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Biosynthetic mode can determine the mechanism of protein quality control

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ABSTRACT

Proteins trafficking through the endoplasmic reticulum (ER) are topologically diverse. As such, multiple pathways collectively termed ER-associated degradation (ERAD) ensure that protein domains located in the lumen, membrane, and cytosol, are properly folded. The continuous nucleoplasm and cytosol also maintain a network of quality control mechanisms. These center on the Doa10, San1, and Ubr1 ubiquitin ligases. Unlike in the ER, the necessity for multiple pathways here is unclear. With all three factors localized in the nucleus, at least in part, how substrates are individually recognized is unknown. In this study, we show that the mode of biosynthesis can determine the system used for quality control. Targeting and integrating a misfolded protein to the ER membrane makes it an exclusive substrate of Doa10 whereas the soluble form of the same protein makes it a substrate of the San1/Ubr1 E3 system.

1. Introduction

Nascent polypeptides fold into their correct three-dimensional structures as a prerequisite for function. Many must also assemble into complex structures composed of numerous proteins and occasionally nucleic acids. The process can be compromised by genetic mutation, transcriptional and translational errors, cell stress conditions, or stochastic effects caused by the high density of proteins found in all cells. To prevent the accumulation of potentially toxic aberrant proteins, quality control mechanisms are deployed throughout the cell to monitor the folded states of both newly synthesized and existing polypeptides.

Currently, the best understood quality control mechanisms are found in the endoplasmic reticulum. Its needs are complex because newly synthesized proteins must be retained in the ER during maturation to stay in contact with chaperones and folding enzymes. Failure to fold is detected by ERAD pathways and defective molecules are sorted for export from the ER and degraded by the ubiquitin–proteasome system (UPS). In budding yeast, the Hrd1 pathway recognizes folding defects in luminal and transmembrane domains (ERAD-L and ERAD-M, respectively). Membrane proteins bearing cytosolic lesions are recognized by the Doa10 pathway (ERAD-C) [1,2]. Hrd1 and Doa10 are ER membrane localized E3 ubiquitin ligases that organize receptor complexes responsible for substrate recognition, ubiquitination, and membrane extraction [3–5]. The recognition of misfolded proteins in the nucleus and cytosol are understood in less detail but are also composed of multiple pathways. Interestingly, Doa10 recognizes and degrades some misfolded cytosolic and nuclear proteins [6]. Its client range also includes some folded nuclear proteins [7]. The Ubr1 E3 enzyme, best known for recognizing "N-end rule" degrons (degradation signals), also ubiquitinates some misfolded proteins [8–10]. Finally, the San1 E3 enzyme processes both nuclear and cytosolic proteins and may function exclusively in quality control because of its ability to directly bind unfolded proteins [11–13]. These enzymes do not function redundantly for most substrates examined. This is somewhat curious because they are known to function in the same compartment. This raises the key question of what characteristics are used by each for substrate recognition. To better understand how E3 enzymes select their substrates, a membrane-anchored Doa10 substrate was converted to a soluble protein by deleting its signal sequence and transmembrane domain. Surprisingly, the resulting protein no longer uses the Doa10 pathway and becomes entirely dependent on the quality control pathways directed by San1 and Ubr1.

2. Materials and methods

2.1. Plasmids used in this study

Plasmids were constructed using standard cloning protocols [14]. All genes encoding expression constructs were confirmed by nucleotide sequencing. All substrates in this study contain an engineered HA epitope tag at their C-termini. Substrate expression plasmids were constructed by placing coding sequences under the control of the strong constitutive *TDH3* (encoding glyceralde-

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Table 1
Primers used in this study.

