A signal comprising a basic cluster and an amphipathic α-helix interacts with lipids and is required for the transport of Ist2 to the yeast cortical ER

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Summary
The yeast integral membrane protein Ist2 is encoded by a bud-localised mRNA and accumulates at patch-like domains of the cell periphery, either at the cortical ER or at ER-associated domains of the plasma membrane. Transport of IST2 mRNA and local protein synthesis are not prerequisite for this localisation, indicating that Ist2 can travel through the general ER to membranes at the cell periphery. Here, we describe that the accumulation of Ist2 at the cortical ER requires a cytosolicly exposed complex sorting signal that can interact with lipids at the yeast plasma membrane. Binding of the Ist2 sorting signal to lipids and rapid and efficient transport of Ist2 from perinuclear to cortical ER depend on a cluster of lysine residues, the formation of an amphipathic α-helix and a patch of hydrophobic side chains positioned at one side of the amphipathic α-helix. We suggest that a direct interaction of the Ist2 sorting signal with lipids at the plasma membrane places Ist2 at contact sites between cortical ER and plasma membrane. This provides a physical link of an integral membrane protein of the cortical ER with the plasma membrane and might allow direct transport of proteins from cortical ER to domains of the plasma membrane.

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Introduction
The endoplasmic reticulum (ER) is an organelle that forms contacts with many other membranes, and these contacts are specialised for traffic of both material and information (Levine and Loewen, 2006). One example of such direct transport is the exchange of lipids between the ER and plasma membrane. Experiments in Saccharomyces cerevisiae show that a non-vesicular exchange of lipids occurs between the ER and plasma membrane (Baumann et al., 2005; Raychaudhuri et al., 2006; Schnabl et al., 2005). The yeast cortical ER is in contact with the plasma membrane at many positions, where such a direct exchange of molecules between cortical ER and plasma membrane might occur (Perktold et al., 2007; Pichler et al., 2001). However, little is known about the molecular components of ER-to-plasma-membrane contact sites and the sorting of ER membrane proteins to these or to other domains of the yeast cortical ER.

The yeast S. cerevisiae has two main ER types: the perinuclear ER and the cortical ER (Voeltz et al., 2002). The cortical ER is a peripheral meshwork of membrane tubules underlyng the plasma membrane, with only a few cytoplasmic tubules connecting the cortical ER to the perinuclear ER (Koning et al., 1993; Preuss et al., 1991; Prinz et al., 2000). However, the entire ER is one continuum, as shown by free diffusion of luminal GFP and the mobility of integral ER membrane proteins (Luedeke et al., 2005). The movement of the translocon component Sec61, the SNARE Sec22 and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmg1) between the cortical and perinuclear ER of mother cells is only ten times slower than the free diffusion of GFP in the ER lumen (Luedeke et al., 2005). Fluorescence loss in photobleaching experiments in mother cells revealed that half of the Sec61 exchanges between cortical and perinuclear ER in less than 20 seconds. Between the ER of mother and daughter cells this exchange occurs within minutes (Luedeke et al., 2005).

Based on mRNA transport and local translation, the cortical ER of daughter cells is the main site for synthesis of a number of membrane proteins encoded by localised mRNAs (Aronov et al., 2007; Shepard et al., 2003; Takizawa et al., 2000). One of these localised mRNAs encodes the polytopic membrane protein Ist2, which localises to patch-like domains of the cell periphery (Juschke et al., 2004; Takizawa et al., 2000). By fluorescence microscopy, these domains colocalise with the ER protein Dpm1 (Juschke et al., 2004). However, cell fractionation and protease protection experiments indicate a localisation of Ist2 at the plasma membrane (Juschke et al., 2004). Transport of Ist2 to these peripheral domains occurs independently of the classical secretory pathway on a route that bypasses the Golgi (Juschke et al., 2004;
Juschke et al., 2005). This transport requires the cytosolically exposed C-terminal domain of Ist2 (Juschke et al., 2005). Without this domain, Ist2 accumulates at punctuated structures of the perinuclear and cortical ER (Franz et al., 2007; Juschke et al., 2005). When attached to a cytosolically oriented C-terminus of a membrane protein, a protein sorting of the C-terminal 69 amino acid signal is sufficient for sec-independent transport to the cell periphery (Franz et al., 2007). Transport of Ist2 can operate independently of IST2 mRNA localisation (Franz et al., 2007), indicating that the Ist2 sorting signal leads to the accumulation of Ist2 at the cortical ER from where the protein may reach ER-associated domains of the plasma membrane.

In this study, we found that a certain amount of Ist2 localises for a short period at the perinuclear ER of mother cells, and we identified specific residues required for rapid movement from the perinuclear to the cortical ER. These residues are part of a sorting signal that binds lipids at the plasma membrane, suggesting that the interaction between the Ist2 sorting signal and lipids localises Ist2 at the cortical ER.

