Septin-dependent compartmentalization of the endoplasmic reticulum during yeast polarized growth

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Introduction

The ER, the major intracellular membrane system of eukaryotes, ensures the biosynthesis of all lipid precursors, as well as the membrane insertion and the translocation through the lipid bilayer of most membrane and secreted proteins (for reviews see Matlack et al., 1998; Meldolesi and Pozzan, 1998; McMaster, 2001; Ma and Hendershot, 2001). As a calcium-storing organelle, the ER plays crucial roles in signal transduction and the regulation of calcium-dependent processes, such as the control of myosin II activity during muscle contraction (Meldolesi and Pozzan, 1998). However, ER function reaches beyond the metabolism and impacts the structural organization of the cell, at least through the formation of the nuclear envelope (Baumann and Walz, 2001). As such, it is the eukaryotic organelle par excellence.

The ER is formed of an oxidizing environment enveloped by a single lipid bilayer. It assembles into sheets and reticulated tubules that appear continuous with each other by electron microscopy (Baumann and Walz, 2001). Furthermore, photobleaching experiments showed that ER components freely diffuse throughout the entire ER lumen, diffusion of ER membrane proteins was restricted at the bud neck. Ultrastructural studies and fluorescence microscopy revealed the presence of a ring of smooth ER at the bud neck. This ER domain and the restriction of diffusion for ER membrane proteins through the bud neck depended on septin function. The membrane-associated protein Bud6 localized to the bud neck in a septin-dependent manner and was required to restrict the diffusion of ER membrane proteins. Our results indicate that Bud6 acts downstream of septins to assemble a fence in the ER membrane at the bud neck. Thus, in polarized yeast cells, diffusion barriers compartmentalize the ER and the plasma membrane along parallel lines.

Polarized cells frequently use diffusion barriers to separate plasma membrane domains. It is unknown whether diffusion barriers also compartmentalize intracellular organelles. We used photobleaching techniques to characterize protein diffusion in the yeast endoplasmic reticulum (ER). Although a soluble protein diffused rapidly throughout the ER lumen, diffusion of ER membrane proteins was restricted at the bud neck. Ultrastructural studies and fluorescence microscopy revealed the presence of a ring of smooth ER at the bud neck. This ER domain

The ER is formed of an oxidizing environment enveloped by a single lipid bilayer. It assembles into sheets and reticulated tubules that appear continuous with each other by electron microscopy (Baumann and Walz, 2001). Furthermore, photobleaching experiments showed that ER components freely diffuse throughout the entire ER of fibroblasts (Dayel et al., 1999; Nikonov et al., 2002). Thus, the consensus has emerged that eukaryotic cells contain a single ER. In turn, ultrastructural studies established that the continuous ER membrane is highly organized and forms differentiated domains, such as the nuclear envelope and the rough and smooth ER (Baumann and Walz, 2001). However, we still know little about how these structures differentiate from each other. We also know little about the involvement of the ER in complex cellular processes such as cell polarization and cell division. Particularly, we do not know how the ER is cleaved at or before cytokinesis.

In most cells, the ER is tightly associated with the cytoskeleton, and it colocalizes extensively with microtubules in animal cells (Barr, 2002; Du et al., 2004). This tight association of ER and cytoskeleton suggests that cell polarization might strongly impact on ER organization. Over the last decades, cell polarization has been mainly apprehended as the asymmetric distribution of plasma membrane markers. In epithelial cells, neurons, and yeast, this asymmetry takes the form of functionally and structurally distinct plasma membrane domains that are separated by diffusion barriers (Faty et al., 2002; Boiko and Winckler, 2003). Whether and how the compartmentalization of the plasma membrane affects the internal organization of the cell has not been studied much, and little is known about how cell polarity impinges on ER organization.

The budding yeast Saccharomyces cerevisiae has provided an excellent model to study cell polarity and its molecular mechanism (Pruyne and Bretscher, 2000a,b). This unicellular
organism divides by budding; i.e., it polarizes its growth to produce a daughter cell de novo. The restriction of cell growth to the developing bud depends on the polarization of exocytosis and the actin cytoskeleton. Actin cables are nucleated at the bud cortex in a formin-dependent manner and align along the mother-bud axis. These cables serve as tracks for the myosin-dependent delivery of exocytic vesicle to the bud. Thereby, they ensure the polarized delivery of new membrane, cell wall remodeling enzymes, and cell wall material during bud growth. During this process, the yeast plasma membrane is compartmentalized into a bud and a mother domain that are separated by a septin-dependent diffusion barrier (Barral et al., 2000; Takizawa et al., 2000). Septins are GTPases that assemble into membrane-associated filaments. In yeast, these filaments form a ring at the cortex of the bud neck (for review see Fatty et al., 2002). Among other functions, this ring establishes the lateral diffusion barrier that helps maintain the compartmentalization of the yeast plasma membrane.

Recent studies suggest that cell polarity not only affects the plasma membrane but also deeper functions of the cell such as protein synthesis. In yeast, at least 24 mRNAs are specifically translated in the bud (Shepard et al., 2003). Localization of these transcripts depends on actin and the type V myosin Myo4 and its binding partner She3 (Jansen, 2001). Little is known about how these mRNAs are maintained in the bud upon transport. However, the fact that at least 18 of them encode membrane proteins (Shepard et al., 2003) suggests that the ER in the bud might play some role in RNA anchoring. In this study, we focused our attention on the impact of cell polarity on the organization of the yeast ER.

Results

The bud and mother ERs form separate diffusion domains

The yeast ER is organized into a reticulate network that covers the cell cortex and is connected with the nuclear envelope by cytoplasmic tubules (Preuss et al., 1991; Prinz et al., 2000). To gain further insights into yeast ER organization, we performed fluorescence loss in photobleaching (FLIP) experiments on cells expressing Sec61-GFP. Sec61 is the major subunit of the translocon and resides in the ER membrane (Gorlich et al., 1992). Upon repeated photobleaching of a small cortical area, fluorescence loss in other ER regions indicates exchange of Sec61-GFP molecules with the bleached area. This technique permits the evaluation of exchange rates between different regions of the ER. A small area of the mother cortex was photobleached in medium- to large-budded cells. As seen in Fig. 1 A (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1), photobleaching led to rapid fluorescence loss over the entire cortex of the mother cell ($t_{1/2} = 14 ± 2 s$; Fig. 1 C). Fluorescence was lost with similar kinetics in the perinuclear ER ($t_{1/2} = 19 ± 8 s$). In contrast, fluorescence decayed slower in the bud ER ($t_{1/2} = 119 ± 36 s$, $n = 8$). This phenomenon was fully reproducible and the delay of fluorescence loss in the bud was highly significant (Fig. 1 E). It also did not depend on the existence of absence of ER tubules between the perinuclear ER and the bud cortex (Fig. 1, compare A and B; and Video 2, available on June 28, 2017).

