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Cell Reports
Article

The Prion-like Domain in the Exomer-Dependent Cargo Pin2 Serves as a *trans*-Golgi Retention Motif

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http://dx.doi.org/10.1016/j.celrep.2014.02.026

SUMMARY

Prion and prion-like domains (PLDs) are found in many proteins throughout the animal kingdom. We found that the PLD in the *S. cerevisiae* exomer-dependent cargo protein Pin2 is involved in the regulation of protein transport and localization. The domain serves as a Pin2 retention signal in the *trans*-Golgi network (TGN). Pin2 is localized in a polarized fashion at the plasma membrane of the bud early in the cell cycle and the bud neck at cytokinesis. This polarized localization is dependent on both exo- and endocytosis. Upon environmental stress, Pin2 is rapidly endocytosed, and the PLD aggregates and causes sequestration of Pin2. The aggregation of Pin2 is reversible upon stress removal and Pin2 is rapidly re-exported to the plasma membrane. Altogether, these data uncover a role for PLDs as protein localization elements.

INTRODUCTION

Prion proteins can exist in a normally folded state or an aggregated state. The aggregated state is able to drive normally folded proteins into aggregation. Induction of the yeast Sup35 prion *[PSI+]* can occur spontaneously, but is greatly facilitated if the cell has previously achieved a *[PIN+]* state (Derkatch et al., 1997). This *[PIN+]* state can be reached by the overexpression of a number of different factors that contain a prion domain or prion-like domain (PLD) (Derkatch et al., 2001). Thus, efficient induction of prions may require the presence of other prions.

Genome-wide analyses indicate that 0.3% (in humans) to 24% (in plasmodium) of cellular proteins contain a prion domain or PLD (Michelitsch and Weissman, 2000; Osherovich and Weissman, 2002; Singh et al., 2004). RNA-binding proteins are overrepresented among the PLD-containing proteins (Michelitsch and Weissman, 2000). Obviously, not all prion or PLDs cause disease, and they may instead act as scaffold or interaction domains. Yet, in most instances, their precise role remains elusive.

Transport to the plasma membrane and secretion are essential processes in eukaryotic cells. Cargoes destined for the plasma membrane will be sorted into transport carriers for either direct delivery or delivery via endosomes. Evidence for the direct route exists in yeast and in mammalian cells. TGN46-containing transport containers, which are dependent on protein kinase D and are devoid of vesicular stomatitis virus glycoprotein (VSVG) or collagen, have been identified in HeLa cells, indicating a specific sorting mechanism at the *trans*-Golgi network (TGN) (Wakana et al., 2012). In *Saccharomyces cerevisiae*, the chitin synthase Chs3 and the mating-response protein Fus1 require Chs5 and Chs5p-Arf1p-binding proteins (ChAPs) collectively termed exomer for their export from the TGN to the plasma membrane (Trautwein et al., 2006; Wang et al., 2006). Chs3 and Fus1 necessitate a combination of regulated endocytosis and exocytosis to achieve their precise localization at the bud neck for Chs3 and to the bud tip for Fus1 in a cell-cycle-dependent manner (Barfield et al., 2009; Valdivia et al., 2002).

The ChAP family consists of Bch1, Bch2, Bud7, and Chs6, which can associate with Chs5 to form oligomers of heterotetrameric complexes. These complexes consist of two Chs5 molecules and two ChAPs, and either two identical or two different ChAPs can be bound to Chs5 (Paczkowski et al., 2012; Trautwein et al., 2006). The ChAPs may act as adaptor molecules to interact and recruit cargo to specific sites at the TGN from which they reach the plasma membrane. Although previous studies identified Chs3 and Fus1 motifs that were necessary for export from the TGN, none of these motifs were sufficient (Barfield et al., 2009; Rockenbauch et al., 2012; Starr et al., 2012). In addition, the interaction motifs were not conserved between the two cargo proteins. Thus, the interaction between the cargoes and exomer appears to be rather complex.

Given the lack of conserved motifs between Chs3 and Fus1, other interaction sites must be important for controlling the export of these proteins in a temporally and spatially controlled manner. These interaction sites potentially could adopt an appropriate conformation upon interaction with the ChAPs, and then the linear transport signal might be recognized. In support of this notion, it was shown that all ChAPs are able to interact with Chs3, although only Chs6 is essential for its plasma membrane localization (Trautwein et al., 2006). Examples of such interactions include Src homology domains that recognize phosphorylated Tyr in proteins (Groffen et al., 1983; Moran et al., 1990), the interaction of the ArfGAP Glo3 with SNAREs and cargo (Rein et al., 2002; Schindler et al., 2009), and PLD-containing proteins that are important for processing body and stress granule assembly (Alberti et al., 2009; Decker et al., 2007; Gilks et al., 2004; Michelitsch and Weissman, 2000; Vessey et al., 2006) or are often found in cytoskeletal elements (Alberti et al., 2009; Michelitsch and Weissman, 2000).

Chs3 is a multispanning transmembrane protein, and Fus1 only becomes exomer dependent upon mating. To better

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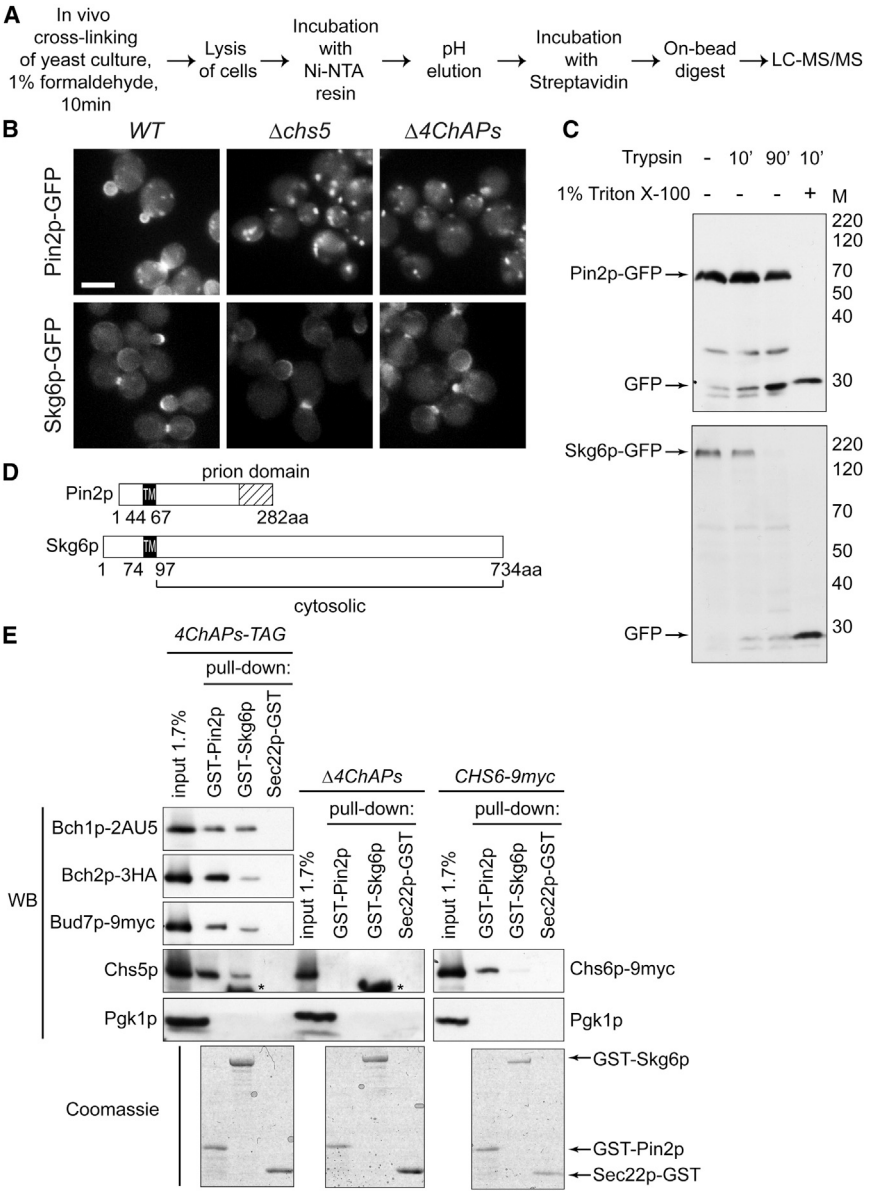

Figure 1. Pin2 Is an Exomer-Dependent Cargo

(A) Flowchart of biochemical screen for exomer cargo.

