RESEARCH ARTICLE

A nuclear ubiquitin-proteasome pathway targets the inner nuclear membrane protein Asi2 for degradation

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ABSTRACT

The nuclear envelope consists of inner and outer nuclear membranes. Whereas the outer membrane is an extension of the endoplasmic reticulum, the inner nuclear membrane (INM) represents a unique membranous environment containing specific proteins. The mechanisms of integral INM protein degradation are unknown. Here, we investigated the turnover of Asi2, an integral INM protein in Saccharomyces cerevisiae. We report that Asi2 is degraded by the proteasome independently of the vacuole and that it exhibited a half-life of ~45 min. Asi2 exhibits enhanced stability in mutants lacking the E2 ubiquitin conjugating enzymes Ubc6 or Ubc7, or the E3 ubiquitin ligase Doa10. Consistent with these data, Asi2 is post-translationally modified by poly-ubiquitylation in a Ubc7- and Doa10-dependent manner. Importantly, Asi2 degradation is significantly reduced in a sts1-2 mutant that fails to accumulate proteasomes in the nucleus, indicating that Asi2 is degraded in the nucleus. Our results reveal a molecular pathway that affects the stability of integral proteins of the inner nuclear membrane and indicate that Asi2 is subject to protein quality control in the nucleus.

KEY WORDS: ERAD, Nuclear membrane, Nuclear proteasome, Protein degradation, Ubiquitylation

INTRODUCTION

The confinement of the genome within the nucleus by the nuclear envelope is a hallmark of eukaryotic cells. The nuclear envelope is composed of two membrane layers, the inner and the outer nuclear membrane, which are connected at the sites of nuclear pore complexes (NPCs) (Hetzer, 2010). The outer nuclear membrane (ONM) is a direct extension of the endoplasmic reticulum (ER) membrane and its protein composition closely resembles that of the ER membrane. In contrast, the inner nuclear membrane (INM) contains many specific proteins that are involved in nuclear processes, such as chromatin organization, control of gene expression and DNA damage repair (Heessen and Fornerod, 2007; Mikhail et al., 2008; Oza et al., 2009; Schober et al., 2009). Although the ONM and INM form a continuous membrane system, the NPCs that assemble at the junction of the two membranes establish a physical barrier limiting diffusion and free exchange of integral membrane proteins between the ER or ONM and INM. Several models have been proposed to mechanistically explain the transport of membrane proteins from the site of their synthesis and membrane insertion at the ER to their final destination in the INM. These include the diffusion and nuclear retention model, nuclear localization signal (NLS)-mediated transport through peripheral channels of the NPC (reviewed in Zuleger et al., 2012) and, more recently, NLS-mediated transport through the central NPC channel (Meinema et al., 2011). In contrast to their nuclear targeting, the fate of the integral membrane proteins once they have reached the INM is less clear. Whether integral INM proteins are subject to turnover, and if so, what degradative pathway is involved, remains an intriguing and unsolved biological question.

Protein degradation is a key regulatory mechanism controlling cellular processes like gene transcription and cell cycle progression, and is also crucial in protein quality control pathways, which protect cells from accumulation of aberrant proteins (Goldberg, 2003). The majority of cellular proteins exhibit characteristic rates of turnover with their various half-lives ranging from a few minutes to a few days (Belle et al., 2006; Ciechanover, 2007; Yen et al., 2008). However, studies in Caenorhabditis elegans and rat brain have revealed that some components of the nuclear membrane – the scaffold nucleoporins embedded in the nuclear pore membrane – are extremely long-lived proteins that appear stable for the entire life-span of a metazoan organism and do not have any significant turnover (D’Angelo et al., 2009; Savas et al., 2012). It remains unclear whether the stability of these proteins is due to the inability of the degradation machinery to gain access to them, or because the components involved in nuclear-envelope-associated protein degradation are missing in the nucleus.

Cells possess two major sites of protein degradation, the lysosome (or its yeast analog the vacuole) and the proteasome, a large multi-catalytic protease complex that degrades poly-ubiquitylated proteins (Ciechanover, 2007). Proteasomal degradation substrates include misfolded or damaged proteins, as well as properly folded proteins whose levels need to be downregulated, such as cell cycle regulators or transcription factors. Protein ubiquitylation is required as a signal for proteasomal targeting and is mediated by three classes of enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (Ubc or E2) and a substrate-specific ubiquitin-protein ligase (E3). Although a large portion of the proteasomes localizes to the nucleus (Enenkel et al., 1998; Peters et al., 1994; Russell et al., 1999) and protein degradation clearly can occur in the nucleus, the best-characterized protein quality control degradation pathways are localized in the cytoplasm (Goldberg, 2003). There are only a limited number of studies providing evidence for degradation-mediated protein quality control in the nucleus (Furth et al., 2011; Gardner et al., 2005; Iwata...
et al., 2009). In particular, the molecular pathways that might govern the turnover of integral membrane proteins of the INM are not known.

