Erratum

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The yeast endocytic protein Epsin 2 functions in a cell-division signaling pathway

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Summary

The epsins are a family of adaptors involved in recruiting other endocytic proteins, binding of ubiquitylated cargo and induction of membrane curvature. These molecules bear a characteristic epsin N-terminal homology (ENTH) domain and multiple peptide motifs that mediate protein-protein interactions. We have previously demonstrated that the ENTH domain of epsin is involved in Cdc42 signaling regulation. Here, we present evidence that yeast epsin 2 (Ent2) plays a signaling role during cell division. We observed that overexpression of the ENTH domain of Ent2 (ENTH2), but not Ent1, promoted the formation of chains of cells and aberrant septa. This dominant-negative effect resulted from ENTH2-mediated interference with septin assembly pathways. We mapped the ENTH2 determinants responsible for induction of the phenotype and found them to be important for efficient binding to the septin regulatory protein, Bem3. Supporting a physiological role for epsin 2 in cell division, the protein localized to sites of polarized growth and cytokinesis and rescued a defect in cell division induced by Bem3 misregulation. Collectively, our findings provide a potential molecular mechanism linking endocytosis (via epsin 2) with signaling pathways regulating cell division.

Introduction

It is well established that endocytosis and signaling are functionally linked (von Zastrow and Sorkin, 2007). For example, endocytosis can contribute to the regulation of ligand availability and receptor levels at the cell surface (von Zastrow and Sorkin, 2007). It is through this mechanism that endocytosis leads to receptor downregulation (von Zastrow and Sorkin, 2007) and shapes extracellular morphogen gradients (Gonzalez-Gaitan, 2003). However, endocytosis is also required for signaling activation; it is now recognized that localization of ligand-receptor complexes at endosomal compartments ['signaling endosomes' (von Zastrow and Sorkin, 2007)] is required for the initiation of specific signaling events (Di Guglielmo et al., 2003). Although the exact mechanism is unknown, endocytosis also plays a crucial role in the activation of the Notch-DSL pathway (Nichols et al., 2007). Moreover, we have recently demonstrated that the endocytic adaptor, epsin, directly modulates Cdc42-dependent signaling (Aguilar et al., 2006). This function, shared with intersectin (Jenna et al., 2002), is mediated by inhibition of Cdc42 GTPase activating proteins (GAPs), i.e. by inhibition of the Cdc42-signaling inhibitors (Aguilar et al., 2006).

Accordingly, we modeled the epsin molecule as bi-modular. Thus, whereas the epsin N-terminal homology (ENTH) domain acts as a signaling module by binding and inactivating Cdc42 GAPs (Aguilar et al., 2006), the relatively unstructured epsin C-terminus binds to other elements of the endocytic machinery (AP2, EH-domain containing proteins and clathrin) as well as ubiquitylated cargo (Wendland, 2002). We speculate that this bi-modular architecture allows epsin to temporally and spatially coordinate endocytosis with Cdc42-dependent signaling. Moreover, we propose that the C-terminal end directs the localization of the signaling module to sites of receptor activation (Maldonado-Baez et al., 2008) (and our own unpublished observations), and dictates the dynamics and regulation of the ENTH domain. Reciprocally, the ENTH domain cooperates with the endocytic module by binding membrane lipids (Itoh et al., 2001) and by inducing membrane curvature at sites of nascent endocytosis (Ford et al., 2002).

Several studies suggested that with respect to the timing of membrane recruitment (Toret et al., 2008) and interactions with the endocytic machinery (Rosenthal et al., 1999; Aguilar et al., 2003) epsin paralogs behave similarly. No data are available, however, regarding potential signaling redundancy among epsin family members. This represents an important gap in the conceptual framework for an emerging concept of signaling by the endocytic machinery, and by epsins in particular. To address this issue, we used a dominant-negative approach, overexpressing the signaling modules of the two yeast epsin paralogs (Ent1 and Ent2). This allowed us to evaluate the paralog-specific signaling functions for epsins without the complications or masking effects from the multiple interactions of the endocytic module.

Our results indicate that ENTH2, but not ENTH1, has specific functions related to cell division pathways. Specifically, our data

suggest that ENTH2 impairs cell division by interfering with the regulation of the septin organization protein Bem3. Interestingly, overexpression of ENTH2 did not produce any measurable endocytosis defect. Our results also indicate that Ent2 plays a role in cell division by downregulating Bem3 activity. In summary, our data indicate that epsin paralogs share some signaling properties, but they also have specific functions. This work reveals a novel link between the endocytic machinery and the regulation of cell division pathways.

Results

Overexpression of ENTH2 in yeast cells induces defects in cell division

To gain insight into the specific signaling properties of the yeast epsins, Ent1 and Ent2, we used a dominant-negative approach consisting of overexpressing their ENTH domain signaling modules. We expressed ENTH1 or ENTH2 in W303 cells from a methionine-repressible *MET25* promoter in a high-copy (2 μ) plasmid (Rönicke et al., 1997; Sikorski and Hieter, 1989). Under overexpression conditions (no methionine for 36 hours), ENTH2 led to significantly reduced growth compared to cells overexpressing ENTH1 or bearing empty vector (Fig. 1A).

The morphology of cells grown in medium without methionine was examined with the cell-wall stain calcofluor white. Empty vector transformants and ENTH1-overexpressing cells showed overall normal morphology (Fig. 1B, left and middle panels, respectively). Only 1% of ENTH1 cells exhibited a mild phenotype (small or slightly elongated buds; see Fig. 1B, middle panel insets) consistent with some degree of Cdc42 hyperactivation (Ziman et al., 1991) and probably the result of the Cdc42 GAP-inhibitory activity of the ENTH domain (Aguilar et al., 2006). By contrast,

ENTH2-overexpressing cells displayed a severe cell-division phenotype characterized by the presence of chained cells (Fig. 1B, right panel; supplementary material Movie 1). Bright patches of calcofluor white indicated an abnormal concentration of chitin at bud necks and peripheral regions (Fig. 1B, arrows).

Since the overexpression of ENTH2 led to reduced growth, we stained ENTH2-overexpressing cells with the vital dye Methylene Blue to assess cell viability. Our results indicated that more than 60% of the chained cells were Methylene-Blue-positive (Fig. 1C) and therefore considered non-viable. These observations suggested that the reduced growth induced by ENTH2 overexpression (Fig. 1A) correlates with a decrease in cell viability linked to abnormalities in cell division.

