Sec3p is needed for the spatial regulation of secretion and for the inheritance of the cortical endoplasmic reticulum.

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Abstract

Sec3p is a component of the exocyst complex that tethers secretory vesicles to the plasma membrane at exocytic sites in preparation for fusion. Unlike all other exocyst structural genes, SEC3 is not essential for growth. Cells lacking Sec3p grow and secrete surprisingly well at 25°C, however late markers of secretion, such as the vesicle marker Sec4p and the exocyst subunit Sec8p, localize more diffusely within the bud. Furthermore, sec3Δ cells are strikingly round relative to wild-type cells and are unable to form pointed mating projections in response to α factor. These phenotypes support the proposed role of Sec3p as a spatial landmark for secretion. We also find that cells lacking Sec3p exhibit a dramatic defect in the inheritance of cortical ER into the bud, while the inheritance of mitochondria and Golgi is unaffected. Overexpression of Sec3p results in a prominent patch of the ER marker Sec61p-GFP at the bud tip. Cortical ER inheritance in yeast has been suggested to involve the capture of ER tubules at the bud tip. Sec3p may act in this process as a spatial landmark for cortical ER inheritance.
Introduction

The genetic dissection of membrane traffic in *S. cerevisiae* has facilitated our understanding of the molecular mechanism of vesicular transport. At the post-Golgi stage of the exocytic pathway, more than a dozen gene products are required for the different steps of the transport reaction. The rab GTPase Sec4p and its nucleotide exchange factor Sec2p are required for the polarized delivery of secretory vesicles to sites of secretion (Walch-Solimena et al., 1997). This transport step utilizes actin cables and the type V myosin motor, Myo2p (Johnston et al., 1991) (Govindan et al., 1995) (Pruyne et al., 1998). Actin cables are oriented towards sites of active surface growth. These are the bud tip, early in the yeast cell cycle, and the mother-bud neck, late in the cycle (Adams and Pringle, 1984). Following delivery to sites of surface growth, secretory vesicles fuse with the plasma membrane in a reaction mediated by a SNARE complex consisting of the yeast t-SNAREs Sso1/2p and Sec9p, together with the v-SNARE Snc1/2p (Brennwald et al., 1994) (Grote et al., 2000). Most of the other gene products required for post-Golgi membrane traffic are components of a large, evolutionarily conserved, complex called the exocyst (TerBush et al., 1996). The exocyst is composed of 8 subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p. It acts after polarized vesicle delivery, but prior to SNARE complex formation and fusion (Walch-Solimena et al., 1997) (Grote et al., 2000). The molecular aspects of its role in exocytosis are not fully understood, however several lines of evidence support a model in which the exocyst acts to tether secretory vesicles to specialized exocytic sites on the plasma membrane prior to docking and fusion (Guo et al., 1999).

The exocyst is peripherally associated with the plasma membrane (Bowser et al., 1992), highly concentrated at sites of cell surface growth (TerBush and Novick, 1995) (Finger et al., 1998). In the case of Sec3p, this polarized localization is independent of the secretory pathway. Sec3p has therefore been postulated to function as a spatial landmark, marking exocytic sites. The localization of Sec3p is
dependent on a direct interaction with the Rho1p (Guo et al., 2001) and Cdc42p GTPases (Zhang et al., 2001), as well as an interaction with Bud4p (Osman et al., 2002), but is independent of the actin cytoskeleton (Finger et al., 1998). In contrast, another subunit of the exocyst, Sec8p requires both ongoing secretion (Finger et al., 1998) and a functional cytoskeleton (Ayscough et al., 1997) for its localization. A third subunit, Sec15p associates with secretory vesicles and directly interacts with Sec4p in its GTP-bound form (Guo et al., 1999). The working hypothesis is that the exocyst complex assembles as vesicles carrying Sec4p-GTP arrive at sites marked by Sec3p. The assembled complex then acts to tether incoming vesicles to these exocytic sites. We show here that SEC3 is unique among the exocyst genes in that it is not essential for growth or secretion. Phenotypic analysis of sec3Δ cells strongly supports the proposed role for Sec3p as a spatial landmark for exocytosis.

A prior study has shown that certain sec3 mutants appear to accumulate endoplasmic reticulum (ER) tubules in addition to secretory vesicles (Finger and Novick, 1997). Biochemical analysis of these mutants, however, showed no defect at the level of ER to Golgi transport and only a minor defect in transport out of the Golgi (Finger and Novick, 1997). A recent study on the dynamics of ER in yeast has offered a possible clue to the cause of this phenotype (Fehrenbacher et al., 2002). The ER in yeast is found in a cortical network that makes several tubular connections to the nuclear envelope. At an early stage of bud growth, ER tubules move to the apical tip of the bud and appear to become attached to the cortex at that site. It was proposed that this attachment could direct the inheritance of ER by serving to pull tubules into the bud as the cell cycle progresses (Fehrenbacher et al., 2002). Given the proposed role of Sec3p as a spatial landmark for exocytosis as well as the appearance of ER accumulation in sec3 mutant cells, we explored the possibility that the apical concentration of Sec3p may function as an attachment site for cortical ER. Indeed, we show here that sec3Δ mutant cells exhibit a dramatic defect in ER inheritance. By
tethering both secretory vesicles and cortical ER, Sec3p may help to orient the entire secretory pathway and thereby facilitate polarized growth.
Materials and Methods

Yeast Strains, Media, and Reagents

*S. cerevisiae* strains used in this study are listed in Table I. YPD (rich medium) and SD (synthetic medium) were prepared as described (Sherman, 1991). For SC media 40µg/ml adenine, 20µg/ml histidine, 40µg/ml leucine, 40µg/ml lysine, 20µg/ml methionine, 10µg/ml tyrosine, 40µg/ml uracil were added. Synthetic complete medium lacking sulfate (SC-S) was prepared similar to the SC medium but all sulfate salts were replaced with the corresponding chloride salts and no methionine was added. To select for cells containing a *URA3* marker based plasmid uracil was omitted from the SC medium. For all experiments described here the strains were grown in SC medium at 25°C overnight to an OD600 of 0.2-0.5.

Pro-Mix from Amersham Pharmacia was used for the ^35^S-labeling experiments. For the immunoprecipitation of Bgl2HA we used the monoclonal antibody 12CA5 and protein G-sepharose beads (Amersham Pharmacia). The enzyme to degrade the yeast cell wall was Zymolyase 100T from Seikagaku Corporation. All lysates were prepared in the presence of protease inhibitors 6µg/ml antipain, 2µg/ml aprotinin, 2µg/ml chymostatin, 8µg/ml leupeptin, 12µg/ml pepstatin A and 1mM PMSF (Sigma Chemical Corporation).