Primer	Construct	Sequence (5′ → 3′)
RP205	pRP6	GTCAGGATCCATGATACCCGATATAAGTAGAGGC
RP206	pRP6	TTATCTAGATTATTTATCGTCATCGTCCTTGTAATCTTCACTATGCGTTATAACCATTG
RP12	pRP22	AACAATGGTTATAACGCATAGTGAATACCCATATGATGTTCCAGATTACGCTTCTA

hyde-3-phosphate dehydrogenase) in yeast centromeric vectors. Oligonucleotide primers used in plasmid construction can be found in Table 1.

pRP12 and pRP80: pSK138 (Ng plasmid collection) digested with *Bam*HI and *Xba*I to release the WS-HA open reading frame. For generating pRP12, the fragment was ligated into pDN420, a pRS313- based plasmid containing the *TDH3* promoter and the *ACT1* terminator (Ng plasmid collection). WS-HA fragment along with promoter and terminator was released from pRP12 by digesting it with SalI and SacII. Released fragment was ligated into a pRS315- based plasmid, generating pRP80.

pRP6 and pRP22: Encodes Ste6*Cp-HA driven by *TDH3* promoter. A 750-bp fragment containing the Ste6*Cp-FLAG was amplified using RP205/RP206 primers and pSK138 as the template. The fragment was cloned into the pYes2.1 vector (Invitrogen, Carlsbad, CA) to generate pRP6. Ste6*Cp-FLAG fragment was released from pRP6 by digesting the plasmid with *Bam*HI and *Xba*I. Digested fragment was ligated into pDN420, generating pRP10. For generating pRP22, pRP10 FLAG-tag was replaced with HA-tag by site-directed mutagenesis using primer RP12 [15].

YEp105: YEp105 (pCUP1-mycUb) contains a c-*myc*-tagged ubiquitin allele expressed from the *CUP1* promoter. YEp105 was provided by Mark Hochstrasser (Yale University) [16].

2.2. Strains and antibodies

Saccharomyces cerevisiae strains used in this study are described in Table 2. Anti-HA monoclonal antibody (HA.11) and anti-myc monoclonal antibody (9E10 c-myc) were purchased from Covance Research Products (Princeton, NJ). Anti-Kar2p and anti-Sec61p antibodies were provided by Peter Walter (University of California, San Francisco, CA). Anti-HA affinity matrix was purchased from Roche (Nutley, NJ).

2.3. Metabolic pulse-chase assay

Cells were grown in synthetic complete media (SC) lacking methionine, cysteine, and components for plasmid selection where applicable. 3.0 OD600 units of cells were labeled with 82.5 μCi of [^{35}S]-methionine/cysteine (EasyTag TM EXPRESS ^{35}S, Perkin Elmer, Waltham, MA) and chased with excess cold amino acids for times indicated. Protein immunoprecipitation and resolution by SDS–PAGE was carried as described [17]. Gels were exposed to phophor screens for 24–48 h and scanned and quantified using the Typhoon TM phosphorimager and ImageQuantTM TL software (GE Healthcare Life Sciences, Uppsala, Sweden). All data plotted reflect three independent experiments with the SD of the mean indicated.

2.4. Cycloheximide chase assay and Western blotting

Cells were grown to mid-log phase in synthetic media. Cessation of protein synthesis was initiated by adding cycloheximide to 200 μg/ml to begin the chase. At each time point, the chase was terminated by transferring an aliquot of cells into 1 ml ice cold 10% trichloroacetic acid (TCA). Detergent lysates were prepared by mechanical cell disruption and TCA precipitation as described previously [17]. Crude cell lysates were separated by SDS–PAGE and transferred to nitrocellulose. The membrane was blocked for 30 min at 37 °C (5% non-fat milk in PBST [phosphate buffered saline containing 0.1% Triton X-100]) before incubation with the primary antibody in the same buffer (2 h at room temperature or overnight at 4 °C). The membrane was subsequently washed with PBST twice for 13 min at RT and probed with secondary antibody at a dilution of 1:10,000–1:20.000 (HRP-conjugated -mouse IgG [Pierce Biotechnology] or HRP-conjugated -rabbit IgG [Jackson ImmunoResearch Laboratories, Inc.]) in PBST with 0.5% non-fat milk for 1 h at RT. Membrane was washed thrice with PBST buffer

Table 2
Strains used in this study.

