ER-associated retrograde SNAREs and the Dsl1 complex mediate an alternative, Sey1p-independent homotypic ER fusion pathway

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ABSTRACT The peripheral endoplasmic reticulum (ER) network is dynamically maintained by homotypic (ER–ER) fusion. In Saccharomyces cerevisiae, the dynamin-like GTPase Sey1p can mediate ER–ER fusion, but sey1Δ cells have no growth defect and only slightly perturbed ER structure. Recent work suggested that ER-localized soluble N-ethylmaleimide–sensitive factor attachment protein receptors (SNAREs) mediate a Sey1p-independent ER–ER fusion pathway. However, an alternative explanation—that the observed phenotypes arose from perturbed vesicle trafficking—could not be ruled out. In this study, we used candidate and synthetic genetic array (SGA) approaches to more fully characterize SNARE-mediated ER–ER fusion. We found that Dsl1 complex mutations in sey1Δ cells cause strong synthetic growth and ER structure defects and delayed ER–ER fusion in vivo, additionally implicating the Dsl1 complex in SNARE-mediated ER–ER fusion. In contrast, cytosolic coat protein I (COPI) vesicle coat mutations in sey1Δ cells caused no synthetic defects, excluding perturbed retrograde trafficking as a cause for the previously observed synthetic defects. Finally, deleting the reticulons that help maintain ER architecture in cells disrupted for both ER–ER fusion pathways caused almost complete inviability. We conclude that the ER SNAREs and the Dsl1 complex directly mediate Sey1p-independent ER–ER fusion and that, in the absence of both pathways, cell viability depends upon membrane curvature–promoting reticulons.

INTRODUCTION

In all eukaryotes, the endoplasmic reticulum (ER) consists of a branched network of tubules and ribosome-dense sheets (cisternae) that is continuous with the outer nuclear envelope. In Saccharomyces cerevisiae, the majority of the ER network resides at the cell cortex along the plasma membrane (peripheral ER), with several tubules connecting to the outer nuclear envelope (nuclear ER). Functionally, the ER mediates multiple processes, including protein synthesis and secretion, lipid synthesis, and calcium regulation (reviewed in Schuldiner and Weissman, 2013). Recent research has identified contact sites between the ER and most other organelles, implicating the ER in additional processes, including lipid droplet biogenesis, lipid transfer, and mitochondrial division (reviewed in English and Voeltz, 2013).

ER tubes are shaped and maintained by the reticulons (Rtn1p and Rtn2p in yeast) and the reticulon-like protein Yop1p (Voeltz et al., 2006; reviewed in Chen et al., 2013). Rtn1p or Yop1p is sufficient to form tubules in proteoliposomes in vitro (Hu et al., 2008) and likely functions by forming a curvature-inducing wedge in the lipid bilayer (Voeltz et al., 2006; Shibata et al., 2008; Zurek et al., 2011). Simultaneous deletion of rtn1, rtn2, and yop1 results in an almost entirely cisternal, nontubulated peripheral ER (West et al., 2011). Surprisingly, however, this triple mutant displays only a minor growth defect (Voeltz et al., 2006; Chen et al., 2012).

Over time, the ER network must alter its structure dynamically, while maintaining lumenal continuity, by forming new branch points or by merging existing branches at three-way tubule junctions via...
Homotypic (ER–ER) fusion (Lee and Chen, 1988). Homotypic ER fusion is mediated in mammals by the dynamin-like GTPase atlastin and in yeast by its functional orthologue Sey1p (Hu et al., 2009; Orso et al., 2009). Sey1p is enriched at three-way junctions in the peripheral ER and binds to the reticulons Rtn1p and Yop1p (Hu et al., 2009). Surprisingly, sey1Δ mutants have no growth defect and relatively normal ER structure, similar to rtn1Δ and yop1Δ single mutants. sey1Δ rtn1Δ and sey1Δ yop1Δ double mutants, however, display abnormal, nontubulated peripheral ER with enlarged cisternae (Hu et al., 2009). Despite the lack of a strong growth or structural defect, sey1Δ mutants exhibit a decreased (but not abolished) rate of homotypic ER fusion in vivo, whereas rtn1Δ rtn2Δ yop1Δ mutants, which have extremely perturbed ER structure, exhibit a wild-type homotypic ER fusion rate (Anwar et al., 2012). Consistent with a direct role in ER–ER fusion, Sey1p is sufficient to mediate proteoliposome fusion in vitro (Anwar et al., 2012). Another protein, Lnp1p, also resides at three-way tubule junctions and appears to antagonize Sey1p fusogenic activity, possibly by promoting ring closure (Chen et al., 2012).

Together these findings demonstrate that Sey1p mediates homotypic ER fusion, but the relatively weak sey1Δ phenotypes suggest there is a second, partially redundant, Sey1p-independent homotypic ER fusion pathway. Patel et al. (1998) demonstrated that ER membranes isolated from ufe1Δ-1 cells were defective in an in vitro ER–ER fusion assay. More recently, strong growth and ER structure defects were demonstrated for double mutants of sey1Δ and the three ER-bound retrograde (Golgi-to-ER) SNAREs (soluble ΔN-ethylmaleimide-sensitive factor attachment protein receptors) sec20, ufe1, and use1 (Anwar et al., 2012; Rogers et al., 2013). Moreover, sey1Δ ufe1Δ-1 double mutants had an even slower rate of homotypic ER fusion than sey1Δ or ufe1Δ-1 single mutants (Anwar et al., 2012). These data suggest that SNARE proteins mediate the hypothesized Sey1p-independent homotypic ER fusion pathway.

