i> (1999)
pLM22	4.2kb XhoI-SpeI fragment containing 6 $\times$ His-tagged SEC24 in pRS316	Miller <i>et al.</i> (2003)
pLM23	4.2kb XhoI-SpeI fragment containing 6 $\times$ His-tagged SEC24 in pRS313	Miller <i>et al.</i> (2003)
pLM132	Sec24-B1 (Sec24R230A) mutation in pLM23	Miller <i>et al.</i> (2003)
pLM133	Sec24-B2 (Sec24R235A) mutation in pLM23	Miller <i>et al.</i> (2003)
pLM134	Sec24-B3 (Sec24R230A,R235A) mutation in pLM23	Miller <i>et al.</i> (2003)
pLM135	Sec24-B4 (Sec24R559M) mutation in pLM23	Miller <i>et al.</i> (2003)
pLM136	Sec24-B5 (Sec24R561M) mutation in pLM23	Miller <i>et al.</i> (2003)
pLM174	Sec24-C1 (Sec24R342A) mutation in pLM23	Miller <i>et al.</i> (2003)
pLM299	Sec24-A1 (Sec24W897M) mutation in pLM23	Miller <i>et al.</i> (2005)
pLM308	D71E73A (Yor1D71A, E73A) mutation in pEAE83	This study
pLM309	Δ F (Yor1 Δ F670) mutation in pEAE83	This study
pLM310	D1472E1474A (Yor1D1472A, E1474A) mutation in pEAE83	This study
pLM311	D691D693A (Yor1D691A, D693A) mutation in pEAE83	This study
pLM312	D729A (Yor1D729A) mutation in pEAE83	This study
pEAE93	GFP-tagged YOR1 in pRS316	Epping and Moye-Rowley (2002)
pLK101	D71E73A (Yor1D71, E73A) mutation in pEAE93	This study
pLK102	D1472E1474A (Yor1D1472, E1474A) mutation in pEAE93	This study
pRS315-PDR1-3	pdr1-3 in pRS315	Carvajal <i>et al.</i> (1997)
pRL001	pEAE83 (URA3 marker replaced with TRP1)	This study
spQC19	L1162C (Yor1L1162C) mutation in pEAE83	This study
spQC44	F481C (Yor1F481C) mutation in pLM280	This study
spQC20	F481C, L1162C (Yor1F481C, L1162C) mutations in pEAE83	This study
spQC24	F481C, L1162C (Yor1F481C, Δ F670, L1162C) mutations in pLM309	This study
spQC46	D71E73A (Yor1D71, E73A, F481C, L1162C) mutations in spQC20	This study

viously (Miller *et al.*, 2002), except the urea wash was omitted. Briefly, membranes were washed twice with buffer B88 (20 mM HEPES, pH 6.8, 250 mM sorbitol, 160 mM potassium acetate, and 5 mM magnesium acetate), and 125 μ g of membranes per reaction were incubated with COPII proteins (10 μ g/ml Sar1p, 10 μ g/ml Sec23p/24p, and 20 μ g/ml Sec13/31p) either in the presence of 0.1 mM GTP with a 10 $\times$ ATP regeneration system or 0.1 mM GDP. Vesicles were separated from donor membranes by centrifugation at 16,000 rpm for 5 min, and the vesicle fraction was further concentrated by high-speed centrifugation at 55,000 rpm for 20 min. Vesicle pellets were resuspended in SDS sample buffer and heated at 55°C for 5 min before separation by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and analyzed by immunoblotting. HA-tagged Yor1p was detected with a monoclonal anti-HA antibody (Covance, Princeton, NJ), and control proteins Erv46p and Sec22p were detected with polyclonal antibodies, gifts from C. Barlowe (Dartmouth Medical School) and R. Schekman (U. C. Berkeley), respectively.

Radiolabeled semi-intact cells were prepared essentially as described previously (Kuehn *et al.*, 1996). Briefly, cells were grown to mid-log phase in synthetic complete medium, and a total of 5.0 OD₆₀₀ of cells were harvested and starved of methionine/cysteine for 10 min before addition of Express protein labeling mix (~70 μ Ci/OD₆₀₀ of cells; MP Biomedicals, Irvine, CA). Cells were labeled for 15 min at 30°C, and then they were metabolically killed and converted to spheroplasts. Cells were gently lysed, washed once with low acetate B88 (20 mM HEPES, pH 6.8, 250 mM sorbitol, 50 mM potassium acetate, and 5 mM magnesium acetate) and twice with B88 before incubation with COPII proteins (10 μ g/ml Sar1p, 10 μ g/ml Sec23p/24p, and 20 μ g/ml Sec13/31p) in a final reaction that contained 2.5 OD of cells either in the presence of 0.1 mM GTP with a 10 $\times$ ATP regeneration system or 0.1 mM GDP. Vesicles were separated from donor membranes by centrifugation at 16,000 rpm for 5 min, solubilized with 1% SDS (final concentration), and diluted with immunoprecipitation (IP) buffer (50 mM Tris, pH 7.5, 160 mM NaCl, 1% Triton X-100, and 2 mM Na₂S₂O₃). Proteins were immunoprecipitated using monoclonal anti-HA antibodies (Covance) precoupled to protein G-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), or polyclonal antibodies against Gas1p, Sec22p or Vph1p (gifts from R. Schekman) coupled to protein A-Sepharose beads (GE Healthcare). Immune complexes were separated by SDS-PAGE and analyzed by PhosphorImager analysis using a Storm PhosphorImager (GE Healthcare). Proteins were quantified using ImageQuant software (GE Healthcare).

Blue Native Gel Electrophoresis (BNGE)

In total, 5.0 OD₆₀₀ of cells expressing wild-type HA-tagged Yor1p or HA-tagged mutant Yor1p were collected during mid-log phase, washed with 250 μ l of lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1 mM dithiothreitol [DTT]), and lysed by vortexing with glass beads for 10 min at 4°C. The lysate was transferred to a fresh tube, membranes collected by centrifugation at 15,000 rpm for 5 min and proteins solubilized with 100 μ l of lysis buffer with 1% digitonin. After incubation on ice for 30 min, the soluble fraction was separated from the insoluble material by centrifugation at 5000 rpm for 2 min, and the supernatant was mixed with 10 $\times$ BNP sample buffer (5% Coomassie G250, 10% 6-aminocaproic acid, 50 mM Bis-Tris, and 10% glycerol). Samples were run on a 7% acrylamide Bis-Tris gel (50 mM Bis-Tris, 6.55% 6-aminocaproic acid, and 13.9% glycerol) by using a two-buffer system consisting of a cathode buffer (50 mM Tricine, 15 mM Bis-Tris, and 0.02% Coomassie G250) and anode buffer (50 mM Bis-Tris). Proteins were transferred to PVDF overnight at 20 V and analyzed by immunoblotting with a monoclonal anti-HA antibody (Covance).

Limited Proteolysis

Cells expressing either wild-type Yor1p-HA or HA-tagged Yor1p mutants were harvested during mid-log phase. In total, 10.0 OD₆₀₀ of cells were collected, resuspended in 200 μ l of B88 buffer, and glass bead lysed at 4°C for 5 min. Cell lysates were subjected to centrifugation at 16,000 rpm for 5 min, and the membrane pellet was resuspended in 100 μ l of B88 and divided into four 25- μ l reactions (2.5 OD₆₀₀/reaction). Each reaction was treated with a final concentration of 0, 25, 50, or 100 ng/ μ l trypsin (Sigma-Aldrich) for 10 min on ice. Digestion was terminated by addition of 0.2 μ g/ml (final concentration) soybean trypsin inhibitor (Sigma-Aldrich) to all reactions and incubated on ice for 15 min. Proteins were separated by SDS-PAGE, transferred to PVDF, and the pattern of Yor1p fragments analyzed by immunoblot by using an anti-HA antibody.

For cells bearing thermosensitive alleles, radiolabeled semi-intact cells were used as the source of membranes. Cells were grown to mid-log phase in complete synthetic medium at 30°C, and then they were harvested, resuspended in fresh medium lacking methionine/cysteine to an OD₆₀₀ of 5, and incubated for 10 min with gentle shaking at 37°C. Cells were metabolically labeled at 37°C for 10 min by adding 60 μ Ci of Express protein labeling mix (MP Biomedicals) per OD₆₀₀ unit of cells. Spheroplasts prepared from these labeled cells were resuspended in 100 μ l of B88 and divided into four 25- μ l reactions. Each reaction was treated with a final concentration of 0, 100, 200, or 400 ng/ μ l trypsin for 10 min on ice. Digestion was terminated by addition of soybean trypsin inhibitor to all reactions followed by incubation on ice for

15 min and by two washes with B88. After solubilization with SDS (1% final concentration) and heating to 55°C for 5 min, the resulting protein extracts were diluted with 5 volumes of IP buffer (50 mM Tris, pH 7.5, 160 mM NaCl, 1% Triton X-100, and 2 mM Na₂S₂O₃) and cleared by centrifugation. Yor1p fragments were immunoprecipitated from the cleared supernatant and analyzed by SDS-PAGE and PhosphorImager analysis as described above.