Strain	Genotype	Source
W303	*MAT***a**, *leu2-3,112*, *his3-11*, *trp1-1*, *ura3-1*, *can1-100*, *ade2-1*	P. Walter (UCSF)
SMY723	*MATα*, *cdc48-3*, W303 background	T. Rapoport (Harvard)
RPY65	*MATα*, pRP6, W303 background	This study
RPY69	*MATα*, *cdc48-3*, pRP6, W303 background	This study
RPY145	*MAT***a**, *pRP22*, W303 background	This study
RPY151	*MAT***a**, *ubc7::KANMX*, *pRP12*, W303 background	This study
RPY152	*MAT***a**, *doa10::KANMX*, *pRP12*, W303 background	This study
RPY151a	*MAT***a**, *pRP12*, W303 background	This study
RPY366	*MAT***a**, *san1::KANMX*, pRP22, W303 background	This study
RPY367	*MAT***a**, *ssa1::KANMX*, *ssa2::KANMX*, pRP22, W303 background	This study
RPY368	*MAT***a**, *ydj1::KANMX*, pRP22, W303 background	This study
RPY369	*MAT***a**, *doa10::KANMX*, pRP22, W303 background	This study
RPY531	*MAT***a**, *ubr1::KANMX*, pRP22, W303 background	This study
RPY532	*MAT***a**, *san1::KANMX*, *ubr1::KANMX*, pRP22, W303 background	This study
RPY540	*MAT***a**, *pdr5::KANMX*, pRP12, W303 background	This study
RPY541	*MAT***a**, *pdr5::KANMX*, pRP22, W303 background	This study
RPY542	*MAT***a**, *ubc7::KANMX*, *pRP222*, W303 background	This study
RPY560	*MAT***a**, *sse1::KANMX*, *pRP22*, *W303 background*	This study
RPY640	*MAT***a**, *pRP22* and YEp105 W303 background	This study
RPY641	*MAT***a**, *san1::KANMX*, pRP22 and YEp105 W303 background	This study
RPY642	*MAT***a**, *ubr1::KANMX*, pRP22 and YEp105 W303 background	This study
RPY643	*MAT***a**, *san1::KANMX*, *ubr1::KANMX*, pRP22 and YEp105 W303 background	This study

for 10 min at RT. The membrane was incubated with HRP substrate (SuperSignal West Pico substrate, Pierce Biotechnology, Rockford, IL) for 2–5 min and signal was visualized. If required, membrane was stripped by incubating in 100 mM glycine, pH 2.5 at 55–60 °C for 30 min followed by another round of protein detection.

2.5. Alkali extraction assay

Cell pellets were resuspended in 1.2 ml of ACE buffer (10 mM potassium phosphate, pH 7.0 containing 1 mM PMSF and protease inhibitor cocktail [PIC, Sigma–Aldrich, St. Louis, MO]) and mechanically disrupted on a vortex mixer using 0.5 mm zirconium beads. Cell debris and nuclei were pelleted down by low speed centrifugation (at 800*g* for 5 min at 4 °C). 750 μl of cleared lysate was mixed with 750 μl of Na_2CO_3 (200 mM, pH 11.5) and incubated on ice for 30 min. After centrifugation at 100,000*g* at 4 °C for 30 min, the pellet was dissolved in 100 μl of TCA resuspension buffer. Proteins in the supernatant were precipitated by adding TCA. Substrate protein is detected from total (T), pellet (P), and supernatant (S) fractions by using anti-HA antibody. Sec61p and Kar2p serve as controls for integral membrane and soluble proteins, respectively.

2.6. Substrate ubiquitination assay

Cells expressing misfolded proteins and overexpressed ubiquitin were resuspended in 1 ml of 10% TCA chilled on ice. After bead beating, precipitated proteins were collected by centrifugations at 14,000 rpm for 10 min at 4 °C. The pellet was resuspended in TCA resuspension buffer (3% SDS, 100 mM Tris–base, 3 mM DTT). Relative substrate levels were measured by immunoblotting. Lysates were mixed with 700 l of IPS II (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, and 1% Triton X-100), 6 l of protease inhibitor cocktail (Roche, Nutley, NJ), 6 l of 100 mM PMSF, 40 l of anti-HA affinity matrix and 10 mM NEM (Sigma–Aldrich). Substrate proteins were immunoprecipitated after normalization using anti-HA antibody, separated by SDS–PAGE, and transferred onto nitrocellulose. Total substrate proteins were detected using anti-HA antibody and ubiquitinated substrates were detected by using anti-Myc antibody.