SNAREs mediate vesicle fusion during cargo trafficking between organelles (reviewed in Delic et al., 2013). To fuse vesicles, SNARE proteins on the incoming vesicle bind to SNAREs on the target membrane and form a four-helix coiled-coil SNARE complex that forces the lipid membranes into close apposition (Sutton et al., 1998). In vivo, unique sets of SNARE proteins (usually four SNAREs) mediate the different vesicle- trafficking steps, although some SNAREs are used in multiple pathways (Burri and Lithgow, 2004). Although SNAREs are sufficient to fuse vesicles in vitro (Weber et al., 1998), vesicle trafficking in vivo additionally requires v-SNAREs, Rab GTPases, and SM (Sec1/Munc18) proteins, and tethering complexes (reviewed in Hong and Lev, 2014). In retrograde vesicle trafficking, the v-SNARE Sec22p on cis-Golgi-derived cytotoxic coat protein I (COPI)-coated vesicles binds the t-SNAREs Sec20p, Ufe1p, and Use1p on the ER to catalyze vesicle–ER fusion (reviewed in Spang, 2013). Retrograde trafficking additionally requires the Rab GTPase Ypt1p, the SM protein Sly1p, and the Dsl1 tethering complex (Ossig et al., 1991; Jedd et al., 1995; Kraynack et al., 2005; Li et al., 2005; Kamena et al., 2008). The Dsl1 tethering complex is a CATCHR (complex associated with tethering containing helical rods) family member comprising the subunits Tip20p, Dsl1p, and Sec39p/Dsl3. This complex is thought to mediate the initial connection between the ER membrane and an incoming vesicle. Specifically, the Dsl1 complex is anchored to the ER membrane through interactions of two of its subunits (Tip20p and Sec39p) with two ER t-SNAREs (Sec20p and Use1p; Figure 1A; Andag et al., 2001; Andag and Schmitt, 2003; Ren et al., 2009; Tripathi et al., 2009; Zink et al., 2009). The third Dsl1 complex subunit, Dsl1p, connects Tip20p to Sec39p and contains a flexible 80-residue segment called the “lasso.” The lasso, located at the tip of the 20-nm-tall Dsl1 complex, binds two subunits of COPI coats, α-COP (Cop1p) and δ-COP (Ret2p). By binding multiple ER SNAREs and tethering the incoming vesicle near the ER membrane, the Dsl1 complex appears to promote the assembly of productive trans-SNARE complexes; it may also facilitate vesicle uncoating (Ren et al., 2009; Zink et al., 2009; Diefenbacher et al., 2011).

Although it appeared from previous work (Anwar et al., 2012; Rogers et al., 2013) that the retrograde SNAREs Sec20p, Ufe1p, and Use1p mediate a redundant, Sey1p-independent homotypic ER fusion assay, the flexible lasso domain of Dsl1p binds to the COPI coat (not shown; adapted from Ren et al., 2009).}

**FIGURE 1**: SNARE-mediated homotypic ER fusion requires DSL1. (A) The Dsl1 complex (Dsl1p-Sec39p-Tip20p) binds to the N-terminal regulatory domains of the SNAREs Use1p and Sec20p through interactions with Sec39p and Tip20p, respectively. The flexible lasso domain of Dsl1p binds to the COPI coat (not shown; adapted from Ren et al., 2009). (B) Growth assays (see Materials and Methods) of wild type (MY14008), dsl1ΔL (MY14012), sey1Δ (MY14016), sey1Δ dsl1ΔL (MY14020), dsl1ΔE (MY14024), and sey1Δ dsl1ΔE (MY14028), grown on YEPD for 2 d at 30°C or 6 d at 18°C. Each spot from left to right represents a 10-fold dilution.
fusion pathway, several fundamental questions remained. First, it is possible that the synthetic growth and ER structure defects are due to downstream effects of perturbed retrograde vesicle trafficking. Second, it is unclear whether the SNAREs act in isolation or in combination with their normal vesicle-trafficking accessory proteins, including the Dsl1 tethering complex and the COPI coat. Third, it remained possible that the Sey1p-mediated and putative SNARE-mediated homotypic ER fusion pathways are not truly redundant but might mediate distinct subsets of homotypic ER fusion events. In this study, we have further characterized SNARE-mediated homotypic ER fusion and determined that it requires the Dsl1 complex but not the COPI coat, is not a result of perturbed retrograde vesicle trafficking, and is only partially redundant with Sey1p-mediated homotypic ER fusion.

RESULTS

SNARE-mediated homotypic ER fusion requires DSL1

Cells containing both a sey1 deletion and a mutant retrograde SNARE allele (sec20-1, ufe1-1, use1-10AA, or use1-0laya) have strong synthetic growth and ER structural defects not seen in either single mutant (Anwar et al., 2012; Rogers et al., 2013). We hypothesized that homotypic ER fusion is mediated by two parallel pathways, one Sey1p mediated and the other DSL1 mediated, and that only disruption of both pathways results in severe ER–ER fusion and growth defects.

To assess whether the SNAREs act with their normal accessory proteins or alone, we first created sey1Δ dsl1Δ double mutants and assayed possible synthetic growth defects. Dsl1p, like the other subunits of the Dsl1 complex, Tip20p and Sec39p/Dsl3p, is encoded by an essential gene. We assayed two dsl1 mutants in this study: dsl1ΔE and dsl1ΔL (Figure 1A). The C-terminal E domain is the most highly conserved region of Dsl1p (Zink et al., 2009), while the flexible L (lasso) region mediates α-CP (Cop1p)- and β-CP (Ret2p) binding (Andag et al., 2001; Andag and Schmitt, 2003; Zink et al., 2009); remarkably, neither the E domain nor the lasso is needed in vivo for normal growth (Zink et al., 2009; R. W. Baker and F. M. H., unpublished data). Interestingly, sey1Δ dsl1ΔE double mutants exhibited a strong synthetic growth defect at 30°C and an even more severe defect at 18°C, whereas sey1Δ dsl1ΔL mutants exhibited no growth defect at 30°C and a minor growth defect at 18°C (Figure 1B). These data suggest that SNARE-mediated ER–ER fusion requires Dsl1p but not the COPI-binding lasso.