Doa10 is an integral membrane E3 ubiquitin ligase that partially localizes to the INM, where it participates in the degradation of a soluble non-membrane-bound nuclear transcription factor Matz2 (Deng and Hochstrasser, 2006). Doa10 also mediates degradation of the mutant kinetochore protein Ndc10-2 (Furth et al., 2011; Ravid et al., 2006). Furthermore, Doa10 has a well-established role as one of the two major ubiquitin ligases facilitating ER-associated degradation (ERAD). ERAD primarily targets damaged or misfolded proteins in the ER membrane and lumen to the proteasomes, but it also regulates the physiological levels of some correctly folded wild-type proteins (Hegde and Ploegh, 2010).

Doa10 functions with E2 enzymes Ubc6 and Ubc7 (Swanson et al., 2001), which have also been found at the INM (Deng and Hochstrasser, 2006). Ubc6 is an integral membrane protein, whereas Ubc7 lacks transmembrane domains and is tethered to the membrane through an interaction with the integral membrane protein Cue1. Interestingly, Ubc6 is itself a substrate for Doa10-dependent degradation (Swanson et al., 2001; Walter et al., 2001). Membrane extraction of ubiquitylated ER membrane protein substrates is mediated by the ATPase Cdc48 protein complex or in some cases by the proteasome itself (Vembar and Brodsky, 2008). Cdc48 is known to enter the nucleus in a cell-cycle-dependent manner (Madedo et al., 1998). Mammalian cells possess TEB4 (also known as MARCHE6) (Kreft et al., 2006), an ortholog of Doa10, and there are also orthologs of Ubc6 and Ubc7 in mammalian cells (Kostova et al., 2007; Vembar and Brodsky, 2008).

In this study, we analyze the degradation of Asi2, an integral INM protein in Saccharomyces cerevisiae. Asi2 is a 33 kDa protein with two membrane-spanning segments and a large 26-kDa N-terminal domain oriented towards the nucleoplasm (Zargari et al., 2007). Asi2 is a negative regulatory component of the amino-acid-induced Ssy1–Ptr3–Ssy5 (SPS) sensing pathway (Forsberg et al., 2001; Ljungdahl and Daignan-Fornier, 2012). In the absence of inducing amino acids, Asi2 functions together with two other INM proteins, Asil and Asis, to prevent the transcription factors Stp1 and Stp2 binding to promoters (Boban et al., 2006; Zargari et al., 2007). We report that Asi2 is ubiquitylated in a Ubc6-, Ubc7- and Doa10-dependent manner and is subsequently targeted for degradation by proteasomes in the nucleus. This study represents the first example of a mechanism for the turnover of an integral component of the INM.

**RESULTS**

### Asi2 exhibits turnover independently of SPS sensor signaling

In order to characterize the degradation of the INM protein Asi2, we first investigated the turnover rate of functional Asi2 proteins carrying N-terminal Myc or HA epitope tags (Zargari et al., 2007). During cycloheximide (0.36 mM) treatment for up to 120 min, to inhibit new protein synthesis, we followed the decrease in protein levels and determined the half-life of Myc-tagged and HA-tagged Asi2 (Fig. 1). Asi2 protein levels were analyzed by immunoblotting with anti-Myc or anti-HA antibodies. Asi2–Myc exhibited a half-life of 43 min (Fig. 1A). A slightly lower degradation rate (half-life, 51 min) was measured for HA-epitope tagged Asi2 (Fig. 1B). Asi2 resolves as two specific protein bands, suggesting that Asi2 is subject to post-translational modification (Zargari et al., 2007); the nature of the modification is currently unknown. Although Asi2 has two potential N-glycosylation sites, treatment with endoglycosidase-H (endo-H) does not affect the electrophoretic mobility of either of the Asi2 bands (Zargari et al., 2007).

Asi2 functions as a negative regulator of the amino-acid-induced SPS-sensor-dependent pathway that regulates gene expression (Zargari et al., 2007). Although the precise mechanism has yet to be determined, Asi2 appears to function by restricting access of the transcription factors Stp1 and Stp2 to SPS-sensor-regulated promoters. In cells lacking Asi2, Stp1 and Stp2 inappropriately bind promoters and ectopically induce gene expression (Boban et al., 2006; Zargari et al., 2007). We examined the possibility that the stability of Asi2 is coupled to amino-acid-induced signaling. If so, we posited that, under inducing conditions in medium containing the amino acid leucine (+Leu), Asi2 would exhibit decreased stability. This would remove Asi2 as an inhibitory component and would be expected to contribute to SPS sensor signaling. Using two approaches we compared Asi2–HA protein stability in non-induced and amino-acid-induced cells. First, we tested Asi2–HA protein stability in the absence and presence of extracellular amino acids (Fig. 1C). We observed only a minor difference in Asi2 degradation rates. In the second approach, we examined Asi2–HA protein stability in a ssy5Δ mutant, which lacks the Stp1- and Stp2-processing endoprotease component of the SPS sensor (Andréasson et al., 2006; Andréasson and Ljungdahl, 2002; Pifirmann et al., 2010) and is defective in SPS-sensor-dependent signaling (Fig. 1D). Again Asi2 protein stability was only nominally increased in the ssy5Δ mutant as compared to the wild-type (Fig. 1D). Taken together, these results indicate that Asi2 protein degradation is independent of the SPS sensor signaling.