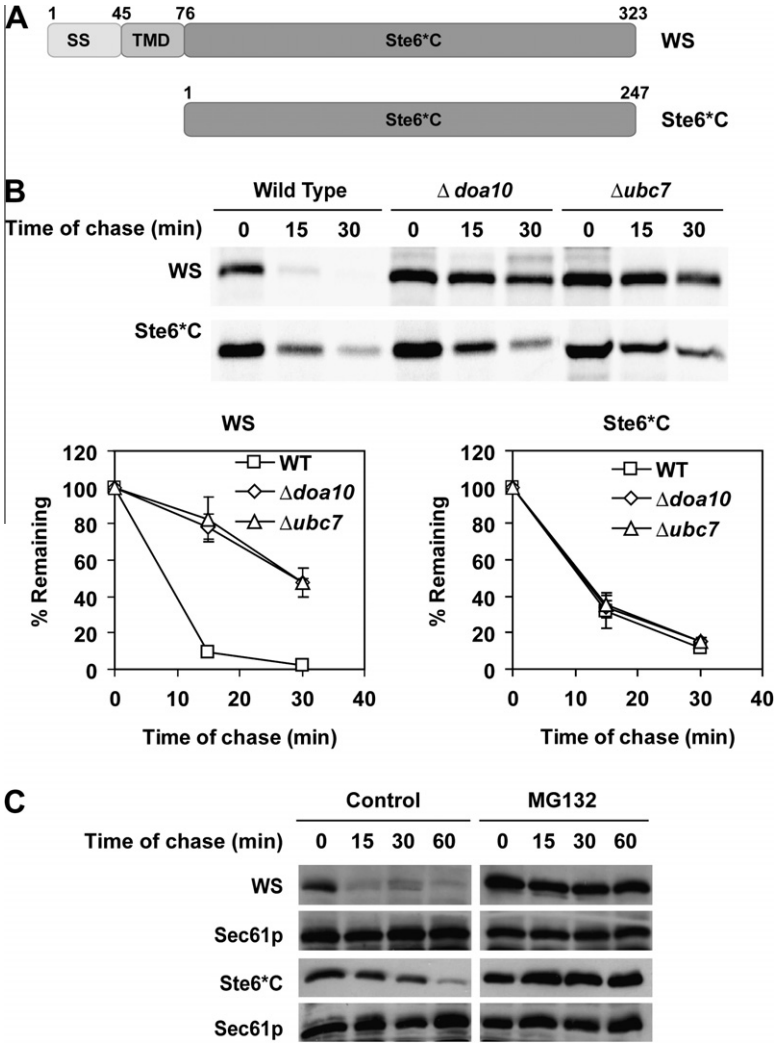

Fig. 1. Untethering the WS Doa10 determinant from the ER membrane changes its quality control mechanism. (A) Schematic representation of WS and Ste6*C substrate proteins. "ss" denotes the cleaved Kar2 signal sequence and "TMD" denotes the transmembrane domain from Wsc1. Each number above the diagrams indicates the amino acid position. (B) Wild type, Δ*doa10* and Δ*ubc7* strains expressing WS and Ste6*C were pulse labeled for 5 min with [^{35}S]-methionine/cyteine followed by cold chase for indicated times. Proteins were immunoprecipitated and resolved by SDS–PAGE and quantified using a phosphorimager. Representative phosphor screen scans are shown. Error bars represent the SD of three independent experiments. (C) Cycloheximide decay experiments were performed using drug-sensitized Δ*pdr5* cells in the absence and presence of MG132 (20 μM). Cell lysates were prepared and proteins resolved by SDS–PAGE. WS and Ste6*C substrates were detected by anti-HA antibody. The blot was stripped and re-probed with anti-Sec61 antiserum as a loading control.

