ER network formation requires a balance of the dynamin-like GTPase Sey1p and the Lunapark family member Lnp1p

Shuliang Chen1,2, Peter Novick1,3 and Susan Ferro-Novick1,2,3

Although studies on endoplasmic reticulum (ER) structure and dynamics have focused on the ER tubule-forming proteins (reticulons and DP1/Yop1p) and the tubule fusion protein atlastin, nothing is known about the proteins and processes that act to counterbalance this machinery. Here we show that Lnp1p, a member of the conserved Lunapark family, plays a role in ER network formation. Lnp1p binds to the reticulons and Yop1p and resides at ER tubule junctions in both yeast and mammalian cells. In the yeast Saccharomyces cerevisiae, the interaction of Lnp1p with the reticulon protein, Rtn1p, and the localization of Lnp1p to ER junctions are regulated by Sey1p, the yeast orthologue of atlastin. We propose that Lnp1p and Sey1p act antagonistically to balance polygonal network formation. In support of this proposal, we show that the collapsed, densely reticulated ER network in lnp1Δ cells is partially restored when the GTPase activity of Sey1p is abrogated.

The ER possesses a unique and highly conserved morphology that reflects the many essential roles that it plays in all eukaryotic cells1,2. Within each cell, it typically forms one contiguous structure of interconnected sheets and tubules arranged into a polygonal network that makes numerous connections with the outer membrane of the nuclear envelope. This network is spread throughout the cell, all the way to its cortex, by a dynamic process of tubule extension and tubule fusion, leading to the formation of new polygons. At the steady state, polygon formation is balanced by ring closure: the sliding of an ER junction along the side of a polygon until ultimately the polygon is lost3.

ER tubules and networks are generated and stabilized by the reticulons, DP1/Yop1p and atlastin. These components are conserved throughout evolution2,4-6. The reticulons are transmembrane proteins2,4 that work synergistically with the reticulon-interacting protein, DP1/Yop1p, to add positive curvature to the bilayer of the ER membrane2,4,7. Consistent with this role, these proteins are concentrated on tubular elements of the ER and the highly curved edges of ER sheets2,4,8. The dynamin-like GTPase atlastin binds to the reticulons and DP1/Yop1p and promotes ER tubule fusion. It localizes to the three-way junctions that interconnect the tubular elements of the ER to form the ER network5,9,10. In humans, mutations in either atlastin-1 or REEP1, a member of the DP1/Yop1p protein family2, lead to a distal axonopathy of corticospinal neurons11,12. However, when orthologues of the reticulons, DP1/Yop1p and atlastin are all disrupted in the same haploid yeast strain, growth is not affected. These observations suggest that neurons, which are highly elongated, may be particularly sensitive to defects in ER morphogenesis.

In this study, we report that a member of the conserved Lunapark protein family13, Lnp1p, defined by two transmembrane domains and a zinc finger motif, is required for ER network formation. Lnp1p works in synergy with the reticulons and Yop1p, but in antagonism to Sey1p (yeast orthologue of atlastin). It resides at three-way ER tubule junctions in both yeast and mammalian cells and it interacts with the reticulons, Yop1p and Sey1p. Sey1p negatively regulates the interaction of Lnp1p with the reticulons and is necessary for the localization of Lnp1p to ER junctions. We propose that Lnp1p counterbalances Sey1p-directed polygon formation by promoting polygon loss through ring closure.

RESULTS

Lnp1p is required to maintain the cortical ER network and localizes to ER tubule junctions

To identify components of the machinery required to generate the cortical ER network in yeast, we screened the Saccharomyces cerevisiae deletion library using Rtn1p fused to GFP (Rtn1p–GFP) as a marker...
Figure 1: Cortical ER morphology is abnormal in the inp1 mutant. (a) Yeast cells expressing Sec61p-GFP and Rtn1p-RFP (SFNY 2111, SFNY 2112) were grown to early log phase in YPD medium at 25°C. Subsequently, the cells were collected and directly examined under the fluorescence microscope (left). The percentage of cells with abnormal ER morphology was quantified (right) from cells expressing Rtn1p-RFP. Error bars are s.e.m. for three separate experiments. *P < 0.001, Student’s t-test.

(b) Alignment of the transmembrane domain and zinc finger motif of the Lunapark protein from yeast to man. Hydrophobic amino acids are shown in green and charged amino acids in purple. The four cysteines that form an atypical Cys-4-type zinc finger motif are highlighted in blue. The beginning and end of the transmembrane domains are marked on the S. cerevisiae sequence. (c) The zinc finger motif plays a role in ER morphology. The same as in a, only the following number of cells was quantified (right): n = 334, wild type (SFNY 2088, SFNY 2094); n = 391, inp1-1 (SFNY 2116, SFNY 2120). Error bars represent s.e.m. for three separate experiments. *P < 0.001, Student’s t-test. The arrowheads in a and c point to areas of the cortex that lack cortical ER. Scale bar, 3 μm.