### Asi2 is degraded by the ubiquitin-proteasome system independently of the vacuole

Different pathways could account for Asi2 protein degradation. First, we tested whether vacuolar function is necessary for Asi2 degradation. We analyzed Asi2 protein stability in a pep4Δ mutant yeast strain with impaired vacuolar function. Pep4 is a vacuolar aspartic protease required for proteolytic activation of many vacuolarzymogens, such as carboxypeptidase Y (CPY) (Ammeter et al., 1986; Woolford et al., 1986). Zymogens and proteins normally degraded in the vacuole are stabilized in the pep4Δ deletion mutant. We found that Asi2 stability was not affected in the pep4Δ mutant, indicating that the vacuole is not involved in Asi2 protein degradation (Fig. 2A).

Next, we tested whether Asi2 is degraded by the proteasome. We examined Asi2 protein stability in a yeast strain carrying a temperature-sensitive cin3-1 mutation, which impairs the activity of the Rpt6 (Cim3) ATPase in the proteasomal regulatory particle (Ghislain et al., 1993; Schork et al., 1995). At the restrictive temperature of 37°C, Asi2 protein levels were significantly increased and Asi2 turnover was greatly slowed in the cin3-1 mutant (half-life, 81 min), as compared to the wild-type (half-life, 35 min). These findings are consistent with Asi2 being a substrate for proteasome degradation (Fig. 2B).

As proteins are usually targeted to the proteasome by post-translational poly-ubiquitination modification, we examined whether Asi2 is ubiquitylated. To enrich ubiquitylated forms of Asi2 in the cell, Asi2–HA was expressed in a cin3-1 mutant (see above) with or without overexpression of ubiquitin. Anti-ubiquitin immunoblot analysis of the immunoprecipitated Asi2 clearly showed that Asi2 is modified by poly-ubiquitylation (Fig. 2C).
Notably, ubiquitylated species of Asi2 were detected both in cells overexpressing ubiquitin from a plasmid and in cells expressing endogenous levels of ubiquitin (Fig. 2C, lanes 1 and 3, respectively). Taken together, these data indicate that Asi2 is targeted for degradation by the ubiquitin-proteasome pathway.

**Asi2 degradation is impaired in mutants lacking ubiquitylation components Doa10, Ubc6 and Ubc7**

Given that Asi2 is an integral component of the INM, we tested the requirement of the ER-associated E3 ubiquitin ligase Doa10, which is known to also partially localize to the INM, for its degradation (Deng and Hochstrasser, 2006). We examined...
whether Asi2 degradation required Doa10 and its associated E2 enzymes Ubc6 and Ubc7 (Swanson et al., 2001). We also examined Asi2 stability in a mutant lacking E3 ubiquitin ligase Hrd1, an integral membrane protein of the ER involved in ubiquitylation of several ERAD substrates (Vembar and Brodsky, 2008), which functions primarily with E2 enzyme Ubc7 (Bays et al., 2001; Deak and Wolf, 2001). In contrast to Doa10, Hrd1 has not been found in the INM (Deng and Hochstrasser, 2006). Although the deletion of HRD1 did not significantly alter Asi2 stability, Asi2 was stabilized in the deletion mutant lacking DOA10 (Fig. 3A). Furthermore, the stability of Asi2 was similar in the doa10Δ mutant and in the doa10Δ hrd1Δ double mutant cells. These data indicate that a Doa10-dependent pathway contributes to targeting Asi2 for degradation. Next, we examined stability of Asi2 in ubc6Δ, ubc7Δ single and ubc6Δ ubc7Δ double mutants. Consistent with our finding that Asi2 is stabilized in a doa10Δ mutant, Asi2 was also stabilized in mutants lacking either Ubc6 or Ubc7 (Fig. 3B). These latter observations are consistent with yeast two-hybrid data indicating that Ubc6 and Ubc7 might interact in a protein complex (Chen et al., 1993). However, we noted that Asi2 is more stable in the ubc7Δ mutant than in ubc6Δ. The underlying basis for the greater stability in the ubc7Δ mutant is not clear.

To test the possibility that the elevated levels of Asi2 in ERAD mutants were due to secondary and unanticipated consequences of increased ASI2 transcription, we assessed ASI2 mRNA levels in doa10Δ and ubc7Δ mutants using real-time quantitative PCR (Fig. 3C). ASI2 mRNA levels were similar in wild-type cells and
doa10Δ and ubc7Δ mutants. The results indicate that the elevated levels of Asi2 in the cells lacking Doa10 and Ubc7 are due to enhanced Asi2 protein stability rather than elevated transcription.