Cross-linking of TM Domains

Cells expressing cysteine-substituted forms of Yor1p-HA were grown to mid-log phase, and then they were harvested and converted to spheroplasts. Spheroplasts were washed twice in 20 mM HEPES, pH 7.4, and incubated with increasing concentrations of M8M (prepared as a 100 $\times$ stock in dimethyl sulfoxide) as indicated. Cells were cross-linked for 15 min at room temperature, and then they were harvested, resuspended in 100 μ l of 1% SDS, 50 μ l of 3 $\times$ SDS sample buffer without β -mercaptoethanol or DTT. Cells were disrupted by glass bead lysis (15 min; 4°C), heated to 55°C for 5 min, and proteins were separated by SDS-PAGE followed by immunoblot analysis using anti-HA antibodies.

Pulse-Chase Analysis of Yor1p Stability

Cells were grown to mid-log phase in complete synthetic medium, and then they were harvested, resuspended in fresh medium lacking methionine/cysteine, and incubated for 15 min with gentle shaking at either 30°C (for cells lacking a temperature-sensitive allele) or at 37°C (for cells bearing a temperature-sensitive allele). Cells were metabolically labeled for 5 min by adding 30 μ Ci of Express protein labeling mix (MP Biomedicals) per OD₆₀₀ unit of cells. A 10 $\times$ chase solution (10 mM L-cysteine, 50 mM L-methionine, 4% yeast extract, and 2% glucose) was added and 2 OD aliquots of cells harvested at different times. At each time point, cells were transferred to chilled tubes, and sodium azide was added to a final concentration of 20 mM. Cells were washed once with 20 mM sodium azide and resuspended in 100 μ l of 1% SDS. Glass beads were added, and cells were lysed by vortexing for 15 min at 4°C. Cell lysates were heated at 55°C for 5 min, diluted with 5 volumes of IP buffer (50 mM Tris, pH 7.5, 160 mM NaCl, 1% Triton X-100, and 2 mM Na₂S₂O₃), and cleared by centrifugation. Yor1p and Gas1p were immunoprecipitated from the cleared lysate and analyzed by SDS-PAGE and PhosphorImager analysis as described above.

RESULTS

The yeast ABC transporter Yor1p is a polytopic membrane protein that functions at the plasma membrane as a pleiotropic drug pump to clear toxins from the cytosol. A mutant form of Yor1p, Yor1p- Δ F, which carries a deletion of a phenylalanine residue at position 670, is trapped in the ER, destabilized *in vivo*, and degraded by the cytoplasmic ubiquitin/proteasome system (Katzmann *et al.*, 1999). To independently confirm that ER-export of Yor1p- Δ F is impaired, we used an *in vitro* vesicle budding assay that recapitulates COPII vesicle formation from purified ER membranes. Mitochondrial membranes were isolated from cells expressing an HA-tagged copy of either wild-type Yor1p or Yor1p- Δ F. These purified membranes were incubated with the COPII components Sar1p, Sec23p/Sec24p, and Sec13p/Sec31p in the presence of either GTP or GDP. This incubation is sufficient to generate transport vesicles, which can be separated from donor membranes by differential centrifugation and specific capture of cargo proteins into vesicles analyzed by immunoblotting (Barlowe *et al.*, 1994). Whereas wild-type Yor1p was efficiently packaged into COPII vesicles, very little Yor1p- Δ F was detected in the vesicle fraction (Figure 1B). Two proteins that cycle between the ER and Golgi, Sec22p and Erv46p served as positive controls to demonstrate efficient vesicle biogenesis in this assay. The specific absence of Yor1p- Δ F in COPII vesicles generated *in vitro* is consistent with its *in vivo* ER localization and suggests that the mutant protein has engaged the ER quality control checkpoint that prevents deployment of aberrant cellular proteins.

We next developed assays to probe the folding, assembly state, or both of Yor1p. We first examined the migration of Yor1p by using BNGE, which allows the preservation of native structures by solubilizing proteins with mild deter-

Figure 1. Yor1p- Δ F is excluded from COPII vesicles and is misfolded. (A) Schematic diagram of the yeast ABC transporter, Yor1p, which, like CFTR, has two membrane-spanning domains (MSD1 and MSD2), two nucleotide binding domains (NBD1 and NBD2), and an analogous phenylalanine residue (F670) in NBD1 that is necessary for protein stability and trafficking. Yor1p also has two previously identified terminal diacidic motifs (D₇₁E₇₃ and D₁₄₇₂E₁₄₇₄) and two additional acidic motifs in NBD1 (D₆₉₁D₆₉₃ and D₇₂₉) that may interact with COPII coat machinery in facilitating ER export. (B) Capture of Yor1p into ER-derived COPII vesicles was examined in an *in vitro* vesicle budding assay by using microsomal membranes expressing HA-tagged forms of Yor1p as the donor membranes. Membranes were incubated with COPII proteins in the presence of GTP (+) or GDP (–), and vesicles were separated from the total membrane (T) fraction by centrifugation. Cargo proteins were detected by immunoblot analysis. (C) Yor1p and Yor1p- Δ F were analyzed by BNGE after solubilization with either digitonin or SDS as indicated followed by immunoblotting to detect HA-tagged forms of Yor1p. The amount of material loaded following solubilization with SDS represents a 1/5 dilution compared with that loaded for digitonin-solubilized samples. (D) Microsomal membranes expressing HA-tagged forms of Yor1p were subjected to limited proteolysis by treating membranes with increasing amounts of trypsin followed by SDS-PAGE and immunoblotting to detect HA-tagged proteolytic fragments. (E) Membranes expressing HA-tagged forms of cysteine-modified Yor1p were cross-linked with increasing concentrations of M8M before SDS-PAGE and immunoblot analysis. Yor1p expressed from two separate plasmids, each bearing single cysteine substitutions showed no cross-linked species (left), whereas Yor1p containing both F481C and L1162C mutations was readily cross-linked into a higher-molecular-weight species (middle). Yor1p- Δ F containing the same substitutions instead presented as a very high-molecular-weight aggregate, with neither the wild-type nor cross-linked species detectable (right).

gents (Schagger and von Jagow, 1991). Solubilized proteins are coated with Coomassie Blue, which imparts a net negative charge, allowing proteins to migrate uniformly in one-dimensional nondenaturing gel electrophoresis. Microsomal membranes containing HA-tagged forms of Yor1p were solubilized with digitonin, and protein complexes analyzed by BNGE followed by immunoblotting. Wild-type Yor1p was detected as a single band of ~350 kDa, whereas Yor1p- Δ F formed a high-molecular-weight smear of ~670 kDa (Figure 1C). An additional sample of Yor1p was solubilized with SDS and reducing agents, which also yielded a single band of ~350 kDa, suggesting that this species corresponds to monomeric Yor1p that, under these electrophoretic conditions, migrates as a larger protein than its expected ~150 kDa. Similar aberrant protein migration profiles are characteristic of other yeast multispanning membrane proteins, Gap1p (Kota and Ljungdahl, 2005) and Pma1p (Lee *et al.*, 2002). The precise nature of the high-molecular weight smear that corre-

sponds to Yor1p- Δ F is not clear; the mutant protein may form large aggregates or may be in complex with other proteins, including cytoplasmic or ER chaperones.

As a separate assay to probe the folding and assembly of Yor1p, we subjected membranes containing either wild-type or mutant Yor1p to limited proteolysis. Microsomal membranes were treated with increasing concentrations of trypsin and analyzed by immunoblotting. Yor1p presented a persistent ~160-kDa band and a single major cleavage product of ~60 kDa with increasing amounts of trypsin (Figure 1D, left). The pattern of Yor1p- Δ F cleavage products was markedly different, with both the 160- and 60-kDa bands further digested into smaller products with increasing trypsin (Figure 1D, right). The increased susceptibility of Yor1p- Δ F to trypsinolysis supports the hypothesis that this mutant protein is improperly assembled, thereby exposing additional trypsin cleavage sites that are obscured in the correctly folded protein.