The text mentions that there are two genes in yeast encoding reticulons, RTN1 and RTN2. Rtn1p is very abundant, whereas Rtn2p is expressed only in the presence of high salt. The advantage of using Rtn1p–GFP is that it is much brighter than the other markers that have previously been used to study the cortical ER network in yeast and it specifically labels the tubular ER, as well as the edges.
Figure 2 Lnp1p resides at three-way junctions. (a) The ER network collapses in lnp1 mutant cells. Wild-type (SFNY 2088) and mutant cells expressing Rtn1p-GFP were grown to early log phase in YPD medium at 25 °C and collected. Live cells were directly examined under the fluorescence microscope and the images were deconvolved. The outlined area is enlarged in the bottom panel. For the lnp1-1 mutant (SFNY 2116), the zinc finger mutations are C223A C226A C244A and C247A. For the lnp1-5 mutant (SFNY 2142), the zinc finger mutation is C223A. For the lnp1-2 mutant (SFNY 2113), the zinc finger mutations are C223A and C226A. (b) Lnp1p-3×GFP is present on puncta that line the tubular ER. Cells expressing Lnp1p-3×GFP and Rtn1p-RFP (SFNY 2093, top) or Lnp1p-3×GFP and DsRed-HDEL (SFNY 2100, bottom) were grown to early log phase in YPD medium at 25 °C, collected and the live cells were directly examined under the fluorescence microscope. (c) Lnp1p-3×GFP localizes to three-way junctions. Cells expressing Lnp1p-3×GFP and Rtn1p-RFP (SFNY 2093) were grown and examined as in a. The outlined area is enlarged in the bottom panel. (d) Lnp1p-3×GFP partially overlaps or is adjacent to Sey1p-RFP at three-way junctions. Yeast cells expressing Lnp1p-3×GFP and Sey1p-RFP (SFNY 2143) were grown and examined as in a. The outlined area is enlarged in the bottom panel. (e) Human Lnp1GFP localizes to the three-way junctions of the ER marked by Rtn4b-mCherry in COS-7 cells. The outlined area is enlarged in the bottom panel. Scale bars in b and the peripheral images in a,c,d are 3 μm. Scale bars in the enlarged images in a,c,d are 1 μm. The scale bar in e (top) is 10 μm, and the scale bar in the enlarged images in e (bottom) is 5 μm.
subsequent studies we disrupted this ORF in our strain background. When two different ER markers (Rtn1p–RFP and Sec61p–GFP) were used to analyse the ER in a strain deleted for YHR192W, the morphology was found to be abnormal in approximately 91% of the mutant cells (Fig. 1a, left and right). Large sectors of the cortex lacking cortical ER were observed and regions where the ER network seemed to have collapsed were also seen in the mutant (Fig. 1a,c, left). A blast search of YHR192W revealed it to be the sole yeast member of a conserved protein family named Lunapark. This protein family is defined by the presence of two amino-terminal predicted transmembrane domains and a carboxy-terminal zinc finger motif (Fig. 1b). Adjacent to the zinc finger, in animal homologues, is the sequence LNPARK, hence the gene name LNP1.

An lnp1 mutant (lnp1-1), in which the four cysteine residues of the zinc finger (Fig. 1b, blue) were mutated to alanine, exhibited the same ER phenotype as lnp1Δ, indicating a functional role for this motif (Fig. 1c). To examine the consequences of disrupting this motif in more detail, we constructed strains that contain one (lnp1-5, C223A), two (lnp1-2, C223A C226A) and four (lnp1-1, C223A C226A C244A C247A) cysteine mutations in the zinc finger motif. Interestingly, the ER morphology became increasingly more aberrant as the number of cysteine mutations increased (Fig. 2a and Supplementary Fig. S1b,c). Large cortical sectors devoid of ER appeared and the polygons became smaller on average with each additional mutation until they could no longer be resolved by light microscopy. These findings suggest that the ER became more densely reticulated with each mutation in Lnp1p.

Thin-section electron microscopy revealed short sections of ER between small, closely spaced openings in lnp1Δ cells (Fig. 3), confirming that the ER was densely reticulated in the mutant.

Next, we performed co-localization studies with Lnp1p–3×GFP and the ER marker proteins Rtn1p–RFP, Sey1p–5×RFP and the ER retrieval sequence HDEL fused to DsRed. This analysis revealed that Lnp1p–3×GFP specifically resides on puncta that line the tubular ER (Fig. 2b), and at junctions where the tubular ER meets the nuclear envelope (Supplementary Fig. S2a). When we deconvolved an area of the ER at the periphery of the cell using OpenLab software, we were able to resolve the polygonal network marked by Rtn1p–GFP (Fig. 2c, middle bottom panel). Lnp1p–3×GFP was found at the three-way junctions of the ER network (Fig. 2c, bottom), partially overlapping or adjacent to Sey1p–5×RFP (Fig. 2d). In mammalian cells, Lnp1 also localized to the three-way junctions of the ER (Fig. 2e), suggesting that its function is conserved from yeast to higher cells. Interestingly, in the absence of Lnp1p, the ER junctions were no longer clearly resolved and Sey1p–5×RFP seems to spread throughout the densely reticulated ER network (Supplementary Fig. S2b).

**Lnp1p acts in synergy with Rtn1p, but in antagonism to Sey1p**

To begin to address the relationship of Lnp1p to the reticulons and Yop1p, we disrupted LNP1 in strains that lacked the reticulons and/or Yop1p. When we disrupted RTN1 in haploid cells that were also disrupted for LNP1, we observed a growth defect (Fig. 4a, left panel, see white circles and doubling times in Supplementary Fig. S3a). This growth defect was further enhanced when YOP1 was deleted in the lnp1Δ rtn1Δ mutant (Fig. 4a, middle panel, see white squares; Supplementary Fig. S3a), and was most pronounced when LNP1 was disrupted in a strain that lacked both reticulon genes (RTN1 and RTN2) and YOP1 (Fig. 4a, right panel, see white triangles; Supplementary Fig. S3a). No growth defect was observed when lnp1Δ was deleted in combination with rtn2Δ, yop1Δ or an rtn2Δyop1Δ double deletion (Supplementary Fig. S3a). Thus, the enhancement of the growth defect seems to depend on the loss of Rtn1p. Consistent with these genetic studies, we observed an enhancement of the cortical ER defect in lnp1Δrtn1Δ, lnp1Δrtn1Δyop1Δ and lnp1Δrtn1Δrtn2Δyop1Δ mutant cells (Fig. 4b). As previously reported, disruption of YOP1 in mutant cells that lacked one or both reticulon genes also enhanced the cortical ER defect (Fig. 4b); however, no growth defect was associated with this phenotype (Fig. 4c). Together these findings indicate that Lnp1p acts in synergy with Rtn1p to structure the cortical ER.