Strain Construction, and Molecular Biology Techniques

Standard techniques were used for the sporulation and the tetrad and complementation analysis of yeast strains (Sherman, 1991). To transform yeast cells a lithium acetate method was used (Gietz et al., 1992). Heterozygous diploids were constructed in our strain background by replacing one copy of the exocyst genes with the kanMX-module. The kanMX cassette together with 200-300 base pairs homologous to
the 5’ and 3’ region of the disrupted gene was amplified by PCR from strains of the yeast deletion collection (ResGen Invitrogen Corporation). To serve as a template the cells were treated with Zymolyase 100T 120units/ml for 15 minutes at 37°C. The cells were harvested at low speed in a bench top microcentrifuge and heated for 5 minutes at 92°C. The pellets were resuspended in a standard PCR reaction mixture containing the corresponding 3’ and 5’ primers (20mers). After transformation of the PCR products the yeast cells were recovered 3 hours in liquid YPD medium at 25°C and then spread onto YPD plates containing 300µg/ml Geneticin (Gibco BRL Life Technologies) as the selective antibiotic. For single step chromosomal tagging of BGL2 the vector pFA6a-3HA-His3MX6 (Longtine et al., 1998) was chosen. Long oligonucleotide primers (65bp) with 45bp homology to the 3’ end of BGL2 were designed to remove the stop codon of BGL2 and fuse in frame the HA tag as described (Longtine et al., 1998).

To tag the genomic copy of SEC61 with GFP, the last 576 base pairs of SEC61-coding sequence, the coding sequence of GFP (S65G, S72A) (Cormack et al., 1997), and the first 455 base pairs of the 3' noncoding sequence of SEC61 were amplified by PCR using the primer pairs pSEC61-1 / pSEC61-2 (5' - CAACGCGGATCCACACACCCCCAATCATGTTGCAGAGT-3' / 5' - CAGTGAATAATTCTTCACCTTTAGACATCAAATCAGAAAATCCTGGAACG-3'), pSEC61-3/pSEC61-4 (5' - CGTTCCAGGATTTTCTGATTTGATGTCTAAAGGTGAAGAATTATTCACTG-3' / 5' - CTTTGGATATTATTTTCATTTTATATTCATTTGTACAATTCCATCCATCCATGGG-3'), and pSEC61-5/pSEC61-6 (5' - CCATGATATTGGATGTTCTACAAATGAATATAAAATGAAAAATAATATCCAAAG-3' / 5' - CAACGCGGATCCATCCGTCCCGGAAAAGAGGAGACAGA-3'), respectively. These three fragments served as templates for the fusion PCR using pSEC61-1 as the 5' outer oligo, pSEC61-6 as the 3' outer oligo, and pSEC61-3 as the linking oligo. The PCR product, a fusion of the 3’ part of SEC61-coding...
sequence, the in-frame GFP sequence and the SEC61 terminator, was digested with BamHI and subcloned into pRS305, a yeast integrating vector containing URA3. The resulting plasmid, pYDY101, was cut at a unique PmlI site within the SEC61-coding sequence and used for yeast transformation. The construct was verified by sequencing.

Plasmids expressing the Sec3pΔN and Sec3pΔC regions tagged with four HA epitopes were constructed as follows: first, a plasmid was made by replacing the 1.4 kb HindIII-KpnI region in 3ScaI-ApaIpNB419 (Finger and Novick, 1997) with the 1.6 kb HindIII-KpnI fragment of pSEC3-4xHA (Finger et al., 1998). The resulting plasmid was then used as template for PCR amplification. The Sec3pΔN-expressing plasmid, pGPD-SEC3ΔN-4xHA, was generated by inserting the PCR product which carries an ATG codon followed by the in-frame 3’ end of the SEC3-4xHA gene into the p415GPD vector (ATCC). The Sec3pΔC-expressing plasmid, pGPD-SEC3ΔC-4xHA, was generated by inserting the PCR product which contains the 5’ end of the SEC3 gene and the PCR product that carries the in-frame four consecutive HA epitopes into the p415GPD vector (ATCC).

Detailed description of the constructs used for the in vivo localization of different organelles can be found in the following publications: pRH475 carrying the ER marker hmg1-GFP (Cronin et al., 2000), SEC7-3xGFP as a Golgi marker (Rossanese et al., 2001) and pYDY104 for the mitochondrial targeting of red fluorescent protein (mito-RFP; (Du et al., 2001)).

**Invertase Secretion**

Yeast cells were grown at 25°C to an OD₆₀₀ of 0.2-0.5 in SC medium (2% glucose). Ten OD₆₀₀ units of cells were harvested and washed in 5ml of SC medium containing only 0.1% glucose. The cell pellet was resuspended in SC 0.1% glucose at 1 OD₆₀₀ unit of cells/ml to induce expression of invertase. At several time-points after invertase derepression samples of 1ml were taken. After the addition of
20mM sodium azide, these samples were kept on ice. At the end of the time-course the cells were pelleted, washed three times and resuspended in ice-cold distilled water containing 10mM sodium azide. Half of the cell suspension was used to determine the extracellular invertase activity. The other half was lysed by two freeze thaw cycles in liquid nitrogen in the presence of 1% Triton X-100. These permeabilized cells were used to determine total cellular invertase activity. Invertase activity was measured as described (Goldstein and Lampen, 1975).

**Pulse Chase Analysis of Proteins Secreted Into the Medium**

Cells were grown overnight to OD$_{600}$ of 0.2-0.5 in SC medium at 25°C. The cells were harvested and washed twice in SC-S. The cells were re-suspended in SC-S at 2 x 10$^8$ cells/ml and incubated for another 10 minutes at 25°C. Per time-point 100µCi of labeling mixture (Pro-Mix) containing $^{35}$S Methionine and $^{35}$S Cysteine was added. During the 5 minute labeling the cells were either kept at 25°C or shifted to 37°C. Labeled cells were chased by adding cold methionine, cysteine and ammonium sulfate to a final concentration of 0.2mM, 0.25mM and 3mM, respectively. Aliquots of 400µl of cell suspension were removed during the chase and combined with 10µl 1M sodium azide, 10µl 1M sodium fluoride and kept on ice. The cells were then pelleted at low speed in a benchtop microfuge at 4°C. To completely remove the cells this step was repeated. Supernatants were transferred to fresh tubes containing TCA (final concentration 10%) and deoxycholate (final concentration 0.01%). These samples were incubated on ice for at least 30 minutes. The proteins were precipitated at 4°C (high speed centrifugation in benchtop centrifuge for 10 minutes). TCA precipitated proteins were washed twice with acetone at –20°C, air dried, and re-suspended in SDS sample buffer (100mM Tris pH 6.8 2% SDS, 10% glycerol, 3% 2-mercaptoethanol, 0.005% Bromphenolblue). Samples were boiled for 5 minutes and electrophoresed on 8% polyacrylamide gels.
**Secretion of Bgl2HA**

Wild-type and sec3Δ cells expressing a HA-tagged version of Bgl2p were grown to an OD$_{600}$ of 0.2-0.5. Labeling of these cells was carried out as described above except that 200µCi of $^{35}$S labeling mixture was used per time-point. Labeled cells and a two times concentrated spheroplast buffer stock were combined to obtain cell suspensions in 100mM Tris pH 7.4, 1.4M sorbitol, 10mM sodium azide, 10mM sodium fluoride, 30mM βME and 0.05mg/ml Zymolyase. Cell suspensions were incubated for 30 minutes at 37°C. The released cell wall material was then separated from intact cells by centrifugation. The supernatants were boiled for 5 minutes after the addition of 1% SDS. For immunoprecipitation the samples were diluted 20 fold with IP-buffer (50mM Hepes pH 7.4, 150mM KCl, 1mM EDTA, 0.1% βME, 0.5% IGEPAL and protease inhibitors). Bgl2HA was immunoprecipitated using the 12CA5 monoclonal antibody.