2.7. Indirect immunofluorescence microscopy

Indirect immunofluorescence was performed as described previously [13] with minor modifications. Briefly, cells were fixed with 3.7% formaldehyde at 30 °C for 90 min and spheroplasted by Zymolyase digestion (1 mg/ml zymolase 20T [US biological, Marblehead, MA], 0.1 M potassium phosphate, pH 7.5, 1.4 M sorbitol). Spherolplasts were applied to each well of a poly-L-lysine-coated slide for 10 min and washed. Slides were immersed in methanol for 6 min and in acetone for 30 s at −20 °C. Each well was blocked with PBST containing 5% bovine serum albumin. Primary antibodies and secondary antibodies were incubated in this buffer incubated for 90 min each. Slides were washed twice with PBS after each application. Primary antibodies HA.11 mAb (Covance, Princeton, NJ) and polyclonal anti-Kar2p were diluted to 1:200 and 1:500, respectively. Kar2p is an ER resident protein and an established marker for the ER and nuclear envelope [18]. Secondary antibodies Alexa Fluor 488 goat anti-mouse and Alexa Fluor 596 goat anti-rabbit were diluted to 1:500. Nuclei were visualized by DAPI (4′,6′-diamidino-2-phenylindole) staining. Samples were examined by confocal microscopy using Axio Imager.M1 microscope with 100× 1.4 NA oil Plan-Aprochromat objective (Carl Zeiss MicroImaging, Inc., Oberkochen, Germany). Images were archived by LSM Image Browser and Adobe Photoshop.

3. Results

Ste6p is a multi-spanning transmembrane protein that functions to transport the a-factor pheromone across the plasma membrane [19]. A mutant version called Ste6-166p (also known as Ste6*) introduces a termination codon near the C-terminus resulting in a truncated, misfolded form retained in the ER and degraded by ERAD [20]. We previously reported that the misfolded C-terminal domain is the recognition determinant of E3 enzyme Doa10. To understand the minimum content required for substrate recognition, we constructed two new variants called WS and Ste6*C. WS contains an N-terminal cleaved signal sequence from Kar2p, the transmembrane domain from the single-span Wsc1 protein, and cytosolic domain from Ste6-166p (Fig. 1A). The Wsc1 transmembrane was chosen because it lacks ERAD determinants [21]. Ste6*C contains only the cytosolic domain of Ste6-166p (Fig. 1A). Lacking any ER targeting segments, Ste6*C is predicted to be a cytosolic protein. To determine if these proteins are targeted for clearance by cellular quality control mechanisms, metabolic pulse-chase