Results

Insertion of Ist2 into the perinuclear ER of mother cells

Is all Ist2 synthesised at the cortical ER of daughter cells as a consequence of mRNA localisation, or does synthesis also take place at the perinuclear ER of mother cells? Since GAL1-promoter-induced, fluorescent-protein-tagged Ist2 appeared at peripheral patches without any staining of the perinuclear ER (Fig. 1A) (Juschke et al., 2004; Juschke et al., 2005; Takizawa et al., 2000), we argued that if Ist2 is inserted into the perinuclear ER, the protein leaves this part of the organelle before maturation of the fluorescent proteins. To analyse this, we localised GAL1-induced Ist2 with an N-terminal hemagglutinin (HA) tag by indirect immunofluorescence. HA-Ist2 was synthesised 30 minutes after galactose induction, as detected by western-blotting (Fig. 1B). After 40 minutes, ist2Δ cells frequently showed HA-Ist2 staining at buds, and at patches in the periphery of budded and unbudded cells (Fig. 1C). 40% of the unbudded cells and 18% of budded cells showed additional perinuclear HA-Ist2 staining (indicated with arrows in Fig. 1C, quantification at the 0-minute time point in Fig. 1D). The transport of IST2 mRNA to the bud cortical ER is probably responsible for the reduced number of budded cells with perinuclear HA-Ist2 staining compared with unbudded cells with this staining. Detection of GAL1-induced YFP-Ist2 at the perinuclear ER with an antibody against the C-terminal domain of Ist2 confirmed that a detectable pool of YFP-Ist2 is also inserted into the perinuclear ER (supplementary material Fig. S1). This pool is not detected by YFP fluorescence in living cells (Fig. 1A), probably because of the slow maturation of YFP.

How long does this pool of Ist2 remain at the perinuclear ER? To address this question, we stopped protein synthesis by adding cycloheximide, took a sample every three minutes, fixed the cells with formaldehyde and localised HA-Ist2 by immunofluorescence with antibodies against HA. Within the first 3 minutes, the number of unbudded cells with perinuclear ER staining was reduced from 40% to 21%, indicating that Ist2 reached the cell periphery rapidly (Fig. 1D). Repression of HA-Ist2 transcription by glucose resulted in a similar disappearance of HA-Ist2 from the perinuclear ER. After a 10 minute treatment with glucose, the number of unbudded cells with detectable HA-Ist2 at the perinuclear ER dropped from 50% to 26% (not shown). These data confirm the cycloheximide results and demonstrate that some of the newly synthesised Ist2 spends a limited time at the perinuclear ER before it accumulates at the cell periphery.

Basic residues at the C-terminus of Ist2 are required for trafficking to the cortical ER

Ist2 from S. cerevisiae comprises 946 residues and has eight transmembrane domains and a domain, named DUF590, of unknown function (Fig. 2A) (Galindo and Vacquier, 2005). The cytosol-oriented C-terminal domain of Ist2 contains a complex protein-sorting signal comprising the C-terminal 69 residues of Ist2 (Franz et al., 2007). This signal consists of two parts. Its N-terminus contains a cluster of single amino acid repeats with multiple threonine, histidine and serine residues (T/H/S cluster) (Fig. 2A). Its C-terminus consists of a basic region, which is rich in lysine and leucine residues (K/L-sequence). The C-terminal 11 residues of the K/L-sequence (K936-L946) are predicted to form an
amphipathic α-helix (Sapay et al., 2006), of which the three most C-terminally located lysines and L946 fit the consensus of a di-lysine ER-retrieval signal (Fig. 2B).

To identify the residues that are essential for the function of the Ist2 sorting signal, we screened for mutants that mislocalized to a perinuclear or punctuated peripheral distribution. Among other mutations, we identified five single residue exchanges in the K/L-sequence that caused such mislocalization (G937E, L938Q, L939P, K941E and L942Q) (Fig. 2B). These mutations fall into three different categories: change from a basic into an acidic residue, loss of a hydrophobic side chain and change of a secondary structure by the introduction of a helix-breaking proline residue. An alignment of a selection of closely related yeast species revealed that the K/L sequence is better conserved than the upstream T/H/S cluster (Fig. 2B).

We tested the effects of these three types of mutation individually. Using fluorescence microscopy it is not possible to discriminate whether a protein is at the plasma membrane or at the underlying cortical ER. Therefore, we use the term ‘cell periphery’ to describe localization at plasma membrane or cortical ER. To determine whether the isolated Ist2 alleles localize to the general ER or to membranes at the cell periphery, we used sec23-1 mutant cells, which fail to form COPII vesicles and accumulate proliferated ER structures (Novick et al., 1980). Since the proliferated cortical ER of these cells detaches from the cell periphery, a trapping of mutated Ist2 at the general ER becomes visible. The sec23-1 phenotype was monitored by the expression of GAL1-induced hexose transporter (Hxt1-CFP). At the non-permissive temperature of 37°C Hxt1-CFP was trapped at proliferated ER structures, whereas at 25°C, Hxt1-CFP reached the plasma membrane, from where it entered the vacuole via endocytosis (Fig. 3A). At permissive and non-permissive conditions, GAL1-induced YFP-Ist2 localized exclusively to the cell periphery (Fig. 3A). A deletion of the K/L sequence (residues 929-946) resulted, at 25°C, in an accumulation at perinuclear ER and at peripheral dots, indicating that the K/L sequence is required for sorting from the
perinuclear to the cortical ER. At 37°C, YFP-Ist2L942Q and Hxt1-CFP were trapped at proliferated ER structures (Fig. 3A).

A mutation of C-terminally located K931, K933, H934, K935, K936, H940, K941, K943, K944, K945 to A (basic-all) resulted in an accumulation in ill-defined large dots and other intracellular structures at 25°C (Fig. 3B). Upon the shift to 37°C, most of the protein was trapped at the proliferating ER. Next, we exchanged either four of the N- or C-terminal basic residues with alanines. Mutation of K931, K933, H934, K935, K936 to A (basic-N) caused at 25°C a weak staining of structures resembling perinuclear ER in addition to the accumulation at the cell periphery. Mutations of H940, K941, K943, K944, K945 to A (basic-C) showed no phenotype, suggesting that if the N-terminal part of the basic cluster is present, the C-terminal part has only a minor contribution to Ist2 sorting. Taken together, these results suggest that positive charges are indeed important for the peripheral accumulation of Ist2 and that the lysine residues have a position-dependent function.