**Localization Analysis**

In vivo localization of Hmg1p-GFP, Sec7p-GFP and mitochondrial RFP was performed as described previously (Du et al., 2001) (Rossanese et al., 2001).

For Sec8-GFP localization 4-8 OD$_{600}$ units of yeast cells were harvested and resuspended in 1ml of fresh SC medium and further incubated at 25°C or shifted to 37°C. After various incubation times the cells were centrifuged in a benchtop microfuge at low speed for 30 seconds at room temperature. The cell pellet was immediately fixed in cold methanol and incubated at –20°C for 10 minutes. After a centrifugation step the cells were briefly resuspended in cold acetone. The fixed cells were washed three times in phosphate buffered saline before analysis under the fluorescence microscope.
For Sec4p immunofluorescence, yeast cells were grown to an optical density of OD$_{600}$ 0.2-0.5 in SC medium. Aliquots of 20 ml were added to 2.5ml of 1M KPO$_4$ pH 6.5 buffer. Immediately, 2.5 ml of formaldehyde solution (37%) was added. For fixation the cells were incubated for 2 hours at room temperature. The cells were then washed three times in spheroplasting medium 0.1M KPO$_4$ pH 6.5, 1.4M sorbitol (SP). To digest the cell wall, cell suspensions were incubated in SP containing 20mM βME and 0.2mg/ml Zymolyase for 30 minutes at 30°C. The cells were washed twice in SP and then allowed to bind to polylysine coated glass slides. Before adding the primary antibody the cells were blocked in antibody solution (Phosphate buffered saline, 1% bovine serum albumin, 0.1% Triton X-100). Monoclonal antibodies to Sec4p were used as the primary antibody followed by a Texas Red conjugated goat anti-mouse secondary antibody. Stained cells were visualized using the RITC filter set of a Zeiss fluorescence microscope.

Indirect immunofluorescence staining using HA-specific antibody was performed essentially as described previously (Du et al., 2001).

To visualize filamentous actin structures, cells were grown to early exponential phase at 25°C in SC and fixed in SC medium containing 3.7% formaldehyde for 30 minutes at 25°C. The cells were then stained with 20U/ml Texas Red-phalloidin (Molecular Probes) as described (Finger and Novick 1997).

**Electron Microscopy**

Yeast cells were grown at 25°C to an OD$_{600}$ of 0.4 in SC medium. The cells were harvested and resuspended in 1.5% KMnO$_4$ and left for 20 minutes at room temperature. After 5 washes with distilled water, cells were stained with 2% uranyl acetate for 4 hours at room temperature in the dark. The
samples were dehydrated by incubation with 50, 70, 95 (2 times 5 min each) and 100% Ethanol (2 times 15 min). The cells were then washed briefly in acetone before embedding in Spurr resin (Electron Microscopy Science, Fort Washington, PA). For polymerization the cell pellets in Spurr were cured for 48 hours at 80°C. Ultrathin (60nm) sections were cut on a Reichert ultramicrotome and collected on formvar- and carbon-coated grids. The samples were post-stained with 2% uranyl acetate and lead citrate and examined in a Philips Tecnai 12 electron microscope.
Results

Sec3p is not essential for growth

Sec3p is a protein of 1336 amino acids containing amino and carboxy terminal domains separated by a potential coiled-coil domain (amino acids 322 to 465) (Haarer et al., 1996). The C-terminal domain binds to Sec5p, another subunit of the exocyst, while the N-terminal domain binds Rho1p and Cdc42p (Guo et al., 2001). Cells expressing Sec3p lacking the Rho1p/Cdc42p binding domain grow quite well (Guo et al., 2001; Haarer et al., 1996). Truncation of the carboxy terminal domain of Sec3p, results in poor growth on rich medium, yet relatively good growth on synthetic complete medium (Haarer et al., 1996) (Osman et al., 2002). When the SEC3 promoter is replaced with the regulated GAL1 promoter, cells are able to grow slowly on repressing medium, suggesting that SEC3 may not be essential (Finger and Novick, 1997). Nonetheless, the Saccharomyces genome database lists SEC3 as an essential gene.

We have systematically determined if each member of the exocyst complex is essential for growth. We created heterozygous diploids by disrupting the 8 exocyst genes with the kanMX cassette, leaving only the start and stop codons (see Materials and Methods). Geneticin resistant clones were sporulated and more than 12 tetrads were dissected on synthetic complete (SC) and YPD medium. Consistent with an essential role for the exocyst in secretion, 7 of the 8 heterozygous diploids did not give rise to Geneticin resistant haploids after dissection. In contrast, tetrads derived from the sec3Δ/SEC3 diploids gave rise to 2 wild-type haploids and 2 slower growing sec3Δ::kanMX clones on SC media (Fig. 1A). At 37°C, the sec3Δ cells were tightly blocked in growth. We conclude that, unlike all other exocyst genes, SEC3 is not essential for growth.
As was shown for certain sec3 truncations (Haarer et al., 1996), sec3Δ cells grew better on SC versus YPD medium at 25°C (Fig. 1B). After dissection of the sec3Δ/SEC3 strain on YPD only about 20% of the sec3Δ spores gave rise to a colony. The first sec3Δ colonies were observed only 5 days after dissection (our unpublished results) as compared to 3 days on SC medium (Fig. 1A). Therefore, SC medium was used in all the experiments described below. The reasons for the observed growth difference of sec3Δ cells on SC versus YPD are unclear. The discrepancy with the Saccharomyces genome database regarding the essentiality of SEC3 probably reflects differences in methodology rather than a strain difference as dissection of the heterozygous diploid obtained from the genome project also gave rise to viable sec3Δ cells. The sensitivity of sec3Δ cells to YPD may explain why disruptants directly selected on YPD Geneticin plates were not obtained in the yeast genome project.

sec3Δ cells accumulate only a small fraction of their secretory cargo

To determine the extent to which secretion is inhibited in a sec3Δ strain, the export of invertase was monitored at 25°C. In wild-type cells, 90 min after a shift to derepressing medium, only 9.2% +/-3.8 (n=4) of invertase activity was intracellular (Fig. 2A), whereas, in sec3Δ cells the intracellular pool was 31% +/-2 (n=4). Given that Sec3p is a subunit of the exocyst, this is a relatively weak effect on secretion. For comparison, several of the temperature-sensitive exocyst mutants accumulate more than 95% of their invertase in the intracellular pool at their non-permissive temperature (Novick et al., 1980). At 37°C, more than 75% of the invertase remained intracellular in sec3Δ cells (Fig. 2A). Therefore sec3Δ cells are temperature-sensitive for both growth and secretion.