Asi2 was only partially stabilized in a doa10Δ mutant (see Fig. 3A), thus additional E3 ligases are likely to be involved in targeting Asi2 for degradation. San1 was recently discovered as an E3 ubiquitin ligase involved in degradation of aberrant nuclear proteins (Gardner et al., 2005; Rosenbaum et al., 2011). Therefore, we tested Asi2 stability in a doa10Δ hrd1Δ mutant lacking SAN1. The deletion of SAN1 did not enhance the stability of Asi2 (Fig. 4A). Therefore it is unlikely that San1 participates in Asi2 degradation. Consequently, an additional and yet to be
Asi2 functions in concert with two other INM proteins, Asi1 and Asi3, presumably in a protein complex at the INM (Zargari et al., 2007). Asi1 and Asi3 possess RING domains similar to those found in E3 RING-finger ligases. Therefore we tested the possibility that Asi1 and Asi3 are involved in ubiquitylation of Asi2 by examining Asi2 protein stability in a double asi1A asi3A mutant. Consistent with the Asi proteins functioning together within a complex, Asi2 degradation was enhanced in the asi1A asi3A mutant as compared to wild-type (Fig. 4B). This result clearly indicates that neither Asi1 nor Asi3 function as E3 ligases that ubiquitylate Asi2.

**Asi2 is poly-ubiquitylated in a Ubc7- and Doa10-dependent manner**

To test whether Asi2 protein stabilization in doa10Δ and ubc7Δ deletion mutants (see Fig. 3) is a direct consequence of impaired Asi2 ubiquitylation in the mutant strains lacking specific E3 or E2 enzymes, we examined Asi2 ubiquitylation status in the mutants lacking the E3 ligase Doa10 and the E2 enzyme Ubc7 (Fig. 5). To enrich ubiquitylated species and thus facilitate detection of ubiquitylated proteins, the experiment was performed with yeast strains carrying the cim3-1 mutation, which impairs proteasomal degradation downstream of protein ubiquitylation. By analyzing the ubiquitylation status of immunoprecipitated Asi2 in immunoblots using anti-ubiquitin antibody we found that Asi2 was less ubiquitylated in the cim3-1 doa10Δ mutant as compared to the cim3-1 mutant expressing functional Doa10 (Fig. 5A). Moreover, Asi2 ubiquitylation was almost completely abolished in the mutant lacking Ubc7 (Fig. 5B). The diminished level of Asi2 ubiquitylation correlates well with the enhanced stability of Asi2 in doa10Δ and ubc7Δ mutants (Fig. 3A,B, respectively). Taken together, the data demonstrate that Asi2 is a substrate for Doa10–Ubc7–Ubc6-dependent ubiquitylation and degradation.

**Asi2 is degraded by proteasomes localized in the nucleus**

In yeast, proteasomes are localized in the nucleus as well as in the cytoplasm (Enenkel et al., 1998; Peters et al., 1994; Russell et al., 1999). The degradation of nuclear substrates requires efficient targeting of proteasomal subunits to the nucleus (Chen et al., 2011). Nuclear targeting of the proteasomes depends on functional Sts1, which interacts with proteasomal components and the nuclear import factor Srp1 (Chen et al., 2011; Romero-Perez et al., 2007). As shown previously (Chen et al., 2011), proteasomes fail to accumulate in the nucleus of cells carrying the temperature-sensitive sts1-2 mutation when grown at the restrictive temperature of 37°C (supplementary material Fig. S1A). Asi2 is substantially stabilized in the stts1-2 mutant grown at the restrictive temperature (half-life, 45 min) as compared to wild-type cells (half-life, 31 min) (Fig. 6), consistent with Asi2 being targeted for proteasomal degradation in the nucleus. As a positive control we assessed the stability of AspPrA, a misfolded protein that has been shown to be degraded in the nucleus (Prasad et al., 2010); AspPrA was stabilized in stts1-2 mutant at the restrictive temperature (supplementary material Fig. S1B). In contrast, and as previously shown (Chen et al., 2011), the cytoplasmic proteasomal substrate Ura3-HA-SL17 (Gilon et al., 1998; Ravid et al., 2006) was not stabilized in the stts1-2 mutant (Ravid et al., 2006) (supplementary material Fig. S1C).

**DISCUSSION**

In this study, we addressed the molecular basis of INM protein turnover by examining the stability of Asi2, a well-characterized integral INM protein in yeast (Zargari et al., 2007). Our results clearly demonstrate that Asi2 is subject to turnover and is targeted to the proteasomes in the nucleus by a
Ubc6–Ubc7–Doa10-dependent ubiquitylation pathway. Although the Doa10-associated integral membrane E2 enzyme Ubc6 is targeted by Doa10-dependent degradation (Swanson et al., 2001; Walter et al., 2001), presumably also in the INM, our study shows for the first time that a bona fide integral INM protein, which is not itself a component of the ubiquitylation machinery, is delivered to the nuclear proteasomes. Based on our data, we propose a model of Asi2 protein degradation in the nucleus (Fig. 7).