Detailed inspection of Methylene-Blue-positive cells revealed abnormal, enlarged and ectopic septa (Fig. 1C). Electron microscope analyses confirmed these observations and showed the existence of ectopic septa across the cell and putative cytosol entrapments (lacunae) within aberrant septum-like structures (Fig. 1D, arrows). Importantly, these studies indicated that ENTH2-overexpressing cells with an overall normal morphology also displayed abnormal septum deposition and lacunae formation (Fig. 1D, right inset). We speculate that these cells may be in early stages of the phenotype or they may contain lower ENTH2 concentrations that are insufficient to trigger the more severe manifestations of this phenotype.

We also used the DNA-specific dye DAPI to stain the nuclei in ENTH2-overexpressing cells. Fig. S1A in the supplementary material shows multinucleated cells, indicating that karyokinesis and nuclear segregation occurred in ENTH2-overexpressing cells. However, we also observed instances that suggest that the most abnormal cells have reduced nuclear inheritance, perhaps because
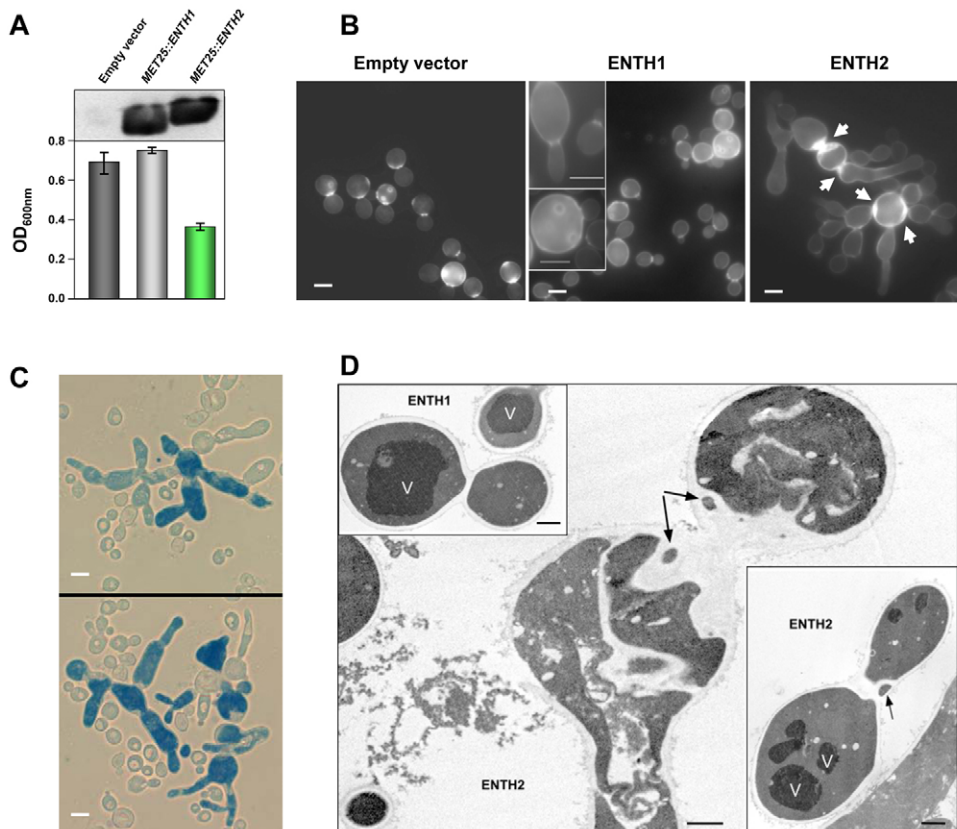

Fig. 1. Overexpression of ENTH2, but not ENTH1, leads to severe abnormalities in cell division. (A) W303 cells overexpressing HA-tagged ENTH1, ENTH2 (from a *MET25* promoter) or transformed with empty pMET25.426 vector were grown for 36 hours in selective media lacking uracil and methionine. Optical density of triplicate cultures was measured at 600 nm and whole cell lysates analyzed by western blotting with an anti-HA antibody. (B) Cells were grown overnight in selective media lacking uracil and methionine, the cell walls were stained with calcofluor white and visualized by epifluorescence microscopy with UV optics. Insets in central panel shows cells with slightly elongated buds or enlarged cells with small buds. Arrows point to abnormal chitin depositions in the bud neck regions. Scale bars: 5 μm. (C) ENTH2-overexpressing cells grown as in B were stained with Methylene blue to reveal dead cells. Two representative fields are shown. Scale bars: 5 μm. (D) Electron microscopy of ENTH1- and ENTH2-overexpressing cells prepared according to the method of Collins et al. (Collins et al., 2000) to preserve the cell wall integrity. Arrows point to putative cytosol entrapments by abnormal septa. Scale bars: 1 μm.

of the presence of abnormal and/or ectopic septa between and within the cells (Fig. 1D).

Characterization of the ENTH2-overexpression phenotype
Penetrance of the ENTH2-overexpression phenotype
We verified that overexpression of ENTH2 induces a variety of morphological abnormalities (supplementary material Fig. S1A). We determined that in unsynchronized cultures 20-40% ENTH2-overexpressing cells fail to achieve cell division (supplementary material Fig. S1B), leading to the accumulation of multinucleated cells. Since the phenotype involves loss of cell viability (i.e. loss of phenotypic cells) the penetrance range calculated for the ENTH2 cell-division defect might be an underestimation of its real values. Furthermore, cells with normal morphology, and no more than two nuclei, can also show signs of phenotypic abnormalities when examined at the ultrastructural level (see Fig. 1D, right inset). Analysis of the distribution of affected cells among the different morphological and cell-division defects revealed that, as opposed to ENTH1, ENTH2 overexpression led to enrichment in the phenotypically advanced categories 2, 3 and 4 (supplementary material Fig. S1C).

Cell-division defects in the ENTH2-overexpression phenotype are independent of growth and expression conditions
To further confirm the ENTH2-specific nature of the phenotype described above, we expressed ENTH2 (and ENTH1) for 16 hours from different vectors (with different auxotropic markers, e.g. *TRP1*, *URA3*) and promoters (e.g. *MET25*, *GAL1*) and analyzed the morphological characteristics of the overexpressing cells. We verified that independently of the growth conditions and promoter-dependent expression strategies used, overexpression of ENTH2 (but not ENTH1) led to deficiencies in cell division (supplementary material Fig. S2A).