To characterise the function of the lysine residues further, we mutated single lysine residues of the N-terminal part of the basic cluster to alanines. The mutation K936A caused partial punctuated accumulation in a few cells at 25°C, whereas the mutation K935A and the double mutation K933A/H934A had no effect (supplementary material Fig. S2A). The K936A mutation, however, increased the trapping of Ist2 if combined with a mutation in the C-terminal part (supplementary material Fig. S2B). Mutations of three lysines or two lysines and a histidine in K931-K935 were tolerated (supplementary material Fig. S3A), whereas a K931A/K933A/H934A/K935A mutation caused some perinuclear accumulation at 25°C (supplementary material Fig. S3B). However, compared with the basic-N mutant, less of the K931A/K933A/H934A/K935A mutant was trapped at the proliferated ER at 37°C (compare Fig. 3B and supplementary material Fig. S3B). These results indicate an additive effect of K936 and the C-terminal part of the basic cluster as well as a position-dependent requirement of at least two lysines and/or one histidine in the N-terminal part in cooperation with several lysine residues in the C-terminal part.

To investigate whether transport of the Ist2 basic-N mutant from the perinuclear ER to the cell periphery was simply delayed or whether the protein could not leave the perinuclear ER, we induced the synthesis of Ist2 basic-N for 120 minutes at 25°C. After a 180 minute shift into glucose medium, Ist2 basic-N was not degraded but maintained its perinuclear localisation (Fig. 3C, indicated by arrow). Under the same conditions, wild-type YFP-Ist2 reached the cell periphery without any visible intracellular accumulation. This localisation and the quantification of Ist2 proteins by western blotting (Fig. 3D), indicated that the mutation of the N-terminal cluster of basic residues did not affect the stability of Ist2 and showed that this mutation abolished trafficking of Ist2 from the perinuclear ER to the cortical ER.

**Mutation of hydrophobic residues traps Ist2 at specific ER domains**

As a second category of mutations causing intracellular accumulations of Ist2, we identified mutations of the hydrophobic positions L938 and L942 (L938Q, L942Q). Most sec23/J cells expressing YFP-Ist2L942Q at 25°C showed punctuated accumulation at perinuclear and peripheral structures and a trapping at the proliferating ER at 37°C (supplementary material Fig. S4). We observed a heterogeneous phenotype, because some cells showed a localisation only at the cell periphery (quantification in supplementary material Table S1). A comparison of two clones with different expression revealed that the degree of punctuated accumulation at 25°C depends on the amount of Ist2 L942Q synthesis (supplementary material Fig. S4). In cells with high expression, the accumulation of Ist2 L942Q in dots was more pronounced. This accumulation in large dots was observed at both 25°C and 37°C. Importantly, we never saw such punctuated localisation for wild-type Ist2, which was induced similarly.

Next, we asked whether the hydrophobicity at position 942 influences the peripheral accumulation. Exchange of L942 to alanine, valine or methionine at 25°C led to accumulation in one or two dots in 72-89% of cells (supplementary material Fig. S5, Table S2). Compared with the L942Q mutant, these dots disappeared in cells shifted to 37°C, although small amounts of Ist2 remained at proliferated ER structures. Exchange of L942 to tryptophan and phenylalanine resulted in only 12% and 25%, respectively, of cells in punctuated accumulation. The mutation to isoleucine had no effect. As a next step, we determined whether the remaining leucines also contribute to the peripheral accumulation of Ist2. Mutation of L938 or L939 to alanine and valine led to punctuated phenotype, whereas double mutation of L938 and L939 to alanine or valine increased this phenotype, whereas double mutation of L938 and L939 to isoleucine was tolerated. Compared with positions 938, 939 and 942, the mutation of L946 had the mildest effect.

To analyse at which membranes the punctuated Ist2 was trapped, we colocalised mCherry-tagged Ist2L942Q (red) with GFP-tagged proteins characteristic for specific organelles (green). mCherry-Ist2L942Q dots showed no overlap with Sed5, Gef1 and Kex2 (supplementary material Fig. S7). Instead of mCherry-Ist2L942Q, we used GFP-tagged Ist2L942Q (green) for colocalisation with Sec7-RFP (red). Similarly to Sed5, Gef1 and Kex2, we observed no overlap between Ist2L942Q and Sec7 (supplementary material Fig. S7). A statistical analysis of Ist2L942Q dots revealed an average distance of five pixels (0.4 μm) between the maximum signal intensity of an Ist2L942Q dot and the nearest Sec7, Sed5, Gef1 or Kex2 dot, indicating that Ist2L942Q was not trapped at either the Golgi (Sec7, Sed5, Gef1 and Kex2) or in endosomes (Gef1 and Kex2) (supplementary material Fig. S8A). Fractionation of mCherry-Ist2L942Q by separation on a 20-60% sucrose density gradient revealed that the majority of Ist2 L942Q cofractionated with rough ER and plasma membrane markers (Sec61 and Pma1) in fraction 8, whereas the Golgi-modified forms of Emp47 migrated in fractions 4-6 (supplementary material Fig. S9A,B). Under these conditions, rough ER and plasma membrane do not separate. Emp47 shuttles between the ER and Golgi (Schröder et al., 1995) and Sed5-GFP showed a similar partial separation from mCherry-Ist2L942Q into Golgi fractions (supplementary material Fig. S9C). No difference was observed for Ist2L942Q compared with the wild type, when separated on a sucrose gradient under these conditions (data not shown).