Finally, we developed a cross-linking assay to probe the assembly of the transmembrane domains (TMDs) of Yor1p. Previous studies on the related ABC transporters P-glycoprotein and CFTR had used cysteine residues, substituted within specific transmembrane domains, to probe the ability to form cross-linked disulfide bonds (Chen *et al.*, 2004). Misfolding mutations, including the $\Delta F508$ lesion, resulted in an inability to form specific cross-links, suggesting that these transmembrane domains fail to assemble correctly. We introduced cysteine residues at specific positions in the 6th and 12th TM domains of Yor1p, equivalent to the sites used for CFTR. Membranes expressing wild-type Yor1p that contained cysteine substitutions at F₄₈₁ (TM6) and L₁₁₆₂ (TM12) were exposed to a methanethiosulfonate cross-linker with a 13-Å spacer arm and the mobility of the protein monitored by nonreducing SDS-PAGE and anti-HA immunoblotting (Figure 1E). On exposure to cross-linker, a species with reduced mobility was detected, similar to that observed for CFTR. This cross-linked protein was also detected with a L₄₇₉C/L₁₁₆₂C substitution pair, analogous to a second cross-linking pair that was used to probe transmembrane domain assembly in CFTR (Pagant, unpublished data). The cross-linked species likely represents an intramolecular modification, because when the individual F₄₈₁C and L₁₁₆₂C substitutions were introduced separately on two different plasmids and cotransformed into cells, no cross-linked proteins were detected (Figure 1E, left). When the paired F₄₈₁C/L₁₁₆₂C substitutions were introduced into Yor1p- ΔF , no cross-linked species were detected; instead, the unmodified protein disappeared and the majority of the protein presented as a very-high-molecular weight aggregate that largely failed to enter the resolving gel. Similar higher order aggregation was also detected for CFTR- ΔF , and it is thought to represent nonspecific cross-linking of exposed cysteine residues (Chen *et al.*, 2004). Thus, like CFTR- ΔF , Yor1p- ΔF seems to contain improperly assembled transmembrane domains.

Identification of the ER Export Motif Used by Yor1p

To gain a better understanding of how Yor1p engages the ER export pathway, a clear definition of the ER export signals used by Yor1p is required. Two independent diacidic motifs have been identified in Yor1p; when either the N-terminal D₇₁XE₇₃ or C-terminal D₁₄₇₂XE₁₄₇₄ were mutated, Yor1p was trapped in the ER, causing cells to become oligomycin sensitive (Epping and Moyer-Rowley, 2002). Similarly, ER exit of CFTR is dependent upon the presence of a diacidic motif, although the putative signal in CFTR is contained within NBD1 (Wang *et al.*, 2004). We investigated the potential roles of several diacidic motifs in mediating ER exit of Yor1p, focusing on the two terminal DXE motifs and two potential diacidic motifs in NBD1 of Yor1p: D₆₉₁XD₆₉₃ and D₇₂₉XY₇₃₁. The latter signal is spatially homologous to the aspartic acid residue of the DXE CFTR export signal, but it only contains a single acidic residue (Figure 1A). We generated HA-tagged mutants where each potential motif was replaced with alanine, and we examined *in vivo* phenotypes and *in vitro* capture into COPII vesicles of each of the mutant proteins (Figure 2).

Yor1p is required for growth in the presence of low concentrations of the mitochondrial poison, oligomycin, and impaired trafficking of Yor1p to the plasma membrane results in oligomycin sensitivity (Epping and Moyer-Rowley, 2002). We replicated the oligomycin-sensitive phenotypes of the D₇₁AXE₇₃A and D₁₄₇₂AXE₁₄₇₄A mutants observed by Epping and Moyer-Rowley (2002); the N-terminal mutant was more sensitive to oligomycin than the C-terminal mu-

tant. Although the D₆₉₁AXD₆₉₃A mutant was extremely sensitive to oligomycin, the D₇₂₉A mutant was relatively resistant to the drug, similar to wild-type Yor1p (Figure 2A). These data suggest that D₇₂₉ residue does not comprise the ER export motif for Yor1p, but the oligomycin sensitivity phenotype alone did not allow us to distinguish between the three remaining motifs as bona fide export signals.

To better define the signal responsible for ER export of Yor1p, we examined uptake of each of the mutants into COPII vesicles generated *in vitro* from radiolabeled permeabilized cells. Wild-type Yor1p was efficiently packaged into COPII vesicles, and of all the diacidic mutants that we tested, only the N-terminal D₇₁AXE₇₃A mutant was impaired in its capture into COPII vesicles (Figure 2B). A control cargo protein, Vph1p, was equivalently packaged in each of these experiments, indicative of general budding efficiency. We further tested the localization of Yor1p mutants by microscopy by using Yor1p-GFP fusions (Figure 2C). As reported previously, wild-type Yor1p fused to GFP was found at the plasma membrane, whereas the N-terminal (D₇₁AXE₇₃A) diacidic mutant showed a perinuclear localization and cortical ER localization, consistent with its retention in the ER (Epping and Moyer-Rowley, 2002). Conversely, the localization of the C-terminal (D₁₄₇₂AXE₁₄₇₄A) diacidic mutant more closely resembled that of the wild-type protein, with strong plasma membrane fluorescence and no detectable internal perinuclear staining. This is consistent with our *in vitro* budding data, but it conflicts with a previous report that used sucrose gradients to show that Yor1p-D₁₄₇₂AXE₁₄₇₄A was ER retained (Epping and Moyer-Rowley, 2002). We note that the oligomycin sensitivity phenotype of this C-terminal mutant is intermediate between wild-type Yor1p and the N-terminal mutant, suggesting some *in vivo* impairment of protein delivery or function. It is possible that the C-terminal mutant protein is turned over more rapidly at the plasma membrane, rendering cells slightly more sensitive to oligomycin. Such destabilization may create a steady-state distribution such that the mutant protein is located both at the plasma membrane and in the ER; the ER pool may be undetectable by GFP fluorescence but more easily resolved by cell fractionation. Consistent with this model, destabilization of CFTR by truncation of the C terminus causes the protein to be rapidly internalized from the plasma membrane and cycled through an endosomal compartment before lysosomal destruction (Sharma *et al.*, 2004).

To confirm that the impaired trafficking of the D₇₁AXE₇₃A mutant results from an abrogated exit signal and not a folding defect, we examined the folding status of the mutant protein (Figure 2D). Yor1p-D₇₁AXE₇₃A showed a similar cleavage profile to that of wild-type Yor1p with a persistent band at ~160 kDa and another at ~60 kDa. Similar experiments with BNAGE also indicated that each of the mutants was properly folded, with migration patterns similar to wild-type Yor1p (Lee, unpublished data). Finally, we probed the arrangement of transmembrane domains of the di-acidic mutant by using cysteine cross-linking, which revealed a similar cross-linking pattern to that of the wild-type protein (Figure 2E). Together, these data confirm that the N-terminal diacidic motif, D₇₁AXE₇₃, is the ER export signal for Yor1p, and that the ER export defects observed when this motif is altered are not caused by misfolding of Yor1p. The other diacidic mutants were also correctly folded, as determined by trypsin sensitivity and BNAGE, suggesting that the oligomycin sensitivity of these proteins may stem from defects in protein function rather than impaired protein trafficking or gross protein misfolding.

Figure 2. Identification of an N-terminal diacidic motif as the ER exit signal for Yor1p. (A) Four-fold serial dilutions of yeast expressing HA-tagged forms of Yor1p were tested for sensitivity to increasing concentrations of oligomycin (1, 2.5, and 5 $\mu\text{g/mL}$). Cells expressing wild-type Yor1p were able to grow under all conditions, whereas cells containing the plasmid alone (pRS316) were sensitive to very low levels of oligomycin. The various diacidic mutants showed a range of oligomycin sensitivities, whereas the ER-retained Yor1p- Δ F was completely oligomycin sensitive. (B) Packaging of Yor1p mutants into COPII vesicles was examined by an in vitro vesicle budding assay. Cells were radiolabeled with [^{35}S]methionine/cysteine for 15 min, permeabilized, and incubated with COPII proteins in the presence of either GTP (+) or GDP (-). HA-tagged wild-type and mutant forms of Yor1p were immunoprecipitated and analyzed by SDS-PAGE and PhosphorImage analysis. The percentage of Yor1p found in the vesicle fraction was quantified relative to the total Yor1p (T) present in the donor membranes. Packaging of D₇₁XE₇₃A was severely impaired, whereas that of the D₆₉₁XD₆₉₃A, D₇₂₉A, and D₁₄₇₂XE₁₄₇₄ mutants was similar to that of wild-type Yor1p. An independent cargo protein, Vph1, was also examined to indicate efficient generation of vesicles in the in vitro assay. (C) Wild-type cells coexpressing pRS315-PDR1-3 and Yor1p-GFP fusions as indicated were examined by epifluorescence microscopy. Only the N-terminal di-acidic mutant showed perinuclear staining consistent with ER retention. Wild-type Yor1p and the C-terminal diacidic mutant both localized to the plasma membrane. (D) Folding and stability of Yor1p and the N-terminal diacidic mutant were analyzed by limited proteolysis. Whole cell lysates containing HA-tagged wild-type or mutant forms of Yor1p were incubated with increasing amounts of trypsin, subjected to SDS-PAGE and Yor1p fragments detected by immunoblotting with an anti-HA antibody. (E) The arrangement of transmembrane domains in the N-terminal di-acidic mutant was probed by cysteine cross-linking as described in Figure 1E.