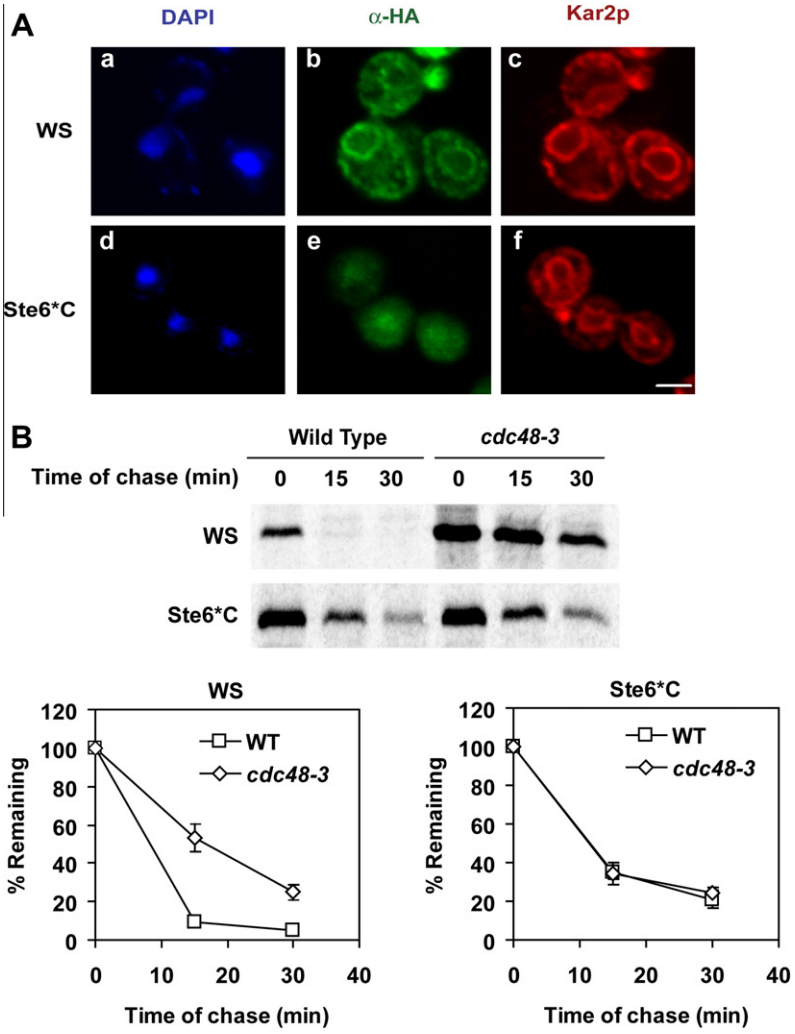

Fig. 2. WS and Ste6*C localization and degradation. (A) Wild type cells expressing substrate proteins were fixed, stained, and visualized as described in Section 2. Substrate proteins were detected using anti-HA antibody in the green channel. Anti-Kar2p was used to stain nuclear envelope/ER membranes and visualized in the red channel. Nuclear DNA is stained by DAPI. Images represent individual optical sections using confocal microscopy. Scale bar, 2 μm. (B) Pulse chase analysis was performed in wild type and *cdc48-3*^{ts} cells expressing WS and Ste6*C as described in Fig. 1B, except stains were grown to log phase at 23 °C and shifted to 37 °C 30 min before labeling.

was performed in wild type, Δ*ubc7* (general ERAD), and Δ*doa10* (ERAD-C) strains expressing them. As shown in Fig. 1B, WS is rapidly degraded in wild type cells and strongly stabilized in Δ*ubc7* and Δ*doa10* strains. This experiment shows that the anchored cytosolic domain is sufficient for degradation by the ERAD-C pathway. By contrast, rapid Ste6*C degradation is observed in the three strains. Despite containing the entire cytosolic domain of Ste6-166p, this result shows that a distinct pathway degrades Ste6*C. To determine the pathway used, we treated sensitized cells expressing the variants with the proteasome inhibitor MG132 [22]. Complete stabilization by MG132 confirms that Ste6*C uses a UPS pathway independent from ERAD (Fig. 1C).

Indirect immunofluorescence experiments were performed to determine the intracellular localization of WS and Ste6*C. As expected, WS is localized to ER membranes and an alkali extraction assay confirms it as a bona fide integral membrane protein (Fig. 2A, compare panels b and c; Fig. S1). Ste6*C, lacking hydrophobic segments, localized diffusely in the cytosol and nucleus (Fig. 2, compare panels d, e, and f). Consistent with these data, WS degradation requires the function of the AAA-ATPase Cdc48p, a core ERAD factor for substrate extraction from membranes (Fig. 2B) [23–26]. Ste6*C, on the other hand, degraded efficiently in the absence of normal Cdc48p function. This pattern is reminiscent of misfolded cytosolic proteins degraded by the CytoQC pathway [6,13,27,28]. We wondered whether Ste6*C is indeed a substrate of CytoQC because Doa10 was reported as one of the E3 enzymes involved in the pathway [6].

We next tested Ste6*C degradation in strains lacking the other CytoQC E3 enzymes San1 and Ubr1. As shown in Fig. 3A, Ste6*C degradation is partly defective in either single mutant, but to the greatest extent in Δ*ubr1*. Eliminating both genes virtually halts Ste6*C degradation. We next measured polyubiquitination of Ste6*C, a requirement for degradation in the CytoQC pathway [6,8,13]. For these experiments, we used the Myc-tagged ubiquitin system to facilitate detection [16]. In wild type cells, polyubiquitinated Ste6*C is readily detected despite its rapid degradation (Fig. 3B, WT lane). By contrast, polyubiquitinated forms are reduced in Δ*san1* cells and nearly undetectable in Δ*ubr1* cells, consistent with Ste6*C degradation rates in these strains (Fig. 3B). In the Δ*san1*Δ*ubr1* mutant, polyubiquitinated forms are eliminated. These data show that making the Ste6* ERAD determinant soluble changes its folding surveillance system from Doa10 to the San1/Ubr1 system.