Ist2L942Q dots colocalised with Scs2-containing ER at bud tips, cortical and perinuclear ER (Fig. 4, indicated by arrows). Scs2 is an integral ER membrane protein that functions as an adaptor for lipid-transfer proteins and in the anchorage of ER at the bud cortex (Loewen et al., 2003; Loewen et al., 2007). Some Scs2, which showed a normal ER distribution in cells overexpressing wild-type Ist2, was recruited to the dots containing Ist2L942Q. An even stronger recruitment into Ist2L942Q dots was observed for the integral ER membrane proteins Dpm1 and Sec63 (Fig. 4).
In Dpm1-GFP coexpressing cells, mCherry-Ist2 L942Q had reproducibly more peripheral signal, whereas all other strains showed more accumulation of Ist2 L942Q in dots. The reason for this variability remains unknown. The distance of the maximum signal intensities between Ist2 L942Q dots and Scs2, Dpm1 or Sec63 signals at the ER were zero to one pixel, indicating that these proteins colocalise (supplementary material Fig. S8B). In comparison with Scs2, Dpm1 and Sec63, the recruitment of Hmg1 to Ist2-L942Q-positive dots occurred less frequently (supplementary material Fig. S8B). The enzyme Hmg1 is involved in the first step of ergosterol lipid biosynthesis (Basson et al., 1986). At the light microscopic level, all tested ER proteins localised to the perinuclear and cortical ER.

Some of the Ist2 L942Q dots were closely adjacent to Erg6, which localises to the ER and ER-derived lipid droplets (Athenstaedt et al., 1999). However, many of the punctuated Erg6-positive lipid droplets were clearly separated from Ist2 L942Q, indicating that Ist2 L942Q did not accumulate at lipid droplets (Fig. 4; supplementary material Fig. S8C). A total of 90% of Sec13 dots, which stain the ER exit sites containing COPII, showed a separation from Ist2 L942Q, with a distance of maximal signal intensities of three or more pixels (Fig. 4; supplementary material Fig. S8C). Sucrose density centrifugation revealed that more than 65% of Sec13-GFP was separated from Ist2 L942Q (supplementary material Fig. S9D).

In summary, the mutation of hydrophobic residues abolished the transport of Ist2 to patch-like domains at the cell periphery. The colocalisation and fractionation experiments clearly indicate that the Ist2 L942Q mutation led to a dot-like accumulation at perinuclear, tubular and cortical ER domains. The intracellular Ist2 L942Q dots were often located at positions where ER tubules emerge from the perinuclear ER in the direction of the cortical ER (colocalisation of Ist2 L942Q with Scs2 and Dpm1) (Fig. 4).

Next, we investigated whether the Ist2 L942Q dots correspond to aggregated protein. Expression of YFP-Ist2 L942Q for 3 hours resulted in a 1.7-fold upregulation of Kar2, whereas expression of wild-type YFP-Ist2 had no effect on Kar2 levels (supplementary material Fig. S10A,B). This suggests that an aggregation of YFP-Ist2 L942Q might cause the observed trapping at the ER. However, antibody staining of endogenous Kar2 in cells expressing mCherry-Ist2 L942Q did not result in a massive recruitment of Kar2 to these structures (supplementary material Fig. S10C,D).
Mutations of single leucines into less hydrophobic and bulky residues in sec23-1 cells at 25°C led to a punctuated accumulation at specific ER domains. At 37°C, most of these punctuated structures were dissolved, and we observed a slight accumulation at intracellular ER structures (supplementary material Figs S5 and S6). Is the observed disappearance of punctuated Ist2 at 37°C just a matter of protein concentration or is it a temperature- and energy-dependent effect? To exclude a temperature-dependent effect on protein concentration, we induced the expression of Ist2 L942V at 25°C for 120 minutes, stopped new transcription of IST2 mRNA by the addition of glucose and kept the cells at 25°C or 37°C (supplementary material Fig. S11). After 60 minutes in glucose at 25°C, most cells maintained one to two Ist2 L942V dots, whereas most of these dots disappeared at 37°C. This mobilisation of trapped Ist2 was reduced in ATP-depleted cells poisoned with 10 mM sodium azide, indicating that higher temperatures mobilise trapped Ist2 L942V in an energy-dependent manner.

Hydrophobic residues function as part of an amphipathic α-helix

We identified a L939P mutation as a third category of mutations causing intracellular accumulations of Ist2. If the hydrophobic residues L938, L939 and L942 function as part of an amphipathic α-helix, the introduction of a helix-breaking proline is expected to abolish the function of this amphipathic α-helix. All four leucines in the K/L sequence are predicted to locate on one side of an amphipathic α-helix as illustrated by a helical wheel projection (Fig. 5). According to the prediction program AmphipaSeek, this short amphipathic α-helix comprises residues K936 to L946 (Sapay et al., 2006). The four hydrophobic side chains of L938, L939, L942 and L946 will be located within an angle of 120 degrees in the helix. According to the Ist2 sequence of Ashbya gossypii (Fig. 2B), we inserted three extra residues (alanines) after position L938. This insertion added one extra helical turn without changing the hydrophobic patch and had no effect on the localisation of Ist2 (Fig. 5A). Similarly, an insertion of four alanines after position G937 resulting in an arrangement of leucines as in wild-type Ist2, had no effect. To test whether the concentration of hydrophobic residues at one side of the helix is required for Ist2 trafficking, we changed the distribution of hydrophobic side chains. As seen in the helical projection, a mutation of LLxxL to LxxLL inserted a positive residue in the hydrophobic patch. This caused an accumulation in dots at 25°C and weak trapping at the proliferated ER at 37°C (Fig. 5B). Such an arrangement of hydrophobic residues exists in the Ist2 sequences of Candida guilliermondii and other more distantly related yeast species (Fig. 2B). Additional spreading of the leucine residues by insertion of one or two alanines between K941 and L942 had a stronger effect with a more pronounced accumulation at proliferated ER at 37°C.