(B) Plasma membrane localization of Pin2, but not Skg6, is dependent on exomer. Fluorescence microscopy of WT, *Δchs5*, or *Δ4ChAPs* cells expressing chromosomally tagged Pin2-GFP and Skg6-GFP. Scale bar represents 5 μm.

(C) The C terminus of Pin2 and Skg6 is cytosolic. Spheroplasts expressing either Pin2-GFP or Skg6-GFP were treated with trypsin ± TX-100 and analyzed by immunoblot.

(D) Scheme of Pin2 and Skg6 topology.

(E) Pin2 and Skg6 bind all four ChAPs. Pull-downs of yeast lysates with purified GST-tagged cytosolic domains of Pin2, Skg6, or Sec22. Pull-downs were performed with 4ChAPs-TAG, *Δ4ChAPs*, or Chs6-9myc lysates. Samples were immunoblotted for Chs5, epitope tags present on the ChAPs, or Pgk1. Asterisks indicate the nonspecific interaction of GST-Skg6 with the anti-Chs5 antibody. Coomassie staining shows the levels of GST-tagged constructs in the pull-downs.

See also Figure S1 and Tables S1 and S2.

understand the exomer-dependent transport pathway, we identified cargo proteins, including Pin2, a single transmembrane domain (TMD) protein with a large cytoplasmic region that contains a PLD. This PLD regulates the traffic of Pin2 under normal growth conditions and is essential for Pin2 retention in internal structures upon stress. We identified a transport mechanism in which a PLD is essential for the temporal and spatial control of intracellular protein localization.

RESULTS

The PLD Protein Pin2 Is an Exomer-Dependent Cargo

In order to better understand exomer-dependent transport to the plasma membrane, we aimed to identify novel cargoes. We appended Chs5 or each of the four members of the ChAP family with a histidine-biotin-histidine (HBH) tag. The HBH tag consists of a biotinylation sequence flanked by two His_6 tags (Tagwerker et al., 2006). This tag allows the purification of proteins or (after crosslinking) protein complexes under denaturing conditions. This tag should allow for easy extraction of membrane proteins, which would represent potential cargo proteins, when bound to the exomer complex. Cells were crosslinked and lysed under denaturing conditions. The crosslinked complexes were affinity purified and analyzed by mass spectrometry (Figure 1A). We identified TMD-containing proteins (potential cargoes) and soluble proteins (potential regulators). We focused on potential cargoes and tested them for their ability to be transported to the plasma membrane in a Chs5-dependent manner. One of the hits that required Chs5 for localization to the bud in small- and medium-sized cells and to the bud neck in large-budded cells was the previously uncharacterized PLD-containing protein Pin2 (Figure 1B). In the absence of Chs5, Pin2 remained in internal structures, similar to what was observed with the other exomer-dependent cargoes (Barfield et al., 2009; Santos and Snyder, 1997; Trautwein et al., 2006). If Pin2 is an exomer-dependent cargo, a deletion of all four ChAPs should phenocopy a *Δchs5* strain. In a *Δ4ChAPs* strain, Pin2 was also found in internal structures (Figure 1B). Therefore, Pin2 represents a exomer-dependent cargo.

It has been reported that all exomer-dependent cargoes localize to the bud or bud neck (Barfield et al., 2009; Chuang

and Schekman, 1996; Santos and Snyder, 1997), suggesting that perhaps all bud-localized proteins are potential exomer clients. However, another candidate from our biochemical screen, Skg6, which localized to the bud and the bud neck in a cell-cycle-dependent manner similar to that observed for Pin2, reached the plasma membrane through an exomer-independent pathway, because deletion of *CHS5* or the four ChAPs had no effect on Skg6 localization (Figure 1B). Thus, the cell-cycle-dependent spatial distribution of the proteins alone cannot be used to discriminate between exomer-dependent and -independent cargoes.

Pin2 and Skg6 Are TMD Proteins with C-Terminal Domains Facing the Cytoplasm

Pin2 and Skg6 are single TMD proteins with unclear topology. To determine the topology of both proteins, we performed trypsin digests of cells expressing chromosomal C-terminal GFP fusions of Pin2 and Skg6 in the presence or absence of 1% TX-100. Pin2-GFP was resistant to trypsin treatment for up to 90 min in the absence of detergent. Solubilizing the plasma membrane rendered Pin2-GFP protease sensitive (Figure 1C). Similar results were obtained for Skg6-GFP. Consistent with these results, phosphoproteome studies reported phosphorylation sites for both Skg6 and Pin2 in the C-terminal part of the proteins (Bodenmiller et al., 2007; Li et al., 2007; Sadowski et al., 2013; Soulard et al., 2010). Therefore, the C terminus of Pin2 and Skg6 face the cytoplasm, and the N terminus of either protein is exposed to the environment. In both proteins, the TMD is relatively close to the N terminus, resulting in small extracellular domains (Figure 1D).

Pin2 and Skg6 Interact with Exomer Components In Vitro

The determination of the topology allowed us to create GST-fusion proteins of the cytoplasmic exposed tails of Pin2 and Skg6, and to revisit their interaction with exomer. We wanted to confirm the interaction because crosslinking only measures proximity. We performed a GST pull-down experiment from yeast lysates in which three of the four ChAPs (Bch1, Bch2, and Bud7) were chromosomally appended with different tags, or Chs6 was myc tagged. The functionality of the tagged proteins was established previously (Trautwein et al., 2006). Pin2 and Skg6, but not the endoplasmic reticulum (ER)-Golgi v-SNARE Sec22, pulled down exomer components (Figure 1E). Interestingly, proximity to and even interaction with exomer appears to be insufficient to describe an exomer-dependent cargo, as Skg6 travels to the plasma membrane in the absence of exomer. It is conceivable, however, that Skg6 can use either pathway to reach the plasma membrane.

As a final proof, we probed the interaction of Pin2 with Chs5. Chs3 and Fus1 depend on the ChAPs for efficient interaction with Chs5 (Rockenbauch et al., 2012; Sanchatjate and Schekman, 2006). Although we could detect a robust interaction between Chs5 and Pin2 in the presence of the ChAPs, this interaction was abolished when the ChAPs were deleted (Figure 1E). Therefore, we conclude that Pin2 is a exomer-dependent cargo.

Either Bch1 or Bch2 Is Sufficient to Support Pin2 Plasma Membrane Localization

Exomer-dependent cargoes require one or two members of the ChAP family for timely exit from the TGN (Barfield et al., 2009; Sanchatjate and Schekman, 2006; Trautwein et al., 2006; Ziman et al., 1998). We tested single and double ChAP deletions for their failure to export Pin2-GFP from the TGN. Only the double deletion *Δbch1 Δbch2* altered Pin2-GFP localization (Figures S1A–S1C). However, this mutant was less potent than *Δchs5*, indicating that the other ChAPs also can contribute to proper Pin2 localization (Figure S1C).

Next, we asked whether either Bch1 or Bch2 would also be sufficient for Pin2 TGN export. Pin2 still reached the plasma membrane even when only one ChAP was present, albeit somewhat less efficiently than in the wild-type (WT; Figures S1D and S1E). At least in the case of Bch2, this small reduction in export efficiency was not due to less binding to Pin2 (Figure S1F). Our data indicate that Bch1 and Bch2 can independently promote export of Pin2 from the TGN to the plasma membrane.

Exomer Binds the Pin2 C Terminus In Vitro

Thus far, we have shown that Pin2 is an exomer-dependent cargo that binds directly to exomer. The interaction of exomer with its cargoes Chs3 and Fus1 is complex and requires more than just a linear sequence motif (Barfield et al., 2009; Rockenbauch et al., 2012). To identify potential interacting regions, we generated three truncations in the cytoplasmic domain containing a GST-Pin2 construct. As shown above, the cytoplasmic domain of Pin2 bound all ChAP proteins (Figures 1E and 2A). In addition, the construct expressing the C-terminal ~120 amino acids of Pin2 precipitated the ChAPs, albeit more weakly. Since GST-Pin2(72-210) was unable to interact with exomer, we conclude that the exomer-binding site resides in the C-terminal 72 amino acids. Thus, exomer recognizes sequences in the Pin2 C terminus, but other sequences in the molecule might still contribute to the binding efficacy.