To address whether Lnp1p and Sey1p act in synergy, we deleted SEY1 in the lnp1Δrtn1Δ double, lnp1Δrtn1Δyop1Δ triple and lnp1Δrtn1Δrtn2Δyop1Δ quadruple mutants. To our surprise, we found that the loss of Sey1p strongly suppressed the growth defects of each of these mutants (Fig. 4c, boxed area). These genetic findings indicate that Lnp1p acts in antagonism to Sey1p. In addition, although the ER network seemed to be less branched in the sey1Δ mutant (Fig. 4d and Supplementary Fig. S3b), disruption of SEY1 largely restored the collapsed, more highly branched network in the lnp1Δ mutant (Fig. 4d and Supplementary Fig. S3b) in over 70% of the cells examined (Fig. 4e). The loss of GTPase activity is sufficient for suppression, as a sey1 mutant defective in GTP-binding (sey1K190A) suppressed the cortical ER defect in the lnp1Δ mutant as efficiently as sey1Δ (Fig. 4d,e). Deletion of SEY1 also partially rescued the cortical ER defect in the lnp1Δrtn1Δ, lnp1Δrtn1Δyop1Δ and lnp1Δrtn1Δrtn2Δyop1Δ mutants (Supplementary Fig. S3c).

**Sey1p–GTP restricts Lnp1p to ER tubule junctions**

To address how Sey1p acts in antagonism to Lnp1p, we examined the localization of Lnp1p–3×GFP in the sey1Δ and sey1K190A mutants.
Interestingly, Lnp1p–3×GFP was no longer restricted to puncta on the cortical ER in these mutants (Fig. 5a, middle and bottom panel). Instead, as for Sec61p–2×RFP and DsRed–HDEL, Lnp1p–3×GFP was spread evenly throughout both the cortical ER and nuclear envelope (Fig. 5a and Supplementary Fig. S2a). Quantification of the phenotype revealed that Lnp1p–3×GFP was spread throughout the ER in most of the sey1Δ and sey1K50A cells examined (Fig. 5b). Unlike the case for Lnp1p, the localization of Rtn1p–RFP and Yop1p–RFP was unaltered in the sey1Δ mutant (Supplementary Fig. S4a,b). A closer examination of the peripheral ER network in both the sey1Δ and sey1K50A mutants revealed that Lnp1p–3×GFP was in the ER sheets and not the ER tubule junctions (Fig. 5c). Interestingly, whereas Lnp1p–3×GFP was in the sheets, Rtn1p–GFP lined the more highly curved edges of the sheets in these mutants. Together these findings indicate that, unlike the reticulons and Yop1p, Lnp1p does not have an intrinsic ability to localize to highly curved membrane domains (Fig. 5c). In addition, these findings show that Sey1p–GTP seems to play an important role in restricting Lnp1p to ER tubule junctions.

The interaction of Lnp1p with Rtn1p is regulated by Sey1p

The observation that Lnp1p acts in synergy with Rtn1p and in antagonism to Sey1p suggests that Lnp1p might physically interact with Rtn1p and/or Sey1p. To begin to address this possibility, we performed immunoprecipitation experiments. Lysates prepared from three different strains containing Lnp1p–3×HA with either Rtn1p–3×FLAG, Sey1p–3×FLAG or the Rtn1p–interacting protein Yop1p (tagged with 3×FLAG) were incubated with anti-HA affinity matrix. The beads were then washed and blotted with anti-FLAG antibody. This analysis revealed that Lnp1p–3×HA specifically interacts with all three proteins, but not with Sec22p, a type II membrane protein that resides on the ER (Fig. 6a). The interaction was dependent on the presence of the HA tag as no Rtn1p–3×FLAG, Sey1p–3×FLAG or Yop1p–3×FLAG were detected in the precipitate when lysate containing untagged Lnp1p was incubated with anti-HA affinity matrix (Supplementary Fig. S4c). The amount of Rtn1p that co-precipitated with Lnp1p was similar to the amount of Sey1p that co-precipitated with Rtn1p (Supplementary Fig. S4d).
Cells with Lnp1p–3×GFP on NE (%)

|          | WT   | sey1Δ | sey1K50A |
|----------|------|-------|----------|
| 0       | 0    | +     | +        |
| 10      | 0    | +     | +        |
| 20      | 0    | +     | +        |
| 30      | 0    | +     | +        |
| 40      | 0    | +     | +        |
| 50      | 0    | +     | +        |
| 60      | 0    | +     | +        |
| 70      | 0    | +     | +        |

The percentage of cells in which Lnp1p–3×GFP is in a punctate cortical ER pattern was quantified. The number of cells examined is listed below. Error bars represent s.e.m. for three separate experiments. n = 348 wild type; n = 336 sey1Δ; n = 243 sey1K50A. **P < 0.0001, Student’s t-test.