The phenotype induced by ENTH2 overexpression is consistent with a dominant-negative effect
We determined three features of the ENTH2 phenotype. First, that the phenotype is dominant and it has been verified in different wild-type genetic backgrounds (e.g. W303, BY4741, SEY6210; data not shown). Interestingly, the extent and severity of the phenotype was higher in epsin-deletion backgrounds (supplementary material Table S3). *ent2Δ* single knockout cells are susceptible to the ENTH2-induced phenotype (supplementary material Table S3) but these cells do not display cell-division defects by themselves. These results, therefore, suggest that ENTH2 exerts dominant-negative effects on putative Ent2-dependent pathways and on redundant cell-division pathway(s) (see Fig. 8 and Discussion). Second, that the extent of the phenotype is ENTH2 dose dependent, i.e. the higher the intracellular concentration of ENTH2 (up to a maximal 100:1 ENTH2 to endogenous Ent2 ratio as detected by western blotting), the higher the abundance of phenotypically affected cells (supplementary material Fig. S2B). Third, as predicted by our approach rationale, full-length Ent2, which is capable of proper localization and regulation, does not interfere with the cell-division process (supplementary material Table S3). As expected, this suppression requires the two modules acting in *cis*, as co-overexpression of the endocytic module (Δ*ENTH2*, i.e. *ENT2^145-613^*) and ENTH2 in *trans* does not inhibit the phenotype (supplementary material Table S3).

Endocytosis is normal in ENTH2-overexpressing cells
The ENTH domain contributes to internalization of cargo by inducing membrane curvature as an early event during endocytic vesicle formation (Ford et al., 2002). Moreover, it is also possible that the ENTH domain through its signaling functions may indirectly interfere with endocytic function leading to cell-division defects. Therefore, we tested the ability of ENTH2-overexpressing cells to perform endocytosis by monitoring fluid-phase uptake, membrane internalization and receptor-mediated endocytosis (Baggett et al., 2003; Urbanowski and Piper, 2001).

ENTH2-overexpressing cells incubated with the water-soluble dye Lucifer Yellow displayed dye accumulation in the vacuolar lumen (Fig. 2A), demonstrating that cells were capable of fluid-phase uptake. Next, we labeled the plasma membrane with the lipophilic dye FM4-64 at 4°C followed by incubation at 30°C to allow internalization. ENTH2-overexpressing cells showed FM4-64 membrane staining of intracellular compartments, particularly of the vacuolar membrane (Fig. 2B), demonstrating the occurrence of plasma-membrane turnover in viable phenotypic cells. We also analyzed the internalization of the G-protein-coupled receptor Ste3 fused to GFP in ENTH2-overexpressing cells. Endocytosis-competent cells rapidly internalize Ste3 from the plasma membrane leading to its accumulation in the vacuole (Urbanowski and Piper, 2001). In agreement with normal Ste3-GFP trafficking, ENTH2-overexpressing cells consistently showed vacuolar accumulation of the fusion protein at steady state (Fig. 2C). Similarly, overexpression of ENTH1 did not affect endocytosis (Fig. 2). Our results indicate that ENTH2-overexpressing cells showed no detectable endocytosis defects in three separate assays (Fig. 2). ENTH2-overexpressing cells also showed clathrin-GFP dynamics indistinguishable from those of cells overexpressing ENTH1 (supplementary material
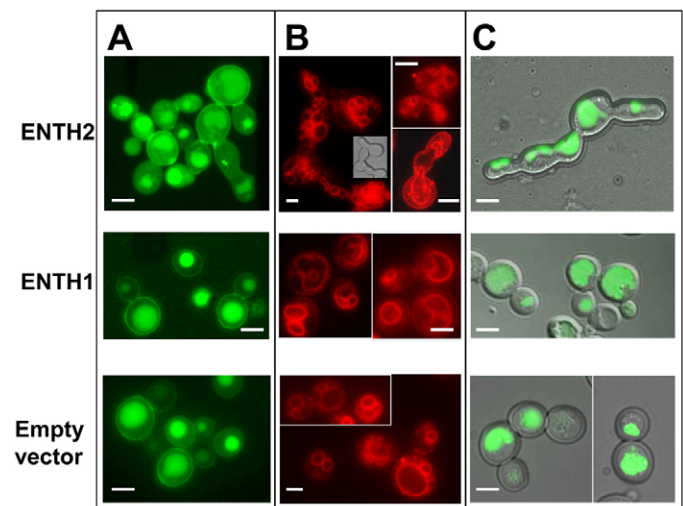

Fig. 2. ENTH domain overexpression does not affect endocytosis. Cells overexpressing the indicated ENTH domains for 6 hours, or transformed with empty vector, were incubated with Lucifer Yellow (for 1 hour at 30°C and then washed with PBS; A) or with the lipophilic dye FM4-64 (for 30 minutes at 4°C, washed and chased for 20 minutes; B). Cells were examined using epifluorescence microscopy with appropriate optics. In B, a DIC image is included to show the severe morphological abnormalities of the ENTH2-overexpressing cell stained with FM4-64. (C) Cells expressing Ste3-GFP were transformed with pMET25::ENTH1, pMET25::ENTH2 or empty vector. Following incubation in selective medium lacking methionine for 6 hours, the cells were examined using epifluorescence microscopy with a GFP filter. Scale bars: 5 μm.

Movie 2). Together, these results suggest that ENTH2 overexpression leads to cell-division defects by affecting either a yet-to-be-found, cargo-specific endocytic pathway and/or a signaling pathway.