Next we wanted to test whether the C-terminal part of Pin2 is necessary and sufficient to cause exomer-dependent export (Figure 2B). First, we generated a construct in which the exomer interaction site identified in vitro was eliminated (Pin2(1-210)-GFP). This construct still reached the plasma membrane in the WT and to a lesser extent in *Δchs5*; however, the polarized localization was lost (Figures 2B and 2C). This phenotype is reminiscent of Chs3 localization in *Δchs5 Δapm1* cells, in which recycling from endosomes to the TGN is blocked (Valdivia et al., 2002). A construct that contained only the first 245 of the 282 amino acids of Pin2 still accumulated at the plasma membrane in an exomer-dependent manner. Therefore, the exomer interaction site might reside in residues 210–245 of Pin2. Trimming the protein further down to 152 residues shifted Pin2 localization entirely to the plasma membrane and the internal pool was depleted, consistent with a defect in endocytosis (Figure 2B). A similar phenotype was previously reported for Chs3 localization in a *Δend3* strain, in which endocytosis was blocked (Chuang and Schekman, 1996; Ziman et al., 1996). Despite a notable plasma membrane localization of Pin2(Δ79-152)-GFP, most of the protein accumulated in the vacuole in WT cells, indicating that the membrane proximal

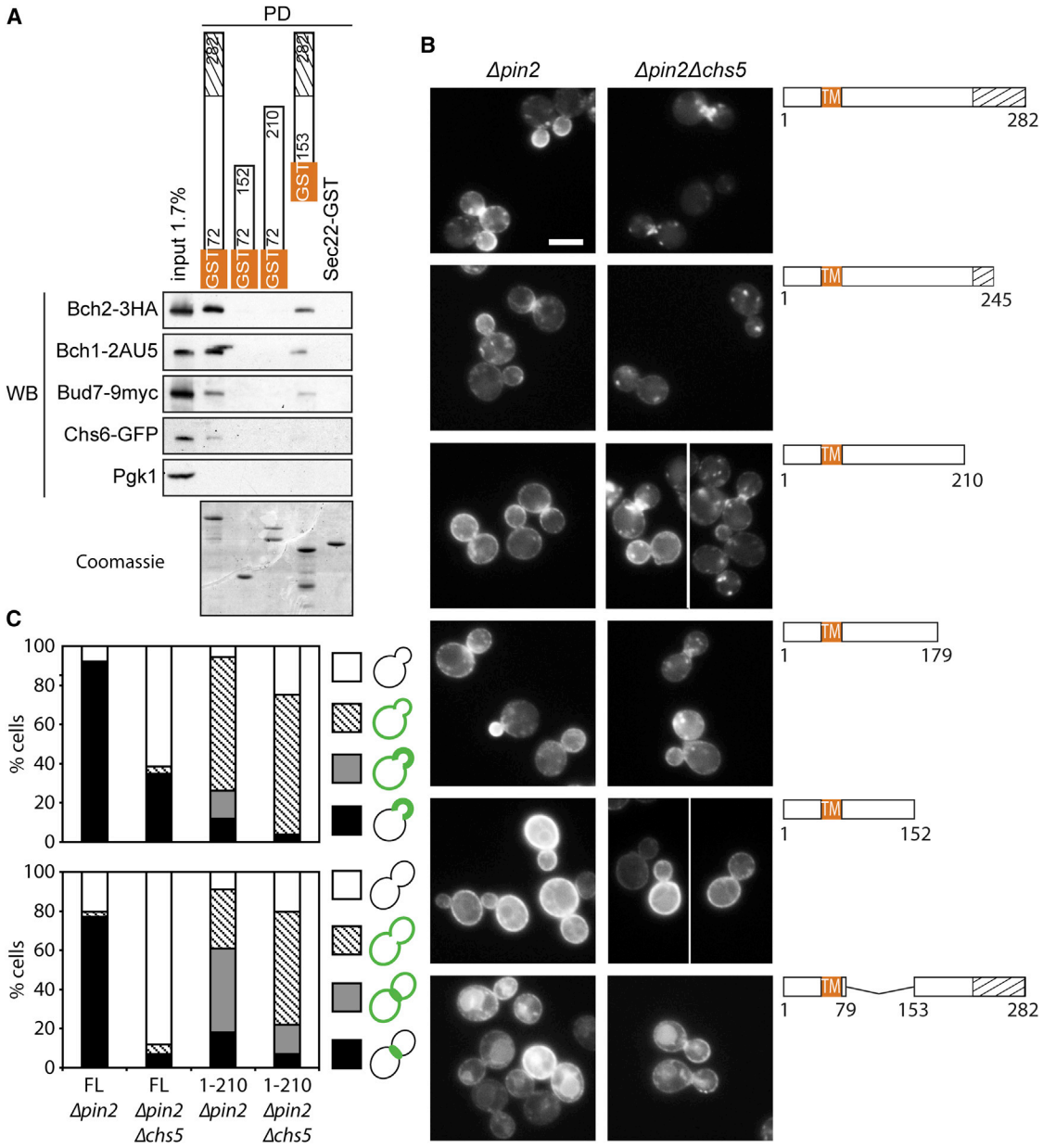

Figure 2. The Pin2 Cytosolic Domain Contains Motifs for Exomer Binding and Endocytosis

(A) Amino acids 211–282 contain an exomer-binding motif. Pull-downs from 4ChAPs-TAG lysates with Pin2-GST variants. GST-Sec22 was used as a negative control. Pull-downs were analyzed by immunoblot. Pgk1 served as control. Coomassie staining showed the levels of GST-tagged constructs in the pull-downs.

(B) Δ 211–245 abolishes exomer dependency. Fluorescence microscopy images of *Δpin2* and *Δpin2Δchs5* cells expressing Pin2-GFP variants. Scale bars represent 5 μm.

(C) Quantification of phenotypes in (B); 100 small and medium budded cells, and 100 large budded cells were quantified.

See also Figure S2 and Tables S1 and S2.

region of Pin2 also may contribute to proper Pin2 localization. Most importantly, the pool that reached the plasma membrane arrived there in an exomer-independent manner, because localization of Pin2(Δ79-152)-GFP in *Δchs5* was indistinguishable from that in WT cells. Therefore, the C-terminal domain is not sufficient to direct Pin2 into the exomer pathway. The effects we observed were not due to large overexpression of the constructs over the endogenous protein (Figure S2A). Taken together, these data indicate that the interaction between Pin2 and exomer might be rather complex, and it is rather unlikely that a short linear sequence within Pin2 would be necessary and sufficient to promote temporal and spatial controlled plasma membrane localization. These data are in agreement with what has been observed for the other exomer