To determine whether Sey1p regulates the interaction of Lnp1p with the reticulons, we examined the interaction of Lnp1p–3×HA with Rtn1p–3×FLAG in strains in which SEY1 was either deleted or overexpressed from the inducible GALI promoter. Interestingly, we precipitated more Rtn1p with Lnp1p when SEY1 was deleted (Fig. 6b, compare lanes 1 and 2), and less Rtn1p with Lnp1p when Sey1p was overexpressed (Fig. 6b, compare lanes 3 and 4). Thus, Sey1p seems to negatively regulate the interaction of Lnp1p with Rtn1p. When we examined the consequences of overexpressing Sey1p on its localization and on the localization of Rtn1p, Yop1p and Lnp1p, we found that Sey1p forms large round structures that predominantly reside at the periphery of the cell (Supplementary Fig. S5). These structures also contained Rtn1p and Yop1p (Supplementary Fig. S5d,e), but not Lnp1p (Supplementary Fig. S5a–c), consistent with the observation that less Rtn1p is precipitated with Lnp1p when Sey1p is overexpressed (Fig. 6b). Electron microscopy analysis demonstrated that the large structures consist of tubular membranes (Supplementary Fig. S5f). These tubular membranes were not present when Lnp1p was overexpressed from the inducible GALI promoter (Supplementary Fig. S5g). Lnp1p was found on the tubular ER and nuclear envelope within 2 h after induction (Supplementary Fig. S6a). After 4 h, the nuclear envelope and cortical ER became aberrant and the localization of Rtn1p and Lnp1p no longer overlapped (Supplementary Fig. S6a,b). Eventually, the overproduction of Lnp1p led to cell death (Supplementary Fig. S6c). Interestingly, this growth defect was suppressed when Lnp1p was overexpressed in a strain that also overexpressed Sey1p (Supplementary Fig. S6d,e). Under conditions of co-overexpression, Lnp1p–GFP and Sey1p–RFP were found to co-localize in large, round peripheral structures. (Supplementary Fig. S6c).

**Sey1p, but not Lnp1p, is defective in cortical ER and nuclear ER fusion**

Atlastin, but not its putative homologue Sey1p, has been directly shown to promote the fusion of ER membranes. As Sey1p and Lnp1p seem to have opposing effects, we examined whether the loss of Lnp1p suppresses the presumed fusion defect in the sey1Δ mutant. To do this, we established an assay that measures ER–ER fusion in vivo during the mating reaction. Yeast cells have two mating types, MATa and MATα. When cells of the opposite mating type come in contact with each other, in
response to pheromone signalling, the wall at the contact site of each cell is degraded (cell wall remodelling) and the plasma membranes of the two cells fuse (Fig. 7a). This allows the ER in the two parent cells to fuse, mixing their lumenal contents, and the nuclei from each cell to fuse forming a zygote. To assess cortical ER–ER fusion, we mated one cell expressing the ER luminal marker DsRed–HDEL with another cell expressing cytosolic GFP (Supplementary Fig. S7). The time at which the plasma membranes fused, as indicated by the transfer of cytosolic GFP between the two parent cells, was defined as $t = 0$ (Fig. 7a). We then measured the time it took to initiate and complete ER lumenal mixing in wild-type, $\text{lnp}1\Delta$, $\text{sey}1\Delta$ and $\text{lnp}1\Delta\text{sey}1\Delta$ cells (Supplementary Fig. S7). This analysis revealed that the timing of the start (Fig. 7b) and completion (Fig. 7c) of ER mixing was similar in wild-type and $\text{lnp}1\Delta$ cells. Mixing, however, was delayed in the $\text{sey}1\Delta$ mutant. This delay was not significantly suppressed in an $\text{lnp}1\Delta\text{sey}1\Delta$ double mutant. Consistent with the hypothesis that Sey1 mediates ER fusion at the contact zone where the two cells fuse, we found that Sey1p–5×RFP localizes to this zone in wild-type zygotEs (Supplementary Fig. S8, top), whereas Lnp1p (Supplementary Fig. S8, middle) and Rtn1p (Supplementary Fig. S8, bottom) do not.

We also assessed nuclear envelope fusion in the strains described above by mixing $\text{MAT}a$ and $\text{MAT}a$ cells that expressed Sec61p–GFP. The cell suspension was plated on a YPD plate for 4 h at 25 °C, and the zygotEs that formed a new bud near the site of cell fusion were scored. The cell suspension was plated on a YPD plate for 4 h at 25 °C, and the zygotEs that formed a new bud near the site of cell fusion were scored.

**Figure 6 Sey1p regulates the interaction of Lnp1p with Rtn1p.** (a) Lnp1p interacts with Rtn1p, Yop1p and Sey1p. Yeast lysates were prepared from cells expressing either Lnp1p–3×HA and Rtn1p–3×FLAG (SFNY 2102), Lnp1p–3×HA and Yop1p–3×FLAG (SFNY 2103), or Lnp1p–3×HA and Sey1p–3×FLAG (SFNY 2104). Lysates were immunoprecipitated with anti-HA antibody and analysed by western blot analysis using anti-FLAG, anti-HA and anti-Sec22p antibodies. Quantiﬁcation of the immunoprecipitates is shown at the bottom. Error bars represent s.d., $n = 3$. (b) Yeast lysates were prepared from cells expressing Lnp1p–3×HA and Rtn1p–3×FLAG when Sey1p was either absent or overexpressed (SFNY 2132, SFNY 2131). Overexpression of Sey1p was induced in YP medium with 0.1% galactose. Lysates were immunoprecipitated with anti-HA antibody and western blot analysis was performed using anti-FLAG antibody. The percentage of the immunoprecipitate (IP) and lysate (Input) loaded on the gel is indicated at the top of each lane. Quantiﬁcation of the immunoprecipitates is shown at the bottom. Error bars represent s.e.m., $n = 3$. *$P < 0.02$, Student’s $t$-test. Uncropped images of blots are shown in Supplementary Fig. S9.
analysed further (Fig. 8a). The mating pairs with unfused nuclei (three versus two nuclei) and the fraction that failed to go on to form diploid cells (Fig. 8b,c) were measured. Cells were determined to be diploid if they were able to sporulate and failed to mate with either MATa or MATα tester strains. This analysis revealed that Sey1p, but not Lnp1p, is required for nuclear fusion; that is, sey1Δ is defective in karyogamy. Thus, Sey1p is required for both cortical ER–ER and nuclear ER fusion. In addition, as for the ER–ER fusion defect, the nuclear fusion defect of the sey1Δ mutant was not strongly suppressed in the sey1Δlnp1Δ double mutant. Therefore, Lnp1p does not seem to antagonize Sey1p by inhibiting a Sey1p-independent ER fusion pathway.