The cell-division phenotype depends on ENTH2-specific determinants distinct from those required for inhibition of Cdc42 GAP activity

Considering the high conservation between the ENTH domains of Ent1 and Ent2 (75% identity, 91% homology; Fig. 3A), it is striking that only ENTH2 triggers the cell-division phenotype described above. We reasoned that the determinants responsible for the ENTH2-specific dominant-negative effect must be present within the very few domain regions not conserved in ENTH1. Thus, we targeted two clusters of less-conserved residues for mutagenesis; each cluster mapped to exposed loops (loops 2 and 7) in a predicted three-dimensional model of ENTH2 (Fig. 3A,B). To decrease the probability of affecting the domain conformation, we mutated the amino acids within ENTH2 to the corresponding ENTH1 residues present at the same positions (Fig. 3A). Two ENTH2 loop mutants were generated: ENTH2$^{T33Q, P34V, D40I}$ and ENTH2$^{N112D, S114E, E118Q}$ (referred to as ENTH2 QVI and DEQ, respectively). Each ENTH2 mutant was tested for its ability to trigger the characteristic ENTH2 cell-division phenotype. Our results showed that ENTH2 QVI induced cell-division abnormalities, whereas the ENTH2 DEQ mutant was impaired for phenotype induction (Fig. 3C; supplementary material Fig. S3A). Protein levels of both ENTH2 mutants were similar, ruling out differential abundance or stability as a factor in the ability to induce the phenotype (Fig. 3D). As expected, overexpression of ENTH2 DEQ did not affect cell growth (supplementary material Fig. S3B). Furthermore, we found that ENTH2-specific residues (130-144) located on the terminal helix-8 are also required for phenotype induction (Fig. 3C).

Importantly, replacing the ENTH1 loop 7 and helix-8 with their ENTH2-specific counterparts produced an ENTH1 mutant (NSE-H8) capable of interfering with cell division (Fig. 3C). Thus, we conclude that ENTH2 loop 7 and helix-8 (NSE-H8) are necessary and sufficient for induction of the characteristic phenotype.

Other established ENTH domain functional mutants, such as lipid-binding or helix-0 mutants (Ford et al., 2002) still induced the dominant-negative effect (supplementary material Table S3). These results are very important, although not surprising, as the ENTH2 determinants involved in these functions are completely conserved in the phenotypically inactive ENTH1 (Fig. 3A).

Since the NSE-H8 region is different from the ENTH1-ENTH2-conserved Y100-T104-patch involved in inhibition of GAP activity (Fig. 3A), we hypothesized that the ENTH2-specific phenotype would not rely on GAP inactivation. Consistent with this, the GAP inhibition-impaired mutants ENTH2^{Y100R} and ENTH2^{T104D} (Aguilar et al., 2006) were as efficient as ENTH2 wild type for phenotype induction (Fig. 3C; supplementary material Table S3). Moreover, we overexpressed the isolated GAP domain from Rga2 (lacking ENTH domain binding regions) (Aguilar et al., 2006) to bypass putative ENTH2-dependent GAP inactivation, and observed no significant effect on the cell-division phenotype (supplementary material Table S3). Further, overexpression of a GAP-domain-containing fragment of Bem3 enhanced, instead of suppressed the ENTH2 phenotype (supplementary material Table S3).

Taken together, these results suggest that the cell-division phenotype depends on ENTH2-specific determinants (NSE-H8) that
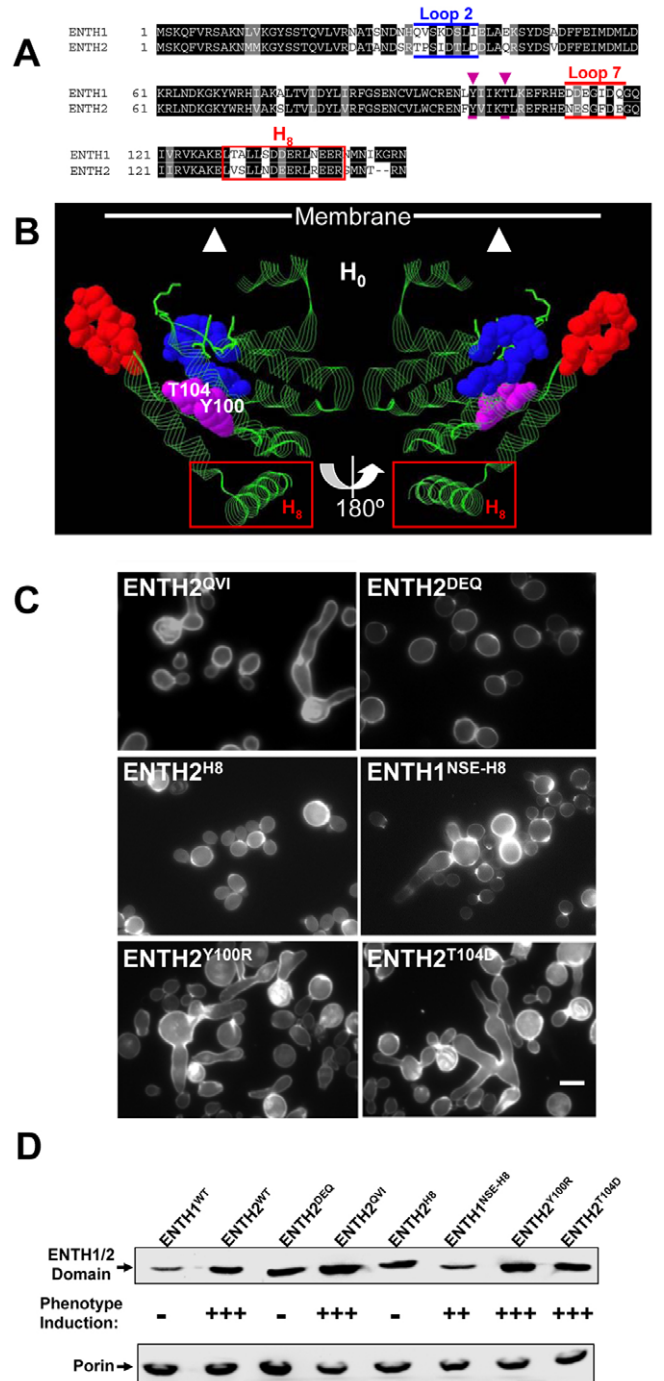

Fig. 3. The ENTH2-overexpression cell-division phenotype depends on determinants located on the ENTH domain terminal loop 7 and helix-8. (A) ENTH1 and ENTH2 domain sequence alignment. Colored bars indicate two loops with a high sequence divergence between ENTH1 and 2 (loop 2: blue; loop 7: red). Purple triangles indicate the Y100-T104 patch involved in Cdc42 GAP inhibition. The red box indicates helix-8 (H$_8$). (B) Two views with a 180 degree rotation of a ENTH2 three-dimensional model. Loops 2 (blue) and 7 (red) as well as residues Y100 and T104 (purple) are highlighted. (C) W303 cells overexpressing different ENTH2 and ENTH1 mutants for 16 hours were processed for microscopy as described in Fig. 1B. Scale bar: 5 µm. (D) Whole cell lysates of cells transformed with the indicated ENTH1 or ENTH2 mutants were analyzed by western blotting with an anti-ENTH domain antibody. The presence of the mitochondrial protein porin (VDAC1), was also investigated and used as loading control. The ability of the different ENTH1/2 mutants to induce the cell-division phenotype given in supplementary material Table S3 is also indicated.

correlate with ENTH2-specific functions unrelated to GAP inhibition.