This model is supported by our finding that Asi2 is stabilized in the sts1-2 mutant, which exhibits impaired nuclear accumulation of proteasomes. This result indicates that a substantial pool of Asi2 is degraded by the proteasomes localized in the nucleus. Consistent with Asi2 being recognized by the ubiquitylation machinery in the nucleus, Doa10 and the associated E2 enzymes Ubc6 and Ubc7 have been found to localize to both the ER membrane and the INM (Deng and Hochstrasser, 2006). Nuclear localization of Doa10 is required for degradation of the transcription factor MATα2 (Deng and Hochstrasser, 2006). In contrast to Doa10, Hrd1 seems to localize exclusively to the ER (Deng and Hochstrasser, 2006). The fact that Hrd1 is physically separated from the INM is consistent with Asi2 not being a

Fig. 5. Asi2 ubiquitylation is decreased in mutants lacking Doa10 and Ubc7. Immunoblot analysis of immunoprecipitation experiments. (A) Strains cim3-1 DOA10 (PLY1348) and cim3-1 doa10Δ (MYB226) carrying the ubiquitin overexpression plasmid (myc-Ub/LEU2 2m) and either a plasmid expressing epitope-tagged Asi2–HA (pMB3) or a plasmid expressing untagged Asi2 (pMB128) were analyzed. Plasmid pMB128 expressing untagged Asi2 serves as a control for specific immunoprecipitation. Immunoprecipitation was performed using anti-HA antibody (IP: α-HA) and immunoblotting (IB) was performed using anti-HA or anti-ubiquitin antibodies, as indicated. To verify that a similar amount of protein was used as a starting material for the immunoprecipitation, input samples immunoblotted with anti-Dpm1 antibody are shown. (B) An experiment performed as in A using cim3-1 DOA10 (PLY1348) and cim3-1 ubc7Δ (MBY225) strains.

Fig. 6. Asi2 protein is stabilized in the sts1-2 mutant exhibiting impaired proteasome accumulation in the nucleus. Cycloheximide (CHX) chase of Asi2–HA (pMS01) in a STS1 (NA10) and sts1-2 (NA25) strain was assessed by immunoblotting with anti-HA and anti-Pgk1 antibodies. Cells were grown at 26°C to an OD600 of 0.8, transferred to medium pre-warmed to 37°C (OD600 of 0.7) and incubated at 37°C for 4 h before CHX was added. Steady-state protein levels at t=0 (P=0.009, two-tailed Student’s t-test) and the percentage of remaining Asi2 after CHX addition are shown. Data represent mean±s.d. (n=3). P-values of data in time course are listed in supplementary material Table S4. Asi2 protein half-life: 31 min (STS1) and 45 min (sts1-2).
substrate for Hrd1-dependent ubiquitylation and with the degradation of Asi2 in the nucleus.

Asi2 protein stabilization correlated well with the decrease in Asi2 ubiquitylation in the doa10Δ and ubc7Δ mutant strains. However, the fact that Asi2 is still degraded in doa10Δ mutants suggests that a Doa10-independent pathway exists that is capable of targeting Asi2 for degradation. This additional pathway could function in parallel with Doa10, or become engaged in Asi2 turnover only after Doa10 function is impaired. We found that the E3 ligase San1, previously reported in soluble nuclear protein quality control, is not involved in this parallel pathway. Delivery of integral membrane proteins to the proteasome includes a membrane extraction step, which, in many cases, is mediated by the Cdc48–Ufd1–Npl4 complex (Wolf and Stolz, 2012). In support of our results, Asi2 has been shown to be enriched in an npl4 mutant strain in a proteome-wide screen for ubiquitylated membrane proteins (Hitchcock et al., 2003). Newly synthesized proteins are subject to protein quality control (Duttler et al., 2013). A minor fraction of these, comprising mostly cytoplasmic proteins, is ubiquitylated co-translationally (Duttler et al., 2013). A recent study indicates that Sts1 links proteasomes to the Srp1-bound nascent polypeptide chains and therefore might be involved in co-translational protein degradation (Ha et al., 2014); however, further investigation is necessary to clarify the potential role of Sts1 in this process. Although we cannot fully exclude that a small pool of Asi2 is degraded in the ER immediately after its synthesis, our finding that Asi2 protein continues to be degraded at later time points after new protein synthesis has been inhibited by cycloheximide suggests that Asi2 degradation is not limited to the quality control of newly synthesized Asi2 at the ER. A substantial fraction of the cellular pool of Asi2 is therefore degraded after having been correctly targeted to the INM.

Previous studies have identified Mps2-1, a mutant form of spindle pole body protein Mps2, as a substrate of Doa10-dependent degradation pathway (McBratney and Winey, 2002; Ravid et al., 2006). Whereas wild-type Mps2 is an integral membrane protein that localizes to the site of the spindle pole body insertion into the nuclear envelope (Jaspersen and Winey, 2004), the thermolabile Mps2-1 mutant protein is degraded at the non-permissive temperature in a Doa10-dependent manner (McBratney and Winey, 2002; Ravid et al., 2006). In contrast to our results for Asi2, the published data indicate that mutant Mps2-1 degradation occurs directly after protein synthesis as a protein quality control mechanism in the ER membrane.