Figure 1. Dynamics of the translocon subunit Sec61 throughout the yeast ER during metaphase. (A and B) Diffusion from the mother cortex to the bud cortex is slow. FLIP was performed on a metaphase cell expressing Sec61-GFP. The bleaching region is depicted by an empty rectangle. (A) Pictures were taken every 20 s. (B) Cell showing a cytoplasmic tubule connecting the nucleus and the bud cortex (Video 2). Pictures were taken every 4 s, with relevant frames shown. Note that the movie covers a shorter period of time than in A. (C) Graph showing the kinetics of fluorescence loss for the cell in A. The cartoon depicts the bleaching region (box) and the three areas in which fluorescence was measured (blue, mother cortex; yellow, perinuclear ER; pink, bud ER). (D) Graph for the cell in B. Color code as in C. (E) Graph showing the kinetics of fluorescence loss after averaging eight different FLIP experiments as in A and B. Color code as in C. (F) Diffusion is fast within each compartment. Loss in fluorescence in the cell in Fig. 2 A was measured in four different regions, two on the mother cortex (pink and yellow boxes on the cartoon) and two on the bud cortex (light and dark blue). The yellow and the light blue region are equidistant from the bleaching region. (G) Graph representing the $t_{1/2}$ of the nonbleached compartment over the $t_{1/2}$ of the bleached compartment. Measures were made on the same eight cells as in B. Error bars indicate the SD. Bars, 1 μm. In this and subsequent figures, elapsed times since the beginning of bleaching are indicated in each frame of the movies, in seconds.
Sec61 (Nikonov et al., 2002). Thus, differences in translational activity between mother and bud might cause Sec61 to diffuse slower in the bud than in the mother. This could explain the slow kinetics of fluorescence loss in the bud versus the mother cell. However, photobleaching at the bud cortex (Fig. 2 B and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1) and FRAP experiments (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1) indicated that Sec61 moved at similar rates in the bud and the mother cell. In these experiments, fluorescence loss was rapid in the bud but slow in the mother. Thus, our FLIP and FRAP data suggest that diffusion of Sec61 through the bud neck is restricted and hence that mother and bud ERs form separate diffusion domains.

A remarkably similar situation was observed for the nuclear envelope during late anaphase. In these cells, exchange between mother and bud cortical ERs remained limited. In addition, the mother cortex exchanged material only with the nuclear half located in the mother cell (Fig. 2 C and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1; ratio $t_{1/2}$ perinuclear ER mother/$t_{1/2}$ mother = 2.6, $t_{1/2}$ perinuclear ER bud/$t_{1/2}$ mother = 11.6, $t_{1/2}$ bud cortex/$t_{1/2}$ mother = 13.4, $n = 6$). Concomitantly, the perinuclear ER located in the bud also exchanged rapidly but exclusively with the bud cortical ER (unpublished data). This indicates that in late anaphase the yet undivided nucleus behaved as two distinct domains of diffusion, each exchanging Sec61 molecules only with the cortical ER of the cellular domain in which it was located. Thus, diffusion through the bud neck was limited at all times. At least in the case of the nuclear envelope, a lateral diffusion barrier must have restricted the movement of Sec61-GFP within the continuous ER membrane at the bud neck.

Next, we extended our investigations to other ER proteins. The ER membrane proteins GFP-Sec22 and Hmg1-GFP (Fig. 3, A and B) provided results very similar to those obtained with Sec61-GFP. Sec22 is a v-SNARE required for vesicular transport between the ER and the Golgi (Kaiser and Schekman, 1990; Newman et al., 1990) and GFP-Sec22 localizes almost exclusively to the ER. Hmg1 is one of the two yeast isoforms of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Qureshi et al., 1976). Thus, the ER is separated into mother and bud domains by an activity that limits the exchange of ER membrane proteins through the bud neck.

**The ER lumen is continuous throughout the cell**

Different results were obtained with the luminal protein GFP-HDEL. Exchange was rapid between the mother and the bud ER (Fig. 3 C and Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1; upon bleaching in the mother: $t_{1/2}$ in the bud/$t_{1/2}$ in the mother = 2 ± 0.4). GFP-HDEL is targeted to the ER lumen by a signal sequence, while a retrieval sequence (HDEL) ensures ER retention (Pelham, 1991). The fast exchange of GFP-HDEL between mother and bud did not take place through the Golgi because it was not slowed down in the sec18-1, sar1-D32G, and cdc48-6 mutants incubated at the restrictive temperature for at least 30 min (Tables I and II and Fig. 3, D and E). Previous studies indicated

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**Figure 2.** Dynamics of the translocon subunit Sec61 during a different cell cycle stage. (A–C) FLIP is applied on cells expressing Sec61-GFP. Color codes and cartoons are as in Fig. 1 C. Relevant frames are shown for each movie. The graphs show the kinetics of fluorescence loss in the three compartments. (A) Photobleaching is applied at the mother cell cortex during metaphase (Video 1). (B) Photobleaching is applied at the bud cortex during metaphase (n = 4; Video 3). (C) Photobleaching is applied at the mother cortex during anaphase (n = 6; Video 4). Bar, 1 μm.
that these mutations abolish the function of the protein within 10 min at the restrictive temperature. Sec18 is the yeast homologue of NSF and is required for vesicle fusion with target membranes during antero- and retrograde transport between ER and Golgi (Spang and Schekman, 1998). Sar1 is required for vesicle budding at the ER surface (Barlowe et al., 1993). The AAA-ATPase Cdc48 is required for ER homotypic membrane fusion events (Latterich et al., 1995). Thus, none of the known pathways for ER membrane dynamics was involved in GFP-HDEL exchange between the mother and the bud.

Further data supported the view that GFP-HDEL freely diffused throughout the entire ER. First, in wild type (WT) and mutants, the bud ER exchanged half of GFP-HDEL–associated fluorescence with the mother ER in 12 s (Fig. 3 C). These fast kinetics exclude that it depended on vesicular transport. Furthermore, movement of GFP-HDEL and Sec61-GFP throughout the ER required neither ATP nor GTP (Fig. 4 A). Indeed, when the FLIP experiments were performed in cells treated with sodium azide to deplete metabolic energy, no change in the dynamics of GFP-HDEL and Sec61-GFP was observed.

This treatment influenced neither exchange of the markers between mother and bud nor the diffusion of either molecule throughout the mother cortex (Fig. 4 A). In contrast, it immediately stopped nuclear movements and the elongation of the nucleus of anaphase cells, indicating that energy depletion was rapid. Therefore, ER membrane fission, fusion, and vesicular trafficking had little impact on the movement of membrane and

Table I. Quantification of ER distribution in EM pictures

<table>
<thead>
<tr>
<th></th>
<th>Mother WT</th>
<th>Bud WT</th>
<th>Bud neck WT</th>
<th>Mother shs1Δ</th>
<th>Bud shs1Δ</th>
<th>Bud neck shs1Δ</th>
</tr>
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<tbody>
<tr>
<td>% rough ER</td>
<td>92 ± 3</td>
<td>72 ± 5</td>
<td>3 ± 6</td>
<td>93 ± 3</td>
<td>64 ± 8</td>
<td>91 ± 12</td>
</tr>
<tr>
<td>% smooth ER</td>
<td>8 ± 3</td>
<td>28 ± 5</td>
<td>97 ± 6</td>
<td>7 ± 3</td>
<td>36 ± 8</td>
<td>9 ± 12</td>
</tr>
<tr>
<td>% surface covered</td>
<td>48 ± 5</td>
<td>75 ± 11</td>
<td>97 ± 5</td>
<td>62 ± 5</td>
<td>79 ± 17</td>
<td>72 ± 27</td>
</tr>
</tbody>
</table>

EM images of medium-budded cells were analyzed using the software ImageJ. Published June 20, 2005.
The ER membrane appeared also continuous at the bud neck (Fig. 4 B) of observed to be continuous through the bud neck (Fig. 4 B) of confocal microscopy and GFP-HDEL as a marker, the ER lumen was continuity of the ER by light and electron microscopy. Using confo-

data indicate that the ER is continuous through the bud neck but that the ER membrane at this location is different from the rest of the ER membrane because it extensively lacks classical ER membrane proteins such as Sec61 and Hmg1.

Analysis of ER organization by high pressure freezing EM led to similar conclusions (Fig. 4 C). In these images, the ER lumen is apparent as a ribosome-free ribbon around the nucleus and at the cell cortex. ER tubules are also observed in the cytoplasm, some of which emerge from the cortical or the perinuclear ER. In rare instances, these ER tubules linked the luminal proteins within the ER. Thus, the ER must be physically continuous throughout the cell, including the bud neck.