**DISCUSSION**

The ER is shaped through the addition of bilayer curvature by the reticulons and DP1/Yop1p (refs 2,4,21). Curiously, although the reticulons and DP1/Yop1p are sufficient for the formation of tubules in vitro5, the simultaneous loss of Rtn1p, Rtn2p and Yop1p does not lead to an appreciable growth defect in yeast. ER tubule interconnections are formed when the dynamin-like GTPase atlastin fuses ER tubules to each other to form a network4. At present, it is unknown whether atlastin is sufficient for forming ER tubular junctions; however, in its absence, long unbranched tubules predominate6. In yeast, the atlastin homologue Sey1p facilitates homotypic ER–ER and nuclear ER envelope fusion during the mating reaction. Further, as yet unidentified components must be needed for ER network formation, as yeast cells lacking Sey1p, the reticulons and Yop1p are not impaired for growth.

Here we show that a member of a conserved protein family, named Lunapark, is a player in ER network formation. Interestingly, as for mutations in the Sey1p homologue atlastin or the Yop1p homologue REEP1, the lnp-1 mutant of *Caenorhabditis elegans* has been linked to neuronal defects22. In yeast, the loss of Lnp1p in an rtn1Δrtn2Δyop1Δ triple mutant leads to a striking growth defect and profound ER morphology defects, indicating that Lnp1p acts in synergy with the reticulons and Yop1p to structure the ER. Lnp1p contains two transmembrane domains and a zinc finger motif that is essential for its function. It interacts with Rtn1p, Yop1p and Sey1p and localizes to ER junctions where it partially overlaps with, or is adjacent to, Sey1p. The localization of Lnp1p to ER junctions is dependent on Sey1p–GTP, which also negatively regulates the interaction of Lnp1p with Rtn1p.

The synergistic genetic interactions between *LNP1* and *RTN1* could indicate that Lnp1p and Rtn1p either have redundant functions, or they act on converging pathways. It seems unlikely that Rtn1p acts redundantly with Lnp1p because it is at least ten times more abundant than Lnp1p (www.yeastgenome.org) and it localizes to the tubular ER and the edges of ER sheets24,25, whereas Lnp1p resides at ER junctions. Furthermore, the phenotypes of lnp1Δ and rtn1Δ mutants, as revealed by electron microscopy, are distinct. Loss of Lnp1p leads to the formation of densely reticulated ER, whereas the loss of Rtn1p results in non-fenestrated sheets4.
which balances the mitochondrial fusion activity of Fzo1p, an outer
The dynamin-like GTPase Dnm1p promotes mitochondrial fission
is not a GTPase and fission of ER tubules has not been directly observed
by live-cell imaging. Alternatively, Lnp1p could also balance Sey1p
function by directing ER polygon loss through ring closure. Lnp1p
might be needed for the sliding of a three-way junction along an ER
tubule to allow a polygon to shrink or perhaps for the final membrane
scission event needed to resolve the residual hole, completing the
process of ring closure. In either case, the loss of Lnp1p would
be predicted to result in the accumulation of excess polygons of
decreasing size. The densely reticulated cortical ER observed by electron
microscopy in the lnp1Δ mutant is consistent with this proposal.

METHODS

Methods and any associated references are available in the online
version of the paper at www.nature.com/naturecellbiology

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

S.F.-N. and P.N. conceived the idea for the screen. S.F.-N., P.N. and S.C. designed the experiments, analysed the data and wrote the manuscript. S.C. performed the research.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Figure 8 The sey1Δ mutant exhibits a defect in nuclear fusion that is
not suppressed by the loss of Lnp1p. (a) Haploid MATa and MATα yeast
cells expressing Sec61–GFP were grown to early stationary phase. Equal
numbers of cells of each mating type were mixed, incubated at 25°C for
2–4 h on a YPD plate and examined for nuclear fusion under the fluorescence
microscope. (b) Quantification of the data in a. The number of cells examined
is listed below. Error bars represent s.e.m. for three separate experiments,
n = 65 wild type; n = 55 lnp1Δ; n = 38 sey1Δ; n = 48 lnp1Δsey1Δ. ∗ P < 0.01, Student’s t-test. (c) The same as in a except zygotes were picked,
transferred to a YPD plate and incubated for 4 days at 25°C. Cells were
considered to be diploid if they sporulated and could no longer mate with
MATa and MATα tester strains. The number of cells examined is listed
below. Error bars represent s.e.m., n = 85 wild type; n = 95 lnp1Δ; n = 123
sey1Δ; n = 127 lnp1Δsey1Δ. ** P < 0.005, Student’s t-test. Scale bar, 3 μm.