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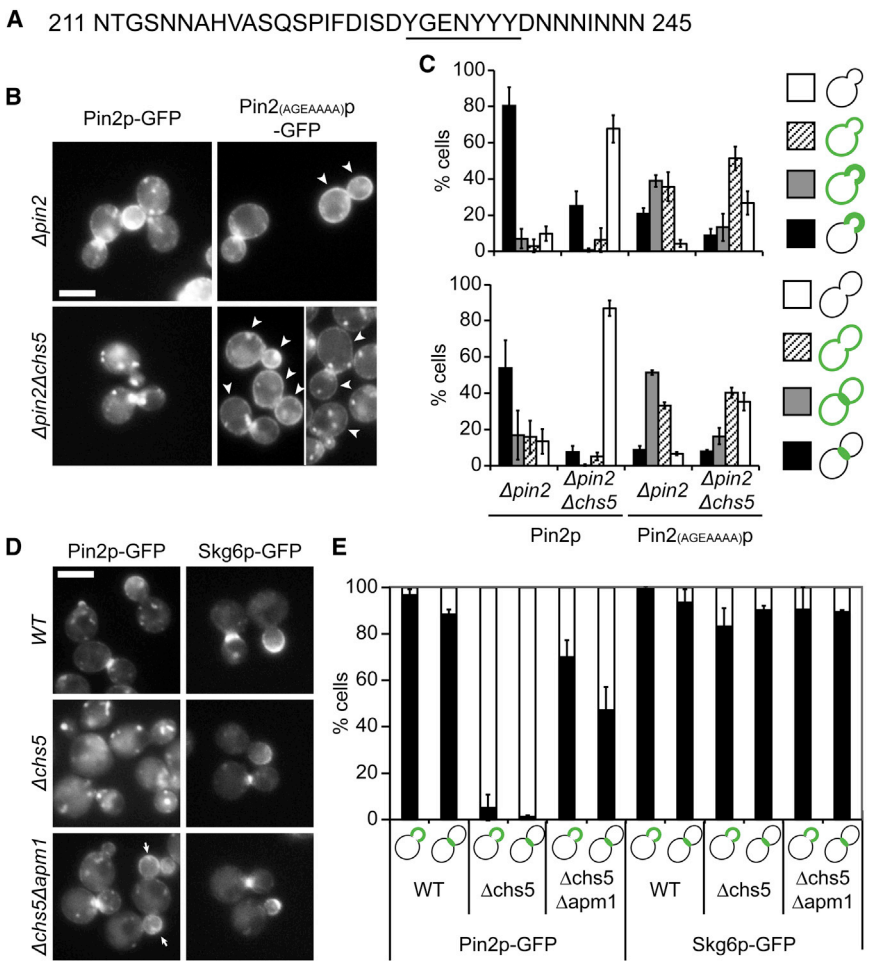


Figure 3. Pin2 Is Retrieved from Endosomes to the TGN by AP-1

(A) Amino acids 211–245 of Pin2 contain a motif required for exomer dependency. Underlined is a Y-rich sequence, a potential AP-1 binding motif.

(B) Mutation of the YGENYYY motif rescues Pin2 export in a *Δchs5* strain. Fluorescence microscopy of *Δpin2* and *Δpin2Δchs5* strain cells expressing GFP-tagged Pin2 or the AGEAAAA mutant. Arrowheads indicate Pin2(AGEAAAA)-GFP at the plasma membrane of the daughter and mother cells.

(C) Quantification of (B) as described in Figure 2C in each of three independent experiments.

(D) *Δapm1* rescues Pin2 export to the plasma membrane in a *Δchs5* strain. Fluorescence microscopy of WT, *Δchs5*, and *Δchs5Δapm1* cells expressing Pin2-GFP and Skg6-GFP. Arrows indicate Pin2-GFP exclusively at the plasma membrane of the daughter cell in *Δchs5Δapm1* cells.

(E) WT, *Δchs5*, and *Δchs5Δapm1* cells were scored as in (C).

Error bars represent SD. Scale bars in (B) and (D), 5 μm. See also Figures S3 and S4 and Tables S1 and S2.

cargoes, Chs3 and Fus1 (Barfield et al., 2009; Rockenbauch et al., 2012).

Pin2 Recycles between Endosomes and TGN

Chs3 and Fus1 have been shown to reach endosomes and to be retrieved from there through an AP-1-dependent pathway (Barfield et al., 2009; Valdivia et al., 2002). In the absence of Chs5 and AP-1, Chs3 and Fus1 arrived at the plasma membrane through an alternative route. We tested whether this recycling is a general feature of exomer-dependent cargoes. As shown above, Pin2(1-210), but not Pin2(1-245), was localized to the plasma membrane independently of Chs5, indicating that the region aa 210–245 may contain an AP-1-binding site (Figure 2B). The μ subunits of AP complexes can bind to the Y-based sorting motif YXXø (where X is any amino acid, and ø is a bulky hydrophobic amino acid) (Ohno et al., 1995). We identified a cryptic Y-based motif, YGENYYY, in the 210–245 peptide (Figure 3A). Although the spacing for the motif was not perfect, we replaced the Ys and N by As. Transport to the plasma membrane of the A mutant was independent of Chs5 (Figures 3B and 3C), indicating that YGENYYY could be a functional adaptor-complex-binding site. To prove that Pin2 indeed undergoes AP-1-dependent recycling, we deleted the μ subunit of the AP-1 complex, *APM1*. In a *Δchs5 Δapm1* mutant, Pin2 was localized mostly at the plasma membrane, whereas Skg6 localization was not affected (Figures 3D and 3E). Moreover, the Pin2(AGEAAAA) mutant protein did not change its localization in a *Δchs5 2Δapm1* mutant, confirming that YGENYYY is required for AP-1-dependent recycling to the TGN (Figure S3). To test whether YGENYYY is essential for AP-1 binding in vitro, we performed a pull-down of Pin2(AGEAAAA)-GST and probed for the AP-1 subunit Apl2. The binding of Apl2 was not significantly reduced in the mutant compared with Pin2 (Figure S4A), indicating that there must be an additional binding site for AP-1. Consistent with Apl2 binding, replacing YGENYYY by AGEAAAA did not grossly alter the conformation of Pin2 as determined by circular dichroism spectroscopy (Figure S4B). Our data are in agreement with the notion that Pin2 cycles between the TGN and endosomes in a manner similar to that observed for Chs3 and Fus1. In addition, the Y-based signal might also be recognized by the AP-2 complex, which promotes endocytosis at the plasma membrane. In contrast to *Δchs5 Δapm1* cells, in which the mother cell was devoid of Pin2, Pin2 (AGEAAAA)-GFP localized to the plasma membrane of mother and daughter cells in both WT and *Δchs5* (Figures 3B and 3D), indicating that endocytosis may be required for proper Pin2 localization.

Endocytosis of Pin2 Is Required for Its Proper Plasma Membrane Localization

Another feature of Chs3 trafficking is that Chs3 localization at the bud neck is dependent on endocytosis (Reyes et al., 2007;

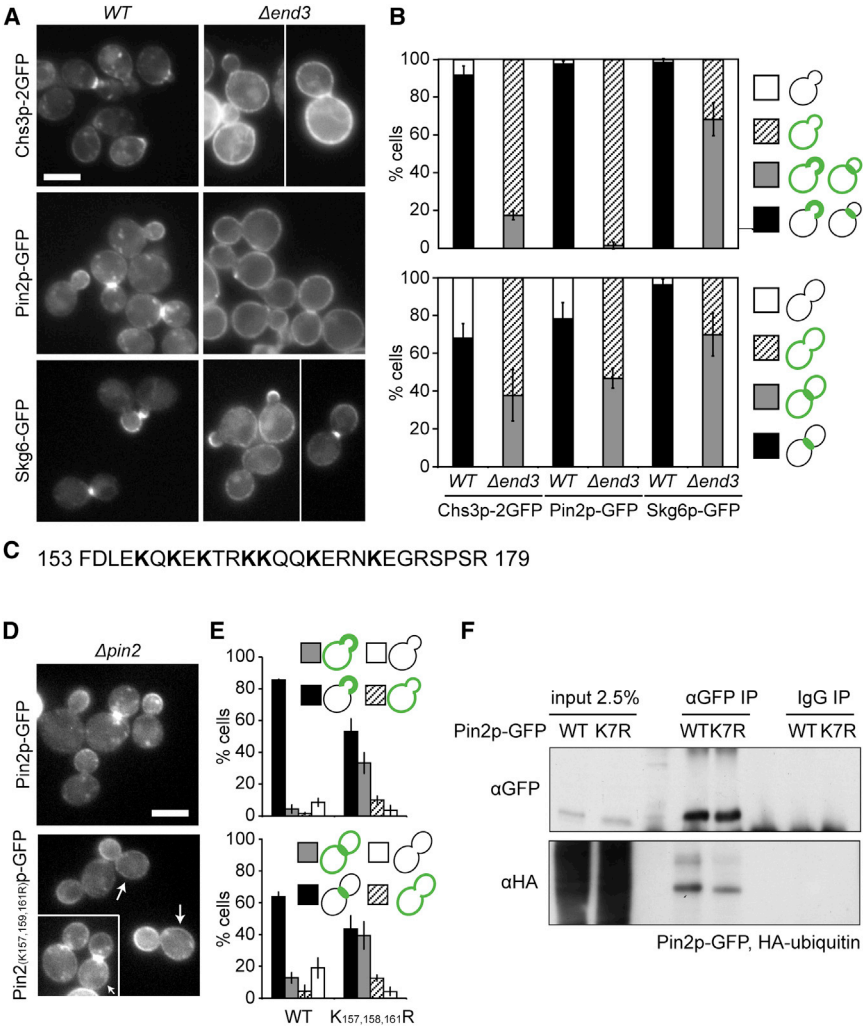


Figure 4. The Polarized Localization of Pin2 Is Dependent on Ubiquitin-Mediated Endocytosis

(A) Endocytosis is required to maintain polarized localization of exomer-dependent cargoes. Fluorescence microscopy of WT and *Δend3* cells expressing Chs3-2GFP, Pin2-GFP, and Skg6-GFP.