The Diacidic ER Export Motif of Yor1p Uses the B-Site on Sec24p

ER export signals function by interacting with Sec24p, which contains three known cargo-binding sites: the A-, B- and C-sites that each recognize distinct motifs. The observation that Yor1p uses a diacidic export motif (DxE) suggests the

B-site of Sec24p as the likely binding site (Mossessova *et al.*, 2003). However, because the superficially similar diacidic motif of Gap1 (DID) does not use the B-site, we also explored a potential role for either the A-site or C-site in Yor1p uptake (Miller *et al.*, 2003). We tested seven defined mutants of Sec24p by examining various drug sensitivities of strains

Figure 3. Identification of the Sec24p B-site as the cargo capture site for Yor1p. Strains expressing WT Sec24p or various A-, B-, or C-site Sec24p mutants as the sole copy of Sec24p were tested for growth defects in the presence of various compounds as indicated. (A) All strains grew normally on YPEG plates. (B) B-site mutants were unable to grow on plates containing oligomycin whereas A- and C-site mutants grew normally. (C) Oligomycin sensitivity of the B-site mutants could be rescued by transforming these strains with an extra episomal copy of Yor1p. (D) The B-site mutants also showed growth defects in the presence of rhodamine. (E) All strains grew normally in the presence of benomyl.

expressing each of these mutants as the sole copy of Sec24p, anticipating that disrupting the Sec24p–Yor1p interaction would result in increased oligomycin sensitivity. Indeed, B-site mutants of Sec24p that displayed normal growth on YPEG (Figure 3A) were unable to grow in the presence of low concentrations ($0.1 \mu\text{g}/\mu\text{l}$) of oligomycin (Figure 3B), but viability could be rescued by introducing an episomal copy of Yor1p (Figure 3C). Similar sensitivity was seen in the presence of rhodamine ($400 \mu\text{g}/\text{ml}$; Figure 3D), which is cleared from the cell predominantly by another ABC transporter, the pleiotropic drug pump Pdr5p, but can also use Yor1p (Rogers *et al.*, 2001). Neither the Sec24p A-site nor C-site mutants were sensitive to oligomycin or rhodamine. All of the Sec24p mutants were able to grow in the presence of benomyl at concentrations that result in lethality of mutants with impaired microtubule function ($10 \mu\text{g}/\text{ml}$; Figure 3E), suggesting that the sensitivity of the B-site mutants to oligomycin and rhodamine is not a general drug intolerance but results from impaired trafficking of specific transporters. These observations suggest that an intact B-site on Sec24p is critical for the trafficking of Yor1p out of the ER and is most likely the site that binds the N-terminal $D_{71}XE_{73}$ motif of Yor1p.

To confirm that the oligomycin sensitivity of the Sec24p B-site mutants resulted from failure of Yor1p to leave the ER, we examined the localization of Yor1p-GFP in these mutants (Figure 4). In wild-type cells and Sec24p A- and C-site mutants, Yor1p-GFP was found exclusively at the plasma membrane (Figure 4), consistent with full oligomycin resistance

of these mutant strains. However, when Yor1p-GFP was expressed in any of the B-site Sec24p mutants, a distinct perinuclear ER fluorescence pattern was observed (Figure 4). This ER retention of Yor1p-GFP is consistent with an inability to be efficiently recognized by the mutant form of Sec24p, causing accumulation in the ER and leading to oligomycin sensitivity. Intracellular localization of Gap1p-GFP was unaffected by the Sec24p B-site mutation (Kung, unpublished data), consistent with *in vitro* vesicle budding data that suggest this site is not used by Gap1p (Miller *et al.*, 2003), and suggesting that the Yor1p trafficking defect is specific. Finally, we examined *in vitro* vesicle budding of Yor1p from urea-washed membranes by using either wild-type Sec24p or a B-site mutant. Although the vesicle budding efficiency was very low, likely the result of urea treatment, Yor1p was packaged into a vesicle fraction in the presence of wild-type Sec24p, but it was absent from vesicles made with the B-site mutant protein (Supplemental Figure 1). Together, these data suggest that the N-terminal diacidic motif of Yor1p acts as an ER export signal that interacts with the B-site on Sec24p to mediate efficient ER egress.

Cytoplasmic Chaperones Regulate the Biogenesis of Yor1p

Given that the diacidic ER export signal for Yor1p resides in a separate domain from the ΔF lesion, it seems unlikely that this particular misfolding mutation directly impacts the interaction between Yor1p and Sec24p in the way that has been proposed for CFTR (Wang *et al.*, 2004). We therefore aimed to understand how various cellular folding and deg-

Figure 4. Yori1p-GFP accumulates in the ER in a Sec24-B mutant. Yori1p-GFP was introduced into strains expressing either wild-type Sec24p or A-, B- or C-site Sec24p mutants as indicated. Localization of Yori1p was examined by epifluorescence microscopy (left) and cell integrity monitored by differential interference contrast microscopy (right). In wild-type cells as well as in the A- and C-site mutants, Yori1p-GFP was located at the plasma membrane, whereas in B-site Sec24p mutant cells, the protein showed perinuclear localization consistent with ER-retention in these cells.

radative pathways might influence ER exit of Yori1p. Because the majority of Yori1p faces the cytoplasm, we hypothesized that protein folding may be assisted by cytoplasmic chaperones, which may in turn influence the recruitment of the COPII machinery. Newly synthesized CFTR in mammalian cells uses heat-shock protein of 90 kDa (HSP90) as a positive folding factor; treatment with geldanamycin (GA), an Hsp90 inhibitor, disrupts the interaction of nascent CFTR with cytosolic HSP90 and enhances its proteasomal degradation (Loo *et al.*, 1998). Yeast possess two cytoplasmic HSP90s, one constitutive (Hsc82p) and one heat-inducible (Hsp82p), which are 97% identical at the amino acid level. Due to their functional redundancy, the expression of only one homologue is required to maintain viability (Borkovich *et al.*, 1989). We analyzed the stability of wild-type Yori1p in a double mutant, *hsc82Δ hsp82Δ*, that expresses a temperature-sensitive mutant protein, *hsp82^{ts}* (G₃₁₃N), which is extremely unstable and rapidly degraded when cells are shifted to the nonpermissive temperature of 37°C (Bohen and Yamamoto, 1993; Fliss *et al.*, 2000). The corresponding wild-type strain for this experiment contains a plasmid-borne copy of wild-type *HSP82*. Cells were grown at permissive temperature and Yori1p degradation was monitored by pulse-chase analysis after shift to 37°C. The rate of wild-type Yori1p degradation was significantly higher in the mutant strain, suggesting that cytoplasmic HSP90 is important in the biogenesis of Yori1p (Figure 5A). Maturation of the cell

wall protein, Gas1p, served as a control to demonstrate that loss of HSP90 function did not lead to general defects in protein secretion. Like Yori1p, Yori1p-ΔF was destabilized in the *hsp82^{ts}* strain, suggesting that HSP90 also plays a role in the attempted folding and biogenesis of this aberrant protein (Figure 5B).

We asked whether these destabilized forms of Yori1p and Yori1p-ΔF could be rescued by perturbation of the ERAD pathway. ERAD of Yori1p-ΔF relies on ubiquitination by the ER-localized E2 ligase, Ubc7p, deletion of which partially stabilizes Yori1p-ΔF (Katzmann *et al.*, 1999). Yori1p-ΔF was slightly restabilized in a *hsp82^{ts} ubc7Δ* double mutant, showing a similar stability to that seen in wild-type cells (Figure 5B, right). Conversely, mutation of *UBC7* in the *hsp82^{ts}* background did not result in the stabilization of wild-type Yori1p, suggesting that perturbation of HSP90 function did not render Yori1p a substrate for the same ERAD accessed by Yori1p-ΔF, but instead it induced degradation via a different mechanism. We examined the folding state of newly synthesized Yori1p by trypsin digestion of membranes isolated from cells that had been radiolabeled after shift to nonpermissive temperature. Yori1p presented a similar cleavage profile in both the wild-type and mutant strains (Figure 5C). These results suggest that Yori1p is able to properly fold in the absence of HSP90 but that it is ultimately destabilized despite this apparent native conformation. We next examined the *in vitro* packaging of Yori1p into COPII vesicles by using membranes from either wild-type or *hsp82^{ts}* cells that had been preshifted to 37°C and pulse-labeled at the restrictive temperature. Yori1p was packaged equally well into vesicles generated from wild-type and mutant membranes, and budding of a control protein, Vph1p, demonstrated equivalent levels of COPII vesicle formation (Figure 5D). These data suggest that the destabilizing effect of *HSP90* disruption does not directly influence the ER exit of Yori1p, which is not degraded by ERAD in the *hsp82^{ts}* strain but likely transits the secretory pathway for degradation in the vacuole.