In summary, it is possible to add at least one extra loop to the α-helical structure without losing its function for trafficking of Ist2 to the cell periphery, as long as there is no disturbance in the hydrophobicity of side chains and the hydrophobic and basic residues are positioned on two opposite sides of an α-helix. To validate whether the extreme C-terminus of Ist2 forms is indeed an α-helix, we determined the secondary structure of a peptide encoding the last 14 residues of Ist2, starting with K933. We performed secondary structure determination with this peptide by circular dichroism (CD) spectroscopy. In aqueous solution (potassium phosphate buffer), the CD spectrum suggested a random structure.
coil conformation (Fig. 5C, blue curve). When solved in increasing concentrations of trifluorethanol (TFE), we recorded a conversion from a disordered state into an α-helical structure at 20% (v/v) TFE. At 40% and 50% TFE, a maximal conversion into an α-helical structure was reached, compared with reference spectra (Chen et al., 1974). However, a rough estimation of the α-helical content for the K933-L946 peptide in 50% (v/v) TFE suggested that parts of the peptide remained unstructured. To confirm that this partially α-helical conformation is required for sorting to the periphery, we tested the secondary structure of a K933-L946 peptide with a L939P mutation, which we had identified in our initial screen (Fig. 2B). In sec23-A cells, newly synthesised Ist2 L939P accumulated at dot-like intracellular ER structures (supplementary material Fig. S12). The presence of a proline at this position abolished the TFE-induced partial conversion into an α-helical structure, supporting the idea that the C-terminus of the Ist2-sorting signal forms an amphipathic α-helix.

The Ist2 sorting signal interacts with membranes

Since many examples are known where basic sequences and amphipathic α-helices mediate membrane binding (Ford et al., 2002; Heo et al., 2006; Lee et al., 2005), we wondered whether the Ist2 sorting signal, which lacks a transmembrane domain, shows any direct interactions with membranes. Therefore, we expressed a fusion of GFP with the last 69 residues of Ist2 in ist2Δ cells. Compared with GFP, which localised at the cytosol and the nucleus, some of the GFP-Ist2 (878-946) accumulated at the entire cell periphery. Compared with the patch-like staining of full-length GFP-Ist2, GFP-Ist2 (878-946) showed a smooth rim staining (Fig. 6A), suggesting that the Ist2 sorting signal binds to the plasma membrane. In addition, large amounts of GFP-Ist2 (878-946) were seen in the cytosol and at the nucleolus (data not shown). The association of the Ist2 sorting signal with the plasma membrane became more obvious after an induction of GAL1-driven GFP-Ist2 (878-946) synthesis followed by 8 hours of repression (Fig. 6B). When attached to the C-terminus of a minimal integral ER membrane protein comprising luminal YFP and the first transmembrane domain from Sec63, the Ist2 sorting signal targeted the vast majority of this reporter to the cell periphery, whereas the same reporter without Ist2 sorting signal showed a strong accumulation at the perinuclear ER (supplementary material Fig. S13).

Cell fractionation of yeast cells expressing GFP and GFP-Ist2 (878-946) showed a binding of some GFP-Ist2 (878-946) to membranes, whereas all GFP remained in the supernatant (Fig. 6C). A flotation of the majority of GFP-Ist2 (878-946) from the bottom of a sucrose-step gradient into lighter fractions confirmed the membrane association of GFP-Ist2 (878-946) (supplementary material Fig. S14A). As marker for the flotation of membranes we used the ER protein Sec61. This association of GFP-Ist2 (878-946) with membranes was abolished by 500 mM potassium acetate, demonstrating an electrostatic interaction between the Ist2 sorting signal and membranes (supplementary material Fig. S14B).

Binding of the Ist2 sorting signal to membranes occurs independently of a protein receptor but requires lipids

We purified GFP-Ist2 (878-946) via an N-terminal His6 tag from bacteria and reconstituted binding of GFP-Ist2 (878-946) to membranes isolated from ist2Δ cells. Similarly to the previous cell fractionation experiments, a significant amount of recombinant GFP-Ist2 (878-946) was found in the membrane fraction. Treatment of membranes with proteinase K before incubation with purified GFP-Ist2 (878-946) had no influence on this binding (Fig. 7A), indicating that lipids, but not proteins, are essential for the interaction between the Ist2 sorting signal and membranes. To confirm an interaction between the Ist2 sorting signal and lipids, we prepared artificial liposomes with an average diameter of 200 nm from a lipid mixture resembling the plasma membrane. Incubation of liposomes with 15 μg purified GFP and GFP-Ist2 (878-946), and recovery of liposomes by centrifugation showed that 30.5% of GFP-Ist2 (878-946) was found in the pellet fraction, whereas GFP remained in the supernatant (Fig. 7B). According to a recently published procedure (Temmerman and Nickel, 2009), we measured the liposome-bound GFP fluorescence by FACS. Rhodamine-phosphatidylethanolamine-labelled liposomes were sorted into the red channel of a FACS and the amount of GFP fluorescence was determined. The GFP fluorescence of GFP-Ist2 (878-946) bound to liposomes was set to 100 (Fig. 7C). Mutation of the hydrophobic residues resulted in a 2.5- to 5-fold reduction of binding with the strongest effect caused by the L939P mutation. Compared with this moderate loss of binding to liposomes, mutation of the first basic cluster to alanines and the rearrangement of basic and hydrophobic residues in the amphipathic α-helix had a much more severe effect.
GFP-Ist2 (878-946) to liposomes. Liposomes were incubated with 15 μg recombinant GFP or GFP-Ist2 (878-946). The reactions were separated into supernatant and pellet fraction, analysed by western blotting and that these interactions trap Ist2 at sites of the cortical ER adjacent to the plasma membrane.