The strong suppression by sey1Δ of the growth defects of the
lnp1Δrttn1Δ, lnp1Δrttn1Δyop1Δ and lnp1Δrttn1Δrttn2Δyop1Δ mutants suggests that Lnp1p balances the function of Sey1p. Furthermore, the suppression of the ER morphology defects of lnp1Δ
by sey1Δ indicates that this balance is important in forming a normal
ER network. One possible role for Lnp1p is to balance the fusion activity
of Sey1p by mediating ER fission. A balance of fission and fusion
events is required to maintain normal mitochondrial morphology.6
The dynamin-like GTPase Dnm1p promotes mitochondrial fission14, which balances the mitochondrial fusion activity of Fzo1p, an outer
mitochondrial membrane GTPase (refs 25,26). Unlike Dnm1p, Lnp1p
is not a GTPase and fission of ER tubules has been directly observed

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METHODS

Screen of the yeast deletion library. The MATa deletion collection (derivative of BY4741, Open Biosystems) was screened in 96-well plates for defects in ER morphology. Briefly, an aliquot (1 µl) of each strain was transferred into 100 µl of YPD medium supplemented with G418 (200 µg ml⁻¹) and incubated for 2–3 days at 23°C. This step was repeated three times before the cells were screened. To screen the cells, all strains were grown in 100 µl of YPD medium for 24 h at 25°C. Subsequent to this incubation, the cells were centrifuged for 5 min at 1,000g, and the pellet was resuspended in 20 µl of transformation buffer (0.2 M LiAc, 40% PEG 3350, 100 mM dithiothreitol, at pH 7.5) containing 10 µg of carrier DNA (salmon sperm) and 100 ng of plasmid DNA (pRS315–RTN1–GFP). The cells were then incubated at 42°C for 30 min and the transformation buffer was removed by centrifugation. The transformants were selected in 150 µl of SC-Leu media. The transformed strain (1 µl) was inoculated into 100 µl of fresh SC-Leu media and incubated at 25°C for 12 h. The morphology of the cortical ER, marked by Rtn1p–GFP, was examined by fluorescence microscopy.

Fluorescence microscopy. Yeast cells (see Supplementary Table S1) were grown at 25°C to early log phase (attenuance (D) of 0.3–0.6 at 600 nm). Approximately 5 D600 µm units of cells were pelleted at 1,000g, resuspended in 250 µl of ice-cold growth medium and examined on an Axio Imager Z1 fluorescence microscope using a ×100 oil-immersion objective. Images were captured with an Axio Cam MRm digital camera and AxioVision software, and deconvolved using OpenLab software.

Electron microscopy. Yeast cells were grown overnight to early log phase (D600 = 0.3–0.6) at 25°C. Approximately 10 D600 µm units of cells were collected using a 0.22 μm filter apparatus, washed with 10 ml of 0.1 M cacydolate (pH 6.8), resuspended in 10 ml of fixative (0.1 M cacydolate and 4% glutaraldehyde, at pH 6.8) and incubated for 1 h at room temperature before the cells were stored overnight at 4°C. The next day, the fixed cells were washed with 50 mM KPi (pH 6.8) and incubated for 1 h on ice, washed with water and then incubated in 2% uranyl acetate (UrAc) for 1 h at room temperature. Samples were dehydrated using a series of ethanol washes, and incubated overnight in Spurr resin. Samples were embedded in fresh Spurr resin, and baked at 80°C for at least 24 h. Sections were stained with lead citrate and uranyl acetate and images were acquired using a Tecnai G2 Spirit transmission electron microscope equipped with a Gatan Ultra Scan 4000 CCD (charge-coupled device) camera.

Mammalian cell culture, transfection and confocal microscopy. COS-7 cells were grown at 37°C in a 5% CO2 incubator in Dulbecco’s modified Eagle’s medium with high glucose and 10% fetal bovine serum. To construct LNP1–GFP, human LNP1 was PCR-amplified from a human complementary DNA (Open Biosystems, NCBI accession number: BC105132), and inserted into pAcGFP1–N1 using the SacI/SacII restriction sites. Transfection of plasmid DNA (pAc–Rtn4β–mCherry and pAc–Lnp1–GFP) into COS-7 cells was performed using Lipofectamine 2000 (Invitrogen). Cells were imaged using an Olympus FV1000 confocal laser scanning microscope and FluoView software.

Immunoprecipitation protocol. Yeast cells grown to D600 µm = 1.0 were collected by centrifugation, washed once with ice-cold water and resuspended in spheroplasting buffer (1.4 M sorbitol, 50 mM NaPi (pH 7.4), 50 mM 2-mercaptoethanol and 10 µg/ml Zymolyase 100T). The resuspended cells were incubated at 37°C for 30 min with gentle shaking, pelleted through a chilled sorbitol cushion (1.7 M sorbitol and 20 mM HEPES, at pH 7.4), and the pellet was resuspended in lysis buffer (25 mM HEPES, 150 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethyl sulphonyl fluoride, 1× protease inhibitor (27) and 1% digitonin, at pH 7.4) using a dounce homogenizer (40 strokes). The lysate was centrifuged at 37,000g for 20 min at 4°C, and the protein concentration of the supernatant was measured using the Bradford assay.

The protein concentration of the lysate was adjusted to 2–4 mg ml⁻¹ with lysis buffer, and 0.5–1.0 ml of the lysate was incubated overnight at 4°C with 10–20 µl of anti-HA affinity matrix (Clone 3F10, Roche). The matrix was pelleted at 2,300g for 30 s, and washed three times with 1 ml of cold lysis buffer that contained 0.2% digitonin. The matrix was heated to 100°C in sample buffer (62.5 mM Tris–HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol, at pH 6.8) for 5 min. The eluted protein was subjected to SDS–PAGE and immunoblotted with anti-HA (1:2,000 dilution, clone HA.11, Covance, MMS-101K), anti-FLAG (1:2,000 dilution, clone M2, Sigma, F 1804) and anti-Sec22p (1:1,000 dilution) antibodies. The anti-Sec22p antibody was generated in our lab. The secondary antibodies used were goat anti-mouse IgG–HRP (1:10,000 dilution, Promega, W402B) or donkey anti-rabbit IgG–HRP (1:10,000 dilution, GE Healthcare, NA9340V).