The intracellular accumulation of invertase in sec3Δ cells at 25°C might indicate a delay rather than a block in secretion. To test this possibility, we followed the release of newly synthesized proteins into the medium by pulse-chase analysis (Fig. 2B). Seven min (5 min pulse, 2 min chase) after the
addition of label, a number of \(^{35}\text{S}\)-labeled protein bands were detected in the medium of both wild-type and \(sec3\Delta\) cells. The maximal signal was reached after an 8 min chase, both for wild type and the \(sec3\Delta\) mutant and the pattern of secreted proteins was similar in both strains. The maximal signal from \(sec3\Delta\) cells was about 30% lower than wild type even though the incorporation of label into protein was comparable. A possible interpretation of this data is that a fraction of the vesicles is blocked for secretion whereas the larger fraction is secreted at a rate close to normal. After a shift to 37°C wild-type cells export a somewhat different set of proteins, while export from \(sec3\Delta\) cells was almost completely blocked (Fig. 2B). The transport block is very rapid, as the \(sec3\Delta\) cells had not been pre-shifted to 37°C.

In \textit{S. cerevisiae}, newly synthesized secretory proteins are exported via at least two different classes of vesicles that are of similar diameter but differ in their density (Harsay and Bretscher, 1995). Invertase is transported in the denser vesicles whereas the Bgl2p exoglucanase is found in the lighter vesicles. To assay export of the cargo of the lighter vesicles, we followed an HA-tagged version of Bgl2p by pulse-chase analysis. Wild-type and \(sec3\Delta\) cells synthesized equal amounts of Bgl2HA as determined by immunoprecipitation of \(^{35}\text{S}\)-labeled Bgl2HA from total cell lysates (our unpublished results). Bgl2HA was secreted equally well from wild-type and \(sec3\Delta\) cells at 25°C (Fig. 2C) and could be detected at the first time-point (5 min pulse) in both strains. From these results we conclude that Sec3p is not essential for the export of cargo from either light or dense secretory vesicles.

**Spatial regulation of exocytosis by Sec3p**

During early stages of the cell cycle, growth is restricted to the apical tip of the bud (Lew and Reed, 1995) (Adams and Pringle, 1984) and this initial period of apical growth is responsible for the elongated shape of \textit{S. cerevisiae}. Consistent with a defect in the spatial regulation of secretion, \(sec3\Delta\) cells were strikingly round; 80% of the \(sec3\Delta\) but only 19% of the wild-type cells (\(n \geq 200\)) had an axial
ratio less than 1.1. Because wild-type diploid cells are more elongated than haploid cells, this phenotype was somewhat more exaggerated in diploid cells where 82% of the sec3Δ/sec3Δ cells and only 6% of the wild-type cells had an axial ratio less than 1.1.

We determined the effects of the loss of Sec3p on the localization of components of the exocytic machinery. In wild type, Sec8p-GFP is localized to buds tips and mother-bud necks (Fig. 3A and 3B for quantitation). A sec3Δ strain with SEC8-GFP as its sole copy of SEC8 grew only slightly slower than the sec3Δ mutant. A similar fraction of sec3Δ and wild-type cells showed a concentration of Sec8-GFP at the bud tip or neck at 25°C. However, in sec3Δ cells, the distribution of Sec8-GFP was broader and more background fluorescence was observed in mother cells (Fig. 3A).

When shifted to 37°C wild-type cells lost the bud tip localization of Sec8-GFP within 10 min, while bud neck localization was less sensitive to the increase in temperature. After 1 hr at 37°C, Sec8-GFP had completely regained its normal distribution (Fig. 3A, B). This behavior may reflect the dependence of Sec8p localization on the actin cytoskeleton, which is also transiently affected by a temperature shift (Delley and Hall, 1999). In sec3Δ cells, Sec8-GFP localization was lost from both bud tips and necks after a 10 min shift to 37°C (Fig. 3A, B) and after 1 hr only a small number of sec3Δ cells were able to repolarize Sec8-GFP (Fig. 3A, B). Thus, at 37°C the localization of the exocyst depends to a large extent on Sec3p.

Using Sec4p as a vesicle marker, we next examined whether secretory vesicles are mistargeted in cells lacking Sec3p. Sec4p is normally concentrated in a small spot at the bud tip (Walch-Solimena et al., 1997). At 25°C sec3Δ cells showed a much broader distribution of Sec4p than wild-type cells (Fig. 4A). Quantification confirmed that the average area of Sec4p staining was 3-4 times larger in sec3Δ than in wild-type cells (Fig. 4B). On the other hand, the average signal intensity in the bud was only slightly higher in the sec3Δ cells.
We also examined the role of Sec3p in polarized surface growth following exposure to mating factor. Treatment of \textit{MATa} wild-type cells with $\alpha$ factor resulted in G1 arrest, followed by the growth of a mating projection (Fig 4C). After 4 hr of treatment most cells exhibited a sharply pointed mating projection with Sec4p highly concentrated at the tip. Addition of $\alpha$ factor to \textit{sec3}\textsuperscript{$\Delta$} cells also induced G1 arrest. However the resulting mating projections were predominantly rounded rather than pointed. Sec4p was distributed across the width of the broad mating projections. The average area of Sec4p fluorescence was 4 fold greater in \textit{sec3}\textsuperscript{$\Delta$} cells than in wild-type cells. Together, our findings demonstrate that the spatial regulation of secretion is affected in \textit{sec3}\textsuperscript{$\Delta$} strains. While the overall polarity of surface growth and secretion is maintained in \textit{sec3}\textsuperscript{$\Delta$} strains, the ability to focus surface growth is lost. Therefore, \textit{sec3}\textsuperscript{$\Delta$} strains are deficient in apical growth at the beginning of the cell cycle and are unable to generate pointed projections during the mating response.

\textbf{\textit{sec3}\textsuperscript{$\Delta$} cells accumulate exocytic vesicles and have mislocalized endoplasmic reticulum.}

Electron microscopy can be used to identify the compartment that accumulates in response to a secretory block (Novick et al., 1981). As secretory vesicles fuse soon after they are formed, few secretory vesicles can be seen in wild-type cells. As expected from our biochemical analysis, \textit{sec3}\textsuperscript{$\Delta$} cells accumulated secretory vesicles at 25°C (Fig. 5C). Vesicles were concentrated preferentially in the bud consistent with the Sec4p localization data. However, a significant number of vesicles were also found in mother cells.

In a prior study, we noted an increase in cytoplasmic ER tubules in two \textit{sec3} alleles, \textit{sec3-4} and \textit{sec3-5} (Finger and Novick, 1997). Although the overall amount of ER and its ultrastructure appeared normal in \textit{sec3}\textsuperscript{$\Delta$} cells, the subcellular localization of the ER was altered (Fig. 5C and D). In wild-type cells, fixed under the conditions used here, the cortical ER is apparent as narrow, darkly stained
structures closely apposed to the plasma membrane in both mother cells and buds (Fig. 5A and B). In the sec3Δ strain, mother cells exhibited normal cortical ER, however an increased number of ER tubules were visible in the cytoplasm, often clustered near the nucleus (Fig 5D). Little or no ER was apparent near the cortex of buds (Fig 5C and D). Quantification showed that 94% (n=33) of wild-type cells had cortical ER in the bud, while only 33% (n=30) of sec3Δ cells had cortical ER in the bud and 27% of sec3Δ cells showed a cluster of ER tubules near the nucleus.