SNARE-mediated homotypic ER fusion requires the entire Dsl1 complex but not the COPI coat

Because dsl1ΔE but not dsl1ΔL mutants exhibited synthetic growth defects with sey1Δ, we screened for additional genetic interactions to gain insight into the function of the Dsl1p E domain. Additionally, we reasoned that this screen might identify novel components of the Sey1p-mediated ER–ER fusion pathway, as any pathway-specific components should, like sey1Δ, display negative genetic interactions with dsl1ΔE but not with dsl1ΔL. To perform the screen, we used synthetic genetic array (SGA) technology. We crossed each strain to a genome-wide set of nonessential deletion mutants (~4300 gene deletion strains) and a large-scale temperature-sensitive mutant collection (~1200 temperature-sensitive strains). We assessed the growth of double-mutant haploids isolated from these crosses using previously published methodology (Baryshnikova et al., 2010; Costanzo et al., 2010). Briefly, we assigned each double mutant an SGA score based on colony size measurements and a multiplicative fitness model. A negative SGA score indicates that the double mutant grew more poorly than expected (synthetic growth defect), whereas a positive score indicates more robust growth than expected.

To identify the most informative genetic interactions, we grouped the interactions into three classes: interactions shared between dsl1ΔΔ and dsl1ΔL, dsl1ΔL-specific interactions, and dsl1ΔΔ-specific interactions (Figure 2A; full data set available in Supplemental Table S1; classes determined via a cutoff score and a difference threshold; see Materials and Methods). As expected, the strongest interactions shared between dsl1ΔΔ and dsl1ΔL consisted of known components of vesicle trafficking. In the dsl1ΔΔ-specific class, we identified five negative interactions: pse1-41, hir1Δ, mps3-1, chk1Δ, and cse2Δ. Four of these genes have nuclear roles: Pse1p interacts with nuclear pore complexes, Mps3p resides at the half-bridge and mediates spindle pole body formation, Hir1p is a subunit of the histone regulation complex, and Cse2p is a subunit of the RNA polymerase II mediator complex. Interestingly, while pse1-41 is by far the strongest dsl1ΔΔ-specific negative genetic interaction, pse1-34 is among the strongest dsl1ΔL-specific positive interactions. The final interaction, chk1Δ, uniquely interacts both negatively with dsl1ΔL and positively with dsl1ΔE. Chk1p is a checkpoint kinase that mediates cell cycle arrest.

Among the 22 dsl1ΔΔ-specific negative interactions (Table 1), eight were related to trafficking (although not exclusively retrograde trafficking), and seven were related to transcriptional regulation. The final seven interactions consist of sey1Δ, whose dsl1ΔΔ-specific negative interaction (Figure 1B) motivated the genome-wide screen; ice2Δ, a gene involved in inheritance of cortical ER (Estrada de Martin et al., 2005) that also has a negative genetic interaction with sey1Δ (Figure 2B); act1-2, an actin allele; ryc2Δ, a pyruvate carboxylase isofom; atg15Δ, a lipase involved in autophagy; and two dubious open reading frames (ORFs), ygr139wΔ and ypr050cΔ. Of note, although act1-2 displayed the strongest negative genetic interaction, 19 other act1 alleles tested displayed no genetic interaction. Allele-specific genetic interactions often result from physical interactions (Sandrock et al., 1997), raising the possibility that Dsl1p and Act1p interact physically, although it also possible that act1-2 specifically disrupts Sey1p-mediated ER–ER fusion. It is possible that any of the aforementioned Dsl1-interacting genes could be new members of the Sey1p-mediated ER–ER fusion pathway; however, we have not yet studied these genes further.

Following similar logic, we reasoned that any additional components in the SNARE/Dsl1-mediated ER–ER fusion pathway should, like dsl1ΔΔ, display negative genetic interactions with sey1Δ. Therefore we examined independently generated sey1Δ SGA data (Costanzo et al., 2010; unpublished data [version 13-04-22], C. Boone, University of Toronto). As expected, the strongest negative synthetic genetic interactions with sey1Δ include use1-1TS (temperature sensitive), a retrograde SNARE implicated previously in SNARE-mediated ER–ER fusion; sec39-1, a Dsl1 complex subunit; and sly1TS, an SM protein required for normal retrograde trafficking (Table 2, interactions with SGA score ≤ −0.25). These results suggest that SNARE-mediated homotypic ER fusion may require the SM protein Sly1p and possibly the entire Dsl1 complex. We discuss the potential role of the remaining five sey1Δ interactions in the Discussion.

Notably, we also observed that sey1Δ exhibited no genetic interactions with the members of the COPI coat (cop1-1, ret2-1, ret3-1, sec21-1, sec26TS, sec29Δ, sec26-2, sec27-1, and sec28Δ), whereas both dsl1ΔΔ and dsl1ΔL exhibited strong interactions with most members (Figure 2B). Additionally, with the exception of sec23-1, other nonretrograde vesicle-trafficking pathway components showed no synthetic interactions with sey1Δ (see Table S1 for a listing of SGA data available for vesicle-trafficking components).
suggest that the SNAREs and the Dsl1 complex together directly mediate Sey1p-independent ER–ER fusion, whereas the COPI coat functions specifically in retrograde vesicle trafficking.