**The ER is continuous through the bud neck**

To investigate this possibility, we characterized the morphology of the ER by light and electron microscopy. Using confocal microscopy and GFP-HDEL as a marker, the ER lumen was observed to be continuous through the bud neck (Fig. 4 B) of most cells. The ER membrane appeared also continuous at the bud neck when C<sub>e</sub>-BODIPY ceramide was used to visualize the ER bilayer (unpublished data). In contrast, both ER membrane markers Sec61-GFP (see Fig. 6 B) and Hmg1-GFP (not depicted) were seen only very rarely and in those cases only transiently at the bud neck (e.g., Fig. 1 B, Sec61-GFP at 28 s). The difference between the luminal and the ER membrane markers was obvious when GFP-HDEL was coexpressed with Hmg1-CFP, in which case an ER domain labeled with GFP and devoid of CFP was observed at the bud neck (Fig. 4 B). Thus, our data indicate that the ER is continuous through the bud neck but that the ER membrane at this location is different from the rest of the ER membrane because it extensively lacks classical ER membrane proteins such as Sec61 and Hmg1.

### Table II. List of mutants tested for compartmentalization of the Sec61-GFP marker or the ssGFP-HDEL

<table>
<thead>
<tr>
<th>Strain</th>
<th>Comp. Sec61</th>
<th>Comp. HDEL</th>
<th>Protein encoded by WT gene</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>cdc12-1</td>
<td>–</td>
<td>NT</td>
<td>Septin</td>
<td>Bud neck</td>
</tr>
<tr>
<td>cdc12-6</td>
<td>–</td>
<td>NT</td>
<td>Septin</td>
<td>Bud neck</td>
</tr>
<tr>
<td>shs1-Δ</td>
<td>–</td>
<td>NT</td>
<td>Septin</td>
<td>Bud neck</td>
</tr>
<tr>
<td>hsl1-Δ</td>
<td>+</td>
<td>NT</td>
<td>SDK</td>
<td>Bud neck</td>
</tr>
<tr>
<td>gln4-Δ</td>
<td>+</td>
<td>NT</td>
<td>SDK</td>
<td>Bud neck</td>
</tr>
<tr>
<td>swe1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Septin checkpoint</td>
<td>Bud neck and nucleus</td>
</tr>
<tr>
<td>cdc12-6 swe1-Δ</td>
<td>–</td>
<td>NT</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>hsl1-Δ gln4-Δ</td>
<td>+/–</td>
<td>NT</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>elm1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Kinase and septin organization</td>
<td>Bud neck</td>
</tr>
<tr>
<td>kcc4-Δ</td>
<td>+</td>
<td>NT</td>
<td>SDK</td>
<td>Bud neck</td>
</tr>
<tr>
<td>cib2-Δ</td>
<td>+</td>
<td>NT</td>
<td>G2/M cyclin</td>
<td>Nucleus, spindle pole bodies, spindle, and bud neck</td>
</tr>
<tr>
<td>sar1-D32G</td>
<td>NT</td>
<td>–</td>
<td>ER vesicle budding</td>
<td>ER</td>
</tr>
<tr>
<td>sec18-1</td>
<td>NT</td>
<td>–</td>
<td>Vesicle fusion</td>
<td>ER, Golgi, and vacuole</td>
</tr>
<tr>
<td>cdc48-6</td>
<td>+</td>
<td>–</td>
<td>Homotypic fusion</td>
<td>Cytoplasm and nucleus</td>
</tr>
<tr>
<td>rvs161-Δ</td>
<td>+</td>
<td>NT</td>
<td>Endocytosis</td>
<td>Bud neck</td>
</tr>
<tr>
<td>rvs167-Δ</td>
<td>+</td>
<td>NT</td>
<td>Actin distribution</td>
<td>Actin cytoskeleton</td>
</tr>
<tr>
<td>bnr1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Actin cable nucleation</td>
<td>Bud neck</td>
</tr>
<tr>
<td>bni1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Actin cable nucleation</td>
<td>Bud tip</td>
</tr>
<tr>
<td>spa2-Δ</td>
<td>+</td>
<td>NT</td>
<td>Cell polarity and cell fusion</td>
<td>Bud tip and bud neck</td>
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<tr>
<td>sph1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Polarized growth</td>
<td>Bud tip and bud neck</td>
</tr>
<tr>
<td>bud6-Δ</td>
<td>–</td>
<td>NT</td>
<td>Polarized growth, bud site selection, and septation</td>
<td>Bud tip and bud neck</td>
</tr>
<tr>
<td>pea2-Δ</td>
<td>–</td>
<td>NT</td>
<td>Polarized growth</td>
<td>Bud tip and bud neck</td>
</tr>
<tr>
<td>hof1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Cytokinesis</td>
<td>Bud neck</td>
</tr>
<tr>
<td>myo1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Cytokinesis</td>
<td>Bud neck</td>
</tr>
<tr>
<td>bni5-Δ</td>
<td>+</td>
<td>NT</td>
<td>Septin organization</td>
<td>Bud neck</td>
</tr>
<tr>
<td>bud3-Δ</td>
<td>+/-</td>
<td>NT</td>
<td>Axial budding</td>
<td>Bud neck</td>
</tr>
<tr>
<td>bud4-Δ</td>
<td>+</td>
<td>NT</td>
<td>Axial budding</td>
<td>Bud neck</td>
</tr>
<tr>
<td>axl2-Δ</td>
<td>+</td>
<td>NT</td>
<td>Axial budding</td>
<td>Bud, bud neck, and vacuole</td>
</tr>
<tr>
<td>axl1-Δ</td>
<td>+/-</td>
<td>NT</td>
<td>Axial budding and cell fusion</td>
<td>Bud neck and cell surface</td>
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<tr>
<td>apl3-Δ</td>
<td>+</td>
<td>NT</td>
<td>α-Adaptin</td>
<td>Bud neck and cell surface</td>
</tr>
<tr>
<td>yap1 B01-Δ</td>
<td>+</td>
<td>NT</td>
<td>Assembly of clathrin cages</td>
<td>Bud, bud neck, and cell surface</td>
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<td>yck2-Δ</td>
<td>+</td>
<td>NT</td>
<td>Casein kinase I</td>
<td>Bud tip and bud neck</td>
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<tr>
<td>bud14-Δ</td>
<td>+</td>
<td>NT</td>
<td>Bud site selection and vacuole maintenance</td>
<td>Bud site, tip, and neck</td>
</tr>
<tr>
<td>bmm1-Δ</td>
<td>+</td>
<td>NT</td>
<td>Cell polarization and bud formation</td>
<td>Bud site, tip, and neck</td>
</tr>
<tr>
<td>bmm2-Δ</td>
<td>+/-</td>
<td>NT</td>
<td>GAP for Rho1</td>
<td>Bud neck and cytoplasm</td>
</tr>
<tr>
<td>bmm3-Δ</td>
<td>+</td>
<td>NT</td>
<td>GAP for Cdc42</td>
<td>Bud site, tip, and neck</td>
</tr>
<tr>
<td>bmm4-Δ</td>
<td>+</td>
<td>NT</td>
<td>Interacts with rho GTPases</td>
<td>Cytoplasm and nucleus</td>
</tr>
<tr>
<td>rom2-Δ</td>
<td>+</td>
<td>NT</td>
<td>GEF for Rho1</td>
<td>Bud site, tip, and neck</td>
</tr>
</tbody>
</table>

At least six FLIP experiments were performed for each genotype (three movies on two independent segregants). For the mutation affecting the ER barrier, six additional movies were performed. NT, not tested.
Photobleaching was dependent smooth ER structure at the bud neck.

The ER is continuous through the bud neck and forms a septin-essential septin in yeast (Carroll et al., 1998; Mino et al., 1998).

Comparison of EM images obtained with the WT and the septin mutant suggested that septins play an important role in the organization of the ER membrane at the bud neck. Indeed, in the cdc12-6 cells shifted to the restrictive temperature, and in the cells lacking Shs1/Sep7, ribosomes nicely aligned along the ER lumen, which was no longer continuous. Thus, differentiation of the ER membrane at the bud neck depended on septin function.