**Cortical ER inheritance is defective in sec3Δ cells**

As it is can be difficult to interpret the full three-dimensional structure of an extended object, such as the ER, from an electron micrograph, we addressed the relationship between the loss of Sec3p and the change in ER localization by fluorescence microscopy, collecting images at a series of focal planes. We compared the localization of the ER marker Hmg1p-GFP (NH2-terminal transmembrane domains of HMG-CoA reductase isozyme 1 (Hmg1p) fused to GFP) in sec3Δ cells with wild type. In wild type, the ER is seen as a network of interconnected tubules closely apposed to the plasma membrane (cortical ER) and as connections to the outer membrane of the nuclear envelope (perinuclear ER) (Prinz et al., 2000) (Preuss et al., 1991). Cortical ER is inherited into daughter cells at early stages of bud growth, well before the perinuclear ER. The first ER elements detected in the bud are cytoplasmic tubules that are often oriented along the mother-bud axis (Du et al., 2001). These ER tubules are frequently directed to the bud tip before they are delivered to the periphery of the bud (Fehrenbacher et al., 2002) (our unpublished data).

We observed a severe delay in the delivery of cortical ER into sec3Δ daughter cells (Fig. 6 A). Approximately 60% of small buds (diameters between 0.3 and 0.5 of the mother cell) were virtually devoid of ER, while more than 90% of wild-type buds of this size contained ER tubules that were
distributed uniformly along the cortex (Fig. 6 A and C). In addition, a significant portion (24%) of small buds in the mutant contained a tubular structure across the bud as the only inherited ER element. Most of these tubules were oriented along the mother-bud axis (Fig. 6A panels b and c). The intensity of these tubules varied from bright, with an intensity similar to that of the cortical ER tubules in the mother cell (Fig. 6A panel c), to dim, an example of which is shown in the upper-left corner of Fig. 6A (panel b). In larger mutant buds (bud bigger than 0.5 of mother cell, but prior to nuclear segregation), a lower but still significant portion (34%) contained little or no ER while an increased portion (44%) contained only tubular staining along the mother-bud axis. In contrast, 100% of wild-type buds, in this phase of the cell cycle, contained cortical ER that was indistinguishable from the mother cell. In large budded M phase cells (containing an elongated nucleus that had not yet divided), a pronounced reduction of cortical ER was observed in 63% of the mutant buds. Faint ER tubules surrounding the perinuclear ER were frequently detected in large sec3Δ buds (Fig. 6A, panel d). Prior to cytokinesis, virtually all sec3Δ daughter cells had obtained normal amounts of cortical ER.

Time-lapse microscopy demonstrated that, despite the inheritance defect, the ER is still highly dynamic in sec3Δ cells (Fig. 6 D, left panels). ER tubules marked by Hmg1p-GFP were seen to move towards the neck and often cross into the bud. However, rather than spreading along the cortex, as they do in wild type (Fig 6 D, right panels), these tubules were often seen to recede back into the mother cell.

To confirm that Sec3p plays a general role in regulating the inheritance of cortical ER rather than a role specific to Hmg1p-GFP, we followed a second ER marker. Sec61p is an ER transmembrane protein required for the translocation and dislocation of proteins across the ER membrane (Johnson and van Waes, 1999). The SEC61 gene was replaced with a SEC61-GFP fusion under the control of the SEC61 promoter and terminator. Fusion of GFP to the cytosolic COOH-terminus (Wilkinson et al., 1996) had no obvious effects on cell viability or growth rate. In wild type, this ER marker showed a localization
pattern similar to Hmg1p-GFP (Fig. 6B, panels e and f; Fig. 6C). In the sec3Δ mutant, we noted the accumulation of Sec61p-GFP in the cytoplasm of some cells. Bright spots were also seen in both mother and daughter cells (Fig. 6B, panels a-d). Nevertheless, Sec61p-GFP revealed a significant fraction of sec3Δ buds that contained no ER or only tubules along the mother-bud axis. A dramatic decrease of cortical ER was also observed in the large buds of M phase sec3Δ cells (Fig. 6B and C). Thus the loss of Sec3p results in a delay in propagating tubules to the bud periphery.

To better define the region of Sec3p needed for ER inheritance, we tested the amino terminal Rho1p binding domain and a sec3 truncation missing the amino terminal domain for their ability to complement the sec3Δ defect (Fig 7A). Expression of the amino terminal domain (Sec3ΔC, residues 1-322) failed to complement the temperature-sensitive growth defect as well as the ER inheritance defect of a sec3Δ strain (Fig 7C). In contrast, expression of an allele missing this region (Sec3ΔN, residues 320-1336) complemented both the growth defect and the ER inheritance defect (Fig 7B).

The inheritance defects described could, in principle, be an indirect consequence of accumulating secretory vesicles. To address this possibility, we examined the inheritance of Hmg1p-GFP in another post-Golgi mutant, sec9-4, grown at the semi-restrictive temperature of 30°C for 12 hr prior to fixation. We chose this temperature because the accumulation of post-Golgi vesicles has been reported in sec9-4 cells grown at 28.5°C (Finger and Novick, 1997) and the doubling time of the sec9-4 mutant is increased to a similar degree (2.0 times that of the wild type at 30°C) as that of sec3Δ cells at 25°C (1.7 times that of wild type at 25°C). Culturing sec9-4 cells at 30°C caused abnormal morphologies, such as widened necks and multiple-budded cells, as well as loss of GFP fluorescence in a fraction of the sec9-4 cells, possibly reflecting cell death. However, under these conditions, ER inheritance occurred normally in more than 90% (n=63) of the small budded sec9-4 cells containing detectable Hmg1p-GFP fluorescence. We have also examined ER inheritance in sec5-24, sec8-9, sec10-2 and sec15-1 at their semi-restrictive
temperatures and found it to be normal (our unpublished results). These results imply that impaired post-Golgi secretion does not by itself, cause a delay in cortical ER inheritance.

Actin has been implicated in ER dynamics in yeast (Prinz et al., 2000) (Fehrenbacher et al., 2002). The defect in ER inheritance in sec3Δ cells could reflect a defect in actin assembly. We examined the pattern of actin assembly in sec3Δ cells at 25°C, yet found it to be normal (Fig. 8). Thus, the effects of the loss of Sec3p on ER segregation are not due to the buildup of secretory vesicles or mislocalization of the actin cytoskeleton.

Mitochondria and Golgi compartments are inherited normally in sec3Δ cells.