Therefore it is unlikely that the observed genetic interactions between sey1Δ and the SNAREs, dsl1ΔE, and sec39-1 are simply a downstream result of perturbed vesicle trafficking. Instead, our data suggest that the SNAREs and the Dsl1 complex together directly mediate Sey1p-independent ER–ER fusion, whereas the COPI coat functions specifically in retrograde vesicle trafficking.
We manually verified the most important SGA results and checked for synthetic growth defects between sey1Δ and candidates that were missing from the SGA arrays. We confirmed the synthetic growth defect in sey1Δ sec39-1 mutants (Figure 3A) and the lack of a COPI interaction (no growth defects in sey1Δ sec27-1, sey1Δ ret2-1, and sey1Δ cop1-1 mutants; Figure 3B and Supplemental Figure S1A). We also tested the third and final subunit of the Dsl1 complex, Tip20p, which was missing from the SGA analysis; sey1Δ tip20-5 mutants exhibited strong synthetic growth defects, implicating the entire Dsl1 complex in ER–ER fusion (Figure 3C).

To test whether Dsl1p, Sec39p, and Tip20p function as a complex during ER–ER fusion, as they do during vesicle trafficking, we tested two additional sey1Δ dsl1 mutants: sey1Δ dsl1-AS33D and sey1Δ dsl1-LS55E/LS8D. In vitro, Dsl1p-AS33D has a weakened interaction with Sec39p and Dsl1p-LS55E/LS8D has a weakened interaction with Tip20p (Ren et al., 2009; Tripathi et al., 2009). The sey1Δ dsl1-AS33D double mutants exhibited a synthetic growth defect (Figure 3D), suggesting that the Dsl1-Sec39 interaction is required for SNARE-mediated ER–ER fusion. On the other hand, sey1Δ dsl1-LS55E/LS8D did not exhibit a synthetic growth defect (Figure 3D). This result is consistent with the ability of the same allele to support retrograde vesicle trafficking, as judged by its wild-type growth rate, and suggests that the dsl1-LS55E/LS8D mutation is not effective in disrupting the Dsl1 complex in vivo. Overall we conclude that the Dsl1 complex needs to be at least partially intact in order to participate in SNARE-mediated ER–ER fusion.

**The Dsl1 complex is directly required for ER–ER fusion**

It remained possible that the synthetic growth defects observed in sey1Δ dsl1Δ double mutants were due to disruption of some other process required for normal growth, other than ER–ER fusion or vesicle trafficking. We therefore examined these mutants for ER structure defects that should arise from disrupted homotypic ER fusion. In wild-type cells, Sec63-GFP marks the entire ER network, which includes the nucleus, tubules extending from the nucleus to the cell cortex, and the tubules and sheets at the cell periphery. Sec63-green fluorescent protein (GFP) distribution appeared similar to wild type in sey1Δ dsl1Δ E single mutants, but sey1Δ dsl1Δ E double mutants exhibited severely disrupted peripheral ER, lacking an organized tubular network and mostly appearing as patches or filaments. Additionally, whereas in wild-type cells the ER forms an almost continuous network close to the cell cortex, and the tubules and sheets at the cell periphery. Sec63-green fluorescent protein (GFP) distribution appeared similar to wild type in sey1Δ dsl1Δ E single mutants, but sey1Δ dsl1Δ E double mutants exhibited severely disrupted peripheral ER, lacking an organized tubular network and mostly appearing as patches or filaments.
to the plasma membrane, in sey1Δ dsl1ΔE mutant cells, we frequently observed large sections of the cortex with no ER and regions of ER internal to the cell.

We also assessed ER structure using Rtn1-GFP, which in wild-type cells localizes specifically to the tubular ER and the edges of sheets at the cell periphery and is excluded from the nucleus. Again, sey1Δ and dsl1ΔE single mutants appeared similar to wild type (Figure 4B). sey1Δ dsl1ΔE double mutants, however, exhibited a striking defect in which Rtn1-GFP accumulated in a few large, bright puncta, often at or near the bud neck (Figure 4B). As a measure of accumulation, the maximum fluorescence intensity of Rtn1-GFP in sey1Δ dsl1ΔE cells was ∼10-fold higher than wild type (Figure 4B, top right), despite having only twofold higher total GFP (Figure 4B, bottom right). We occasionally observed a few long, unbranched ER tubules at the cell periphery (Figure 4B, sey1Δ dsl1ΔE peripheral enlargement), but the bright Rtn1-GFP aggregates near the bud neck usually obscured fluorescence signal at the periphery in most cells. Importantly, similar ER network defects were observed in sey1Δ tip20-5 mutants but not sey1Δ cop1-1 mutants (Figure S1B). Finally, we examined sey1Δ dsl1ΔE cells by electron microscopy and, consistent with our findings by fluorescence microscopy, observed abnormal ER in 53% of cells (random single sections through cells, n = 38; Figure S2A) and dense aggregates of ER-like membranes in 21% of cells (Figures 4C and S2B). Together these data demonstrate a perfect correlation between synthetic growth defects and synthetic ER structure defects, consistent with a causal relationship.

To address whether the Dsl1 complex directly mediates ER–ER fusion, rather than another step that modifies ER structure, we used a direct in vivo ER–ER fusion assay adapted from Anwar et al. (2012). In this assay, we mated cells containing cytosolic GFP and cells containing ER–lumenal mCherry (directed by a signal sequence and C-terminal ER–retention sequence, HDEL). For each mating pair, we collected images at 1-min intervals and determined the duration of the delay between cell fusion (marked by cytosolic GFP transfer) and ER–ER fusion (marked by mCherry transfer; Figure 5A). As previously demonstrated (Anwar et al., 2012), ER–ER fusion required significantly more time in sey1Δ cells than in wild type (mean delay 8.7 vs. 4.9 min, p = 0.01, two-sample t test; Figure 5B). dsl1ΔE cells were not significantly different from wild type (5.3 vs. 4.9 min, p = 0.81). In contrast, ER–ER fusion