Analysis of the diffusion data by in silico modeling

Analysis of ER structure by light and electron microscopy as well as our FLIP data with GFP-HDEL indicated that the yeast ER, like the ER of mammalian cells, is a single and continuous physical entity. However, our FLIP results also indicated that the diffusion of membrane proteins is slowed down in the bud neck. These data suggest the existence of a lateral diffusion barrier in the ER membrane at the bud neck. Alternatively, geometrical constraints might favor the diffusion of luminal over membrane proteins through the bud neck. Comparison of the exchange rate between mother and bud ER for luminal and membrane proteins is not trivial: diffusion in the volume of the ER lumen is not directly comparable to that in the surface of the ER membrane. To circumvent this problem, we developed an in silico modeling approach (see the online supplemental material and Figs. S2 and S3, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1). In this model, the exchange between the mother cortical and the perinuclear ERs was set as a standard for the codiffusion of luminal and membrane markers. This analysis indicated that even after standardization the exchange rate of the membrane protein at the bud neck was still reduced by a factor 16 relative to that of the soluble marker. Two models can account for this reduction: either

Figure 4. The ER is continuous through the bud neck and forms a septin-dependent smooth ER structure at the bud neck. (A) Photobleaching was applied to WT cells expressing Sec61-GFP or ssGFP-HDEL treated or not with 0.1% NaN₃ for 15 min. The left panel shows the ratios of the $t_{1/2}$ of the nonbleached over the $t_{1/2}$ of the bleached compartment for the indicated conditions. The right panel shows a representative kinetics of fluorescence loss over time for cells expressing ssGFP-HDEL treated or not indicated conditions. The right panel shows a representative kinetics of fluorescence loss over time for cells expressing ssGFP-HDEL treated or not with NaN₃. n = 8 for each condition. Error bars indicate the SD. (B) Spinning disk confocal images through the bud neck of a yeast cell expressing ssGFP-HDEL. Blow up of the neck of a cell expressing ssGFP-HDEL (green in the overlay) and Hmg1-CFP (red in the overlay). Arrows point at GFP-HDEL localization to the bud neck. Bars, 1 μm. (C) Transmission electron microscopy images of the bud neck of a WT yeast cell. Magnifications are shown for the indicated regions. (D) Transmission electron microscopy images of the bud neck of a shs1Δ mutant cell. Magnification is shown for the bud neck. Explanatory schemes are shown below each close up.

perinuclear and cortical ERs. In all images, a ribosome-free ribbon continuous with the mother and bud cortical ERs was observed along the plasma membrane of the bud neck. This ribbon had the same thickness as the ER lumen at other cortical locations and around the nucleus. It was still observed in shs1Δ (Fig. 4 D) and cdc12-6 cells shifted to the restrictive temperature for an hour. Within 2 min of the shift, the cdc12-6 septin allele causes the complete disassembly of the septin ring at the bud neck (Dobbelaere et al., 2003; Dobbelaere and Barral, 2004), which causes the dispersion of all known bud neck proteins (Gladfelter et al., 2001). Shs1/Sep7 is a nonessential septin in yeast (Carroll et al., 1998; Mino et al., 1998). Because the same ribbon is observed independent of septin presence, this ribbon must correspond to the ER lumen and not to the exclusion of ribosomes by septin-specific struct-
the morphology of the connections offers a larger volume/surface ratio at the bud neck and thereby favors the exchange of the soluble protein over that of the membrane bound, or the diffusion constant is specifically reduced in the membrane. The first model predicts that the connections between mother and bud cortical ERs should be 16 times thicker than those between cortical and perinuclear ER, which is in contradiction to our EM data. Thus, the second model is most likely correct. Our data indicate the existence of a diffusion barrier slowing down the diffusion of proteins in the ER membrane at the bud neck. This conclusion fits with the existence of a differentiated ER membrane at the same location. Thus, we concluded that a diffusion barrier compartmentalized the ER membrane into mother and bud diffusion domains.

**Septins, septin-dependent kinases (SDKs), and Bud6 are required for the diffusion barrier**

Next, we investigated the molecular basis of this diffusion barrier. Most events at the bud neck depend on septins. Furthermore, our EM data indicated that the smooth ER at the bud neck was disrupted in septin mutants. Thus, we investigated whether the septin ring was involved in the separation of the ER membrane into distinct diffusion domains. *SEC61-GFP cdc12-6* cells shifted to the restrictive temperature (35°C) for at least 15 min were subjected to FLIP studies. In these cells, fluorescence loss in the unbleached compartments (the bud) was only slightly delayed compared with that in the bleached compartment (Fig. 5, A and C; and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1; Table II). The kinetics of fluorescence loss in the unbleached compartments (the bud) showed a half-time of 19.2 s in WT, 20.6 s in *cdc12-6*, and 21.8 s in *bud6Δ* cells. Each point represents one individual experiment. (D) The ratio of fluorescence decay (t_{1/2}) in the nonbleached (bud) versus bleached (mother cortex) compartment (WT vs. *cdc12-6*). Similar results were obtained in cells lacking Shs1 (Fig. 5 D). In all the FLIP movies, the frequency of cytoplasmic ER tubules connecting mother and buds was not increased in septin mutants compared with WT (e.g., ER tubule growing through the bud neck appeared in 10.4% of the frames of movies of *shs1Δ* cells, n = 77 frames, vs. 13.4% in movies of WT cells, n = 88 frames), demonstrating that enhanced exchange through the neck of *shs1Δ* cells was not due to an increased number of cytoplasmic connections. Together, these results indicated that ER compartmentalization depended on septin function. The lack of compartmentalization was not due to the cell cycle delay caused by septin defects because compartmentalization was not restored in *cdc12-6 swe1Δ* cells. The *swe1Δ* mutation releases the cell cycle block caused by septin defects (Barral et al., 1999; Shulewitz et al., 1999; Longtine et al., 2000).

During budding, the septin ring recruits to the bud neck many proteins involved in actomyosin ring assembly, chitin deposition, cell cycle progression, and spindle positioning (De-Marini et al., 1997; Bi et al., 1998; Lippincott and Li, 1998; Barral et al., 1999; Shulewitz et al., 1999; Segal et al., 2000; Kusch et al., 2002; Longtine and Bi, 2003). Using our FLIP assay, we tested whether any of 30 bud neck proteins representative of these different functions would play any role in ER compartmentalization (Table II). Partial defects were observed in cells lacking both SDKs, Hsl1 and Gin4, simultaneously (Fig. 5 D and Table II). Again, these defects were not due to over-activation of the Swe1 kinase (Table II). SDKs are involved in signaling downstream of septins. In addition, Gin4 is required for proper septin ring assembly, whereas Hsl1 is not.
translocon and the formation of a smooth ER domain, and on one hand the exclusion of the septins for the compartmentalization of the ER membrane. Reduction of compartmentalization was similar to that caused by septin mutations (Table II; Fig. 5, B–D; and Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1). Bud6 (Amberg et al., 1997), which is a peripheral membrane protein, has been implicated in actin polarization, microtubule interaction with the cell cortex (Segal et al., 2000), and bud site selection in diploid cells. Together with Pea2, it is a component of the polarizome (Sheu et al., 2000), a complex that also contains either of the formins Bni1 and Bar1, and the polarity factors Spa2 and Sph1. Analysis of ER compartmentalization in 

Bud6 acts downstream of the septins

Several lines of evidence suggest that Bud6 acts downstream of septins in ER compartmentalization. First, analysis of septin organization using Cdc12-GFP, Cdc3-GFP, or Shs1-GFP as reporters indicated that the bud6Δ mutation did not noticeably affect septin organization (unpublished data). Furthermore, investigation of the localization of Ist2-GFP and Spa2-GFP, which both require a functional septin ring to maintain their asymmetric localization to the cortex of large budded cells (Barral et al., 2000; Takizawa et al., 2000), indicated that the septin-dependent diffusion barrier at the plasma membrane was not affected in bud6Δ cells (Fig. 6 A and not depicted). Thus, bud6Δ does not generally interfere with septin function. Second, investigation of Bud6 localization in the shs1Δ strain indicated that Bud6 failed to accumulate (Fig. 6 C, top) or accumulated to a lesser extent (Fig. 6 C, bottom) at the bud neck of small and medium budded cells, consistent with Huisman et al. (2004). These data suggest that Bud6 acts downstream of septins for the compartmentalization of the ER membrane. Remarkably, Sec61-GFP was still excluded from the bud neck of all bud6Δ cells (Fig. 6 B), in contrast to what we observed in the shs1Δ and cdc12-6 cells (Fig. 6 B and compare Videos 8 and 9, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1). Altogether, these data suggest that septins have two independent effects on ER organization at the bud neck cortex. They govern on one hand the exclusion of the translocon and the formation of a smooth ER domain, and on the other hand the assembly of a Bud6-dependent barrier that limits the diffusion of ER membrane proteins.