In S. cerevisiae, mitochondria also form an interconnected tubular network at the cell periphery (Yaffe, 1999). Previous studies have suggested a close association between the ER and mitochondria in both yeast and mammalian cells (Montisano et al., 1982) (Perkins et al., 1997) (Prinz et al., 2000). Moreover, mitochondria are inherited by transmitting tubules along the mother-bud axis. The tubules become immobilized at the bud tip and are then distributed to the periphery (Simon et al., 1997). To address a possible role of Sec3p in this process, we used a mitochondrial targeting sequence fused to RFP (Du et al., 2001). In wild-type cells, about one third of small budded cells contained mitochondrial tubules across the bud, but not at the tip, while one third contained mitochondria tubules that had contacted the bud tip (Fig. 9B). The rest of the small budded cells contained tubules along the mother-bud axis at the base of the neck. For large budded cells in the late S to G2 phases of the cell cycle, an increased fraction of buds had tubules contacting the tip and about 10% of the buds contained tubules in the cortical region. After cells entered mitosis, mitochondrial tubules were found at the periphery in the majority of the buds, with the same distribution as in the mother cell. When mitochondrial distribution was examined in sec3Δ cells, we found that mitochondria tubules were successfully transmitted into the
buds that had failed to inherit cortical ER (Fig. 9A). Quantification of mitochondrial inheritance in sec3Δ cells revealed no delay in transmitting mitochondrial tubules into the bud or in propagating these tubules to the bud periphery (Fig. 9B).

We also investigated the inheritance of early and late Golgi membranes in sec3Δ cells. Early Golgi membranes have been shown to appear in buds at essentially the same stage of bud growth as cortical ER (Preuss et al., 1992) (Rossanese et al., 2001) (Du et al., 2001). We followed the distribution of a HA-tagged version of Och1p, an enzyme that defines one of the earliest Golgi compartments in yeast. We analyzed 63 sec3Δ mutant cells in which the bud contained no Hmg1p-GFP-labeled ER elements or only ER tubules along the mother-bud axis. All had obtained 1 to 5 Och1p-HA-labeled spots, which was comparable to wild type (Fig. 9C). To study the inheritance of late Golgi, we replaced the SEC7 gene with a SEC7-GFP fusion. Sec7p is a peripheral membrane protein that specifically localizes to the late Golgi apparatus (Rossanese et al., 2001). Sec7p-GFP localized to 10-15 spots in wild type and sec3Δ cells (Fig. 9D). Virtually all (68 out of 69) small buds of sec3Δ cells acquired 1 to 4 Sec7-GFP-labeled spots, which was similar to the distribution in wild type. Therefore, we did not detect any significant defects in the structure or inheritance of either early or late Golgi membranes in sec3Δ cells. In total, our results show that Sec3p specifically affects secretory vesicle targeting and ER inheritance.

**Overexpression of Sec3p leads to the formation of a cap of Sec61p at bud tips**

If Sec3p plays a direct role in anchoring the ER at bud tips, overexpression of Sec3p might be expected to alter the distribution of the ER or its binding partner on the ER. Indeed, Sec61-GFP localization exhibited a prominent dot or cap in 89% (n=117) of small to medium sized buds of cells overexpressing Sec3p from a high copy number vector (Fig. 10A). In control cells only 15% of the buds
had a dot or cap of Sec61-GFP (Fig. 10B). Furthermore, the Sec61-GFP dots or caps were generally much brighter in the Sec3p overexpressing cells relative to the control. In contrast to Sec61-GFP localization, Hmg1-GFP localization appeared normal in cells overexpressing Sec3p (Fig. 10C). Electron microscopy of Sec3p overexpressing cells revealed no abnormal accumulation of membrane at the tips of buds (our unpublished data). The Sec3p mediated concentration of Sec61-GFP at the bud tip therefore does not reflect a general effect on ER distribution.
Discussion

In a prior study we had found that the localization of Sec3p to bud tips and necks is maintained even when vesicular transport is blocked or when the actin cytoskeleton is depolymerized (Finger et al., 1998). Sec3p was therefore proposed to act as a spatial landmark for polarized secretion. The findings presented here strongly support a model in which Sec3p marks sub-domains of the plasma membrane for efficient targeting and tethering of secretory vesicles. Furthermore, we now extend that proposal to include a role for Sec3p, and perhaps other components of the exocyst, as an anchor at the bud tip for ER tubules segregated into daughter cells.

Here we show that, unlike all other subunits of the exocyst, Sec3p is not essential for growth or for export of secretory cargo. Furthermore, the pattern of actin assembly as well as the actin dependent polarized transport of secretory vesicles and other organelles appears normal in sec3Δ cells. Indeed, the vesicle marker Sec4p and the GFP tagged exocyst subunit Sec8p are still found concentrated in the bud or close to the mother bud neck under permissive growth conditions. However, in the absence of Sec3p, the vesicles do not appear to find appropriate exocytic sites within the bud, and this results in the fusion of vesicles along the entire surface of the bud. This hypothesis is supported by the broader distribution of Sec4p and the more diffuse localization of Sec8-GFP within the buds of sec3Δ cells relative to wild type buds (Fig. 3 and 4). Such delocalized fusion within the bud would lead to isotropic growth and hence explain the observed round morphology of sec3Δ cells. A similar situation occurs during the formation of mating projections in response to mating factor. Vesicles marked by Sec4p are still delivered in a polarized fashion in sec3Δ cells, but rather than fusing within a well-focused spot, they fuse along a broad front and thereby give rise to a bulbous mating projection. Inefficient targeting may also lead to the failure of a fraction of the vesicles to fuse, as seen in sec3Δ cells under permissive
conditions. At 37°C, sec3Δ cells, similar to conditional mutants defective in other exocyst components, are tightly blocked for growth, fail to export a large fraction of their secretory cargo and accumulate secretory vesicles in a polarized manner. In addition, the polarized localization of Sec8-GFP is lost following a shift of sec3Δ cells to 37°C (Fig. 3A). Sec3p may be critical for the stability of the exocyst at 37°C, while at 25°C the complex may remain sufficiently assembled or active to mediate its essential functions in exocytosis without Sec3p. Thus, a critical function of Sec3p may be to bind and stabilize the exocyst complex at exocytic sites.

We also report the unexpected finding that Sec3p plays a key role in the inheritance of cortical ER. In S. cerevisiae this appears to be a multi-stage process involving transport of cytoplasmic ER tubules along the mother-bud axis, followed by their association with the bud tip and then their propagation along the bud cortex (Du et al., 2001) (Fehrenbacher et al., 2002) (our unpublished data). The daughter cell obtains ER tubules at the cortical region early in S phase, shortly after the initiation of bud growth (Preuss et al., 1991) (Du et al., 2001). In contrast, more than half of sec3Δ mutant buds fail to acquire ER tubules at their cortex even at late S to G2 phases (Fig. 6). Moreover, a significant fraction of sec3Δ cells contains tubules across the bud as the only inherited ER elements. Thus we speculate that Sec3p marks the bud tip destination for ER tubules, similar to its role as a landmark for secretory vesicles. Electron microscopy revealed that many of the sec3Δ cells had an unusually large amount of cytoplasmic ER in the mother cell, in most cases close to the nucleus (Fig. 5D) and a similar phenotype had been noted in a study of sec3-4 and sec3-5 mutant cells (Finger and Novick, 1997). As ER to Golgi transport is unaffected in these cells, it is possible that the structures represent ER tubules that have been retracted back into the mother cell after an unsuccessful attempt to associate with the cortex of the bud. Time-lapse microscopy shows that ER tubules are highly dynamic in sec3Δ cells. Tubules move across the neck, but fail to be stably retained in the bud. Neither mitochondria nor late Golgi, two other
organelles that are transported to the bud tip during the cell cycle (Simon et al., 1997) (Rossanese et al., 2001), require the function of Sec3p for proper inheritance (Fig. 9). It therefore appears that the role of Sec3p is limited to the localization of secretory vesicles and ER tubules.