ENTH2-overexpressing cells show abnormal chitin synthase 2 (Chs2) dynamics

Our data indicate that ENTH2 overexpression does not interfere with endocytosis (Fig. 2) and does not correlate with ENTH domain-mediated GAP inactivation (supplementary material Table S3), but instead leads to abnormal chitin deposition and aberrant septum formation (Fig. 1B-D). Since these latter processes depend on chitin synthase regulation, particularly of Chs2 (Cabib et al., 2001), we examined the localization and dynamics of Chs2-GFP in empty vector transformants, ENTH1- and ENTH2-overexpressing cells. In agreement with normal Chs2-GFP behavior as described by others (Roh et al., 2002), ENTH1-overexpressing and empty-vector-transformed cells had dynamic punctate structures and transient localization at the bud neck during cytokinesis (supplementary material Fig. S4 and supplementary material Movies 3 and 4, respectively). In addition to apparently normal puncta, ENTH2-overexpressing cells had less transient and irregular Chs2-GFP structures (Fig. 4A; supplementary material Movie 5 and see also supplementary material Table S4 for quantitative analysis). We speculate that these structures correlate with abnormal chitin and septum deposition.

ENTH2 dominant-negative effect leads to aberrant septin structures

It has been demonstrated that Chs2 localization requires the assembly of the septin scaffolding proteins at sites of bud emergence and bud neck regions (Longtine and Bi, 2003; Versele and Thorner, 2005). In fact, septin mutant cells have strikingly similar phenotypes to those induced by ENTH2 overexpression (Fig. 1B-D), including chains of cells with aberrant septum formation (Versele and Thorner, 2004; Schmidt et al., 2003; Roh et al., 2002; Cid et al., 1998). Therefore, we asked whether septin assembly is affected by the ENTH2-specific dominant-negative effect. We overexpressed ENTH1 or ENTH2 in cells expressing the septin Cdc3 fused to GFP and analyzed the organization of septin structures. Fig. 4B shows that in empty-vector-transformed and ENTH1-overexpressing cells, Cdc3-GFP normally localized to the bud neck region and formed the characteristic septin collar (Iwase et al., 2006). By contrast, ENTH2 overexpression led to dramatic septin misorganization and mislocalization (Fig. 4C,D). ENTH2-overexpressing cells showed a variety of septin organization defects including the formation of incomplete septin rings (leading to a typical 'bar' phenotype) (see Longtine et al., 2000; Bouquin et al., 2000), ectopic deposition and accumulation of septin fibrillar structures (Fig. 4C). Experiments performed with cells in which ENTH2 expression was induced for 3 hours while released from nocodazole arrest, indicated that the septin defects are among the earlier manifestations of the ENTH2-induced phenotype (Fig. 4D). When septin dynamics were analyzed by live-cell imaging, we observed the formation of filamentous intracellular structures (supplementary material Movie 6). Further supporting these observations, the actomyosin ring component Myo1-GFP that localizes by virtue of its septin-binding activity (Bi et al., 1998) also showed a misorganization-mislocalization pattern in ENTH2-overexpressing cells (supplementary material Fig. S5).
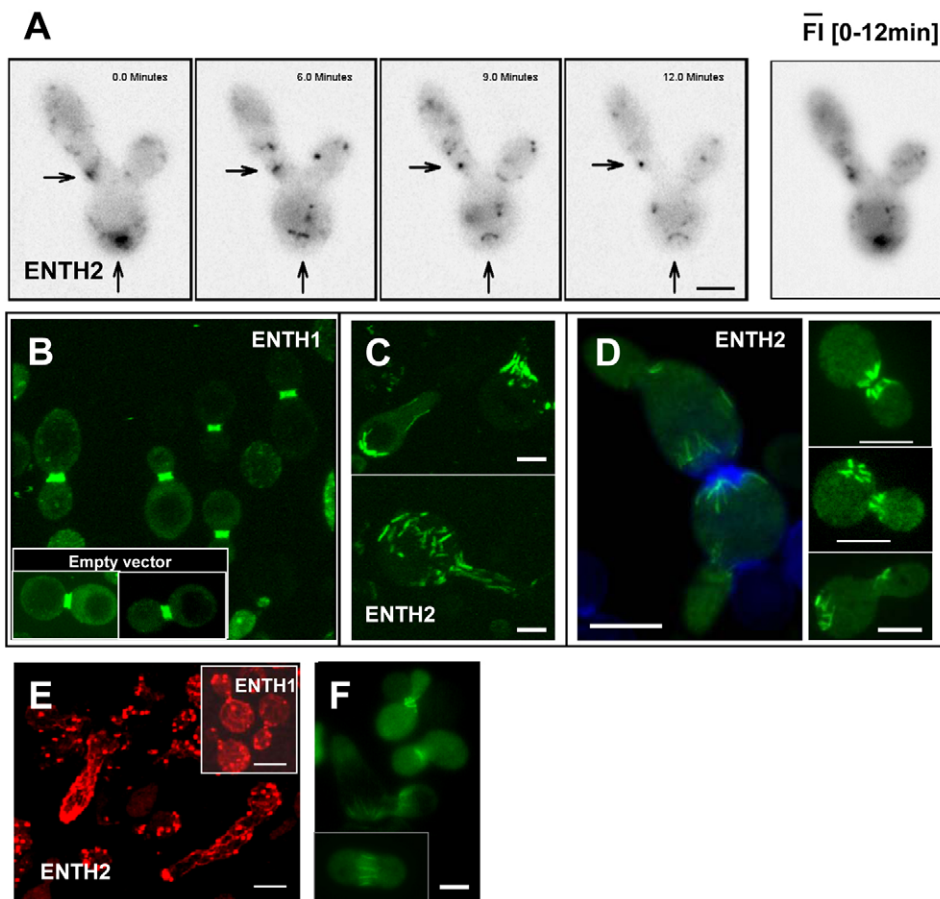