### TABLE 2: SGA scores of sey1Δ-negative genetic interactions.

| Allele | sey1Δ | dsl1ΔE | Description |
|--------|--------|--------|-------------|
| scs3Δ | −0.84  | −0.05  | Protein required for inositol prototrophy |
| use1-TS | −0.65  | −0.80  | ER-localized SNARE required for retrograde vesicular traffic |
| sec39-1 | −0.58  | −0.91  | Component of the Dsl1 tethering complex |
| sly1-TS | −0.54  | −0.50  | SM-family protein involved in ER/Golgi traffic |
| pom33Δ | −0.37  | 0.00   | Transmembrane nucleoporin |
| sec23-1 | −0.36  | −0.05  | COPII coat subunit, also stimulates the GTPase activity of Sar1p |
| yop1Δ | −0.32  | −0.05  | Membrane protein that interacts with Sey1p to maintain ER morphology |
| ice2Δ | −0.31  | −0.29  | Integral ER membrane protein with type-III transmembrane domains |

Sorted list of sey1Δ-negative SGA scores, cutoff at −0.25.
The reticulons are required for viability in a sey1Δ dsl1ΔE background

The reticulons Rtn1p, Rtn2p, and Yop1p are required for normal peripheral ER structure in vivo and can mediate ER tubule formation in vitro (Voeltz et al., 2006; Hu et al., 2008). Previous studies focusing only on Sey1p-mediated ER–ER fusion were unable to explain why sey1Δ rtn1Δ rtn2Δ yop1Δ mutants have only minor growth defects, despite having extremely perturbed ER structure (Voeltz et al., 2006; West et al., 2011). It was posited that either ER–ER fusion is not essential for viability under normal growth conditions in yeast or that there

\[ 4 \times 10^{-13}. \]

Bottom right graph: as above, but the sum of all pixel intensities was measured (including all z-slices). Mean value and p value vs. WT: WT, 0.75 \times 10^6; sey1Δ, 0.57 \times 10^6, p = 0.03; dsl1ΔE, 0.74 \times 10^6, p = 0.62; and sey1Δ dsl1ΔE, 1.7 \times 10^6, p = 3 \times 10^{-4}. All quantifications were performed in background-subtracted, raw images (not deconvolved). (C) Representative electron micrograph of ultrathin-sectioned sey1Δ dsl1ΔE cells (parent diploid MY14907), imaged at 10,000×. Scale bar: 500 nm. Right, 22,500× image of the region outlined in the black box in the left panel. Scale bar: 200 nm.
exists an alternative ER–ER fusion pathway. Despite the identification of an alternative SNARE/Dsl1-mediated ER–ER fusion pathway, sey1Δ dsl1ΔE double mutants, which have lost two ER–ER fusion pathways, remain viable (albeit slow growing). It therefore remained possible that ER–ER fusion is a nonessential process in yeast.

To address this possibility and to explore the relationship, if any, between the reticulons and the SNARE/Dsl1-mediated ER–ER fusion pathway, we dissected ∼100 tetrads from a diploid heterozygous for sey1Δ, rtn1Δ, rtn2Δ, yop1Δ, lnp1Δ, and dsl1ΔE, and quantified colony growth over time of the different genetic combinations, averaged by genotype. We observed a minimal effect of rtn2Δ on the growth rate relative to any comparable genetic combination (Figure S3, A and B; see Table S2 for full growth data), consistent with Rtn2p’s low level of expression under normal growth conditions (De Craene et al., 2006; Voeltz et al., 2006). We therefore averaged colony sizes independently of RTN2. Additionally, our results recapitulated published growth defects of lnp1Δ rtn1Δ and suppression of lnp1Δ rtn1Δ by sey1Δ (Figure S3C; Chen et al., 2012). lnp1Δ was included in our analysis, as Lnp1p was previously shown to antagonize Sey1p activity via an unknown mechanism (Chen et al., 2012). However, there were no synthetic genetic interactions between lnp1Δ and dsl1ΔE, and we therefore do not discuss LNP1 further (Figure S3C).

We next compared growth rates related only to sey1Δ, rtn1Δ, yop1Δ, and dsl1ΔE genotypic combinations (Figure 6A). As expected, all single mutants grew about as well as wild type (compare strain 1 with strains 2–5 in Figure 6A). Among double mutants (strains 6–11), sey1Δ dsl1ΔE exhibited a strong growth defect, as expected from our initial work, and yop1Δ rtn1Δ exhibited a minor growth defect, as previously reported. Surprisingly, rtn1Δ dsl1ΔE exhibited an intermediate growth defect, implying that Rtn1p and Dsl1p share functional redundancy. All triple mutants (strains 12–15) exhibited an intermediate to severe growth defects (see Figure 6B for colony sizes after 6 d of growth). The quadruple-mutant sey1Δ yop1Δ rtn1Δ dsl1ΔE (strain 16) was almost completely inviable (four out of eight spores did not form colonies after 6 d of growth, and the remaining spores formed extremely tiny colonies; Figure 6B), suggesting that there is an unexpected partial redundancy between the function of the reticulons and the two ER–ER fusion pathways, which together are essential for cell viability.

It is thought that the reticulons both generate and maintain ER tubules and thus may act genetically in the same pathway with Sey1p (Hu et al., 2009; Chen et al., 2012). The relationship between SNARE-mediated ER–ER fusion and the reticulons, however, is unclear. Because the sey1Δ yop1Δ rtn1Δ dsl1ΔE quadruple mutant (strain 16) is almost inviable, The Dsl1 pathway must be responsible for the relatively normal growth rate of the sey1Δ yop1Δ rtn1Δ triple mutant (strain 12). We conclude, therefore, that SNARE/Dsl1-mediated ER–ER fusion does not require the reticulons Rtn1p and Yop1p (Figure 6A). Conversely, Sey1p is less able than Dsl1p to support reticulon-independent growth, as cells that are only SEY1+ (yop1Δ rtn1Δ dsl1ΔE, strain 15) have a more severe growth defect than cells that are only DSL1+ (sey1Δ yop1Δ rtn1Δ, strain 12). Notably, however, SEY1 still confers a growth advantage on cells lacking reticulons and wild-type DSL1 (Figure 6B, compare strains 15 and 16). Finally, when dsl1ΔE is combined with sey1Δ, rtn1Δ, or both sey1Δ and rtn1Δ, the resulting growth defects are all similar (Figure 6A, compare strains 8, 11, and 14), suggesting that Sey1p and Rtn1p share a genetic pathway distinct from Dsl1p.