Yori1p-ΔF Uses Multiple ERAD Pathways

Having probed some of the mechanisms of protein folding and ER export of wild-type Yori1p, we determined which ERAD pathway is used in the degradation of the aberrant Yori1p-ΔF. We expressed Yori1p-ΔF in the ERAD mutants, *ubc7Δ* and *sel1Δ/ubx2Δ*, both of which are common to all known ERAD pathways; Ubc7p is the E2 ligase responsible for ubiquitin modification, and Sel1p/Ubx2p is the membrane anchor for the protein extraction apparatus Cdc48p. We examined the stability of Yori1p by pulse-chase analysis in each of these mutants (Figure 6). As reported by Katzmann *et al.* (1999), mutation of Ubc7p resulted in a significant stabilization of Yori1p-ΔF, restoring the protein to essentially wild-type Yori1p levels (Figure 6A). Similarly, deletion of Sel1p caused Yori1p-ΔF to be degraded more slowly than in wild-type cells (Figure 6A). We note that the half-life that we observe for Yori1p-ΔF is significantly longer than that described by Katzmann *et al.* (1999). Because our experimental approaches are largely the same, we suspect that these differences result from the distinct strain backgrounds used in the two studies. In each case, disruption of Ubc7p resulted in the stabilization of Yori1p-ΔF to essentially wild-type Yori1p levels.

Hrd1p/Der3p and Doa10p are components of two different ERAD pathways that are responsible for degrading ERAD substrates with luminal (ERAD-L) and cytosolic (ERAD-C) lesions, respectively. Surprisingly, Yori1p-ΔF was degraded equivalently in each of the *hrd1Δ* and *doa10Δ* mutants, similar to its degradation in wild-type cells (Figure 6B). However, in a *hrd1Δdoa10Δ* double mutant, Yori1p-ΔF

Figure 5. Yor1p and Yor1p- Δ F are destabilized in a *hsp90* mutant strain. (A) Wild-type (HSP82), *hsp82^{ts}* (*hsc82Δ*/HSP82G313N), or *hsp82^{ts}/ubc7Δ* strains expressing an HA-tagged form of Yor1p were grown at 30°C, pulse labeled for 5 min with [³⁵S]methionine/cysteine at 37°C, and chased at 37°C for the times indicated. Cells were subjected to immunoprecipitation for both Yor1p and Gas1p, and the abundance and maturation of these proteins were quantified by SDS-PAGE and PhosphorImage analysis. The results shown are the average of three independent experiments. (B) Wild-type and *hsp82* mutants expressing HA-tagged Yor1p- Δ F were analyzed as described in A. (C) The conformation of Yor1p was probed by limited proteolysis. Cells expressing Yor1p were grown at 30°C and pretreated for 25 min at 37°C before pulse labeling with [³⁵S]methionine/cysteine. Cells were permeabilized, and the washed membranes were incubated on ice for 10 min with increasing concentrations of trypsin (0, 100, 200, and 400 ng/ μ l). Yor1p fragments were immunoprecipitated and visualized by SDS-PAGE and PhosphorImage analysis. (D) Packaging of Yor1p into COPII vesicles was examined by an in vitro vesicle budding assay from cells that had been radiolabeled with [³⁵S]methionine/cysteine at 37°C, permeabilized and incubated with COPII proteins in the presence of either GTP (+) or GDP (-). HA-tagged Yor1p was immunoprecipitated and analyzed by SDS-PAGE and PhosphorImage analysis. The percentage of Yor1p found in the vesicle fraction was quantified relative to the total Yor1p (T) present in the donor membranes. An independent cargo protein, Vph1p, was also examined to indicate efficient generation of vesicles in the in vitro assay.

was slightly stabilized, equivalent to the level of stabilization seen in the *sel1Δ* strain (Figure 6B). These data suggest that Yor1p- Δ F accesses both the ERAD-L and ERAD-C pathways, unlike another misfolded ABC transporter, Ste6p* (Huyer *et al.*, 2004) but similar to the unrelated amino acid transporter Gap1p (Kota *et al.*, 2007).

Stabilization of Yor1p- Δ F in a *ubc7Δ* Mutant Does Not Permit Packaging into COPII Vesicles

We next investigated a possible mechanism by which Yor1p- Δ F might be prevented from entering into COPII vesicles: misfolded or aberrant proteins may be disposed of by ERAD so rapidly that they do not have time to engage the ER export machinery. Moreover, either ubiquitination itself, or simple engagement of the ERAD machinery may mask ER

export motifs that would otherwise drive forward transport. We tested this hypothesis by using the *ubc7Δ* mutant to block the ER ubiquitination of Yor1p- Δ F and by asking whether this stabilization could rescue uptake into COPII vesicles (Figure 7). Stabilization of Yor1p- Δ F did not result in improved folding of Yor1p- Δ F, because newly synthesized Yor1p- Δ F remained highly susceptible to trypsin cleavage in both wild-type and *ubc7Δ* cells (Figure 7A). Similarly, the ability of Yor1p- Δ F to form transmembrane domain cross-links was not restored in a *ubc7Δ* strain (Figure 7B). We next tested capture of this stabilized pool of Yor1p- Δ F into COPII vesicles. Loss of *UBC7* did not result in uptake of Yor1p- Δ F into COPII vesicles; the vacuolar protein Vph1p served as a positive control to demonstrate efficient vesicle formation (Figure 7C). These data suggest that neither the ubiquitina-

Figure 6. Yor1p-ΔF uses multiple ERAD pathways. HA-tagged Yor1p or Yor1p-ΔF was expressed in either a wild-type strain or strains deleted for a variety of ERAD machineries as indicated. Cells were pulse-labeled for 5 min with [³⁵S]methionine/cysteine and chased for the times indicated. Yor1p was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and PhosphorImage analysis. The results shown are the average of three independent experiments.

tion of Yor1p-ΔF nor the engagement of ERAD components downstream of Ubc7p is responsible for the ER retention of Yor1p-ΔF.

It is possible that the *in vitro* vesicle budding assay we use to directly monitor egress from the ER is not sensitive enough to detect a very small increase in the budding efficiency of stabilized Yor1p-ΔF. We therefore used a phenotypic growth assay to probe the function of Yor1p at the plasma membrane under conditions that stabilized Yor1p-ΔF *in vivo*. Consistent with the accumulation of Yor1p-ΔF in the ER, cells that express Yor1p-ΔF as the sole copy of Yor1p are oligomycin sensitive (Katzmann *et al.*, 1999). Strains disrupted for either *YOR1* alone or *YOR1* plus *UBC7* were transformed with plasmids containing wild-type Yor1p or Yor1p-ΔF, with an empty vector serving as a negative control. Strains bearing the empty vector were completely sensitive to oligomycin, whereas the presence of plasmid-borne Yor1p conferred oligomycin resistance (Figure 7D). As expected, Yor1p-ΔF expressed in *yor1Δ* cells was unable to confer oligomycin resistance, and this phenotype was also observed in the *yor1Δ ubc7Δ* double mutant, suggesting that stabilization of Yor1p-ΔF cannot rescue *in vivo* function. We observed a similar phenotype, i.e., no rescue of Yor1p-ΔF function, in a *yor1Δ sel1Δ* double mutant (Pagant, unpublished data).