Fig. 4. Overexpression of ENTH2 leads to septin defects and G2-M cell cycle delay. (A) Chs2-GFP dynamics was analyzed by live-cell imaging in cells overexpressing ENTH2 for 4-6 hours following synchronization with nocodazole. Individual time-points taken from Movie 5 in supplementary material are shown. Arrows point to irregular and less transient Chs2-GFP structures (pseudo-colored black Chs2-GFP signal on white background). The last panel shows the average fluorescence intensity (FI) over a 12-minute interval to highlight the presence and distribution of less transient Chs2-GFP regions. Scale bar: 5 μm. (B-D,F) Cells expressing Cdc3-GFP and overexpressing ENTH1 or empty vector (B) or ENTH2 (C,F) were grown for 16 hours in selective medium lacking methionine and processed for fluorescence microscopy. (D) Cells were arrested with nocodazole for 4 hours and ENTH2 overexpression was induced following release from arrest for 3 hours and imaged. Left panel: cells were also stained with calcofluor white (pseudo-colored blue) to show chitin abnormalities (septum). (E) Cells overexpressing ENTH2 or ENTH1 (inset) domains from the *MET25* promoter were grown in liquid medium lacking methionine for 16 hours. Fixed cells were labeled with Rhodamine-phalloidin and examined using epifluorescence microscopy. Scale bars: 5 μm. (F) W303 Δ*swe1* cells expressing Cdc3-GFP and ENTH2 (see above). Scale bar: 5 μm.

ENTH2 dominant-negative effect leads to a G2-M delay

Septins are crucial scaffolding proteins required for organizing the phosphorylation events that target the G2-M checkpoint protein Swe1 for degradation (Longtine et al., 2000; Versele and Thorner, 2005). Degradation of Swe1 is necessary for the nascent bud to transition from apical growth, characteristic of pre-mitotic phase, to isotropic growth (Longtine et al., 2000; Versele and Thorner, 2005).

Since the ENTH2 dominant-negative effect induces abnormalities in septin assembly (Fig. 4C,D; supplementary material Movie 6), we predicted that it would also interfere with the G2-M cell-cycle transition, manifested as exacerbated apical growth (Longtine et al., 2000; Versele and Thorner, 2005). In agreement with this hypothesis, overexpression of ENTH2 (but not ENTH1) led to hyperpolarized growth (e.g. Fig. 1B,C; supplementary material Movie 1). Because it has been shown that G2-M checkpoint activation leads to hyperpolarization of the actin cytoskeleton (Adams and Pringle, 1984), we stained F-actin in ENTH2-overexpressing cells with Rhodamine-phalloidin (Fig. 4E). We observed hyperpolarized actin structures consisting of actin patches at the tip of very elongated buds, and long actin cables oriented towards the mother cell (Fig. 4E).

Further supporting the hypothesis of a septin-defect-dependent delay of entry into mitosis, we established that bypass of the G2-M checkpoint by deleting *SWE1* decreased the formation of chained cells (Fig. 4F). As expected, *SWE1* deletion did not suppress the upstream ENTH2-dependent septin misorganization defect (Fig. 4F). Collectively, our results suggest that the ENTH2-induced septin defect leads to both G2-M transition delay and Chs2 misregulation-mislocalization.

ENTH2 overexpression affects the localization of the septin-organizing protein Bem3

The septin organization pathway can be divided into three characteristic steps (Longtine and Bi, 2003; Versele and Thorner, 2005): recruitment (step I), ring formation (step II) and ring-to-collar transition or maturation (step III). This pathway requires the concerted and sequential action of over a dozen known proteins (Longtine and Bi, 2003; Versele and Thorner, 2005). We hypothesize that ENTH2 directly or indirectly affects key elements of this pathway. To test this hypothesis, following induction of ENTH2 overexpression, we analyzed the intracellular localization of various proteins known to be involved in each phase of septin assembly, using GFP fusions.

Septin recruitment requires the establishment of sites of polarized growth that concentrate activated Cdc42 (Longtine and Bi, 2003; Versele and Thorner, 2005). These regions are characterized by the presence of the Cdc42 guanine nucleotide exchange factor (GEF), Cdc24 (Longtine and Bi, 2003; Versele and Thorner, 2005). We found that in viable ENTH2-overexpressing cells, Cdc24-GFP normally accumulated at the tip of growing or incipient buds (Fig. 5A, left). Similarly, the step I protein and Cdc42 effector Gic1, localized properly to sites of Cdc42 activation (i.e. sites of polarized growth) such as bud tips and bud emergence sites (Fig. 5A, right), indicating that ENTH2 overexpression does not affect localization of the step I protein. Similar results were obtained with empty vector-transformants and ENTH1-overexpressing cells (supplementary material Fig. S6).

The Cdc42 GAPs Rga1, Rga2 and Bem3 have been shown to be required for ring formation (step II) and it has been suggested that they play an effector role rather than just a GTPase-promoting function (Caviston et al., 2003; Smith et al., 2002; Longtine and Bi, 2003). Upon overexpression of ENTH2 (Fig. 5B, left), ENTH1 or transformation with empty vector (supplementary material Fig. S6) Rga1-GFP assembled correctly at the bud neck region. By contrast, in ENTH2-overexpressing cells, Bem3-GFP was observed in aberrant structures and cortical puncta (Fig. 5B, right; Fig. 5D). These structures were absent in ENTH1- or empty vector transformants (supplementary material Fig. S6, Table S5). As expected, the downstream step III protein Gin4 had an abnormal intracellular localization (Fig. 5C). Importantly, Bem3-GFP abnormal structures significantly colocalized with the Cdc3 septin fused to mCherry (Fig. 5D). In our hands Rga2-GFP was mostly cytosolic, preventing us from analyzing its localization.
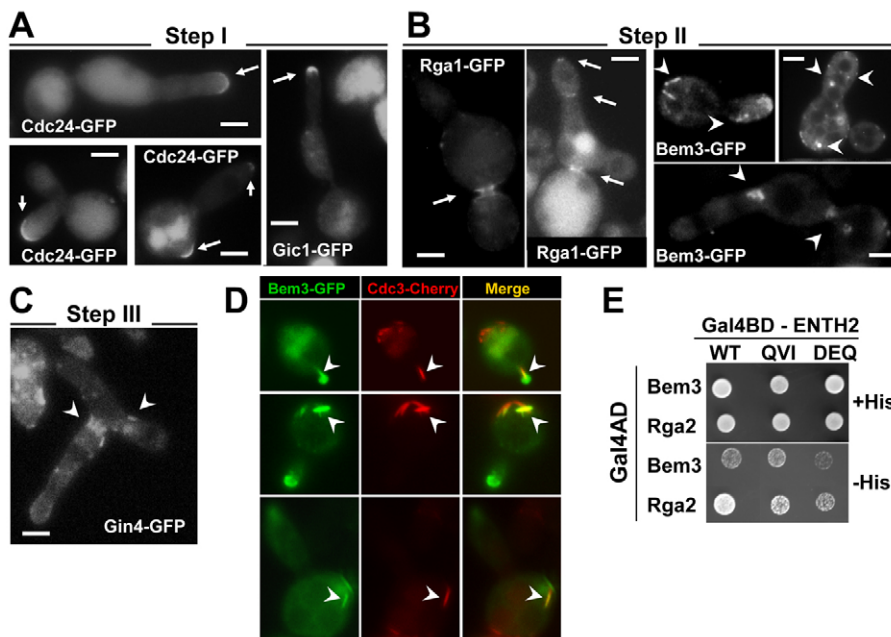