Discussion

In this study, we used photobleaching techniques to investigate ER organization in polarized yeast cells. The diffusion of all tested markers was very fast within the different parts of the ER; i.e., within the ER at the mother cortex, at the bud cortex, and in the nuclear envelope. Remarkably, the yeast ER membrane was separated into distinct domains that reflected cell polarization. This compartmentalization is evidenced by the observation that ER membrane proteins such as the translocon subunit Sec61, the 3-hydroxy-3-methylglutaryl-coenzyme A reductase Hmg1, and the SNARE Sec22 all diffuse very rapidly throughout the bud and mother cortical ERs, but exchange only slowly between these two domains.

Two models can account for such a compartmentalization: either the yeast ER is physically discontinuous, or a diffusion barrier is present within the continuous membrane of the ER at the bud neck. In the first case, residual exchange of ER membrane proteins between the mother and bud must depend on either vesicular transport or transient fusion events. Furthermore, luminal proteins should also be compartmentalized and not to an increased number of ER tubules.

In addition to SDKs, only Bud6 and Pea2 were involved in ER compartmentalization. In bud6Δ and pea2Δ cells the loss of compartmentalization was similar to that caused by septin mutations (Table II; Fig. 5, B–D; and Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200412143/DC1). Bud6 (Amberg et al., 1997), which is a peripheral membrane protein, has been implicated in actin polarization, microtubule interaction with the cell cortex (Segal et al., 2000), and bud site selection in diploid cells. Together with Pea2, it is a component of the polarizome (Sheu et al., 2000), a complex that also contains either of the formins Bni1 and Bar1, and the polarity factors Spa2 and Sph1. Analysis of ER compartmentalization in 

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ized. Our data invalidate both predictions. Indeed, diffusion kinetics of the luminal marker GFP-HDEL and morphological analyses at both the light and electron microscopy levels indicate that the ER lumen is continuous throughout the bud neck. Furthermore, exchange of Sec61 and GFP-HDEL between the mother and the bud was due to passive diffusion because it depended neither on the major players of ER membrane trafficking nor on metabolic energy. Thus, the ER is a physically continuous organelle in yeast like in mammalian cells, and its membrane, but not its lumen, is compartmentalized by a diffusion barrier. Our observation that the membrane of the nuclear envelope is also compartmentalized in late anaphase supports this conclusion, as it indicates that ER compartmentalization at the bud neck does not necessarily require the discontinuity of the ER membrane.

The barrier model was supported by the analysis of our quantitative data by in silico modeling. Indeed, this approach demonstrated that compartmentalization of the membrane markers could not be explained by morphological parameters. The barrier model was also supported by our morphological studies. Indeed, we observe that a specialized ER membrane forms at the bud neck, which is both morphologically (it is a sheet instead of tubules) and structurally different from the rest of the cortical ER. Thus, we conclude that a diffusion barrier compartmentalizes the ER membrane along the same lines as those compartmentalizing the plasma membrane of budded yeast cells. To our knowledge, this is the first report of such an intricate relationship between the organization of the ER and that of the plasma membrane.

What could be the cellular function of this complex ER organization? We see two possibilities: (1) the specialized ER domain at the bud neck might have functions related to cell cleavage and/or (2) the separation of the bud and mother cortical ER membranes might provide support for cell polarization.

At the site of division, a specialized ER domain could play two distinct roles. First, this ER domain might serve as a calcium-storing structure involved in the control of actomyosin contraction. This ER domain could work like the sarcoplasmic reticulum of muscle cells in the control of muscle contraction. In support of this idea, septin defects, which cause the loss of the ring of smooth ER, slow down actomyosin ring contraction during cytokinesis (Dobbelare and Barral, 2004). However, the isolation of mutants that specifically abrogate the formation of the ring of smooth ER at the bud neck will be required to investigate this possibility more rigorously. Second, the ring of smooth ER might be required for ER cleavage at cytokinesis. Further studies will be required to investigate the timing, control, and mechanism of ER cleavage before the completion of cytokinesis.

The compartmentalization of the ER membrane might also play some roles in cell polarity. For example, it could help restrict the synthesis of specialized membrane proteins to one cellular compartment. Most mRNAs that specifically localize to the yeast bud encode membrane proteins and must be translated at the ER surface (Shepard et al., 2003). Thus, ER compartmentalization might help maintain the localized mRNAs in the bud. This mRNA–ER connection seems strengthened by the observation that mRNA and ER transport to the bud neck both involve the type V myosin Myo4 and its associated factor She3 (Estrada et al., 2003). Remarkably, She3 localizes to the bud ER specifically, suggesting that ER compartmentalization might indeed support some structural and functional asymmetry of the ER. However, due to the leakiness of the barrier indicated by our data, it is unclear which kind of asymmetry it helps maintain. Indeed, even though membrane proteins diffused much slower through the bud neck than through the bud or mother cortex, they did eventually translocate from one domain to the other. Thus, the ER diffusion barrier alone cannot maintain very long the asymmetric distribution of polarized factors. Consistently, it has been shown for several products of asymmetrically localized mRNAs that they do not maintain an asymmetric distribution (Shepard et al., 2003), particularly those that are residents of the ER membrane. However, some other products of polarized mRNAs do maintain an asymmetric distribution, the prototype of which is the plasma membrane protein Ist2. In the case of these products, it might be crucial that the ER diffusion barrier slows down their dispersion before they exit the ER, en route to the plasma membrane. Indeed, at least in the case of Ist2, the final localization of the product is determined by the localization of the mRNA (Takizawa et al., 2000). This would not be possible if the product would freely diffuse through the entire ER. Thus, one function of the diffusion barrier in the ER membrane at the bud neck might be to slow down the diffusion to the mother cortex of both polarized mRNAs and their product. Thereby, it permits dynamic processes, such as the movement of Ist2 to the plasma membrane, to remain asymmetric, whereas slower events become isotropic. These observations are reminiscent of the situation recently described in Drosophila melanogaster embryos, where the localization of the Gurken mRNA determines where at the surface of the embryo this growth factor–like signaling molecule is being secreted (Herpers and Rabouille, 2004). It will be interesting to investigate whether diffusion barriers compartmentalize the ER membrane of the D. melanogaster embryo to ensure the accuracy of Gurken targeting, and perhaps to also ensure that each of the distinct nuclei acquire and maintain a distinct identity despite being all in the same cytoplasm. Indeed, the division of the ER might support the organization of the entire cytoplasm into distinct domains, such as during the polarization of yeast cells. Thus, we suggest that the ER might play more profound roles in cellular organization than previously anticipated.

The observation that a diffusion barrier compartmentalizes the ER membrane opens two mechanistic questions: (1) what is the molecular nature of this barrier, and (2) how is it localized to the bud neck? Remarkably, we found only few of the known bud neck proteins to be involved in ER compartmentalization. Thus, the identified candidates might act in a very specific manner.