**FIGURE 5:** ER–ER fusion is severely delayed in sey1Δ dsl1ΔE mutants.

(A) Representative example of the ER–ER fusion assay. Identical genotypes were mated to each other at room temperature; MATα strains expressed GFP-HDEL (pMR6473), MATa strains expressed mCherry-HDEL (pMR6474), MATα strains expressed cytosolic GFP (pMR3619). sey1Δ cells are shown. The first peak in mCherry transfer at 0–2 min corresponds to the transfer of cytosolic sey1Δ cells are shown. The first peak in mCherry transfer at 0–2 min corresponds to the transfer of cytosolic mCherry-HDEL after cell fusion. Arrowhead depicts the point of ER–ER fusion (8 min). Scale bar: 2 μm. (B) Box plot of the times required for ER–ER fusion after cell fusion. Data are pooled from at least two independent experiments for each genotype. Mean values: wild type, 4.9 min (MY14509 × MY14513, n = 16); sey1Δ, 8.8 min (MY14510 × MY14514, n = 16); dsl1ΔE, 5.3 min (MY14511 × MY14515, n = 14); and sey1Δ dsl1ΔE, 23.4 min (MY14512 × MY14516; n = 10). Each box shows the interquartile range (25–75% of the data), black bars represent the median, and outliers are shown as open circles beyond the 1.5 * interquartile range. (C) Percentage of zygotes with unfused ER (binary scoring). The data are pooled from two independent experiments; error bars show ± SE for a binomial distribution. Strains used: wild type (MY14059 × MY14009), dsl1ΔL (MY14061 × MY14013), sey1Δ (MY14063 × MY14017), sey1Δ dsl1ΔL (MY14065 × MY14021), dsl1ΔE (MY14067 × MY14025), sey1Δ dsl1ΔE (MY14071 × MY14031), and sey1Δ dsl1ΔE (MY14071 × MY14071) × wild type (MY14009). All MATα strains expressed GFP-HDEL (pMR6473).
A surprising observation was that cells that are only YOP1+ (sey1Δ rtn1Δ dsl1ΔE, strain 14) have an intermediate growth defect, whereas cells that are only RTN1+ (sey1Δ yop1Δ dsl1ΔE, strain 13) are almost inviable (Figure 6B). Therefore Yop1p but not Rtn1p can contribute substantially to cell growth independently of the other reticulons and of the Sey1p- and SNARE/Dsl1-dependent ER–ER fusion pathways.

**DISCUSSION**

**Characterization of SNARE-mediated homotypic ER fusion**

On the basis of synthetic growth defects and ER–ER fusion experiments in this study, we conclude that SNARE-mediated homotypic ER fusion additionally requires the Dsl1 complex, consisting of Sec33p, Dsl1p, and Tip20p. Furthermore, genetic analysis indicates that the observed synthetic defects are not indirectly caused by perturbed vesicle trafficking; if they were, then other mutations that disrupt vesicle trafficking, such as mutations in vesicle coat components or other SNAREs, should have resulted in growth and ER structure defects in a sey1Δ background (see Table S1 for full list of trafficking components tested). These results imply that SNARE-mediated ER–ER fusion occurs independently of vesicle fusion and relies on direct ER–ER interactions, similar to the model for Sey1p-mediated ER–ER fusion (Figure 7).

In the Dsl1 complex mutants examined in this study, there was a perfect correlation between synthetic growth defects and synthetic ER structure and ER–ER fusion defects. It is conceivable that aberrant ER structures could inhibit the observed rate of ER–ER fusion in our assay, but we note that previous reports demonstrated that rtn1Δ rtn2Δ yop1Δ mutants, which have severe ER structure defects, have a normal rate of ER–ER fusion (Anwar et al., 2012). Therefore we conclude that the SNAREs most likely act with the Dsl1 complex as true fusogens for homotypic ER fusion.

The full composition of the ER–ER fusion SNARE complex is not yet clear. Only three SNARE genes—SEC20, USE1, and UFE1—have shown genetic interactions with SEY1, whereas a normal four-helix SNARE complex requires four SNARE proteins. In retrograde trafficking, Sec22p is the fourth SNARE, but Ykt6p can compensate in cells lacking Sec22p (Liu and Barlowe, 2002). Sec22p/Ykt6p could also redundantly mediate ER–ER fusion, but this is difficult to test, as known sec22 ykt6 double mutants are either inviable or severely slow growing (Liu and Barlowe, 2002; Rogers et al., 2013).

During retrograde trafficking, the Dsl1 complex tethers ER-bound SNAREs to the incoming COPI-coated vesicle and promotes trans-SNARE complex assembly. Our results imply that the Dsl1 complex uses a coat-independent function during ER–ER fusion. In agreement with this conclusion, dsl1Δ-4 mutants accumulate ER and vesicles at the nonpermissive temperature, whereas dsl1Δ-7 mutants accumulate only ER, suggesting that Dsl1p has two different functions (VanRheenen et al., 2001). Furthermore, overexpression of Sec21p (γ-COP), a COPI complex subunit, suppressed the lethality of dsl1Δ-4 but not dsl1Δ-7, supporting a COPI-independent role for Dsl1p (VanRheenen et al., 2001). Mechanistically, because the Dsl1 complex promotes SNARE complex formation and stabilization, this mechanism could apply to ER–ER fusion as well, independent of a vesicle-tethering function (Ren et al., 2009; Diefenbacher et al., 2011). An attractive hypothesis is that the Dsl1p complex might adopt an extended conformation that tethers SNAREs residing on the two opposing ER membranes before fusion.