Rho1p acts through at least two pathways to control the localization of the exocyst. The amino terminus of Sec3p has been shown to bind to Rho1p (Guo et al., 2001) and an allele of Sec3p deleted for the Rho1p-binding domain (Sec3∆N-GFP) is mislocalized, but only when expressed in a wild-type SEC3 background. When expressed as the sole copy, partial localization of Sec3∆N-GFP is seen and the other subunits of the exocyst still localize quite well (Guo et al., 2001) implying a Sec3-independent pathway for exocyst localization. Apparently the truncated allele of Sec3p can localize to some extent by assembling with the rest of the exocyst. We show here that expression of Sec3∆N is able to complement the ER inheritance defect of a sec3Δ strain. A temperature-sensitive rho1 mutant does show a partial defect in ER inheritance following a shift to the restrictive temperature (our unpublished observations). A more complete defect is not seen because this allele may be only partially defective for this function, because other Rho family members are partially redundant or because some of the buds contain ER that had been segregated prior to the temperature shift. In general it is difficult to assay organelle inheritance in mutants such as rho1 that are blocked in bud growth.

An important clue to the role of Sec3p in ER inheritance has come from our observation of the effects of Sec3p overexpression. The formation of a prominent patch of Sec61-GFP at the bud tip in response to Sec3p overexpression, without a parallel change in Hmg1-GFP localization or an accumulation of ER membrane suggests that the patch may represent a selective enrichment of Sec61-GFP within the plane of the ER membrane at the bud tip, rather than an actual increase in the amount of ER membrane at this site. Such a selective enrichment could reflect a physical linkage, either direct or through intermediaries, between Sec61-GFP and the exocyst. Both genetic as well as physical
interactions have been observed between the exocyst and components of the translocon. \textit{SEBI}, encoding the \(\beta\) subunit of the Sec61 translocon complex, was identified as a high copy number suppressor of \textit{sec15-1} (Toikkanen et al., 1996) and was recently shown to suppress defects in the other subunits of the exocyst as well. Overexpression of the other two subunits of the translocon suppresses several exocyst mutants. Co-precipitation experiments indicate a physical linkage between the exocyst and the translocon in both yeast (Toikkanen et al., 2003) and mammalian cells (Lipschutz et al., 2003). In total, these studies support the proposed physical connection between the exocyst at the apical tip of the plasma membrane and the Sec61p-containing translocon complex on the ER membrane. While the efficiency of co-precipitation reported was not high, it is consistent with the observation that only a small portion of the ER is actually anchored at the bud tip and that both the translocon and exocyst complexes have other essential functions. Establishing the details of this interaction between two large, multi-subunit complexes will require further studies.

Taken together our results show that Sec3p is not essential for exocytosis in yeast, but is required for tethering secretory vesicles to specialized sub-domains of the plasma membrane. In a similar manner, Sec3p is also required to stabilize the cortical association of ER tubules that have been extended into the yeast bud. In this way, Sec3p may initiate ER retention and promote distribution along the cortex of the newly forming bud. The Sec3p mediated connection between cortical ER and exocytosis at the bud tip together with the actin-mediated inheritance of Golgi structures may establish an autonomous secretory apparatus in the daughter cells soon after bud initiation and thereby facilitate polarized growth.
Acknowledgements

We thank Randy Hampton, Janet Shaw, Benjamin Glick, Gerry Waters, Charles Boyd and Jeff Coleman for yeast strains and plasmids and Elaine Downie for technical assistance. This work was supported by grants from the N.I.H. to P.N (GM35370) and to S.F.N. (CA 46128, Project 2). A.W. was supported by a fellowship from the Swiss National Science Foundation. Y.D. is an associate of the Howard Hughes Medical Institute.
Abbreviations list

RFP, red fluorescent protein; SC, synthetic complete; YPD, yeast extract peptone dextrose
References


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**Figure Legends**

**Figure 1.** The *SEC3* gene is not essential for yeast growth. (A) A wild-type (left) and heterozygous *sec3Δ:kanMX/SEC3* diploid (right) strain were dissected on SC medium at 25°C. Shown are 7 tetrads for each strain after 3 days of incubation at 25°C. Each row represents a single tetrad. (B) Wild-type and *sec3Δ* haploid strains were grown overnight in SC or YPD medium. Growth was assessed by measuring the optical density (OD$_{600}$) after dilution in the indicated medium. Doubling times were calculated from the exponential growth phase. The values shown represent the average doubling times of four wild-type and four *sec3Δ* strains.

**Figure 2.** The *sec3Δ* strain has a conditional secretion defect. Wild-type (left) and *sec3Δ* mutant cells (right) were tested for their ability to secrete invertase (A), newly synthesized soluble proteins (B), or Bgl2p, a marker protein for light secretory vesicles (C). For panels A and B, experiments performed at 25°C (upper) and 37°C (lower) are shown. (A) For invertase secretion assays the cells were shifted to a low glucose (0.1%) containing medium. Extracellular (white diamonds) and total invertase activity (black squares) were measured. The cells were either continuously grown at 25°C or shifted to 37°C at the 30 min time-point. (B) To follow newly synthesized proteins secreted into the medium, cells were labeled for 5 min with $^{35}$S labeling mix. The cells were then further incubated in chase medium for the indicated times in min. At the end of the incubation, samples were placed on ice in the presence of 20mM NaN$_3$ and 20mM NaF. Proteins in the cell supernatant were TCA precipitated and electrophoresed on 8% SDS PAGE gels. (C) Bgl2HA was extracted from the extracellular cell wall fraction and then immunoprecipitated as described in the Material and Methods. Pulse-chase analysis was done at 25°C as described in B. The indicated times are min after the addition of $^{35}$S labeling mix.
The lane marked with an arrow is an immunoprecipitate from an un-tagged wild-type control strain after 20 min of labeling.

**Figure 3.** The exocyst subunit Sec8-GFP localizes to buds and bud necks in cells lacking Sec3p. (A) Fluorescence images of Sec8-GFP and corresponding DIC pictures are shown for wild-type (left) and sec3Δ cells (right). Cells were grown at 25°C in SC medium or shifted for the indicated times to 37°C. Examples of cells with bud staining are marked with arrowheads and those with neck staining are marked with arrows. (B) Wild-type (grey bars) and sec3Δ cells (black bars) were categorized according to their pattern of Sec8-GFP localization to the bud tip, the bud neck, or delocalized. The graphs depict the percentage of the population within each category. This quantification represents the average from 3 independent experiments.