On the septin side, both disruption of the entire ring and elimination of the nonessential septin Shs1/Sep7 dramatically affected both the ER diffusion barrier and the accumulation of smooth ER in the bud neck. Because the shs1Δ mutation does not dramatically affect cell viability, septin ring assembly, and morphology (Carroll et al., 1998; Mino et al., 1998), but
strongly impacts on ER organization, Shs1 may be specialized for this process. In turn, Bud6 affected only the diffusion barrier and not ER morphology. Thus, the smooth ER at the bud neck does not depend on the presence of the barrier and is not sufficient to form a barrier. Previous data have established that Bud6 is associated with internal membranes (Jin and Ambger, 2000). We propose that Bud6 is a smooth ER protein (Fig. 7), where it might influence protein diffusion.

The role of septins in the recruitment or regulation of ER proteins such as Bud6 might be indirect and mediated at least in part through signaling. Indeed, we found that the SDKs Gin4 and Hsl1 are also involved in the formation of the diffusion barrier. Because SDKs did not seem to function in smooth ER organization, their substrates must be specifically involved in ER compartmentalization. It will be helpful to identify these substrates and to investigate whether Bud6 is one of them. The view that septins act on the ER via signaling mechanisms might explain how they could potentially act at a distance during, for example, the compartmentalization of the nuclear envelope at the bud neck during anaphase.

It is unclear to which extent the observations that we describe here are specific to ER of fungi or relevant for other eukaryotes. At first sight, the fact that the ER of animal cells is mainly cytoplasmic, and not cortical as in fungi, suggests that ER compartmentalization is less likely in metazoans. It will be interesting to perform FLIP experiments on polarized and dividing cells and to investigate whether the role that we ascribe to the cleavage apparatus in the compartmentalization of the yeast ER is conserved during the division of animal cells.

Materials and methods

Strain construction

Yeast strains were constructed by standard genetic techniques. Diploids were isolated on selective medium and subsequently sporulated at 23°C. The background is, unless specified otherwise, S288C. ssGFP-HDEL was expressed from the 2-mu vector pG14 (Lesser and Guthrie, 1993); ssGFP-HDEL has the signal peptide of HDEL has the signal peptide of HDEL and is conserved during the division of animal cells. The different deletions shown in Table I were obtained from the EUROSCARF deletion collection (S288C) and provided to us by M. Peter (ETH, Zurich, Switzerland). Each time that an effect was observed, the mutation was backcrossed several times into our background. The hsl1A, gin4A, shs1A, and swe1A strains are isogenic with S288C. Cdc12-GFP was expressed from a centromeric plasmid (Dobbelnaere et al., 2003). The sec18-1 and cdc48-6 strains were gifts of R. Collins (Cornell University, Ithaca, NY) and S. Jentsch (Max Planck Institute, Munich, Germany), respectively.

FLIP and FRAP experiments

Cells were grown on YPD plates, resuspended in liquid nonfluorescent medium, and immobilized on nonfluorescent medium (Waddele et al., 1996) containing 1.6% agarose. Photobleaching was applied on the area shown on the figures, using a microscope (model LSM510; Carl Zeiss Microimaging, Inc.) and a Plan- Apochromat 100× objective (NA 1.4). For FRAP, scans were collected at 5-s intervals for a minimum of 120 s using the acquisition software LSM510 (Carl Zeiss Microimaging, Inc.). Bleaching regions were irradiated with 250 iterations of 50% laser intensity at 30% output of an argon laser (488 nm) and scans were collected with typically 1% laser intensity at the same conditions. All pictures of FLIP experiments shown in the figures were treated to account for the bleaching due to image acquisition, whereas the movies were left untreated.

Quantification of FLIP experiments

Analysis of the FLIP experiments was performed using the ImageJ 1.29 software (http://rsb.info.nih.gov/ij). The loss of fluorescence over time was measured in different regions of interest (usually the mother cortex, bud cortex, and perinuclear ER). In addition, we measured the intensity of the background. The intensity was measured in different regions of interest (usually the mother cortex, bud cortex, and perinuclear ER). In addition, we measured the intensity of the background.
Online supplemental material
The quantitative transport model is fully described and assessed in the online supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/121/4/DC1.

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References


computational modeling

Computational modeling was performed with the following goals. First, experimental data indicate that exchange of ER membrane proteins between the mother cell and the bud is very slow compared with protein mobility within these two compartments. Thus, we asked whether this difference is solely due to geometrical constraints at the bud neck or to more specific diffusional limitations at the bud neck. For this study, the exchange between the mother cortical ER and the bud ER was compared with the exchanges observed between the mother cortex and the perinuclear ER. Morphologically (as judged by electron and fluorescence microscopy), none of the connections between mother and bud cortices and between the mother cortex and the nucleus appear to be more prominent. Furthermore, we observed that exchange through the bud neck was much more rapid for the luminal protein GFP-HDEL than for Sec61-GFP and Sec22-GFP. Therefore, we asked whether the differences observed between the ER membrane and ER luminal proteins are due to the fact that they move in two- and three-dimensional spaces, respectively, and have different molecular diffusion constants. Finally, our model helped to investigate whether exchanges between mother and bud could be dependent on vector transport.

Model description and parameter identification

Consistent with our sodium azide experiments (Fig. 4), preliminary calculations using simple (first order) models showed that all experimental data could be explained assuming passive diffusion as the mechanism of transport (not depicted). The differences in the decay rates of the nonbleached compartments between membrane and luminal markers (Figs. 1 and 3) could be due to the different molecular diffusion constants of the two protein species Sec61-GFP and GFP-HDEL. The molecular diffusion constant of GFP-HDEL is 20–40 times larger than that of Sec61-GFP (GFP-HDEL: 10–30 μm²/s [Dayel et al., 1999]; Sec61 typical value for membrane proteins: 0.5 μm²/s [Lippincott-Schwartz et al., 2001]). To investigate this, a time-resolved diffusion transport model of the system was developed in order to derive numerical quantities that allow comparison between different FLIP experiments, taking into account influences of dimensionality (two vs. three-dimensional spaces) or differences in the diffusion constants between species. The model is formulated based on physical principles (conservation of mass and momentum). It contains parameters of unknown value that need to be identified from data by means of modeling.

On the time scale of a FLIP experiment (a couple of minutes), the only relevant dynamic processes are mass exchange between the mother cortex and the bud, mass exchange between the perinuclear and the cortical ER of the mother cell, and fluorescence drain due to photobleaching. These processes were fully resolved in the model. Faster dynamics (e.g., individual bleach pulses, switching of the laser, and camera dynamics) were modeled using algebraic (static) equations and slow dynamics (e.g., changes in shape or size of cells and changes in temperature) were neglected. Spatial distributions of protein within the same ER compartment were not resolved because they level out on a much faster time scale than the FLIP cycles (Fig. S1 and Fig. 1 C). Compensation for spontaneous bleaching during imaging was not done in the model itself (in this would unnecessarily add another unknown model parameter), but by normalization of the fluorescence pixel intensities I at each FLIP image (I = I(0)/I(t)); I(0) is the average intensity level in the image background and I(t) is the average intensity at the same time in the same compartment of a control cell to which no FLIP bleaching was applied.