**Discussion**

We identified a sorting signal responsible for the rapid transport of Ist2 from the general ER to specific domains of the cortical ER. A certain fraction of wild-type Ist2 was detected by immunofluorescence at the perinuclear ER for a short period and was transported to the cell periphery within minutes. This transport was faster than the maturation of fluorescence proteins. In living cells the detection of fluorescent-protein-tagged wild-type Ist2 was limited to peripheral membranes without any apparent accumulation at perinuclear or tubular ER or at any other intracellular structures. This was observed under all tested conditions [strong induction by the GAL1 promoter at temperatures between 16°C (not shown) and 37°C in wild-type and sec23-1 cells]. When expressed as a soluble GFP-fusion in ist2Δ yeast cells, the Ist2 sorting signal partially accumulated in a smooth peripheral pattern, suggesting that this sequence interacts directly with the plasma membrane. Since the cortical ER is in close proximity to the plasma membrane at many positions (Perktold et al., 2007; Pichler et al., 2001; Preuss et al., 1991), we suggest that the sorting signal of ER-located Ist2 can bridge the distance between these two membranes.

In vitro reconstitution experiments with purified Ist2 sorting signal and either protease-treated yeast membranes or liposomes confirmed direct binding of the Ist2 sorting signal to lipids. Cell fractionation experiments showed that this interaction is electrostatic, which is consistent with the idea that basic residues of the Ist2 sorting signal interact with negatively charged lipids. Based on the combination of the mutational analysis of the Ist2 sorting signal and the in vitro reconstitution experiments, we suggest that a certain arrangement of a number of lysine residues at the C-terminus of the Ist2 sorting signal creates a surface for the binding of lipids located in the plasma membrane.

Mutation of a cluster of basic residues located N-terminal of the predicted amphipathic α-helix (K931A/K933A/H934A/K935A/K936A) caused a weak perinuclear accumulation of Ist2. Since Ist2 with this mutation is a stable protein, we explain the observed perinuclear localisation with a loss of binding to negatively charged lipids at the plasma membrane. The highly abundant, negatively charged head groups of phosphatidylerine and phosphatidylinositol-4,5-bisphosphate at the cytoplasmic side of the plasma membrane are strong candidates for direct interaction with the Ist2 sorting signal (Behnia and Munro, 2005; McLaughlin et al., 2002).

Additionally, the mutational analysis of the Ist2 sorting signal revealed that an arrangement of the hydrophobic positions L938, L939 and L942 at one side of the amphipathic α-helix is also required for sorting of Ist2. The observed partial transition of an Ist2 peptide from an unstructured sequence into an α-helix in the presence of TFE supports the idea that in a certain environment, for example, at membranes, the C-terminus of the Ist2 sorting signal forms a short three-turn α-helix. Although the precise effect of TFE on peptides remains unclear, it is suggested that TFE mimics certain biophysical properties of a membrane environment (Sonnichsen et al., 1992). Single mutation of L938, L939 or L942 led to a punctuated accumulation of Ist2 at the perinuclear, tubular and cortical ER. High expression and low temperature increased the punctuated accumulation of these Ist2 alleles. Accumulation of these Ist2 alleles at distinct sites distributed over the entire ER might be a consequence of misfolding of the Ist2 sorting signal, which results in a local expansion of the ER and the recruitment of a large subset of ER proteins. This is consistent with the moderate upregulation of Kar2, which is a consequence of the unfolded protein response (Normington et al., 1989). However, a severe misfolding of the Ist2 sorting signal seems unlikely, because energy and elevated temperature dissolved most of the Ist2 L942V accumulations, Ist2 L942Q dots showed no massive recruitment of Kar2, and the majority of Ist2 L942Q cofractionated with ER and plasma membrane markers on sucrose density gradients. Compared with the exchange of lysine residues to alanines and the structural changes of the amphipathic α-helices, the loss of hydrophobic positions had only a moderate effect on the binding of the Ist2 sorting signal to lipids, but caused a severe accumulation at specific domains of perinuclear, tubular and cortical ER.

Since our previous work has shown that a multimerisation domain could replace the N-terminal part of the Ist2 sorting signal (residues 632 Journal of Cell Science 122 (5)
878-928) (Franz et al., 2007), it is likely that the Ist2 sorting signal adopts a specific structure and functions in a multimeric state. In such a scenario, the hydrophobic positions of two or more amphipathic α-helices might form a hydrophobic interface, which leads to proper positioning of lysine residues for multivalent binding to negatively charged lipids. The observation that mutations in hydrophobic residues caused punctuated accumulations at all sites of the ER, and not only at the cortical ER, favours this model. Alternatively, a penetration of the hydrophobic residues into the acyl chain core of membranes might lead to bending of the plasma membrane towards the associated cortical ER (McMahon and Gallop, 2005). In a very speculative model, this might drive a transient fusion of cortical ER and plasma membrane. Thereby, Ist2 could reach the cell surface by diffusion. The observed increased sodium tolerance in cells without Ist2 protein (Entian et al., 1999) is consistent with such a postulated transient connection of ER and plasma membrane.