Stabilization of Yor1p-ΔF in *hsp40* Mutants Rescues Assembly but Does Not Permit Uptake into COPII Vesicles

To investigate whether blocking the ERAD pathway upstream of Ubc7p could lead to export of Yor1p-ΔF from the ER, we analyzed the intracellular fate of Yor1p-ΔF in cells mutated for the ER localized Hsp40s, a class of chaperones known to play roles in protein folding and degradation. Ydj1p is the yeast homologue of Hdj-2, which has been shown to be involved in the ER quality control of mammalian CFTR by assisting in polyubiquitination before degradation

(Younger *et al.*, 2004). However, a second yeast ER-localized Hsp40, Hlj1p, is functionally redundant with Ydj1p in the degradation of human CFTR expressed in yeast cells (Youker *et al.*, 2004). We therefore analyzed the biogenesis of Yor1p in the temperature-sensitive double mutant *hlj1Δ ydj1-151*. Pulse-chase experiments at restrictive temperature showed that Yor1p-ΔF was stabilized in the absence of Hsp40 function in the ER membrane (Figure 8A), consistent with the model that ER-localized Hsp40s divert misfolded proteins for proteosomal degradation. Maturation of the cell wall protein, Gas1p, was identical in wild-type and *hlj1Δ ydj1-151* cells, demonstrating that loss of chaperone function does not lead to general defects in secretory protein biogenesis. Interestingly, the trypsin sensitivity of Yor1p-ΔF synthesized at restrictive temperature in the *hlj1Δ ydj1-151* strain more closely resembled that of the wild-type protein, suggesting that cytoplasmic Hsp40s participate in the unfolding of Yor1p-ΔF before degradation (Figure 8B). However, despite the stabilization and at least partial refolding of Yor1p-ΔF in the *hlj1Δ ydj1-151* mutant, this mutant protein was not packaged into COPII vesicles in an *in vitro* vesicle budding assay (Figure 8C). We used cysteine cross-linking to probe the assembly of the membrane domains of Yor1p-ΔF in the Hsp40 mutants, which revealed that despite the conversion of Yor1p-ΔF to a trypsin-resistant form, the TMDs did not show a wild-type configuration (Figure 8D). Together, these data suggest that Hsp40-containing chaperone systems actively unfold the cytoplasmic domains of Yor1p before degradation without affecting the arrangement of TMDs, and that even after stabilization of aberrant Yor1p, the ER quality control checkpoint is still enforced.

DISCUSSION

Quality control of protein biogenesis within the secretory pathway is a fundamentally important process; the impaired

Figure 7. Biogenesis and trafficking of Yor1p-ΔF in a *ubc7Δ* mutant strain. (A) Permeabilized cells from wild type and *ubc7Δ* strains expressing HA-tagged Yor1p or Yor1p-ΔF were used in an in vitro vesicle budding assay. Total membranes (T) and budded vesicles were collected from reactions that contained either GTP (+) or GDP (−). Yor1p and Vph1 packaging into vesicles was quantified by immunoprecipitation followed by SDS-PAGE and PhosphorImage analysis. (B) Radiolabeled permeabilized cells from a wild-type strain and a *ubc7Δ* strain containing HA-tagged Yor1p or Yor1p-ΔF were incubated on ice for 10 min with increasing concentrations of trypsin (0, 100, 200, and 400 ng/μl). Yor1p fragments were immunoprecipitated from cell lysates and analyzed by SDS-PAGE and PhosphorImage analysis. (C) Whole cell lysates from wild-type or *ubc7Δ* strains expressing cysteine-substituted forms of Yor1p or Yor1p-ΔF were cross-linked with increasing concentrations of M8M as described in Figure 1E. Stabilization of Yor1p-ΔF in the *ubc7Δ* strain did not result in the appearance of the wild-type cross-linked species. (D) Yor1p function at the plasma membrane was tested phenotypically by assessing growth in the presence of oligomycin. Strains bearing deletions in *YOR1* or *YOR1* and *UBC7* were transformed with the plasmids indicated and serial dilutions spotted onto YPEG or YPEG supplemented with oligomycin.

folding and trafficking of proteins is the proximal cause of a number of human diseases. We sought to better understand the interplay between protein folding and forward transport through the secretory pathway by examining how the stability of a misfolded protein within the ER influences uptake into ER-derived COPII vesicles. We explored these pathways by investigating the biogenesis of the yeast ABC transporter Yor1p, a plasma membrane-localized pleiotropic drug pump that is homologous to CFTR with respect to domain arrangement and topology. An aberrant form of Yor1p with an analogous phenylalanine deletion within NBD1, Yor1p-ΔF670, is retained in the ER and degraded by ERAD (Katzmann *et al.*, 1999). We used several in vitro assays to demonstrate that Yor1p-ΔF fails to enter into COPII vesicles and that this aberrant protein is improperly assembled.

We used limited proteolysis to demonstrate that Yor1p-ΔF showed enhanced susceptibility to proteolytic attack, consistent with a partially folded or conformationally destabilized structure similar to that seen for the ΔF form of CFTR (Du *et al.*, 2005). Biochemical and structural analyses of CFTR NBD1 suggest that the ΔF508 mutation does not dramatically alter the fold of the isolated domain but that it may instead disrupt surface topology. This in turn may perturb

an interaction between NBD1 and MSD1, indirectly affecting the interactions between transmembrane helices (Lewis *et al.*, 2005; Thibodeau *et al.*, 2005). Furthermore, F508 in CFTR also contributes to the posttranslational folding of NBD2, likely through interdomain interactions (Du *et al.*, 2005). Because the epitope tag with which we detected Yor1p is located at the C terminus of the protein, trypsinolysis specifically probes the conformational state of the second half of the protein. Thus, the stable ~65-kDa proteolytic fragment observed for wild-type Yor1p likely corresponds to a cleavage event between TM8 and TM9, with the ΔF670 mutation likely destabilizing NBD2, similar to the situation observed for CFTR. We also used cysteine substitution and cross-linking analysis to probe the arrangement of transmembrane domains in Yor1p. The inability of Yor1p-ΔF to form cross-linkable interactions between membrane spanning domains suggests that, like CFTR, multiple interdomain interactions are impaired by this misfolding lesion (Cui *et al.*, 2007).

Together, these in vitro assays suggest that Yor1p-ΔF presents multiple assembly defects: improperly arranged transmembrane segments as well as a destabilized cytoplasmic domain (NBD2). The presence of multiple misfolding lesions is consistent with its redundant use of at least two disposal

Figure 8. Yor1p-ΔF is stabilized in a *hsp40* mutant strain. (A) Wild-type and *hsp40* (*hlj1Δ/ymj1-151*) strains expressing HA-tagged Yor1p or Yor1p-ΔF were grown at 30°C and pretreated for 25 min at 37°C. Then, strains were pulse-labeled for 5 min with [³⁵S]methionine/cysteine at 37°C and chased at 37°C for the times indicated. Yor1p and Gas1p were immunoprecipitated from cell lysates and analyzed by SDS-PAGE and PhosphorImage analysis. The results shown are the average of three independent experiments. (B) Permeabilized cells from wild-type and *hsp40* (*hlj1Δ/ymj1-151*) strains that had been pretreated at 37°C before labeling were used in an in vitro vesicle budding assay. Total membranes (T) and budded vesicles were collected from reactions that contained either GTP (+) or GDP (–). Yor1p and Vph1 packaging into vesicles was quantified by immunoprecipitation followed by SDS-PAGE and PhosphorImage analysis. (C) Radiolabeled permeabilized cells from wild-type and *hsp40* (*hlj1Δ/ymj1-151*) strains expressing HA-tagged Yor1p or Yor1p-ΔF at restrictive temperature were incubated on ice for 10 min with increasing concentrations of trypsin (0, 100, 200, and 400 ng/μl). Fragments were immunoprecipitated from cell lysates and analyzed by SDS-PAGE and PhosphorImage analysis. (D) Radiolabeled permeabilized cells from wild-type and *hsp40* (*hlj1Δ/ymj1-151*) strains expressing cysteine-substituted forms of Yor1p or Yor1p-ΔF at restrictive temperature were subjected to cross-linking with M8M before solubilization and immunoprecipitation. Stabilization of Yor1p-ΔF in the *hsp40* mutant did not result in the appearance of the typical cross-linked species.

pathways. Both the Hrd1p-dependent pathway, which likely disposes of proteins with membrane-localized lesions in addition to ERAD-L clients (Carvalho *et al.*, 2006), and the Doa10p-dependent pathway, which degrades ERAD-C substrates, can divert Yor1p-ΔF for degradation; only when both pathways are disabled is Yor1p-ΔF stabilized. Because this stabilization is only partial, the possibility remains that additional machinery also contributes in vivo to the efficient destruction of aberrant Yor1p. The specific misfolding lesions detected by each of these degradation systems remain to be defined, but they likely result from the exposure of multiple domain interfaces that are usually buried in the native protein. Our evidence that both NBD2 and the transmembrane domains of Yor1p-ΔF are improperly assembled

suggests that both cytosolic and intramembrane lesions are potential signals for degradation by the ERAD-C and ERAD-L/M pathways, respectively. The dual disposal mechanism we see for Yor1p-ΔF is similar to that used by the amino acid permease, Gap1p, which is rapidly degraded in the absence of its chaperone, Shr3p (Kota *et al.*, 2007). Again, the precise nature of the conformational abnormality presented by Gap1p in the absence of Shr3p is not known, but based on our data for Yor1p-ΔF, it seems likely that Gap1p must present multiple folding defects to access both the ERAD-C and ERAD-L/M pathways.

Although misfolded Gap1p and Yor1p-ΔF share the same redundant degradation pathways, these two proteins differ in their ability to be rescued by inhibiting degradation.