Many integral membrane proteins are known to be degraded in the vacuole (lysosome) (Davies et al., 2009; Hegde and Ploegh, 2010). Taking into account that the INM is structurally and functionally distinct from the ER membrane (English and Voeltz, 2013), vacuolar degradation of an INM protein in yeast could be mediated by a process called piecemeal microautophagy of the nucleus (PMN). In yeast, PMN has been shown to occur when a segment of the nuclear envelope is directly sequestered by local invagination of the vacuolar membrane, followed by the release of a nuclear-envelope-derived vesicle into the vacuolar lumen (Roberts et al., 2003). Our data show that vacuolar function is not required for Asi2 turnover under normal growth conditions, which is in agreement with previous reports that PMN is induced to higher levels during starvation but is very low under normal growth conditions (Roberts et al., 2003). The result also indicates that Asi2 is not delivered to the vacuole through membrane trafficking from the ONM or ER membrane.

Unlike in metazoans, where the nuclear envelope is disassembled at the onset of mitosis and re-assembled around separated sister chromatids in telophase, yeast, filamentous fungi and some protists undergo closed mitosis in which the NE remains intact throughout the cell cycle (Anderson and Hetzer, 2008; Cohen et al., 2001; Ribeiro et al., 2002). Thus, the only way for yeast INM proteins to become accessible for the cytoplasmic degradation machinery is by retrograde transport from the INM back to the ONM and ER membrane. Although passive diffusion out of the nucleus has been observed for artificial INM reporter proteins upon blocking karyopherin-mediated transport across the NPC, a native INM protein remained localized in the nucleus and only very slowly redistributed to the ER, even under conditions when active nuclear import was inhibited (Meinema et al., 2013). Moreover, active nuclear export of membrane proteins was never observed in that study (Meinema et al., 2013). In human cells
lacking lamin A, the INM protein emerin is mislocalized to the ER membrane and is targeted for proteasomal degradation (Muchir et al., 2006).

Doa10-mediated Asi2 protein degradation might represent a nuclear protein quality control pathway. Signals that might mediate Asi2 recognition and targeting for degradation are currently unknown. Amphipathic helices have been predicted in some Doa10 substrates, such as in the Deg1 region of MATx2 (Johnson et al., 1998) and in Ndc10 (Furth et al., 2011). The Asi2 region encompassing amino acid residues 49 to 66 is predicted to form an amphipathic helix, hence it is tempting to speculate that this region is involved in targeting Asi2 for degradation. As previously suggested for aberrant nuclear protein substrates of San1 (Gardner et al., 2005), degradation signals in Asi2 might become exposed when damaged or upon changes of its molecular environment at the INM. For instance, a loss of an interaction partner in the nucleus might uncover Asi2 degradation signals. Asi2 functions together with two other integral membrane proteins at the INM, Asi1 and Asi3 (Zargari et al., 2007), which might affect Asi2 protein stability. Indeed, the rate of Asi2 protein degradation is faster in cells lacking Asi1 and Asi3, suggesting that interaction of Asi2 with Asi1 and Asi3 might protect Asi2 from degradation. Moreover, because Asi2 is a component of the SPS sensor pathway (Zargari et al., 2007), we considered it a possibility that Asi2 protein degradation had a regulatory role in SPS sensor signaling. However, the Asi2 protein levels were similar under both inducing and non-inducing conditions for the SPS sensor pathway, indicating that the suppressive role of Asi2 in SPS signaling is not modulated through Asi2 protein stability.

In conclusion, this study addresses for the first time the turnover of a native and functional INM protein. It identifies Asi2 as the first bona fide integral INM protein targeted for the proteasomal degradation in the nucleus and reveals the molecular pathway that mediates Asis2 ubiquitylation. As accumulation of aberrant proteins in the nucleus is a likely cause of several diseases (Brais, 2003; Jana and Nukina, 2003; Orr and Zoghbi, 2001; Peters et al., 1999), elucidating mechanisms of NE-associated protein degradation may contribute to better understanding of disease mechanisms.

**MATERIALS AND METHODS**

**Yeast growth media**

Standard yeast culture media, such as yeast extract, peptone, dextrose (YPD) medium and ammonia-based synthetic minimal dextrose (SD) medium and ammonia-based synthetic complex dextrose (SC) medium, were prepared as described previously (Andréasson and Ljungdahl, 2002). Antibiotic selections were made on solid YPD containing 200 mg/l 5-fluoroorotic acid (5-FOA) was added to SCD medium. Cells were grown in SC at 30°C, unless indicated otherwise.

**Yeast strains**

Yeast *Saccharomyces cerevisiae* strains used are listed in supplementary material Table S1. All strains except *cim3-1* and *sta1*-*2* mutant strains (MBY178, MBY179, NA10 and NA25) are isogenic descendants of the S288c-derived strain A21525/PLY115 (Antebi and Fink, 1992). Recombinant DNA work was performed by standard methods. Genomic manipulation of yeast strains was performed by using homologous recombination of DNA fragments transformed into yeast strains. FGY256 was constructed by transforming strain PLY127 with the *Spa1-SalI DNA fragment containing hrd1::URA3* (Bays et al., 2001). The gene DOA10 was deleted in PLY127 and FGY256 using the PCR-amplified *doa10D::natMX* cassette, creating JKY28 and JKY38, respectively.