(B) WT and *Δend3* cells were scored for the expression of Chs3-2GFP, Pin2-GFP, or Skg6-GFP at the plasma membrane and for the extent of the polarity of cargo localization.

(C) Amino acids 153–179 of Pin2 are required for maintenance of polarized localization at the plasma membrane. Ks are depicted in bold.

(D) Mutation of three out of seven Ks within aa 153–179 causes a partial loss of Pin2 polarity. Fluorescence microscopy of *Δpin2* expressing Pin2-GFP or Pin2(K157,159,161R)-GFP mutant.

(E) Analysis of cells in (D).

(F) Ks within aa 153–179 are ubiquitylated. Immunoprecipitation of GFP-tagged Pin2 or the K7R variant from an HA-ubiquitin-overexpressing strain. HA-ubiquitin was detected with anti-HA antibodies.

Quantifications in (B) and (E) were performed as in Figure 3C. Error bars represent SD. Scale bars in (A) and (D), 5 μm. See also Tables S1 and S2.

Ziman et al., 1996). Therefore, we tested whether Pin2 localization depends on endocytosis. Deletion of *END3* locked Chs3 and Pin2 at the plasma membrane, whereas Skg6 was only mildly affected (Figures 4A and 4B). These results indicate that Pin2 and Chs3 are equally dependent on endocytosis for their proper localization. Moreover, Pin2(AGEAAAA)-GFP was mislocalized over the entire plasma membrane (Figure 3B). Similarly, Pin2(1-152) accumulated at the plasma membrane, whereas internal stores were depleted (Figure 2B). Although Pin2(1-179) also was mostly present at the plasma membrane, internal structures were still observed (Figure 2B). Therefore, information for efficient endocytosis may be located in aa 153–179 of Pin2. The peptide 153–179 of Pin2 contains seven lysines, each of which could potentially be ubiquitylated (Figure 4C). Replacing K157, K159, and K161 by arginines in Pin2 (Pin2(K157,159,161,R)-GFP) caused a strong endocytosis defect (Figures 4D and 4E). To show that Pin2 can be ubiquitylated in this region, we replaced all seven Ks by Rs in a strain that expressed hemagglutinin (HA)-tagged ubiquitin. Pin2 is a target for ubiquitylation, as HA-ubiquitin was covalently attached to Pin2 and addition of this modification was at least partly dependent on the seven Ks (Figure 4F). Given that we had already detected a strong endocytosis defect when only three Ks were mutated, we suggest that aa 153–179 contain sites for ubiquitylation and that this modification serves as a signal for endocytosis. Taken together, these results indicate that Pin2 shares principles of localization with Chs3 that control their temporal and spatial discharge at the plasma membrane.

Pin2 Is a Prion-Inducing Protein

Pin2 was identified in a screen as a protein that when overexpressed can induce the *[PIN+]* prion phenotype, which is a prerequisite for prion formation by Sup35, referred to as the *[PSI+]* prion (Derkatch et al., 2001). In addition, Pin2 contains a Q/N-rich region, referred to as the PLD (Alberti et al., 2009) (Figure 5A). This domain is located in the C-terminal part of the protein facing the cytoplasm (Figure 5B). To confirm the ability of Pin2 to induce *[PIN+]*, we overexpressed Pin2 in a strain that expresses the N-terminal domain of Sup35 fused to GFP (SUP35NM::GFP) (Derkatch et al., 2001), which also contains a prion domain. In the presence of prion-inducing activity, Sup35NM-GFP will aggregate, and green foci and ring-like structures can be observed. Overexpression of Pin2 resulted in Sup35NM-GFP foci, confirming that Pin2 can induce prion formation (Figure 5C). Sup35 is a translational terminator, and loss of Sup35 causes a readthrough in the *ade1-14* nonsense mutation, allowing strains to grow in the absence of adenine

(ade−). Aggregation of Sup35 equally allows strains to grow on ade− plates. Growth on ade− plates was induced by overexpression of *PIN2* only in a construct in which the PLD was present (Figure 5C). Moreover, mutating the Y-motif and the Ks (Pin2 (out)) or replacing Q/Ns by charged E/Ds (Pin2QNtoED) (Figure 5B) strongly reduced prion formation (Figure 5C). In contrast, deleting *CHS5* and hence confining Pin2 to the TGN did not interfere with prion formation.

Next, we tested whether Pin2 itself was able to form SDS-resistant aggregates after overexpression, which is considered a hallmark of a prion protein. Pin2 SDS-resistant aggregates were observed in a PLD-dependent manner (Figure 5D). The SDS-resistant Pin2 aggregates were abolished when the PLD was deleted or mutated. Thus, *[PSI+]* inducibility of Pin2 is dependent on specific sequences within the PLD.

The PLD of Pin2 comprises the Y-based motif. Deletion of the PLD or mutation of the retrieval signal caused Pin2 to be delocalized over the plasma membrane. Thus, it was conceivable that the PLD could be important for retaining the protein inside the cell. To test this possibility, we assessed the formation of SDS-resistant Pin2 aggregates in *Δchs5* (all TGN) and *Δend3* (all plasma membrane) mutant strains. However, under both conditions, SDS aggregates were formed to a similar extent (Figure 5D). Thus, the localization of Pin2 per se is not important for prion formation.

Nevertheless, the PLD of Pin2 may act as a TGN retention signal under normal expression and growth conditions because Pin2QNtoED was exported to the plasma membrane in an exomer-independent and nonpolarized fashion (Figures 5E and 5F). Endogenous expression levels of Pin2 could lead to aggregation, but should not promote the formation of SDS-resistant structures. The plasma membrane localization of Pin2QNtoED was not due to interference with exomer or AP-1 binding, or to partial misfolding (Figure S4). These data indicate that no transport signals are blocked or lost due to the charge changes, but the retention through the prion domain is perturbed.

Pin2 Forms Aggregates in Internal Structures upon Environmental Stress

Use of the *Δchs5* and *Δend3* strains created a nonphysiological all-or-nothing situation. We aimed to find conditions in which we could potentially modulate the localization of Pin2 more immediately and less drastically. Other groups and we have observed that under mild heat stress, Chs3 is quickly internalized from the bud neck, just to reappear delocalized all over the plasma membrane as part of the stress response (Valdivia and Schekman, 2003; Zanolari et al., 2011). Therefore, we probed the localization of Pin2 after exposure to stress (Figure S5A). Under a number of stresses, Pin2 was internalized and accumulated in internal structures. In contrast, Skg6 remained largely unaffected by the stressors, indicating that plasma membrane proteins are not randomly endocytosed upon stress.