Time-lapse imaging of cortical ER fusion. Haploid MATa and MATt cells were grown to log phase at 23°C in SC media. Five-hundred microlitres of each cell culture was mixed and then centrifuged at 1,000g. To prepare a slide of the mating mix, the cell pellet was resuspended in 10 µl of fresh SC media, and applied to a 1.3-mm-thick pad of 3% low-temperature agarose prepared in SC medium. A coverslip was then applied to the sample, sealed with vaseline and incubated at 25°C for 45–60 min. Cell fusion was examined at 25°C on a Axio Imager Z1 fluorescence microscope using a ×63 oil-immersion objective. Images were captured with an Axio Cam MRm digital camera and AxioVision software, and deconvolved using OpenLab software.

Zygote formation assay. Haploid MATa and MATt cells grown to early stationary phase at 25°C were collected by centrifugation at 1,000g. The cells were resuspended in fresh YPD media (D600 µm = 8.0) and incubated at 25°C with shaking for 30 min. MATa cells (3 µl) were mixed with an equal volume of MATt cells and plated onto a fresh YPD plate that was incubated at 25°C for 2–4 h. Cell fusion and zygote formation was then assessed under the microscope. To measure the efficiency of diploid formation, zygotes were picked with a dissection needle and transferred to a fresh YPD plate that was incubated at 25°C for 24 h. Cells were scored as diploid if they were able to sporulate and could not mate with either MATa or MATt tester strains.

Statistical analysis. P values were calculated using Student’s t-test. The statistical significance reported is from independent experiments and presented as mean values. The error bars in the figure legends represent s.d. or s.e.m. as specified in the legends.

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Figure S1 Mutations affecting cortical ER morphology. a, The *pho85A* and *cho1Δ* mutants display defects in cortical ER structure. The deletion library was screened for defects in ER structure as described in the Methods. The scale bar is 3 μm. b, Yeast cells expressing Rtn1p-GFP or Sec61p-GFP were grown to early log phase in YPD medium at 25°C, harvested and the live cells were examined under the fluorescence microscope. c, The percentage of cells with abnormal ER morphology was quantitated in cells expressing Rtn1p-GFP. The number of cells examined is listed below. Error bars represent S.E.M. for three separate experiments, n = 404 *lnp1-2* (C223A, C226A); n = 383 *lnp1-3* (C244A, C274A); n = 322 *lnp1-4* (H246A, H249A). As anticipated, mutations in Lnp1p that are not predicted to disrupt zinc binding (*lnp1-4*) displayed normal ER morphology. *P < 0.01 Student’s t-test. The scale bar is 3 μm.
Figure S2 Lnp1p localizes to the junction between the tubular ER and nuclear envelope. a, Yeast cells expressing Lnp1p-3xGFP and DsRed-HDEL (SFNY 2100, SFNY 2150) were grown to early log phase in YPD medium at 25°C, harvested and the live cells were directly examined under the fluorescence microscope. The boxed area is enlarged in the right panel. The scale bar is 3 μm in merged image and 1 μm in enlarged image. b, ER junctions are no longer resolved in the lnp1Δ mutant. Yeast cells expressing Rtn1p-GFP and Sey1p-5xRFP (SFNY 2144, SFNY 2145) were grown and examined as in Fig. 2a. The boxed area is enlarged in the bottom panel. Scale bar in peripheral image is 3 μm. Scale bar in the enlarged image is 1 μm.
Figure S3 Deletion of \textit{LNP1} does not impair growth in \textit{rtn2Δ} and \textit{yop1Δ} mutants or a \textit{rtn2Δyop1Δ} double mutant. \textbf{a}, Yeast mutants were grown overnight to stationary phase in YPD media, inoculated into fresh YPD media at an OD$_{600} = 0.01$ and incubated at 25°C with shaking (250 rpm). Cell growth was monitored by OD$_{600}$. The doubling time during exponential growth is graphed. Error bars represent S.E.M. \( n = 3 \). *P < 0.0001, **P < 0.0002, ***P < 0.02. \textbf{b}, Deletion of \textit{SEY1} suppresses the ER defect of the \textit{lnp1Δ} mutant. Yeast cells expressing Rtn1p-GFP and DsRed-HDEL (SFNY 2170, SFNY 2171, SFNY 2172, SFNY 2173) were grown to early log phase in YPD medium at 25°C, harvested and the live cells were directly examined under the fluorescence microscope. \textbf{c}, Deletion of \textit{SEY1} suppresses the ER defect of the \textit{lnp1Δrtn1Δ}, \textit{lnp1Δrtn1yop1Δ} and \textit{lnp1Δrtn1Δrtn2Δyop1Δ} mutants. Yeast cells expressing Sec61p-GFP were grown to early log phase in YPD medium at 25°C, harvested and the live cells were directly examined under the fluorescence microscope. The scale bar is 3 μm.
Figure S4 Loss of SEY1 does not affect the localization of Rtn1p and Yop1p. 
a. Yeast cells expressing Rtn1p-RFP and Sec61p-GFP (SFNY 2111, SFNY 2135) were grown to early log phase in YPD medium at 25°C, harvested and the live cells were examined under the fluorescence microscope. 
b. Same as a except Yop1p-RFP and Sec61p-GFP (SFNY 2137, SFNY 2136) were examined. The scale bar is 3 μm. 
c. Anti-HA does not precipitate Rtn1p, Sey1p or Yop1p from lysates that contain untagged Lnp1p. Yeast lysates were prepared from cells expressing the following tags: Rtn1p-3xFLAG; Lnp1p-3xHA and Rtn1p-3xFLAG; Sey1p-3xFLAG; Lnp1p-3xHA and Sey1p-3xFLAG; Yop1p-3xFLAG; Lnp1p-3xHA and Yop1p-3xFLAG. Lysates were immunoprecipitated with anti-HA antibody and analyzed by western blot analysis using anti-FLAG antibody. 
d. Yeast lysates were prepared from cells expressing the following tags: Sey1p-3xHA and Rtn1p-3xFLAG; Rtn1p-3xHA and Yop1p-3xFLAG, or Lnp1p-3xHA and Rtn1p-3xFLAG. Lysates were immunoprecipitated with anti-HA antibody and analyzed by western blot analysis using anti-FLAG and anti-HA antibodies. The percent of the immunoprecipitate and lysate loaded on the gel is indicated at the top of each lane.
Supplementary Information