MBY159-167 was constructed by deleting *asi2* in strains PLY123, FGY205, FGY206, JKY36, PLY127, FGY256, JKY28, JKY38 and FGY217, respectively, using *asi2Δ::kanMX* cassette that was PCR-amplified from plasmid pLG6 by primers prMB310 and prMB311. To construct MBY178 and MBY179, *asi2* was deleted in a *cim3* (CAY220) and *cim3-1* (PLY1348-CMY763) strain, respectively, using a *asi2Δ::kanMX* cassette as described above. CAY220 was constructed by transforming PLY1348 with plasmid pRS16 and a PCR product encompassing a complete *RPT6* (*CIM2*) open-reading frame (ORF) obtained from genomic YMH233 DNA, selecting transfectants at 37°C, followed by curing from plasmid on medium containing 5-FOA. Strain MPY143 was constructed by transforming MBY166 with a *san1::aphMX* cassette that was PCR-amplified using primers prMB515 and prMB516 and plasmid pAG32 as a template. Crosses and subsequent tetrad analysis were performed to verify 2:2 segregation of deletion markers. Primer sequences are listed in supplementary material Table S3.

**Plasmids**

Plasmids were listed in supplementary material Table S2. Plasmid pMS1 (pAZ042-2) was constructed by ligating XmnI/SpeI-cut plasmid pMB303 with *Xhol/StuI*-cut plasmid pRS316. Plasmid pMB108 was constructed using homologous recombination, by transforming yeast with *PvuI*-cut pMB55 and *Xbal/HindIII*-cut pRS317 and selecting *Lys*’ colonies. Plasmid pMB122 was created by ligating a large fragment from *BerG1*-cut pMS1 with the small fragment of similarly cut pPL741. Plasmid pMB128 was created by ligating a large fragment from *Xhol/NolI*-cut plasmid pMB3 with a small fragment of similarly cut pMB122.

**Cycloheximide chase and immunoblot analysis**

Cells were grown in SC at 30°C, unless indicated otherwise, to an optical density of 600 nm (OD600) of 0.6–0.9, pelleted by centrifugation and resuspended in fresh SC to an OD600 of 1.5. After a 20-min incubation at 30°C, cycloheximide (CHX) was added to the culture to a final concentration of 100 μg/ml. Samples were harvested at the indicated time points after addition of cycloheximide by placing 1.5 ml culture aliquots in an ice bath. Total protein was extracted as described previously (Silve et al., 1991). Briefly, cells were harvested by centrifugation, resuspended in 250 μl ice-cold 1.85M NaOH containing 7% β-mercaptoethanol and incubated for 10 min on ice. Protein was precipitated by adding 250 μl cold 50% TCA, followed by 10 min incubation on ice and centrifugation. Protein pellet was washed with 1M Tris and resuspended in sample buffer (100 mM Tris-HCl pH 6.8, 4 mM EDTA, 4% SDS, 2% β-mercaptoethanol, 10% glycerol, 0.02% Bromophenol Blue). Proteins were denatured by incubation at 37°C for 10 min, resolved by SDS-PAGE and analyzed by immunoblotting. The following primary antibodies were used for immunoblotting: anti-Myc (mouse monoclonal 9E10, Roche, dilution 1:2000), anti-HA (rat monoclonal 3F10, HRP-conjugated, Roche, 1:3000, unless otherwise indicated), anti-Pgk1 (mouse monoclonal 22C5, Invitrogen, dilution 1:20,000), anti-Dpm1 (mouse monoclonal 5C5, Molecular Probes, dilution 1:500) and anti-CYF (mouse monoclonal 10A5, Molecular Probes, dilution 1:4000). Secondary antibody was conjugated to IRDye® fluorescent dyes (LI-COR). Immunoactive bands were visualized and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences). In Fig. 1B–D, Fig. 4B, Fig. 6 and supplementary material Fig. S1A enhanced chemiluminescence detection was used and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences). In Fig. 1B–D, Fig. 4B, Fig. 6 and supplementary material Fig. S1A enhanced chemiluminescence detection was used and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences). In Fig. 1B–D, Fig. 4B, Fig. 6 and supplementary material Fig. S1A enhanced chemiluminescence detection was used and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences). In Fig. 1B–D, Fig. 4B, Fig. 6 and supplementary material Fig. S1A enhanced chemiluminescence detection was used and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences). In Fig. 1B–D, Fig. 4B, Fig. 6 and supplementary material Fig. S1A enhanced chemiluminescence detection was used and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences). In Fig. 1B–D, Fig. 4B, Fig. 6 and supplementary material Fig. S1A enhanced chemiluminescence detection was used and the signal was quantified by the Odyssey® Infrared Imaging System (LI-COR Biosciences).
Testing protein ubiquitylation