Fig. 5. ENTH2 overexpression affects the intracellular organization of the septin ring formation protein Bem3. (A-C) The intracellular localization of the indicated GFP fusion proteins involved in different steps of the septin assembly pathway was inspected in cells overexpressing ENTH2. Arrows and arrowheads indicate normal and abnormal structures, respectively. (D) W303 cells with an integrated copy of Cdc3-mCherry and expressing Bem3-GFP were arrested, and overexpression of ENTH2 was induced as described in Fig. 4D. Cells were imaged with appropriate optics. (E) The yeast two-hybrid strain AH109 was co-transformed with plasmids encoding the GAL4 DNA binding domain (Gal4BD)-ENTH2 (WT and mutants, as indicated) and the GAL4 activation domain (Gal4AD)-Bem3 or Rga2 fusions. Transformants were grown on plates containing (+His) or lacking (–His) histidine and supplemented with 20 mM 3-amino-triazole.

Interestingly, even though all Cdc42 GAPs are considered to be step II factors (Caviston et al., 2003; Smith et al., 2002; Longtine and Bi, 2003) and all of them are ENTH domain-interaction partners (Aguilar et al., 2006), our results indicated that they can be differentially affected by ENTH2 overexpression. Thus, these results suggest that the ENTH2 dominant-negative effect takes place at the septin-ring formation step by affecting Bem3 function.

We speculate that Bem3 is an ENTH2 target involved in the development of the phenotype based on the following evidence: (1) Within the septin organization pathway, Bem3 is the most upstream element whose intracellular localization was affected by ENTH2 overexpression. (2) ENTH2-induced Bem3 and septin abnormal structures colocalized (Fig. 5D). (3) Overexpression of the Bem3$^{1-114\Delta}$ constitutively active mutant (Kadota et al., 2004) also led to deficiencies in cell division and to septin misorganization (supplementary material Fig. S7). (4) Yeast two-hybrid studies showed that the phenotype-impaired ENTH2 DEQ mutant was less able to interact with Bem3 but still able to bind Rga2 (Fig. 5E; supplementary material Fig. S8). In agreement with its capacity to induce the cell-division phenotype, the ENTH2 QVI mutant bound Bem3 as well as ENTH2 WT (Fig. 5E; supplementary material Fig. S8).

Yeast epsins concentrate at sites of polarized growth and cytokinesis

To gain insight into whether wild-type full-length Ent2 under endogenous conditions participates in the process of cell division, we first used live-cell imaging to observe the intracellular distribution of Ent2-GFP and Ent1-GFP, throughout the cell cycle. Although the distribution of both epsins was punctate and dispersed over the cell surface, they were concentrated at sites of polarized growth, in agreement with their Cdc42-signaling functions (Fig. 6A-D; supplementary material Movie 7). A subtle, but consistent, difference between Ent1-GFP and Ent2-GFP was observed as the latter was often hyperpolarized to the tip of the growing bud (Fig. 6B, arrows; supplementary material Table S6), whereas Ent1-GFP was more homogenously distributed on the bud or neck (Fig. 6B; supplementary material Table S6). Moreover, differences in Ent1

and Ent2 localization has been previously reported in *ark1Δ prk1Δ* mutants (Watson et al., 2001). We therefore speculate that although primarily overlapping, epsins exhibit functional differences. As the cell cycle progressed into M phase, yeast epsins were more homogeneously distributed at the cell surface and accumulated at the bud neck region (Fig. 6C,D). As expected for a truncation lacking the localization determinants present in the C-terminal module, GFP-ENTH2 showed a mostly diffuse intracellular pattern, although it was slightly enriched in sites of polarized growth and in the bud neck regions (Fig. 6E).

Epsin localization and recruitment did not depend on Bem3 as Ent2-GFP properly localized in *bem3Δ* cells (supplementary material Fig. S9A). Furthermore, ENTH2-GFP partial enrichment at sites of polarized growth was also mostly unaffected by *BEM3* deletion (supplementary material Fig. S9B). Complementary experiments showed that in *ent2Δ* cells Bem3-GFP was recruited to the proper membrane locations (supplementary material Fig. S9C). However, when compared with control strains (supplementary material Fig. S6) *ent2Δ* cells had a less polarized Bem3-GFP puncta and less well-defined bud neck structures (supplementary material Fig. S9C). These results suggest that although both proteins are independently recruited to the plasma membrane, epsin 2 contributes to proper Bem3 organization or regulation at sites of polarized growth and bud neck regions.