Figure S5 Overexpression of Sey1p affects the localization of Lnp1p. 

a, Yeast cells expressing Lnp1p-3xGFP and Rtn1p-RFP (SFNY 2154) were grown to early log phase in YP media containing 2% raffinose at 25°C, then shifted to YP media containing 2% raffinose and 0.5% galactose for 3-6 hr to overexpress Sey1p. Cells were harvested and the live cells were examined directly under the fluorescence microscope. 

b, Same as a except the cells expressed Lnp1p-3xGFP and Yop1p-RFP (SFNY 2155).

c, Yeast cells expressing Lnp1p-3xGFP and Sey1p-5xRFP (SFNY 2156) were grown to early log phase in YP media containing 2% raffinose at 25°C, then shifted to YP media containing 2% raffinose and 0.5% galactose for 3-6 hr. Cells were harvested and the live cells were examined under the fluorescence microscope. 

d, Same as c except the cells expressed Rtn1p-GFP and Sey1p-5xRFP (SFNY 2157). 

e, Same as c, except the cells expressed Yop1p-GFP and Sey1p-5xRFP (SFNY 2158). The scale bar is 3 μm.

f and g, The large structures that accumulate in Sey1p overproducing cells contain tubular membranes. Yeast cells harboring GAL1p-SEY1 (f) or GAL1p-LNP1 (g) were grown at 25°C to early stationary phase in YP media containing 2% raffinose, then shifted to YP media containing 2% raffinose, then shifted to YP media containing 2% raffinose and 0.5% galactose for 4 hr. Yeast cells were harvested and thin section analysis was performed as described in the Methods. In the cross section view, the arrowheads point to segments of ER. Scale bar is 200 nm.
Figure S6  The overexpression of Lnp1p leads to cell death. a, Yeast cells expressing Lnp1p-3xGFP and Rtn1p-RFP (SFNY 2110) were grown to early log phase in YP media containing 2% raffinose at 25°C, then shifted to YP media containing 2% raffinose and 0.5% galactose for 2-4 hr. Cells were harvested and the live cells were examined under the fluorescence microscope. b, Yeast cells expressing Hmg1p-GFP and Rtn1p-RFP (SFNY 2169) were grown to early log phase in YP media containing 2% raffinose at 25°C, then shifted to YP media containing 2% raffinose and 0.5% galactose for 4 hr. Cells were harvested and the live cells were examined under the fluorescence microscope. c, Yeast cells harboring GAL1p-SEY1-RFP and GAL1p-LNP1-GFP (SFNY 2273) were grown to early log phase in YP media containing 2% raffinose at 25°C, then shifted to YP media containing 2% raffinose and 0.5% galactose for 2-4 hr. Cells were harvested and the live cells were examined under the fluorescence microscope. d, Yeast cells expressing Rtn1p-GFP (SFNY 2253, SFNY 2254, SFNY 2256) were grown to early log phase in YP media containing 2% raffinose at 25°C, then shifted to YP media containing 2% raffinose and 0.5% galactose for 2-4 hr. Cells were harvested and the live cells were examined under the fluorescence microscope. e, Yeast cells were grown to early stationary phase in YP media containing 2% raffinose at 25°C. After the cell concentration was adjusted to approximately 1 × 10⁸ cells/ml, the cells were serially (10-fold) diluted and spotted onto YP plates containing 2% raffinose and 0.5% galactose. The plates were incubated at 25°C for 4-5 days. The scale bar represents 3 μm.
Figure S7 Time course of ER fusion. Haploid MATa and MATα yeast cells expressing cytosolic GFP or DsRed-HDEL were grown to log phase in SC media. An equal number of cells of each mating type were mixed and prepared for microscopy as described in the Methods. Time lapse images of cortical ER fusion were acquired on a fluorescence microscope using a 63x oil-immersion objective. Cell-cell fusion was monitored by following cytosolic GFP, and ER-ER fusion was monitored using DsRed-HDEL as an ER marker. Arrowheads point to a mating pair. The scale bar is 3 μm.
Figure S8 Sey1p accumulates at the cell-cell contact zone during zygote formation. Haploid wild type MATα and MATα yeast cells expressing Sey1p-5xRFP (top), Lnp1p-3xGFP (middle), or Rtn1p-RFP and Rtn1p-GFP (bottom) were grown to early stationary phase. Equal numbers of cells of each mating type were mixed, incubated at 25°C for 2-4 hr on a YPD plate and examined. The scale bar is 3 µm.
Figure S9 Full scans
Supplemental Table Legend:

Table S1 Yeast strains used in this study.