At this point, we cannot answer whether Ist2 is transported to ER-adjacent domains of the plasma membrane or whether Ist2 remains at a unique plasma-membrane-associated domain of the cortical ER. Our previous work showed that Ist2 is accessible for external proteases under conditions where other ER proteins remain undigested (Juschke et al., 2004; Juschke et al., 2005). However, an exclusive localisation at cortical ER and a direct interaction with specific domains of the plasma membrane might increase the sensitivity of Ist2 for digestion by external protease.

To our knowledge, the Ist2 sorting signal is the first signal described that mediates a rapid and efficient accumulation of integral membrane proteins at the yeast cortical ER via a direct interaction with lipids of the plasma membrane. This connection might lead to formation of a transient fusion pore, allowing the transfer of Ist2 from ER to plasma membrane. Alternatively, Ist2 might remain at domains of the cortical ER, where it could connect ER and plasma membrane to allow the direct exchange of molecules or information between these two membranes.

Materials and Methods

Yeast strains, media and plasmids
Preparation of media and yeast transformation was carried out as described (Juschke et al., 2005). Yeast strains, media and plasmids are described in supplementary material Table S3.

Sequences and alignments
The sequences refer to Ist2 from S. cerevisiae (P38250), A. gossypii (Q75572), S. bayanus (6.183), S. castelli (71821) (Byrne and Wolfe, 2005) and C. guilliermondii (P38250) (Franz et al., 2004) are isogenic derivate of W303 (Thomas and Rothstein, 1989). YB4741 and its isogenic derivate JY3 (ist2Δ::HIS3MX6) were purchased from Euroscarf. Plasmids are described in supplementary material Table S3.

Random mutagenesis and screening for mislocalised Ist2
To identify the residues in the C-terminal domain of Ist2 that are essential for sorting to the cell periphery, we constructed an integrative plasmid that contains 2205 nucleotides of the 3’ of Ist2 open reading frame followed by EFYF [in psP189] (Damelin and Silver, 2000)]. From this plasmid, we generated by error-prone PCR a library with mutations in the sequence encoding residues 787-942 of Ist2 and recombined these plasmids into the Ist2 locus of a BY4741 wild-type strain. Out of approximately 500 clones, 20 showed perinuclear and punctuated mislocalisation of Ist2-YFP. Sequencing revealed a clustering of mutations in the K.L sequence in clones that mislocalised.

Fluorescence microscopy
Yeast cells expressing constructs under GAL1 control were grown overnight at 25°C in medium supplemented with 2% (w/v) raffinose. For induction, 2% galactose (w/v) was added. GFP-tagged constructs from Fig. 6, as well as all YFP- and CFP-tagged constructs were visualised using an inverted microscope (Leica DM IRE2) equipped with an image processor LCX PL APO CS ×100/1.4 oil-immersion objective (Leica, a camera (Hamamatsu ORCA-ER CCD) and the Openlab software package (Improvision), mCherry-tagged and all other GFP-tagged constructs were visualised using a wide-field epifluorescence microscope (Axio Imager.A1; Carl Zeiss MicroImaging), equipped with a plan-Fluar ×100/1.45 oil-immersion objective (Carl Zeiss MicroImaging), a camera (Cascade:1K; Photometrics) and MetaMorph software (Molecular Imaging). The following filters were used: YFP 510/20 nm (excitation) and 560/40 nm (emission), CFP 436/20 (excitation) and 480/40 nm (emission), GFP 470/40 (excitation) and 525/50 nm (emission) (Leica DM IRE2), mCherry 565/55 nm (excitation) and 650/75 nm (emission) and GFP 470/30 nm (excitation) and 515/30 nm (emission) (Axio Imager). All images were processed with Adobe Photoshop.

Indirect immunofluorescence
Yeast cells were grown to mid-log phase at 25°C in YEP medium supplemented with 2% (w/v) raffinose. After induction for 40 minutes with 2% galactose (w/v), 100 μg/ml cycloheximide (Sigma) was added and samples were fixed at the indicated time points with 2.7% (v/v) formaldehyde for 1 hour at 25°C. After incubation at 30°C for 10 minutes with 0.3% (v/v) β-mercaptoethanol in buffer B (1.2 M sorbitol in PBS), 0.9 mg/ml zymolyase 20T (Seikagaku, Tokyo, Japan) in buffer B was added for 30 minutes. The spheroplasts were spotted onto polylysine-coated slides, dehydrated in 70% (v/v) ethanol at −20°C, and permeabilised with 0.5% (v/v) NP-40 (v/v). The slides were probed with antibodies against HA- (1:1,000, Berkeley Antibody Company), Ist2 (1:1,000, affinity purified rabbit serum raised against GST-Ist2 residues 589-946), or Kar2 (1:500 (Frey et al., 2003]) in PBS with 1% (w/v) BSA for 2 hours, goat-anti-mouse Alexa Fluor 488 (1:400, Molecular Probes) or goat-anti-rabbit Alexa Fluor 488 (1:400, Molecular Probes) for 1 hour, and stained with 0.1 mg/ml 496-diamido-2-phenylindolehydrochloride (DAPI). The slides were mounted with 80% (v/v) glycerol in PBS. For the classification of localisation, at least 100 cells from each of three independent experiments were analysed.