The SNARE/Dsl1-mediated homotypic ER fusion pathway characterized here may have additional components that are not shared with retrograde vesicle trafficking. Such components should exhibit negative genetic interactions with sey1Δ. Only five candidates were identified (Table 2): yop1Δ, ice2Δ, scs3α, pom33Δ, and sec23-1. Yop1p and Ice2p broadly affect ER structure and function and may not be specific to SNARE-mediated ER–ER fusion. Scs3p is required for normal ER membrane biosynthesis and exhibits genetic interactions with many cellular processes, suggesting that it too may not be specific for SNARE-mediated ER–ER fusion (Hosaka et al., 1994; Moir et al., 2012). Pom33p is a transmembrane nucleoporin (Chadrin et al., 2010). Interestingly, some reticulons have additional roles in nuclear pore complex biogenesis in addition to ER tubulation, probably via a common curvature-inducing mechanism (Dawson et al., 2009). Furthermore, Rtn1p and Yop1p physically
interact with Ndc1p, a component of nuclear pore complexes (Casey et al., 2012). Therefore sey1Δ pom3Δ double mutants may have disrupted nuclear pore complex functions. However, as SNARE-mediated ER–ER fusion must occur at the peripheral ER, away from the nucleus, a role for Pom3p at the peripheral ER would be surprising. Finally, Sec23p is a component of the COPII coat that stimulates the GTPase activity of Sar1p and is required for ER-to-Golgi anterograde vesicle trafficking. However, all other anterograde trafficking components, including the rest of the COPII coat, do not genetically interact with sey1Δ (except sar1, for which we have no data; Table S1). It is therefore possible that Sec23p has a novel, vesicle-independent function in SNARE-mediated ER–ER fusion.

Finally, we note that all identified members of SNARE-mediated ER–ER fusion have conserved mammalian orthologues, raising the possibility that this pathway is conserved among eukaryotes (conservation reviewed in Schmitt, 2010).

Functional overlap between Sey1p-mediated and SNARE-mediated ER–ER fusion

An open question is whether Sey1p and SNAREs mediate distinct ER–ER fusion events. sey1Δ and ds1ΔE single mutants exhibit no growth defects, and sey1Δ mutants have only minor defects in ER structure and ER–ER fusion rate compared with sey1Δ ds1ΔE double mutants. This suggests that under normal growth conditions the two pathways are largely, but not entirely, redundant and independent. However, it is possible that one pathway is favored under certain environmental conditions, such as different osmolarities or temperatures, or that they mediate fusion at ER domains that have subtle differences.

The function of ER structure and fusion in yeast cells

An early mystery in our study was why sey1Δ ds1ΔE double mutants are viable if both ER–ER fusogens have been disrupted. We reasoned that this could be because 1) the ds1ΔE mutation only partially disrupts SNARE-mediated ER–ER fusion; 2) there is a third, redundant ER–ER fusion pathway; or 3) ER–ER fusion is not an essential process for yeast viability under normal growth conditions. The first possibility is difficult to test, as all of the genes in the SNARE/Ds1 pathway are essential and cannot be deleted. The second possibility, that there is a third, albeit inefficient, fusogen, is supported by the observation that Yop1p is required for most of the remaining growth in the sey1Δ ds1ΔE double mutant. Therefore Yop1p may be an essential member of a third ER–ER fusion pathway, or Yop1p itself may function as both a reticulum and a weak fusogen. The latter interpretation is supported by the finding that Yop1p is sufficient to form both tubules and three-way ER junctions in vitro, whereas Rtn1p only forms tubules and no junctions (Hu et al., 2008). The third possibility, that ER–ER fusion is not an essential process under our growth conditions, remains formally plausible, as even sey1Δ rtn1Δ yop1Δ ds1ΔE mutants occasionally formed tiny colonies after ~6 d of growth at 30°C (four out of eight sporulations). However, this conclusion assumes there is no fourth fusogen, that ER–ER fusion will not spontaneously occur without a fusogen, and that the ds1ΔE mutation fully disrupts SNARE-mediated ER–ER fusion.

A remaining problem is identifying the most proximal cause of the sey1Δ ds1ΔE growth defect. Disrupted ER–ER fusion could affect multiple downstream processes, including ER inheritance, lipid homeostasis between the nuclear and peripheral ER, and protein recycling. In such cases, one should be able to restore normal growth by rescuing the downstream processes without rescuing ER–ER fusion itself. More indirectly, accumulated ER could physically block exo/endocytosis at the plasma membrane, or organelle or nuclear segregation, as was observed for sey1Δ mutants during nuclear congestion during mating (Rogers et al., 2013). Discerning these mechanisms should prove fruitful in future studies.

MATERIALS AND METHODS

Strains and general yeast methods

Strains and plasmids used are listed in Table S3. Standard methods including cell culture and transformations were performed as
described by Amberg et al. (2005). In general, many of the slow-growing strains described in this study were prone to accumulating intermediate suppressors of growth rate if used for many generations. To avoid this confounding issue, we sometimes generated desired genotypes for an experiment from a heterozygous diploid parent as stated in the figure legends but did not freeze the haploids as separate strains.

**Growth assays**

Cultures were grown to saturation in yeast extract/peptone/dextrose (YE PD) at 23°C (except 30°C for the assay in Figure 1B) and then 0.2 OD<sub>600</sub> unit of cells was pelleted and resuspended in 200 μl dH<sub>2</sub>O. Five 10-fold serial dilutions were made in a 96-well plate and then spotted on YEPD plates and grown at various temperatures as indicated.