We next determined the chaperone requirements of Ste6*C. Cytosolic Hsp70 encoded by the *SSA* gene family, the Hsp70 regu-

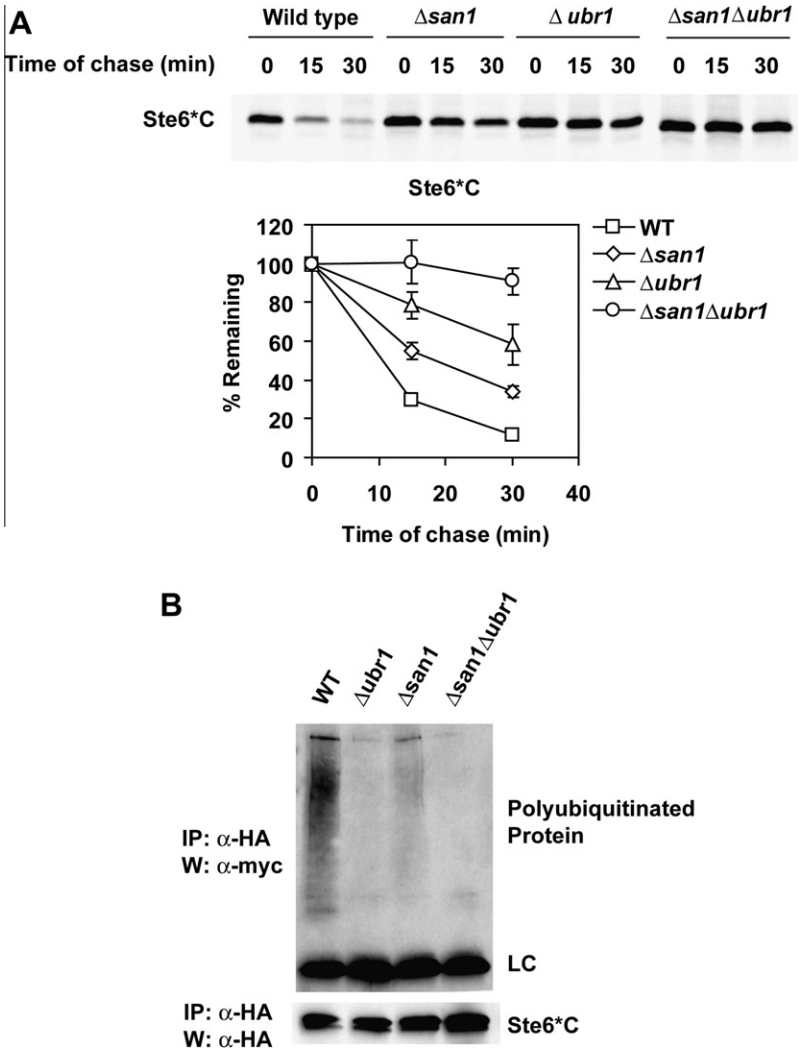

Fig. 3. Ste6*C is a cytosolic quality control substrate. (A) Turnover of Ste6*C in wild type, Δ*san1*, Δ*ubr1*, and Δ*san1*Δ*ubr1* was determined by pulse chase analysis. Cells were pulse-labeled for 5 min and chased for the times indicated at 30 °C. Immunoprecipitated proteins were resolved by SDS–PAGE and quantified using a phosphorimager. (B) Cells expressing Ste6*C were immunoprecipitated from the cell lysates using anti-HA antibody and complexes resolved by SDS–PAGE were transferred to nitrocellulose. Ubiquitinated proteins were detected using anti-myc antibody. Protein amount in wild type, Δ*san1*, Δ*ubr1*, and Δ*san1*Δ*ubr1* was normalized using Lycor system for immunoprecipitation.