The ubiquitination assay was performed based on the protocol published previously (Furth et al., 2011) with some modifications. Yeast strains impaired in proteasomal degradation (cin1-1 mutant) expressing HA-tagged Asn2 from a 2μ plasmid (pMB3) or untagged Asn2 (pMB128) and, where indicated, carrying the ubiquitin overexpression plasmid ( Yep181- CUP1-myc-Ub-LEU2 2μ) were grown in selective SC. Overnight cultures (28°C) were diluted to OD600 0.27–0.30 in selective SC medium containing 100 μM CuSO4 and cells were incubated at 28°C for 2 h, followed by incubation at restrictive temperature of 37°C for 2–3 h. Around 5×106 cells (OD600 of 20) were harvested by centrifugation at 4°C, and the cell pellet was washed in 1 ml ice-cold water. Cells were resuspended in 800 μl ice-cold water, 250 μl solution of ice-cold 1.85 M NaOH and 7% β-mercaptoethanol was added and incubated on ice for 15 min, followed by addition of 250 μl ice-cold 50% TCA, incubation on ice for 15 min and centrifugation (10 min, 15,000 g, 4°C). The protein pellet was washed with 500 μl Tris. At this point samples could be frozen at −80°C. Samples were thawed on ice and protein pellet was resuspended in 200 μl denaturation solution [2% SDS, 2 mM EDTA, 25 mM Tris-HCl pH 7.5, Protease Inhibitor Cocktail (Roche), 0.5 mg/ml Pefabloc (Roche), 100 μM MG132 (Enzo) and 5 mM N-ethyl-maleimide (NEM)]. Proteins were denatured at 65°C for 10 min and insoluble cell debris was removed by two rounds of centrifugation (5 min, 15,000 g, room temperature). Cleared protein lystate was diluted 1:5 with IP-dilution buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 1.2% Triton X-100, Protease Inhibitor Cocktail, Pefabloc, MG132 and NEM in concentrations as above). Lysate was incubated at 4°C for 1 h with gentle rotation, followed by removal of insoluble material by 10 min centrifugation. 20 μl of sample (input) were mixed with 20 μl of sample buffer (8% SDS, 200 mM Tris-HCl pH 6.8, 8 mM EDTA, 20% glycerol, Bromophenol Blue, 4% β-mercaptoethanol) and frozen until analysis by immunoblotting. The rest of the sample was mixed with 25 μl of anti-HA affinity matrix (clone 3F10, Roche) that was previously prepared by mixing with 10–20 μl IP-dilution buffer. Samples were immunoprecipitated for 1.5 h at 4°C with gentle rotation, matrix was pelleted by centrifugation. Washings were performed as follows: two times with washing solution A (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.4% SDS), once with washing solution B (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 350 mM NaCl, 1% Triton X-100) including a 10-min incubation period at 4°C with gentle rotation, and once with washing solution A. Immunoprecipitated protein was eluted by adding 50 μl sample buffer (100 mM Tris-HCl pH 6.8, 4 mM EDTA, 5% SDS, 8M urea, 2% β-mercaptoethanol) to the pelleted matrix followed by 10 min incubation at 65°C. Around 10–20 μl of samples was separated on SDS-PAGE gels (6–16% gradient). For analysis of immunoprecipitated Asn2–HA, eluate was diluted 1:20 with sample buffer. Immunoblotting analysis was done using anti-HA antibody (1:1000, a gift from the Ogris laboratory, MFPL, Vienna, Austria) and anti-ubiquitin P4D1 antibody (1:1000, Santa Cruz Biotechnology). Protein levels in input samples were assessed using anti-Dpm1 antibody (1:500, Molecular Probes, clone 5C5). Immunoreactive bands were visualized by enhanced chemiluminescence detection.

RNA isolation and real-time PCR

Approximately 107 yeast cells exponentially growing in SC medium were harvested by centrifugation. RNA was isolated using a RiboPure™ Yeast Kit and treated with TurboDNase (Ambion) according to manufacturer’s instructions. 3 μg of isolated RNA was examined by gel electrophoresis on a 1% agarose gel containing 0.5 μg/ml of 28S and 18S as markers. RNA integrity was confirmed using samples without reverse transcriptase as a negative control. The qPCR was prepared using Kapa Sybr®Fast qPCR Master Mix (KapaBiosystems). cDNA mixtures were diluted 1:20 and 5 μl was used in a total qPCR reaction volume of 20 μl. The following primer pairs were used: KR65as12fwd and KR66as12rev, TAF10fwd and TAF10rev (supplementary material Table S3). Assays were conducted in triplicates on a Corbett Research RotorGene machine.

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Competing interests

The authors declare no competing interests.

Author contributions

M.B. conceived of, performed and analyzed experiments, prepared figures and co-wrote the manuscript; M.P. performed and analyzed experiments, and prepared figures; A.S. performed and analyzed experiments, and prepared figures; P.O.L. conceived of and analyzed experiments, and co-wrote the manuscript; R.F. conceived of and analyzed experiments and co-wrote the manuscript.

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Supplementary material

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