Generation of yeast cell extracts and western blotting
Denaturing cell lysis and fractionation of yeast cells in supernatant and 25,000 g pellet were done as described (Franz et al., 2007; Frey et al., 2001). For fractionation, we used yeast lysis buffer [20 mM HEPES-KOH pH 7.6, 250 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 0.1 mM PMSF, Complete protease inhibitor (Roche)]. Western blotting was performed using antibodies against HA- (1:3,000), Tub2- (1:500, rabbit serum raised against GST-Tub2 residues 407-457), Gtf44 (1:10,000, affinity-purified rabbit serum raised against GTF44, gift from Dirk Görlich, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany), Ist2 (1:25,000, Sec61-1:7500 (Frey et al., 2001)), Kar2 (1:20,000 (Frey et al., 2001), Emp47 (1:5000, gift from Howard Riezman, University of Geneva, Switzerland (Schroder et al., 1995)) or glucose-6-phosphate dehydrogenase (G6PDH, 1:35,000, Sigma). For quantification of protein bands from western blots and Coomassie-blue-stained gels, the exposed blot or the stained gel were scanned followed by picture processing using the ImageJ software.

Cell fractionation by sucrose density centrifugation and flotation
ist2Δ cells constitutively expressing either Pma1-GFP, Sec5-GFP or Sec13-GFP were grown in selective medium containing 2% (w/v) raffinose. Expression of mCheerry-labeled proteins was induced by the addition of 2% (w/v) galactose for 2 hours. Cells from 10 OD600 units were then treated with 10 mM NaN3 and NaF for 10 minutes, harvested, washed and resuspended in 1 ml of a buffer containing 100 μM sucrose, 10 mM Tris-HCl pH 7.6, 5 mM MgCl2 and Complete protease inhibitor (Roche). After lysis on ice with glass beads, the lysate was centrifuged (500 g, 10 minutes, 4°C) followed by another centrifugation (1200 g, 2 minutes, 4°C). 700 μl of the supernatants were loaded on a 20-60% linear sucrose gradient (buffered with 10 mM Tris-HCl pH 7.6 and 5 mM MgCl2). After centrifugation (100,000 g, 16 hours, 4°C) in an SW40-Rotor (Beckman), fractions were collected from the top. For flotation, a 25,000 g pellet of 100 OD600 yeast cells was resuspended in 2 μl sucrose in yeast lysis buffer, overlaid with a sucrose step gradient (see supplementary material Fig. S14), applied to ultracentrifugation (Beckman SW60-Rotor, 50,000 r.p.m., 16 hours, 4°C) and equal fractions were collected from top to bottom.
Peptide synthesis and CD spectroscopy

The peptide was synthesized by residues 933-946 from wild-type In2 or mutant In2.
L939P were obtained from Peptide Speciality Laboratories (Heidelberg, Germany).
The amount of peptide was determined by weighing the yield of peptide synthesis
on a micro scale. CD spectra were recorded on a Jasco J-715 spectropolarimeter
using a bandwidth of 0.5 nm and a scanning speed of 200 nm per minute.
The instrument was calibrated with (1S+)-(1R)-10-camphorsulfonic acid. Measurements
were carried out at 25°C using a 0.1 cm path length quartz cuvette, with a peptide
concentration of 150 μM in 5 mM potassium phosphate buffer at pH 7.6. Spectral
units were transformed into molar ellipticity per residue.

Protein purification

Recombinant GFP and GFP-fusion proteins were purified from BL21-E.coli using
an N-terminal 6x-His tag. Protein expression was induced at 30°C by adding 1 mM IPTG.
After 2.5 hours the cells were harvested, resuspended in bacterial lysis buffer (50 mM
Tris-HCl pH 7.5, 250 mM NaCl, 2 mM imidazol, 0.5 mM EDTA, 10 mM β-
mercaptoethanol) and lysed with a homogenizer (Emulsiflex C5, Avestin). After
ultracentrifugation (Beckman 45 Ti rotor, 32,000 rpm, 45 minutes, 4°C) the
supernatant was incubated for 1 hour at 4°C with Ni-NTA-beads (Quagen), the beads
were washed several times with lysis buffer and the protein was eluted by incubation
for 3 hours with lysis buffer containing 500 mM imidazol. Eluted protein was dialysed
against a buffer with 25 mM HEPES-KOH pH 7.4, 250 mM potassium acetate.

Binding to proteinase K-treated membranes

Yeast cells from 30 OD600 units were fractionated into supernatant and pellet as
described above, except the yeast lysis buffer contained 50 mM potassium acetate
and no proteinase inhibitors. The pellet fraction was then treated either with buffer
or 50 μg/ml proteinase K (Merck) for 30 minutes at 37°C, washed three times,
icubated for 3 hours with 200 μg/ml recombinant GFP or GFP-Ist2 (878-946) and
fractionated in supernatant and 25,000 μg pellet.

Liposome generation and binding experiments

All lipids were purchased at Avanti Polar Lipids. Lipids including 1 mol% rhodamine-
labeled phosphatidylethanolamine (16:0) were dissolved in chloroform and dried in
(v/v) fatty-acid-free BSA (Roche) in HK buffer for 1 hour (25°C), washed, and the
liposome pellet (25,000 g) was resuspended in HK buffer and

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