**SGA experiments and analysis**

*ds1ΔE::NatMX (MY15059), ds1ΔLasso::NatMX (MY15060), and Dsl1Δ·::NatMX (MY15058) strains were created using parent strain Y7092, and SGA screens were performed and analyzed as previously described (Tong and Boone, 2006; Baryshnikova et al., 2010).** *SGA screens with temperature-sensitive alleles were performed at 26°C. To separate the alleles into classes, we used a cutoff score (requiring at least one SGA score with an absolute value ≥ 0.25) and a difference threshold (requiring that SGA scores differed by at least an absolute value of 0.25 to be classified as specific). Additionally, for the alleles in the shared class, we required that both SGA scores had an absolute value of at least 0.25.

**Image acquisition and analysis**

All microscopy was performed on a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA), based on a Nikon TE2000 (Melville, NY) with an inverted 100x NA 1.4 objective, a 50-W mercury lamp, and a Photometrics Cool Snap HQ CCD camera (Photometrics, Tucson, AZ). All images were deconvolved using the Applied Precision SoftWoRx imaging software.

**ER structure microscopy**

Cells were imaged live in growth medium at room temperature on a standard glass slide and #1.5 coverslip. Typically, imaging began at the bottom of a cell (nearest the objective and touching the coverslip) and 30–40 slices were imaged with 0.15-μm z-spacing. Exposure times were typically 0.5–1.0 s, except sey1Δ ds1ΔE cells and sey1Δ tips20-5 cells expressing Rtn1-GFP (Figures 4B and S1B) were imaged at 0.05–0.1 s to avoid pixel saturation.

**ER–ER fusion assays**

Cultures were grown overnight to early to mid-log phase in synthetic complete medium lacking leucine (SC –leu) at 30°C. Cells from each strain to be mated (0.01 OD<sub>600</sub> unit) were added onto a pretreated 0.17-mm Delta T Culture Dish (Biotechnics, Butler, PA). Immediately before the cells were added, the Delta T dishes were pretreated by coating with 25 μl of concanavalin A (0.1 mg/ml in 20 mM sodium acetate, pH 5.8) for 15 min and then being washed twice with 50 μl of 20 mM sodium acetate (pH 5.8). Cells were allowed to settle for 15 min before being washed with 200 μl of SC –leu to remove unstuck cells. Finally, 2 ml of SC –leu was added to the dish, and matings were imaged at room temperature. Mating pairs were imaged at 1-min intervals with minimal exposure times (usually 0.1 s) and at a single focal plane to reduce photobleaching and phototoxicity. After imaging, raw movies (not deconvolved) were scored for the time between cell fusion and ER–ER fusion. Cell fusion was identified by a rapid transfer of cytoplasmic GFP into the adjacent mating partner that usually equilibrated in less than 2 min. The first time point with cytoplasmic GFP in both cells, even if not yet equilibrated, was marked as the time of cell fusion. After cell fusion, mCherry-HDEL accumulated slowly in the mating partner’s cytoplasm and ER, presumably due to cytoplasmic mCherry-HDEL and protein recycling. Eventually mCherry-HDEL transfer shifted to a rapid equilibration phase (see Figure 5A and Anwar et al., 2012). The first time point with this shift to fast equilibration was marked as the time of ER–ER fusion.

Fixed-cell assays (Figure 5C) were performed as previously described (Rogers et al., 2013). Briefly, cultures were grown at 30°C to mid-log phase, and 0.5 OD<sub>600</sub> unit of cells was mixed and mated on a 0.45-μm nitrocellulose filter (EMD Millipore, Billerica, MA) for 3 h at 23°C. Cells were then washed into 900 μl of 1x phosphate-buffered saline (PBS), and 100 μl of 20% paraformaldehyde dissolved in distilled H<sub>2</sub>O was added. Cells were fixed at room temperature for 15 min; this was followed by one wash in 1x PBS, 4’,6-diamidino-2-phenylindole staining (2 μg/ml in PBS) for 15 min, two more washes in 1x PBS, and, finally, resuspension in 100–200 μl 1x PBS. Cells were imaged on the same day. GFP-HDEL was used in the fixed-cell assay rather than mCherry-HDEL, as GFP-HDEL intensity and localization is preserved better after fixation. Imaging on the same day ensures that the membranes stay intact and GFP-HDEL does not artifactually diffuse to equilbrium. ER was scored as unfused when GFP-HDEL appeared markedly brighter in one-half of the zygote than the other.

**Electron microscopy**

Cells were prepared for transmission electron microscopy as described in Gammie and Rose (2002). Briefly, the workflow included a glutaraldehyde fixation, potassium permanganate staining, sodium periodate treatment, uranyl acetate staining, and embedding in LR White resin. Specifically to our protocol, –5 OD<sub>600</sub> units of mid-log phase sey1Δ ds1ΔE cells (from diploid parent MY14907) were fixed in 2% glutaraldehyde for 30 min at room temperature. Cells were stained with 4% potassium permanganate for 4 h at 4°C. Ultrathin sections (~80 nm) were placed on a nickel slotted-grid (Formvar film, FF-2010-Ni, Electron Microscopy Sciences, Hatfield, PA) and imaged directly, without lead citrate staining.

** Colony size time-course analysis**

Strain MY14454 was sporulated, and 100 tetrads were dissected on YEPD plates. The plates were incubated at 30°C and imaged after 2, 3, 4, 5, and 6 days of growth. Colony size was measured in ImageJ by applying a binary threshold mask to outline colonies. After 6 days, plates were replica plated to determine genotypes. Genotypes for lethal or extremely tiny colonies were inferred by assuming 2:2 segregation of genes. We excluded from our analysis tetrads that did not exhibit 2:2 segregation for all genes or tetrads that did not permit unambiguous determination of all four spores (e.g., only two cells were viable in the tetrad).

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