We chose Li^+ treatment for further analysis. To investigate the kinetics of this internalization, we performed a time-course analysis of Li^+ exposure and analyzed Pin2 localization. Five minutes after addition of LiCl, clusters of Pin2 were present at the plasma membrane, and after 15 min most of Pin2 was internalized (Figures 6A and 6B). Pin2 stayed internalized even after overnight (O/N) treatment. The internalization event was signal dependent, because interfering with either the Hog1 or Slt2 mitogen-activated protein kinase (MAPK) signaling pathways caused a delay in endocytosis of Pin2 (Figure S5B). Similar clustering of the GFP signal was observed with Pin2(out); however, the uptake of this construct was largely reduced (Figures 6A, 6B, and S6A). To determine whether this retention for prolonged periods was dependent on aggregate formation, we used Pin2QNtoED (Figure 5B). Similar to what was observed for the Pin2(1-210) and Pin2(out) mutant proteins, the steady-state localization of Pin2QNtoED was predominantly at the plasma membrane, independently of the presence of Chs5 (Figures 5E and 5F). This indicates that even under normal growth conditions, the PLD contributes to the retention of Pin2 in internal structures. The initial uptake kinetics of the PLD mutant under LiCl were similar to those of Pin2, implying that we did not interfere with ubiquitin-dependent endocytosis signals. However, Pin2QNtoED was less efficiently retained in internal structures (Figures 6A and 6B). Therefore, the PLD in Pin2 is necessary for its internal retention. To demonstrate that Pin2 aggregates in internal structures, we performed Blue native gel electrophoresis and detected a strong increase in aggregates in the megadalton range in a Li^+-treated lysate compared with the untreated control (Figure S6B). These internal structures correspond to the TGN, as they colocalized with the TGN marker Sec7p (Figure S6C). Our results are consistent with the Pin2 PLD acting as a TGN retention signal under both nonstress and stress conditions. Under stress, the equilibrium of Pin2 would be shifted toward the aggregated state.

Pin2 Aggregation Is Reversible

Normally a cell would try to prevent proteins from aggregating. The PLD of Pin2 comprises an essential part of the exomer interaction surface as well as the Y-based motif. Thus, aggregate formation could potentially modulate the functionality of these transport motifs and provide a very efficient sequestering mechanism to prevent degradation of Pin2. Pin2 would be sequestered in internal structures for the duration of the stress, and after stress release, it would readily appear at the plasma membrane in a polarized fashion. To test this hypothesis, we first treated cells O/N with Li^+ to internalize Pin2 (Figures 6C and 6D) and then washed out the Li^+. At 5 min after washout, Pin2 reappeared at the plasma membrane of the bud, and the process was completed within 30 min (Figures 6C and 6D). As expected, Pin2(out) and Pin2QNtoED were less efficiently retained intracellularly than Pin2. Thus, the PLD-mediated aggregation of Pin2 is reversible and may serve as a novel temporary retention mechanism upon stress and during the cell cycle. Consistent with this notion, overexpression of molecular chaperones that could resolve harmful aggregates (Cashikar et al., 2005; Duennwald et al., 2012; Haslbeck et al., 2005) had no effect on Pin2 localization (Figure S7).

PLD Expansion Causes More Efficient TGN Retention under Stress

So far, we had manipulated the PLD in such a way as to make it less functional. We reasoned that increasing the Q/N content should increase the TGN retention, particularly under Li^+ stress.

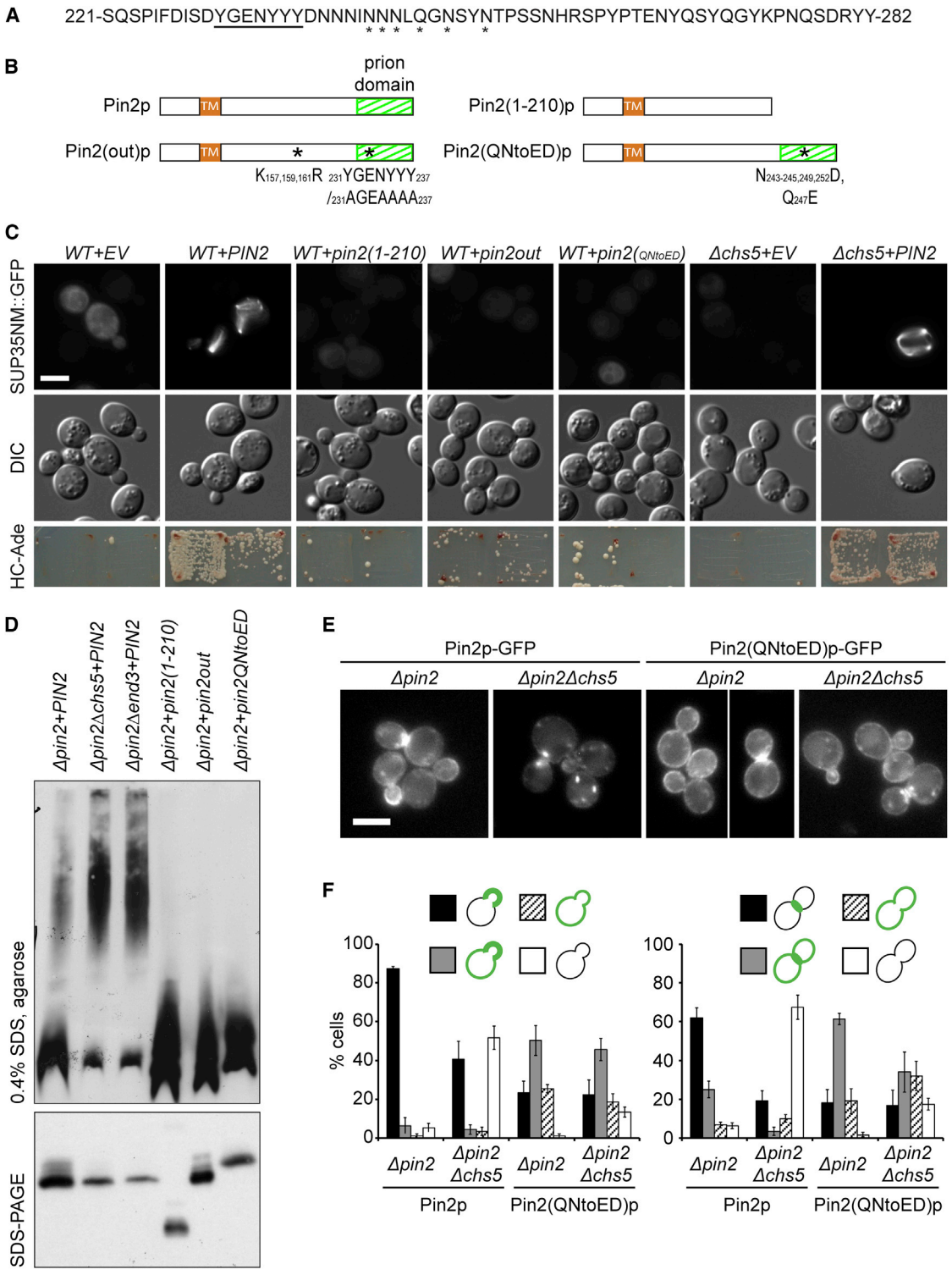


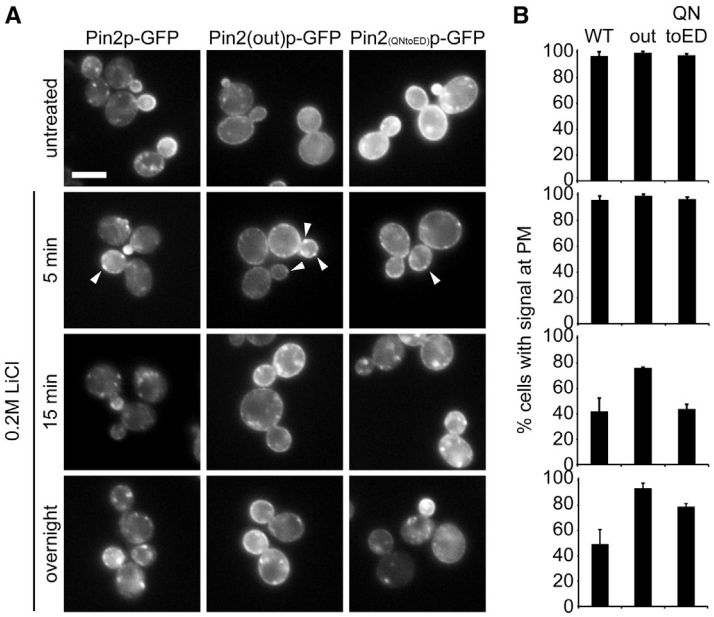
Figure 5. The PLD of Pin2 Acts as a TGN Retention Signal

(A) Amino acid sequence of the Pin2 PLD. The Y-rich motif is underlined. Asterisks indicate residues mutated to disrupt aggregation of the PLD.