Blocking ERAD of Yor1p- Δ F did not permit capture into COPII vesicles, nor did it restore oligomycin resistance. However, when misfolded Gap1p was stabilized by introducing ERAD mutations into an *shr3 Δ* strain, partial recovery of amino acid transport function was observed (Kota *et al.*, 2007). This difference in protein recovery may stem from the nature of the specific folding defects associated with each individual protein. Aberrant Gap1p produced in the absence of its chaperone may represent a mixed population of folding intermediates, some of which can attain a folding state compatible with COPII engagement if the degradation pathways are impaired. Conversely, the specific genetic lesion that causes misfolding of Yor1p- Δ F may render it incapable of proper assembly even given prolonged residence time in the ER. Thus, the ability of an aberrant protein to be rescued by impeding its destruction seems likely to be dependent on the specific nature of the misfolding defect, which will in turn influence the folding, degradative and trafficking pathways that each specific protein can access. Indeed, different misfolding variants of a single protein may behave distinctly; a variety of transthyretin mutants showed distinct secretion phenotypes that were not entirely correlated with the degree of global protein stability, even in the absence of stabilization through ERAD inhibition (Sekijima *et al.*, 2005). Furthermore, even when considering a single protein, different mechanisms of stabilization may result in different outcomes. Attempts to rescue CFTR- Δ F by inhibiting its destruction have met with mixed results; early experiments using proteasome inhibitors did not result in rescue of CFTR- Δ F (Kopito, 1999) but more recent experiments in lung epithelial cells suggest that inhibition of either the proteasome or the protein extraction apparatus, p97/VCP/Cdc48p, can rescue delivery of CFTR- Δ F to the plasma membrane (Vij *et al.*, 2006). Indeed, the ability for a misfolded protein to be rescued by simple stabilization may also depend on the cell type, with the specific complement of cellular chaperones associated with a given tissue likely to play an important role. This seems particularly true for polytopic membrane proteins such as Yor1p and CFTR, which are subject to complex quality control processes involving multiple chaperone machineries (Younger *et al.*, 2006).

One additional mechanism that may contribute to the differing abilities of misfolded proteins to be rescued by inhibition of ERAD is the presentation of an appropriate ER export signal. Such signals tend to be small peptides with limited structural features that may be obscured or improperly presented in the context of a misfolded protein. The failure to present an appropriate export motif could account for the distinct intracellular fates of misfolded variants of transthyretin; the different mutants may display or obscure their ER export signals independently of the thermodynamics of the particular protein fold (Sekijima *et al.*, 2005). In membrane proteins, which interact directly with Sec24p, the presentation of an appropriate export motif in the context of a misfolded protein is likely to be a more complicated affair. For CFTR, the diacidic ER export signal is located in the same domain as the misfolding lesion, suggesting that the structural destabilization induced by the Δ F508 lesion may directly impair the presentation of the signal to Sec24p (Wang *et al.*, 2004). In Yor1p, the diacidic export motif is located in a different cytosolic domain from the misfolding lesion, suggesting the misfolding event has a more indirect effect on protein traffic. One key question that remains unanswered from our study is whether the Δ F lesion impacts the interaction between Sec24p and Yor1p directly. We attempted to address this issue by isolating "prebudding com-

plexes" that contain cargo proteins bound to Sar1p/Sec23p/Sec24p and represent an early cargo-capture phase of vesicle biogenesis (Kuehn *et al.*, 1998). However, we were unable to reproducibly detect wild-type Yor1p in these complexes, perhaps due to the difficulty in solubilizing a protein with so many transmembrane domains (Kung and Miller, unpublished data). Alternative approaches such as reconstitution of Yor1p into detergent micelles or synthetic liposomes may allow us to more directly assess the binding between Yor1p and Sec24p and ask how the Δ F lesion impacts this interaction. In addition to directly impeding interaction with Sec24p, another mechanism by which a misfolding lesion may impact forward traffic is by indirectly preventing capture into vesicles through the recruitment of cytoplasmic chaperones at early stages of protein synthesis. We have demonstrated that cytoplasmic HSP90s play a role in productive biogenesis of wild-type Yor1p. One can imagine a model whereby correctly folded Yor1p no longer interacts with HSP90, release of which in turn exposes the Sec24p interaction motif. Thus, action of Sec24p would be sterically inhibited by HSP90, and a molecular handoff would occur between the folding chaperones and the forward transport machinery. Detailed coimmunoprecipitation experiments, in combination with kinetic analysis of these interactions during Yor1p biogenesis, will be required to fully map the sequential and competitive interactions between the various cytoplasmic chaperone machinery, the ER export apparatus and newly synthesized Yor1p. Furthermore, the action of these chaperones may be very complex: recent evidence suggests that HSP90 can influence CFTR biogenesis in both a positive and negative manner, likely modulated by distinct cochaperones (Wang *et al.*, 2006).

In addition to HSP90s, the ER-localized HSP40s, Ydj1p and Hlj1p, are also candidates for interfering with ER export of Yor1p. Through recruitment of cytoplasmic HSP70s, these chaperones may impair maturation of misfolded proteins either by sequestering the aberrant protein in a distinct ER subdomain or by triggering the degradation pathway. In our experiments, the action of these HSP40s promoted the trypsin-sensitive conformation of Yor1p- Δ F, suggesting an active role in unfolding the cytoplasmic domains before exposure to the ubiquitination and retrotranslocation machinery. A similar role in actively unfolding cytosolic domains of misfolded proteins (both soluble and membrane-bound) before proteasomal degradation has been suggested for the Ssa1p-Ydj1p chaperone complex (Taxis *et al.*, 2003; Park *et al.*, 2007). This action may be required for tightly folded domains that could not be denatured solely by the 19S cap of the proteasome (Taxis *et al.*, 2003). We note that despite some restoration of a more wild-type conformation of the C-terminal domain, Yor1p- Δ F still failed to enter into COPII vesicles in the HSP40 mutant, likely because the arrangement of its transmembrane segments was still aberrant.

In summary, we have developed Yor1p- Δ F as a model yeast misfolded protein, and we have defined some of the chaperones, ER export machinery, and degradation apparatus that engage both the wild-type and aberrant forms of the protein. It is clear from our data that aberrant Yor1p accesses multiple degradation pathways and that stabilization of this misfolded protein by blocking its degradation does not confer transport competence. This observation has important implications for potential treatments for diseases caused by improper ER retention and degradation. A growing body of evidence suggests that different misfolded proteins are handled differently by the multiple processes that comprise ER quality control. It seems likely that, like the multiple pathways of protein degradation from the ER, diverse mecha-

nisms that prevent forward transport are likely to impact the efficiency of ER export in the context of protein misfolding. Such mechanisms may include active ER retention, steric hindrance of interaction with Sec24p, prevention of capture of prebudding complexes into a vesicle proper, or failure to engage additional sorting machinery. Given the wide array of ER export signals and binding sites on Sec24p, it is easy to imagine that cells use a variety of mechanisms to ensure that aberrant proteins are not deployed within the secretory pathway. A deeper understanding of the interplay between the degradative pathways and the ER export machinery will be required to fully elucidate how misfolded proteins are subjected to quality control within the secretory pathway.