The system was abstracted as shown in Fig. 5.2 A. The three distinct ER compartments correspond to the perinuclear ER (yellow), the cortical ER of the mother cell (blue), and the cortical ER of the bud (pink). Each compartment contains a certain amount (mass) of fluorescent protein as a function of time [I(t)], and both contain a certain volume [V0, Vc, and Vb]. The compartments are assumed to be connected by passive diffusion. This assumption is supported by our sodium azide experiment (Fig. 4). Each connection is characterized by its effective cross section area (parameter ρ)

Figure S1. FRAP analysis of Sec61-GFP through the bud neck. [A] Bleaching the mother cortex, as depicted in the cartoon as in Fig. 2. Pictures of relevant time points. Graph shows kinetics of fluorescence recovery. t1/2 of bleached compartment = 163 ± 93, n = 5. [B] Bleaching the bud cortex. Same color code as in Fig. 2. t1/2 of bleached compartment = 118 ± 27, n = 3. [C] Bleaching along a longitudinal axis. t1/2 of the bleached compartment (light green) = 19 ± 15, n = 3. Bar, 1 μm.
for the membrane protein A, its characteristic length L, and an effective molecular diffusion constant D. Because none of these transport parameters is known, they were combined into a single variable \( k = DA/L^2 \), which has the physical units of volume/time and can be thought of as an effective "rate of transport."

The experimental bleaching and image acquisition cycle was modeled as depicted in Fig. S2 B. Each FLIP experiment consists of many repetitions of the cycle "scan–bleach–scan–reset," characterized by the respective time durations \( t_s \), \( t_b \), and \( t_r \). The mathematical formulation of this "cycle function" is:

\[
 h(t) = \begin{cases} 
 1 & \text{if mother cortex is bleached and} \\
 0 & \text{if bud is bleached.}
\end{cases}
\]

Mathematically, they form a third order ordinary algebraic-differential equation model that was solved numerically using Matlab/Simulink (The Mathworks, Inc.) version 6.1.0.450 (R12.1) and a globally fourth order accurate Dormand-Prince (ode45) integration scheme with variable time step size adjusted to guarantee a relative tolerance (i.e., maximum error of the model solution) of \( 10^{-5} \).

Quantitative values for the three unknown parameters \( k_1 \), \( k_2 \), and \( v \) (either \( k_2 \) or \( v \), depending on which compartment was bleached) were obtained by fitting the solution of the above system of equations to experimental FLIP data, minimizing the total quadratic deviation between the model solution and the measurements of both pre- and postbleach points. This was done using Matlab (The Mathworks, Inc.) and was a two-dimensional Nelder-Mead simplex minimization method. Minimization was done subject to the constraints \( k_1 > 0, k_2 > 0, \) and \( v > 0 \).

The initial value for \( k_1 \) was set to 0.05, and the one for \( k_2 \) in 0.5. Varying these values we found that for any initial \( k_1 \) in the range of 0.005 to 1.0 and \( k_2 \) between 0.1 and 10.0, the same fit \( k_2 \) (the same model parameter value) resulted. Even changing to an initial \( k_1 \) of 1.0 while \( k_2 \) was 0.1 did not change the outcome. If initial values outside above mentioned intervals were chosen, the model failed to fit the experimental data. This suggests that our model fit corresponds to the true (global) optimum of the fitting quality. The starting value for \( v \) (i.e., the effective bleached volume) was estimated from the first three data points of each experiment (fluorescence loss during the first bleaching sequence) in order to increase the robustness of the minimization. The two volumes \( V_m \) and \( V_b \) were determined from the initial condition (steady state in both the model and the real system before the first bleaching pulse), and \( V_b \) was normalized to unity because we are only interested in relative comparisons and the FLIP data themselves were normalized before analysis too. The independent FLIP experiments were analyzed using this model. A sample outcome is shown in Fig. S3. The summarized results are in Table S1.
Figure S3. Example of model fit to experimental FRAP data. The solid lines correspond to the model prediction of the total fluorescence in the cortical ER of the mother cell, the dotted lines to the model prediction for the cortical ER of the bud, and the dashed lines to the perinuclear ER of the mother cell. Circles mark experimental postbleach scans and crosses mark the experimental prebleach scans. All data are processed and normalized as described in the main text.

Relative strength of the two connections: ER compartmentalization

No significant directionality could be seen. Diffusion from the mother to the bud seems equally fast as diffusion from the bud to the mother. Therefore, transport rates were considered independent of the direction and all values were averaged over the two bleach cases (mother bleached/bud bleached) using \( n = 8 \) experiments for each protein. For Sec61-GFP, we found \( \lambda_{1,\text{GFP-HDEL}} = 0.00345 \pm 0.000162 \) and \( \lambda_{2,\text{GFP-HDEL}} = 14.623 \pm 6.487 \) and for GFP-HDEL, \( \lambda_{1,\text{GFP-HDEL}} = 0.0551 \pm 0.0240 \) and \( \lambda_{2,\text{GFP-HDEL}} = 13.644 \pm 4.718 \), where the values are given as mean \( \pm \) SD. We can state that for Sec61-GFP the connection between the cortical ER of the mother cell and its perinuclear ER is \( \approx 4,200 \) times stronger than the one between the control ER of the mother cell and the cortical ER of the bud. For GFP-HDEL, the ratio is \( \approx 270 \). Note that these ratios have nothing to do with the ratios of recovery half-times because the latter do not account for the different volumes nor the fact that one protein diffuses in the luminal space whereas the other one is restricted to the membrane. Moreover, \( \lambda \) by definition not only contains the cross section but also the diffusion constant. Both a decreased ER cross section as well as an increased viscosity could thus be the reason for the observed transport barrier in the bud neck.

This compartmentalization effect would have consequence that the mother cortex and the perinuclear ER would behave very much like a single compartment separated from the bud. To validate this prediction, we developed a second model with only two independent compartments, representing the mother cell (both perinuclear and cortical ER combined into the same compartment) and the bud. If it is true that the connection within the mother cell is orders of magnitude stronger than the one between mother and bud, the latter will dominate the dynamics of the whole system (transport rate-limiting connection and a two-compartment model should approximate the experimental data about equally well as the three-compartment model. The model was observed, implemented, and applied following the same steps and using the same methods as the above-described three-compartment model. Being a two-compartment model, the number of parameters is reduced by one to a single \( \lambda \) (only one connection is modeled) and \( \lambda \). We found that the mean model fit error per data point for the two-compartment model was 5.54 \( \pm \) 1.73% and the maximum error we found in a single case was 9.0%. The two-compartment model had a mean error of 4.72 \( \pm \) 1.36% and a maximum of 7.7%. The two-compartment model thus describes the data almost equally well and the two ER parts in the mother cell indeed seem to behave like a single compartment, as predicted by the \( \lambda \) values of the full three-compartment model.

Note that the two-compartment model is only a control. The three-compartment model is closer to the biological reality and is needed to gain an idea of the relative strengths of the two connections (the two-compartment model only models one connection).

Together, these results provide evidence that the two ER compartments in the mother cell behave much like a single compartment, whereas the bud's ER is only weakly connected to it. This weakening of the connection as: ER compartmentalization because the latter do neither account for the different volumes nor the fact that one protein diffuses in the luminal space whereas the other one is restricted to the membrane. Moreover, \( \lambda \) by definition not only contains the cross section but also the diffusion constant. Both a decreased ER cross section as well as an increased viscosity could thus be the reason for the observed transport barrier in the bud neck.

Table S1. Summary of results of model fit

<table>
<thead>
<tr>
<th>Protein</th>
<th>( \lambda_1 ) mean ( \pm ) SD</th>
<th>( \lambda_2 ) mean ( \pm ) SD</th>
<th>Mean fitting error (RMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sec61-GFP</td>
<td>0.00225 ( \pm ) 0.000170</td>
<td>14.53 ( \pm ) 6.74</td>
<td>4.451%</td>
</tr>
<tr>
<td>mother bleached</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bud bleached</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP-HDEL</td>
<td>0.02435 ( \pm ) 0.00090</td>
<td>14.71 ( \pm ) 7.27</td>
<td>5.838%</td>
</tr>
<tr>
<td>mother bleached</td>
<td>0.06668 ( \pm ) 0.01903</td>
<td>14.07 ( \pm ) 3.89</td>
<td>3.652%</td>
</tr>
<tr>
<td>bud bleached</td>
<td>0.03042 ( \pm ) 0.00790</td>
<td>13.12 ( \pm ) 6.58</td>
<td>4.950%</td>
</tr>
</tbody>
</table>

The model fitting quality is quantified using the mean RMS error, which is the square root of the mean of all squared deviations between measured points and the model predictions at those points (i.e., the mean modeling error per data point). We consider the model predictions of the full three-compartment model and the model predictions of the three-compartment model with the mother cell's cortical ER was equal to 1, the RMS error can also be interpreted as a relative error in percent. Given our modelling errors of 4 to 6%, we consider the model fits to be good.