(B) Schematic representation of Pin2 with the indicated PLD: WT, Pin2(1-210), Pin2(out), and Pin2(QNtoED) mutant.

(C) Overexpression of Pin2, but not of prion domain mutants, induces *[PSI+]*. *[PSI+]* induction was detected by the appearance of SUP35NM-GFP foci and ring-like structures, and growth on HC-Ade medium in strains overexpressing Pin2 variants.

(legend continued on next page)



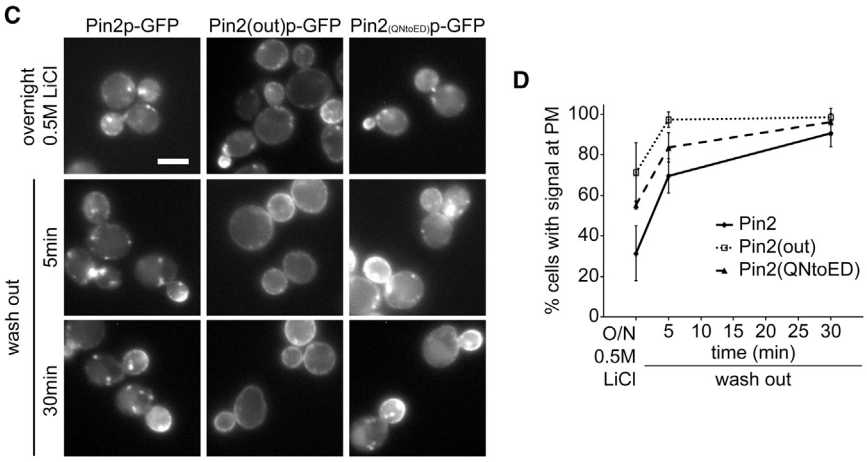


Figure 6. The PLD Confers Retention of Pin2 in Internal Compartments in LiCl

(A) Upon exposure to Li^+, Pin2 is internalized and maintains this localization through the PLD. Fluorescence microscopy of *Δpin2* cells expressing Pin2-GFP, Pin2(out)-GFP, and Pin2QNtoED-GFP treated with LiCl. Arrowheads indicate Pin2 clusters at the plasma membrane.

(B) Cells in (A) were quantified for Pin2-GFP expression at the plasma membrane.

(C) Pin2 is rapidly reexported to the plasma membrane upon environmental stress relief. Strains described in (A) were incubated O/N with LiCl. After harvesting, cells were resuspended in medium w/o LiCl and imaged at indicated time points after washout.

(D) Analysis of the data in (C) as described in (B). For quantifications in (B) and (D), 30–100 small and medium budded cells were quantified as in Figure 3C.

Error bars represent SD. Scale bars in (A) and (C), 5 μm. See also Figures S5–S7 and Tables S1 and S2.

We added two repeats of an N-rich region of Pin2 or Q-rich repeats from Sup35 that are part of the aggregation domain (Figure 7A) (Kushnirov et al., 1988; Liu and Lindquist, 1999). These constructs should not form amyloids under the conditions tested because the frequency with which *[PSI+]* elements appear is roughly 10^6 times lower than the phenotype we were assaying with TGN retention (Liu and Lindquist, 1999). Although under nonstress conditions both of the expanded PLDs trafficked in the same way as Pin2, in the presence of Li^+ they were more efficiently retained in the TGN (Figures 7A, 7B, and S6C). We conclude that reversible aggregation through the PLD is a determinant for the spatial distribution of Pin2.

DISCUSSION

We have identified an exomer-dependent cargo that is localized in a temporally and spatially controlled fashion like the other well-characterized cargo, Chs3. Moreover, Pin2 and Chs3 have very similar trafficking requirements in that they both need constant endocytosis and recycling through the TGN to maintain their proper localization at the plasma membrane (Figure S2). Similar trafficking requirements were also observed for Fus1, but only in response to a mating factor (Barfield et al., 2009). Thus, from the three exomer-dependent cargoes a common regulatory pathway emerges: all three cargoes require constant endocytosis, recycling from endosomes to the TGN, and exocytosis in an exomer-dependent manner for their proper localization at the plasma membrane. However, not all polar localized proteins are bona fide exomer-dependent clients. The protein Skg6 is localized in a polarized fashion at the bud tip and can even interact with exomer, but it does not rely on exomer for its localization. Since we detected the interaction by in vivo crosslinking and by pull-downs, Skg6

(D) Accumulation of Pin2 SDS-resistant aggregates is PLD dependent. Agarose gels of SDS-treated extracts of overexpressed Pin2 or Pin2 variants. Anti-Pin2 immunoblot.

(E) Disruption of Pin2 aggregation causes loss of exomer dependency and polarity at the plasma membrane. Fluorescence microscopy of *Δpin2* and *Δpin2Δchs5* strain cells expressing Pin2-GFP or Pin2QNtoED-GFP. Scale bar represents 5 μm.

(F) Analysis of data in (E) for plasma membrane localization and polarity of the signal as in Figure 3C.

Error bars represent SD. Scale bars in (C) and (E), 5 μm. See also Figure S4 and Tables S1 and S2.

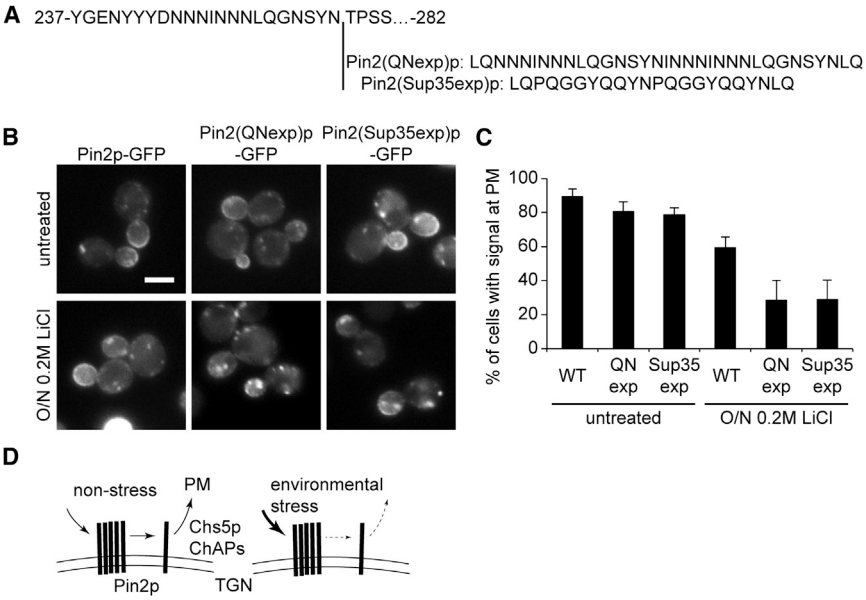


Figure 7. Expansion of the Pin2 PLD Promotes TGN Retention

(A) Sequence of the Pin2(QNexp) and Pin2 (Sup35exp) variants. Two Pin2 Q/N-rich region repeats or two Sup35 repeats were inserted after residue 252 of the Pin2 PLD.

(B) Fluorescence microscopy of *Δpin2* cells expressing Pin2-GFP, Pin2(QNexp)-GFP, or Pin2 (Sup35exp)-GFP treated with 0.2 M LiCl O/N. Scale bar represents 5 μm.

(C) Cells in (B) were scored for the presence of Pin2 at the plasma membrane as in Figure 3C. Error bars represent SD.

(D) Equilibrium shift model of Pin2 toward aggregate formation.

See also Figure S6 and Tables S1 and S2.

may still be able to use the exomer-dependent transport route, but it certainly can also exploit the more classical route via early endosomes.

Although Pin2, Chs3, and Fus1 are exomer-dependent cargoes, they all use somewhat different recognition signals for their interaction with exomer. Our studies confirm that they are complex and may involve at least two different regions of the protein. At least one of these cargoes may be unstructured and may fold only after interaction with exomer.