**Figure 4.** Defect of apical growth in sec3Δ cells during early bud growth and shmoo formation. Immunofluorescence of Sec4p (upper panels of A and C) in wild-type (left) and sec3Δ cells (right). Corresponding DIC are shown in the lower panels. (A) Sec4p is concentrated at incipient bud sites (arrowheads), and in small and medium sized buds. (B) The Sec4p positive area in yeast buds was measured using NIH Image. For this analysis three wild-type and sec3Δ clones, obtained after dissection from the same heterozygous diploid were analyzed. The different clones were grown, fixed, and stained in parallel as described in the Materials and Methods. (C) Sec4p localizes to areas of cell surface growth during shmoo formation. Wild-type (left) and sec3Δ cells (right) were treated with 1µM of α factor for 4 hours at 25°C.
Figure 5. The sec3Δ cells accumulate secretory vesicles and exhibit mislocalized endoplasmic reticulum. Wild-type (A, B) and sec3Δ cells (C, D) grown in SC medium at 25°C were processed for electron microscopy as described in the Materials and Methods. Bar: 1µm. Black arrowheads point to cortical ER in the daughter cells. Note the absence of cortical ER in the large bud of the sec3Δ cell (C). ER tubules extending into the bud (arrow) can be seen in B. An example of ER accumulation in the mother of a sec3Δ cell is shown in D (white arrowhead).

Figure 6. The sec3Δ mutant is defective in the inheritance of cortical ER. Hmg1p-GFP (A) and Sec61p-GFP (B) were used as markers to localize the ER in vivo. Fluorescence micrographs of sec3Δ (upper panels) and wild-type cells (lower panels) are shown. The corresponding DIC pictures show the size of the corresponding yeast buds. The distribution of cortical ER in cells at three different stages of bud formation in sec3Δ and wild type were compared and quantified (C). The three categories were defined as follows (examples of each group are shown in A and B): Small buds between 0.3 and 0.5 times the diameter of the mother cell (arrowheads), buds with a diameter larger than half the mother cell that have not yet received a nucleus (arrows) and large buds where the nucleus has migrated into the bud but is still connected between the mother and daughter cell (diamond arrows). DIC images were taken to measure the diameters of the bud and the mother cells. For each group the fraction of cells with no detectable ER, ER tubules extending into the bud and cortical ER tubules was determined. The number of budded cells examined for each category is indicated in parenthesis. Video supplements to Figure 6A were prepared from z-series images taken at 0.2-µm intervals. (D) Dynamics of Hmg1-GFP labeled ER was monitored by time-lapse imaging. The ER tubule detected in the bud of a sec3Δ cell (panel a) appeared to recede back into the mother cell within
48 sec (panel b). In contrast, ER tubules that were segregated into a wild-type bud (panel d) were delivered to the bud tip after 48 sec (panel e). The DIC images (panels c and f) displayed the size of the buds at the beginning of the experiment. Video supplements to Figure 6D were prepared from images collected at 12-sec intervals for 10 min.

**Figure 7.** The amino terminal Rho1p binding domain of Sec3p is not required for cortical ER inheritance. (A) Diagram of Sec3p domain structure as well as the Sec3p∆N and Sec3p∆C regions tagged with four HA epitopes (denoted by gray oval). The numeral positions of amino acid residues of Sec3p are indicated. (B and C) Expression of Sec3p∆N but not Sec3p∆C complements the ER inheritance defect of sec3Δ cells. ER distribution and DIC images of representative budded cells (arrows) of a sec3Δ strain carrying Sec3p∆N-expressing (B) or Sec3p∆C-expressing (C) plasmids were shown.

**Figure 8.** The distribution of the actin cytoskeleton is normal in sec3Δ cells. Actin localization in wild-type and sec3Δ cells expressing the ER marker Hmg1p-GFP were compared by Texas Red-phalloidin staining. As in wild type, most of the sec3Δ cells contain polarized actin patches and cables. Arrows point to the buds.

**Figure 9.** The inheritance of mitochondria, early and late Golgi is not defective in sec3Δ cells. (A) sec3Δ cells expressing Hmg1p-GFP and RFP fused to a mitochondrial targeting sequence were used to directly compare the inheritance of cortical ER and mitochondria into the bud. (B) Quantification of mitochondrial inheritance into the bud of sec3Δ and wild-type cells. The distinction between different bud sizes was made as described in the legend to figure 6C. Four different types of mitochondrial
distributions in the bud can be distinguished (see schematic drawings in the table and (Simon et al., 1997)). The number in parenthesis indicates the number of cells analyzed. (C) Distribution of the cis-Golgi marker Och1p-HA (left) in the bud of sec3Δ mutant cells was compared to the localization of Hmg1p-GFP (center). DIC pictures of the corresponding cells are shown on the right. Arrows point to mutant buds with no cortical ER. (D). The inheritance and distribution of the late Golgi was normal as shown by the localization of Sec7p-GFP in sec3Δ cells.

**Figure 10.** Overexpression of Sec3p leads to an accumulation of Sec61p-GFP but not Hmg1p-GFP at the bud tip. Representative images of Sec61p-GFP (A and B) and Hmg1p-GFP (C and D) in a Sec3p overproducer, NY2463 (sec3::kanMX, pRS426-SEC3; panels A and C) and in a control strain, NY1211 (SEC3; panels B and D) are shown. Arrows point to either small buds or medium sized buds.
A  

SEC3/SEC3  

sec3Δ/SEC3  

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B  

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A wild-type 25°C  

sec3Δ 25°C  

wild-type 37°C  

sec3Δ 37°C  

Invertase activity (nmol glucose per OD600 unit/min)  

Bgl2HA  

B g  

wild-type 25°C  

sec3Δ 25°C  

wild-type 37°C  

sec3Δ 37°C  

C  

Bgl2HA  

wild-type  

sec3Δ  

Bgl2HA  

Bgl2HA
A wild-type  sec3Δ

25°C

10 minutes 37°C

1h 37°C

B

bud neck non-polar

bud neck non-polar

bud neck non-polar

bud neck non-polar
Figure 6

A. B. Hmg1p-GFP DIC Hmg1p-GFP DIC Sec61p-GFP DIC Sec61p-GFP DIC

sec3Δ sec3Δ

wild type wild type

C.

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D. sec3Δ wild type
Figure 7

A. Coiled-coil Rho1p/Cdc42p Binding Domain

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<th>Rho1p/Cdc42p Binding Domain</th>
<th>4x HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>322</td>
<td></td>
</tr>
</tbody>
</table>

B. Hmg1-GFP

sec3Δ + sec3ΔN

C. Hmg1-GFP

sec3Δ + sec3ΔC
### Figure 9

#### A.

<table>
<thead>
<tr>
<th>Mito-RFP</th>
<th>Hmg1p-GFP</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

#### B.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Small Buds</th>
<th>Large Buds with no nuclei</th>
<th>Large Buds with nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEC3</td>
<td>41% (n=73)</td>
<td>29% (n=73)</td>
<td>30% (n=73)</td>
</tr>
<tr>
<td>sec3Δ</td>
<td>31% (n=83)</td>
<td>39% (n=83)</td>
<td>30% (n=83)</td>
</tr>
</tbody>
</table>

#### C.

<table>
<thead>
<tr>
<th>Och1p-HA</th>
<th>Hmg1p-GFP</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

#### D.

<table>
<thead>
<tr>
<th>Sec7p-GFP</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 10

A. Sec61p-GFP

B. Sec61p-GFP

C. Hmg1p-GFP

D. Hmg1p-GFP