From our model, we have numerical values for four different transport rates: soluble GFP-HDEL across the bud neck (\( \lambda_{1,\text{GFP-HDEL}} \)) and within the mother cell (\( \lambda_{2,\text{GFP-HDEL}} \)) as well as membrane-associated Sec61-GFP across the bud neck (\( \lambda_{1,\text{Sec61-GFP}} \)) and within the mother cell (\( \lambda_{2,\text{Sec61-GFP}} \)). We can now determine the ratio of the transport rates of the two species for each connection as: \( \lambda_{1,\text{GFP-HDEL}}/\lambda_{1,\text{Sec61-GFP}} = 14.81 \) and \( \lambda_{2,\text{GFP-HDEL}}/\lambda_{2,\text{Sec61-GFP}} = 0.934 \). Using the connection within the mother cell as a normalization standard, we find: \( \lambda_{1,\text{GFP-HDEL}}/\lambda_{1,\text{Sec61-GFP}}/\lambda_{2,\text{GFP-HDEL}}/\lambda_{2,\text{Sec61-GFP}} = 15.9 \). This finding means that the soluble GFP-HDEL is exchanged \( \sim 16 \) times faster than the membrane-associated Sec61-GFP in the bud neck than it is in the mother cortex.
within the mother cell. In the model, the transport rate is defined as $\frac{\Delta Q}{\Delta t}$. The effective diffusion coefficient of Sec1-GFP in the intra-mother connection is derived by $D$. The one of GFP-HDEL is $\times$ times larger, hence $a$. Similarly, the ratio of diffusion coefficients across the bud neck is termed $a$. For the standardized ratio, we thus find: $k_{GFP-HDEL}/k_{Sec1-GFP} = (a/1)/a = 1$, where $a$ is the diameter of the respective connection. The relative speed of diffusion of the two species across the bud neck is the same as in the mother cell, the connections in the bud neck would need to be 1.6 times thicker than the connections in the mother cell. From our EM data we know that this is not the case. The only explanation that is compatible with our observations is that the relative speed of diffusion is different in the two connections, meaning that the exchange of Sec1-GFP is slowed down 16-fold across the bud neck.

Altogether our calculations indicate that in no model Sec1-GFP and GFP-HDEL would be very likely to move together through the bud neck. In case of vascular-driven movement, one would have to postulate that incorporation of GFP-HDEL is favored over the packaging of Sec1-GFP. The molecular function of such a specific intraER traffic would be quite obscure. Alternatively, a diffusion barrier in the ER membrane would explain why GFP-HDEL moves more rapidly through the bud neck than Sec1-GFP. This model is in very good agreement with our morphological data that suggest the presence of a cylinder of smooth ER linking the mother and bud ER.

**Shapes of mother-bud connection: exclusion of vesicular transport**

Using the definition of $a$, and some basic geometric considerations, allow rough statements about the geometric shape of the mother-bud ER connection and about whether vesicular transport could also explain the measurements. We consider three cases: (1) mother and bud ER are connected with a set of tubules, (2) they are connected by a cylindrical ring of smooth ER, or (3) they are not directly connected, but vesicular transport takes place between them. For tubular connections with $N$ tubules of mean radius $r$, the total cross section of the connection is given by $N \times \pi \times r^2$. This is the area through which GFP-HDEL diffuses. The total membrane crosssection of the connection is $2 \times N \times \pi \times r$. This is the "area" through which Sec1-GFP moves. Furthermore, we let the diffusion constant of GFP-HDEL be $\times$ times larger than the one of Sec1-GFP. The ratio of transport rates of the two species then becomes: $k_{GFP-HDEL}/k_{Sec1-GFP} = (a/2)^2 / (a/1)$. Similarly, the ratio of diffusion coefficients across the bud neck is termed $a$. For the standardized ratio, we thus find: $k_{GFP-HDEL}/k_{Sec1-GFP} = (a/2)^2 / (a/1)$, where $a$ is the diameter of the connection.

Second, for a set of cylindrical connection (as suggested by the EM pictures in Fig. 4) with mean outer radius $R$ and mean inner radius $r$, we have a total cross section of $(\pi R^2) - (\pi r^2)$ and a total surface line of $2 \times \pi (R + r)$. The ratio of transport rates hence becomes $k_{GFP-HDEL}/k_{Sec1-GFP} = (a/2)^2 / (a/1)$, where $a$ is the diameter of the connection.

Third, for vesicular transport, we recall that $a$ is "volume per time". If we assume a mean $N$ spherical vesicles to be involved in the transport at any time and a mean vesicle travel time of $t$ seconds, the volume (GFP-HDEL) transport rate is $N \times (4/3) \pi R^3 / t$, and the surface area (Sec1-GFP) transport rate is $N \times (4 \pi R^2 / 3) / t$. The ratio of transport rates thus becomes $k_{GFP-HDEL}/k_{Sec1-GFP} = (r/2)^3 / (r/1)$, provided that luminal and membrane proteins are equally well packaged into vesicles.

From this model to experimental VIP data, we know that $k_{GFP-HDEL}/k_{Sec1-GFP}$ is roughly 15. Assuming unhindered diffusion within the mother cell, the ratio of diffusion constants is about $a^2 \approx 40$ (GFP-HDEL):10–30 pm/s (Dyped et al., 1999). Sec1-GFP typical value for membrane proteins: 0.5 pm/s (Lippincott-Schwartz et al., 2001). Using the result $a = 1/10a$, from the previous section gives a relative speed of diffusion of $a = 640$ across the bud neck. This enables us to estimate the order of magnitude of some geometric properties. For the first case, we get a mean tubule radius of ~0.05 µm and for the second case, a cylinder thickness of ~0.05 µm. For the third case, we find that the mean vesicle radius for vesicular transport would have to be ~0.45 µm. Given the simplifications in our model (we have neglected the precise geometry and the fact that $D$ is only the effective diffusion constant and not the molecular one) and the uncertainties in the literature values of the diffusion constants, these numbers are to be considered rough estimates and could well be a factor of 10 wrong. Still, the 50-nm cylinder thickness in the second case is in nice agreement with our electron micrographs. In light of these numbers, vesicular transport is entirely unrealistic. These calculations are consistent with our experimental results and vesicular transport through the bud neck can be ruled out.

**Model validation, accuracy, and limitations**

Our model is limited by the assumptions made in the process of deriving it. It is important to stress that the model is derived from our experiments and our data. As far as model accuracy is concerned, we notice that the mean RMS model fitting error is $\Delta = 10^{-6}$, the mean error per data point is $d = 0.1$, and the maximum error we found was 0.8%, corresponding to a maximal modeling uncertainty of ~10%. This is of the same order of magnitude as the experimental uncertainty and thus no significant reservation can be made. A more limiting aspect is that $a$ is hardly identifiable from the data if the bud is not bleached. This is due to the fact that the bud and the perinuclear ER are only indirectly connected and changes in the transport rate "perinuclear ER $\rightarrow$ cortical ER" have only minor influences on the fluorescence evolution in the bud. We empirically observed that changing $a$ by a factor of two only changes the other parameters (including the fitting error) at the third position after the decimal point. Any difference of less than a factor of 10 in $a$ must therefore be considered insignificant, given the numerical tolerance of $10^{-6}$ used in solving the model. This inaccuracy is in any case highly sensitive there.

**References**