However, Pin2 appears to have a feature distinct from the other two known exomer cargoes: it contains a PLD, and our studies demonstrate that overexpressed Pin2 can form SDS-resistant aggregates in vivo. Although it is not understood how Chs3 is kept in internal stores after the bud neck constriction is finished, we show here that aggregate formation can act as a retention signal in internal stores, at least in the case of Pin2 expressed at physiological levels. Prion domains do not always form amyloid aggregates that cannot be resolved by the cellular machinery. The intrinsically disordered feature of prion domains can cause aggregation that is easily resolved and also may serve as platform for macromolecular assembly (Malinovska et al., 2013). For example, a number of processing body (P body) components, which are part of the major mRNA decay machinery in yeast and mammals, contain PLDs, which are thought to be essential for functional P body formation (Alberti et al., 2009; Decker et al., 2007; King et al., 2012; Malinovska et al., 2013; Michelitsch and Weissman, 2000). The PLD of Pin2 is rich in Ns, which are not enriched in toxic intermediates, whereas Q-rich domains promote the formation of toxic conformers (Halfmann et al., 2011).

The PLD is required to regulate Pin2 export to the plasma membrane under normal and stress conditions (Figure 7F). It is unlikely that Pin2 is the only protein that uses an aggregation mechanism to control its localization. In a census for PLDs in *S. cerevisiae*, a number of proteins involved in vesicular traffic were identified (Michelitsch and Weissman, 2000). However, to our knowledge, regulated trafficking depending on the prion domain has not been demonstrated for any of these proteins. In mammalian cells, Pmel17 forms benign amyloid fibers in melanosomes to sequester melanin (Berson et al., 2003). Although the aggregate formation in this case is dependent on cleavage by a metalloprotease, the transport of Pmel17, such as export from the ER and endocytosis from the plasma membrane, is critical for sorting and its function (Fowler et al., 2006; Theos et al., 2006). A pathway analogous to the exomer route from the TGN to the plasma membrane has also been identified in metazoans (Wakana et al., 2012). Again, the number of proteins that take this transport route is rather small to date, and the identification of more cargoes may also reveal the function of prion retention in this pathway. Since 0.3%–2.5% of cellular proteins, depending on the organism, contain a PLD, regulation similar to that observed in Pin2 is likely to be found in other proteins.

What would be the function of the prion-dependent retention mechanism? During normal growth and under stress, it regulates the amount of Pin2 that is present at the plasma membrane. Moreover, this mechanism prevents the degradation of Pin2 because it remains in the TGN (at least under stress conditions) for at least 16 hr. Under such conditions, retention seems to be important, because Pin2 was released from internal stores as soon as 5 min after the end of the stress. This release mechanism is much faster than resynthesis and transport of Pin2. Here, we identify a retention mechanism for a prion-like protein.

The retention of Pin2 by the PLD could be brought about through two nonexclusive mechanisms. Since part of the exomer-interaction domain is located in the PLD, TGN export signals could be masked. Alternatively, the prion-dependent Pin2 aggregate in the TGN could be too large to get into transport vesicles. The latter possibility would not only restrict Pin2 from plasma membrane localization but would also protect Pin2 from degradation in the vacuole. We also cannot exclude the possibility that other proteins are part of Pin2 aggregates in the TGN.

Similarly to Pin2, Chs3 also reacts to stress. Upon cell-wall stress, Chs3 is rapidly endocytosed and then released at the plasma membrane in a nonpolarized fashion (Valdivia and

Schekman, 2003). This release from internal stores is dependent on the small GTPase Rho1 and the protein kinase Pkc1 (Valdivia and Schekman, 2003). Regulation of the stress response may not be a conserved feature among exomer-dependent cargoes, and may be more related to their function, as a mutant in *PKC1* did not interfere with Pin2 trafficking under Li^+ stress or the release from it (data not shown).

At the moment, why Pin2 would have to be retained in internal stores upon stress is unclear. We speculate that it might sense stress, and that a fraction might be continuously released to the plasma membrane to check the environment. Consistent with this hypothesis, Pin2 was found to interact with various components of the cell-wall integrity pathway in a high-throughput analysis (Schlecht et al., 2012; Tarassov et al., 2008), and deletion of MAPKs of stress-sensing pathways delayed endocytosis of Pin2 upon stress.

EXPERIMENTAL PROCEDURES

Strains, Yeast Genetic Methods, and Plasmid Construction

The yeast strains and plasmids used in this study are listed in Tables S1 and S2. Details are provided in the Supplemental Experimental Procedures.

Microscopy

Cells were grown to OD_{600} 0.2–0.7 in yeast extract peptone dextrose (YPD) or Hartwell's complete (HC) medium supplemented with adenine, harvested, and mounted. Images were acquired with an Axiocam mounted on a Zeiss Axioplan 2 fluorescence microscope, using appropriate filters.

GST Pull-Downs

For pull-downs, 5 μg of GST-Pin2 and Sec22-GST, and 0.5 ml of GST-Skg6 *E. coli* lysate were bound to 10 μl GSH agarose (Sigma Aldrich). *4TAG*, *Δ4ChAPs*, or *CHS6-9myc* cells (10 OD_{600}) were harvested, spheroplasted, and lysed in 1 ml B150Tw20 (20 mM HEPES pH 6.8, 150 mM KAc, 5 mM $Mg(Ac)_2$, 1% Tw-20) + protease inhibitors. Lysates (900 μl) were incubated for 1 hr at 4°C with 10 μl GSH agarose with GST-tagged protein. Pull-downs were washed 3× in B150Tw20 and 1× 20 in mM HEPES pH 6.8, 150 mM NaCl. Proteins were eluted with 35 μl SDS sample buffer at 68°C.

Denaturing Immunoprecipitations

pin2Δ cells (20 OD_{600}) carrying YEp112 HA-ubiquitin and pGFP33 PIN2 or pGFP33 pin2K7R were harvested, spheroplasted, and lysed in 200 μl 20 mM HEPES pH 6.8, 200 mM KCl, 1 mM $MgCl_2$, 2% TX-100, 1 mM dithiothreitol buffer with protease inhibitors (Søgaard et al., 1994). Lysates were cleared (10 min, 10,000 × *g* centrifugation), 1% SDS was added to the supernatants, and samples were heated for 3 min at 95°C. Extracts were diluted 10× to 20 mM HEPES pH 6.8, 200 mM KCl, 1 mM $MgCl_2$, 0.5% TX-100, 0.1% SDS final concentration, and spun for 10 min at 10,000 × *g*. Extracts (1.9 ml) were incubated at 4°C O/N with 5 μg anti-GFP antibody (Torrey Pines) or 5 μg rabbit immunoglobulin G (Dianova) bound to 10 μl ProtA-Sepharose. Samples were washed 3× with 20 mM HEPES pH 6.8, 200 mM KCl, 1 mM $MgCl_2$, 0.5% TX-100, and 1× with 20 mM HEPES pH 6.8, 200 mM NaCl. Proteins were eluted with 35 μl SDS sample buffer at 68°C.

[PSI+] Induction Assay

[PSI+] induction assay was carried out as described previously (Derkatch et al., 2001). Briefly *[pin-][psi-]* 74-D694 or Δ*chs5* was transformed with pSUP35NM::GFP-HIS3 and p426GPDleu2d plasmids. Strains were replica plated for 35 generations on HC-Leu-Ura-His medium and then on HC-His $+Cu^{2+}$ medium to induce SUP35NM::GFP. Cells were analyzed by fluorescence microscopy and for growth on HC-Ade medium to confirm *[PSI+]* induction.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.026.

ACKNOWLEDGMENTS

We thank I. Derkatch, S. Liebman, J. Shorter, S. Nothwehr, and M. Hall for strains and reagents. I.G. Macara is acknowledged for comments on the manuscript. We thank P. Jenö and S. Moes (Mass Spectrometry Facility) and T. Sharpe (Biophysics Facility) from the Biozentrum for support. This work was supported by the Boehringer Ingelheim Fund (to A.M.R.), the Swiss National Science Foundation (31003AB-126021 and 31003A-141207 to A.S.), and the University of Basel.

Received: September 8, 2013
Revised: January 30, 2014
Accepted: February 16, 2014
Published: March 20, 2014

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Please cite this article in press as: Ritz et al., The Prion-like Domain in the Exomer-Dependent Cargo Pin2 Serves as a *trans*-Golgi Retention Motif, Cell Reports (2014), http://dx.doi.org/10.1016/j.celrep.2014.02.026

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