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REFERENCES

- Ahner, A., and Brodsky, J. L. (2004). Checkpoints in ER-associated degradation: excuse me, which way to the proteasome? *Trends Cell Biol.* 14, 474–478.
- Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* 77, 895–907.
- Bohen, S. P., and Yamamoto, K. R. (1993). Isolation of Hsp90 mutants by screening for decreased steroid receptor function. *Proc. Natl. Acad. Sci. USA* 90, 11424–11428.
- Borkovich, K. A., Farrelly, F. W., Finkelstein, D. B., Taulien, J., and Lindquist, S. (1989). hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell. Biol.* 9, 3919–3930.
- Bukau, B., Weissman, J., and Horwich, A. (2006). Molecular chaperones and protein quality control. *Cell* 125, 443–451.
- Carvajal, E., van den Hazel, H. B., Cybularz-Kolaczowska, A., Balzi, E., and Goffeau, A. (1997). Molecular and phenotypic characterization of yeast PDR1 mutants that show hyperactive transcription of various ABC multidrug transporter genes. *Mol. Gen. Genet.* 256, 406–415.
- Carvalho, P., Goder, V., and Rapoport, T. A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126, 361–373.
- Chen, E. Y., Bartlett, M. C., Loo, T. W., and Clarke, D. M. (2004). The DeltaF508 mutation disrupts packing of the transmembrane segments of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 279, 39620–39627.
- 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). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 63, 827–834.
- Cross, F. R. (1997). 'Marker swap' plasmids: convenient tools for budding yeast molecular genetics. *Yeast* 13, 647–653.
- Cui, L., Aleksandrov, L., Chang, X. B., Hou, Y. X., He, L., Hegedus, T., Gentzsch, M., Aleksandrov, A., Balch, W. E., and Riordan, J. R. (2007). Domain interdependence in the biosynthetic assembly of CFTR. *J. Mol. Biol.* 365, 981–994.
- Denning, G. M., Ostedgaard, L. S., and Welsh, M. J. (1992). Abnormal localization of cystic fibrosis transmembrane conductance regulator in primary cultures of cystic fibrosis airway epithelia. *J. Cell Biol.* 118, 551–559.
- Drumm, M. L., Wilkinson, D. J., Smit, L. S., Worrell, R. T., Strong, T. V., Frizzell, R. A., Dawson, D. C., and Collins, F. S. (1991). Chloride conductance expressed by delta F508 and other mutant CFTRs in *Xenopus* oocytes. *Science* 254, 1797–1799.
- Du, K., Sharma, M., and Lukacs, G. L. (2005). The DeltaF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat. Struct. Mol. Biol.* 12, 17–25.
- Ellgaard, L., and Helenius, A. (2001). ER quality control: towards an understanding at the molecular level. *Curr. Opin. Cell Biol.* 13, 431–437.
- Epping, E. A., and Moyer-Rowley, W. S. (2002). Identification of interdependent signals required for anterograde traffic of the ATP-binding cassette transporter protein Yor1p. *J. Biol. Chem.* 277, 34860–34869.
- Fliss, A. E., Benzeno, S., Rao, J., and Caplan, A. J. (2000). Control of estrogen receptor ligand binding by Hsp90. *J. Steroid Biochem. Mol. Biol.* 72, 223–230.
- Guldener, U., Heck, S., Fielder, T., Beinbauer, J., and Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24, 2519–2524.
- Huyer, G., Piluek, W. F., Fansler, Z., Kreft, S. G., Hochstrasser, M., Brodsky, J. L., and Michaelis, S. (2004). Distinct machinery is required in *Saccharomyces cerevisiae* for the endoplasmic reticulum-associated degradation of a multi-spanning membrane protein and a soluble luminal protein. *J. Biol. Chem.* 279, 38369–38378.
- Ismail, N., and Ng, D. T. (2006). Have you HRD? Understanding ERAD is DOAble! *Cell* 126, 237–239.
- Katzmann, D. J., Epping, E. A., and Moyer-Rowley, W. S. (1999). Mutational disruption of plasma membrane trafficking of *Saccharomyces cerevisiae* Yor1p, a homologue of mammalian multidrug resistance protein. *Mol. Cell. Biol.* 19, 2998–3009.
- Kincaid, M. M., and Cooper, A. A. (2007). Misfolded proteins traffic from the endoplasmic reticulum (ER) due to ER export signals. *Mol. Biol. Cell* 18, 455–463.
- Kopito, R. R. (1999). Biosynthesis and degradation of CFTR. *Physiol. Rev.* 79, S167–S173.
- Kota, J., Gilstring, C. F., and Ljungdahl, P. O. (2007). Membrane chaperone Shr3p assists in folding amino acid permeases preventing precocious ERAD. *J. Cell Biol.* 176, 617–628.
- Kota, J., and Ljungdahl, P. O. (2005). Specialized membrane-localized chaperones prevent aggregation of polytopic proteins in the ER. *J. Cell Biol.* 168, 79–88.
- Kuehn, M. J., Herrmann, J. M., and Schekman, R. (1998). COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature* 391, 187–190.
- Kuehn, M. J., Schekman, R., and Ljungdahl, P. O. (1996). Amino acid permeases require COPII components and the ER resident membrane protein Shr3p for packaging into transport vesicles in vitro. *J. Cell Biol.* 135, 585–595.
- Lee, M. C., Hamamoto, S., and Schekman, R. (2002). Ceramide biosynthesis is required for the formation of the oligomeric H⁺-ATPase Pma1p in the yeast endoplasmic reticulum. *J. Biol. Chem.* 277, 22395–22401.
- Lee, M. C., Miller, E. A., Goldberg, J., Orci, L., and Schekman, R. (2004). Bi-directional protein transport between the ER and Golgi. *Annu. Rev. Cell Dev. Biol.* 20, 87–123.
- Lewis, H. A. *et al.* (2005). Impact of the deltaF508 mutation in first nucleotide-binding domain of human cystic fibrosis transmembrane conductance regulator on domain folding and structure. *J. Biol. Chem.* 280, 1346–1353.
- Li, C., Ramjeesingh, M., Reyes, E., Jensen, T., Chang, X., Rommens, J. M., and Bear, C. E. (1993). The cystic fibrosis mutation (delta F508) does not influence the chloride channel activity of CFTR. *Nat. Genet.* 3, 311–316.
- Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B., and Riordan, J. R. (1998). Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. *EMBO J.* 17, 6879–6887.
- Miller, E., Antonny, B., Hamamoto, S., and Schekman, R. (2002). Cargo selection into COPII vesicles is driven by the Sec24p subunit. *EMBO J.* 21, 6105–6113.
- Miller, E. A., Beilharz, T. H., Malkus, P. N., Lee, M. C., Hamamoto, S., Orci, L., and Schekman, R. (2003). Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell* 114, 497–509.
- Mossessova, E., Bickford, L. C., and Goldberg, J. (2003). SNARE selectivity of the COPII coat. *Cell* 114, 483–495.
- Otte, S., and Barlowe, C. (2004). Sorting signals can direct receptor-mediated export of soluble proteins into COPII vesicles. *Nat. Cell Biol.* 6, 1189–1194.
- Park, S. H., Bolender, N., Eisele, F., Kostova, Z., Takeuchi, J., Coffino, P., and Wolf, D. H. (2007). The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system. *Mol. Biol. Cell* 18, 153–165.
- Riordan, J. R. *et al.* (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245, 1066–1073.

- Rogers, B., Decottignies, A., Kolaczowski, M., Carvajal, E., Balzi, E., and Goffeau, A. (2001). The pleiotropic drug ABC transporters from *Saccharomyces cerevisiae*. *J. Mol. Microbiol. Biotechnol.* 3, 207–214.
- Schagger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 199, 223–231.
- Sekijima, Y., Wiseman, R. L., Matteson, J., Hammarstrom, P., Miller, S. R., Sawkar, A. R., Balch, W. E., and Kelly, J. W. (2005). The biological and chemical basis for tissue-selective amyloid disease. *Cell* 121, 73–85.
- Sharma, M. *et al.* (2004). Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J. Cell Biol.* 164, 923–933.
- Taxis, C., Hitt, R., Park, S. H., Deak, P. M., Kostova, Z., and Wolf, D. H. (2003). Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. *J. Biol. Chem.* 278, 35903–35913.
- Thibodeau, P. H., Brautigam, C. A., Machius, M., and Thomas, P. J. (2005). Side chain and backbone contributions of Phe508 to CFTR folding. *Nat. Struct. Mol. Biol.* 12, 10–16.
- Vashist, S., Kim, W., Belden, W. J., Spear, E. D., Barlowe, C., and Ng, D. T. (2001). Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. *J. Cell Biol.* 155, 355–368.
- Vij, N., Fang, S., and Zeitlin, P. L. (2006). Selective inhibition of endoplasmic reticulum-associated degradation rescues DeltaF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. *J. Biol. Chem.* 281, 17369–17378.
- Wang, X., Matteson, J., An, Y., Moyer, B., Yoo, J. S., Bannykh, S., Wilson, I. A., Riordan, J. R., and Balch, W. E. (2004). COPII-dependent export of cystic fibrosis transmembrane conductance regulator from the ER uses a di-acidic exit code. *J. Cell Biol.* 167, 65–74.
- Wang, X. *et al.* (2006). Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. *Cell* 127, 803–815.
- Ward, C. L., Omura, S., and Kopito, R. R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 83, 121–127.
- Wuestehube, L. J., and Schekman, R. W. (1992). Reconstitution of transport from endoplasmic reticulum to Golgi complex using endoplasmic reticulum-enriched membrane fraction from yeast. *Methods Enzymol.* 219, 124–136.
- Youker, R. T., Walsh, P., Beilharz, T., Lithgow, T., and Brodsky, J. L. (2004). Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. *Mol. Biol. Cell* 15, 4787–4797.
- Younger, J. M., Chen, L., Ren, H. Y., Rosser, M. F., Turnbull, E. L., Fan, C. Y., Patterson, C., and Cyr, D. M. (2006). Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell* 126, 571–582.
- Younger, J. M., Ren, H. Y., Chen, L., Fan, C. Y., Fields, A., Patterson, C., and Cyr, D. M. (2004). A foldable CFTR(Delta)F508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. *J. Cell Biol.* 167, 1075–1085.
- Zhang, Y., Nijbroek, G., Sullivan, M. L., McCracken, A. A., Watkins, S. C., Michaelis, S., and Brodsky, J. L. (2001). Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. *Mol. Biol. Cell* 12, 1303–1314.