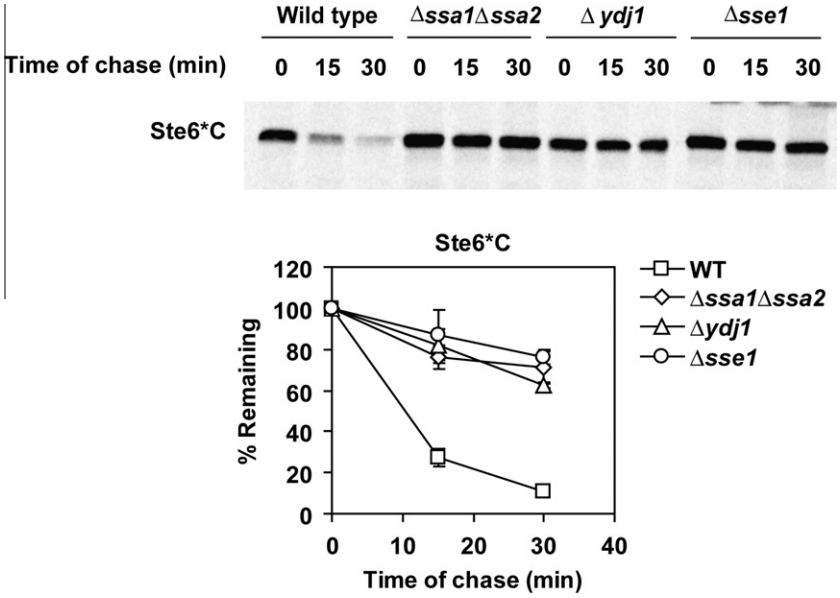

Fig. 4. Degradation of Ste6*C is dependent on Hsp70 chaperone cycle. Turnover of Ste6*C in wild-type, Δ*ssa1*Δ*ssa2*, Δ*ydj1*and Δ*sse1* cells was determined by metabolic pulse-chase analysis as in Fig. 1.

lator and DnaJ class chaperone Ydj1, and Sse1, the Hsp90 cochaperone and Hsp70 nucleotide exchange factor, are required for the quality control of multiple model cytosolic proteins [27,28]. Their role in in Ste6*C degradation was tested in Δ*ssa1*Δ*ssa2*, Δ*ydj1*, and Δ*sse1* strains. Each of the strains is defective in CytoQC but yet viable [13]. As shown in Fig. 4, Ste6*C is strongly stabilized in all CytoQC chaperone mutants. Taken together, these data show that the protein biosynthetic mechanism can determine the quality control system used to monitor folding.

4. Discussion

It is well established that protein quality control systems are composed of multiple pathways, even within the same cellular compartment. In the endoplasmic reticulum, the ERAD-L (luminal), ERAD-M (membrane), and ERAD-C (cytosolic) pathway are deployed in budding yeast to handle the topological diversity of substrates. Similarly, proteins synthesized in the cytosol have at least three pathways to monitor folding [8–13]. Of these, the most enigmatic is the Doa10 pathway because it also serves as the E3 enzyme for ERAD-C [2,7,29]. In addition, its client substrates include folded soluble proteins outside the ER [7]. This includes to transcription factor Matα2. Interestingly, anchoring Matα2 to the ER membrane did not change its requirement for Doa10 [30]. Based on these reports, we expected that performing the converse experiment with the Ste6-166p misfolded determinant would similarly not change the E3 requirement. However, when untethered, the resulting Ste6*C protein degrades independently of Doa10 (Fig. 1). This occurs despite Ste6*C localizing to the nucleus where much of Doa10 resides (Fig. 2). We show that Ste6*C requires the activities of both San1 and Ubr1. This contrasts with the model substrates Δ2GFP and ΔssPrA, which are dependent on San1 almost exclusively [13].

Why does the Ste6*C E3 enzyme requirement depend on the targeting mode when Matα2 does not? The simplest explanation is their different conformational states. The Ste6-166p determinant is misfolded while Matα2 is fully folded. Degrons (degradation signals) on folded proteins like Matα2 are generally discrete and therefore explains the specificity for Doa10p [31]. Misfolded proteins like Ste6-166p on the other hand, display general features like disordered segments and exposed hydrophobic stretches recognized by chaperones involved in the recognition process. With co-translational targeting and membrane insertion of Ste6-166p and WS, the C-terminal determinant is synthesized at the ER membrane where the Doa10 is in close proximity. Ste6*C is synthesized as a cytosolic protein and subject to whatever quality control mechanisms are in effect. In this case, the San1/Ubr1 system efficiently recognizes and degrades Ste6*C. However, the use of this pathway is not simply due to a "first to encounter" mechanism because the Doa10 pathway cannot substitute in its absence (Figs. 2 and 3A).

Together, these data emphasize the interdependence of the Doa10, San1, and Ubr1 pathways to provide comprehensive protein quality control in the cytosol/nucleus. To our knowledge, the Ste6* C-terminal domain is the first substrate that can be ubiquitinated by all three E3 enzymes depending on its mode of biosynthesis. This shows that substrate selectivity can depend on spatial context in addition to sequence and conformational features found in degrons.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.080.

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