Full-length Ent2 rescues the defect in cell division induced by Bem3$^{Δ1-114}$ overexpression

Kadota and collaborators found that Bem3 septin-organizing activity can be enhanced by deletion of the N-terminal 1-114 fragment, suggesting that this region plays a self-regulatory role (Kadota et al., 2004). The Bem3$^{Δ1-114}$ active mutant suppresses the growth, morphological and septin abnormalities displayed by *bni1Δ cla4-75-td* mutants (Kadota et al., 2004). However, we observed that overexpression of the activated Bem3$^{Δ1-114}$ mutant in a wild-type background led to a cell-division phenotype with a 12-30% penetrance (supplementary material Fig. S7; Fig. 7). Moreover, co-overexpression of Bem3$^{Δ1-114}$ and ENTH2 led to a more severe phenotype than either of the two independently (Fig. 7;
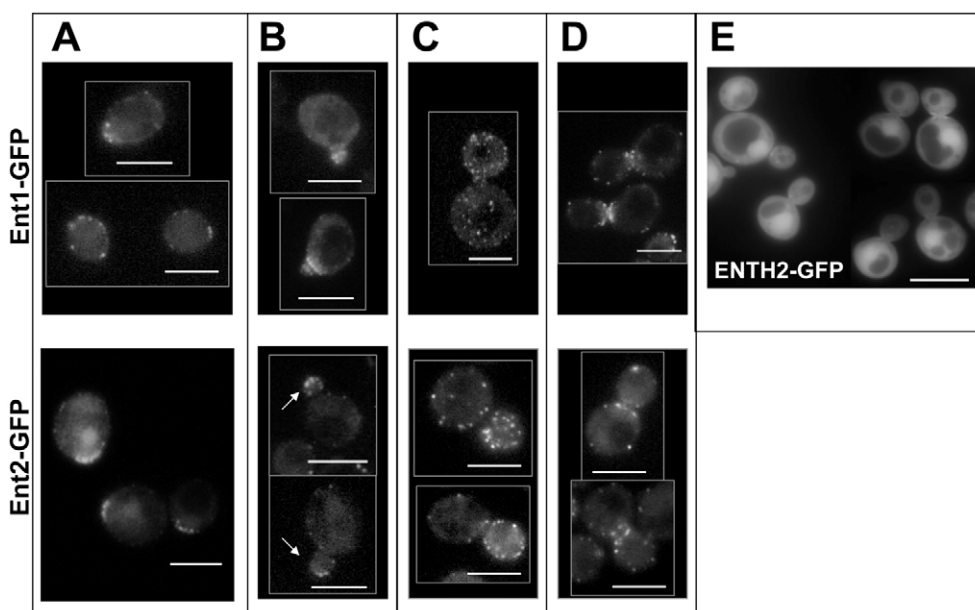

Fig. 6. Intracellular localization of yeast epsin-GFP fusions during the cell cycle. Cells expressing genome-integrated epsin-GFP fusions were cell cycle synchronized using nocodazole. After arrest release, cells were observed by epifluorescence microscopy using appropriate filters. (A-D) Cells at different stages of the cell cycle were identified by morphological characteristics. Scale bars: 5 μm. (E) Cells expressing ENTH2-GFP from a single copy plasmid. Epifluorescence microscopy revealed a diffuse localization at the plasma membrane, in the cytosol and even in the nucleus, suggesting the presence of weak localization determinants.
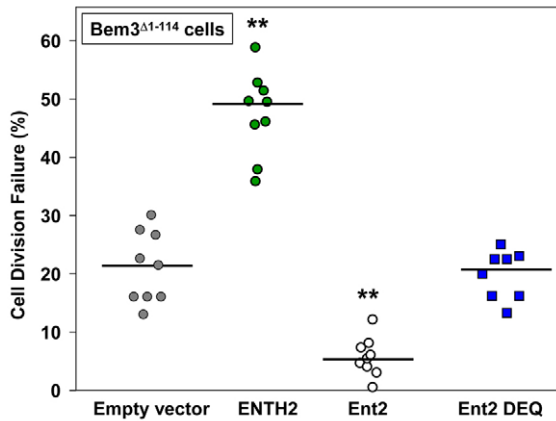
Fig. 7. Ent2 rescues the cell-division phenotype induced by Bem3$^{\Delta 1\text{-}114}$. The percentage of failed cell division attempts for Bem3$^{\Delta 1\text{-}114}$-expressing cells transformed with the indicated vector or construct was estimated as described in Fig. S1B in the supplementary material, by counting more than 200 cells. Counts were repeated nine times for each medium (each is indicated by a horizontal lines). Statistical significance of phenotype enhancement (ENTH2) or rescue (Ent2) with respect to cells transformed with empty vector was assessed using the Wilcoxon test (**$P<0.05$).
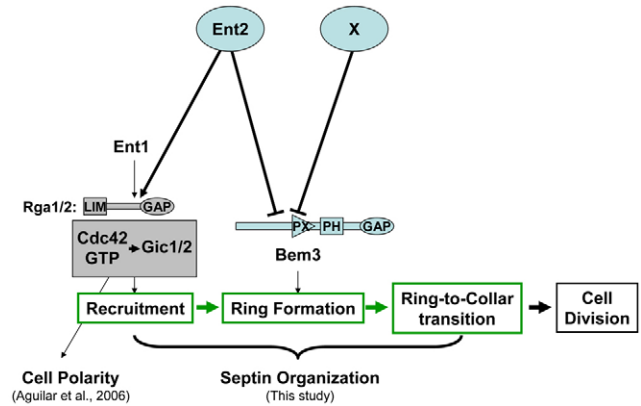

Fig. 8. Model for yeast epsin function in cell polarity and division. Yeast epsins, Ent1 and Ent2, bind Cdc42 GAPs Rga1 and Rga2 to activate Cdc42 signaling towards cell polarity (Gic1/2-dependent) establishment pathways (Aguilar et al., 2006). From this study we propose that epsins (particularly Ent2) affect septin organization pathways by interacting with another GAP, Bem3, which is also involved in septin ring formation. This novel septin-regulatory circuit would be redundant with another pathway (X), yet to be identified.

supplementary material Table S3). These results suggested that ENTH2 overexpression does not inhibit Bem3 function as it enhanced rather than suppressed the phenotype induced by the active Bem3$^{\Delta 1\text{-}114}$ mutant. Since ENTH2 exerts a dominant-negative effect, we hypothesize that the enhancement of the Bem3$^{\Delta 1\text{-}114}$ phenotype results from preventing endogenous Ent2 to interact with Bem3$^{\Delta 1\text{-}114}$. Therefore, we predicted that favoring the formation of the Ent2-Bem3$^{\Delta 1\text{-}114}$ complex should alleviate the cell-division phenotype induced by the activated Bem3 mutant. In agreement, full-length Ent2 overexpression suppressed the phenotype induced by Bem3$^{\Delta 1\text{-}114}$ (Fig. 7). Importantly the Ent2 DEQ mutant, impaired in binding to Bem3, failed to rescue the Bem3$^{\Delta 1\text{-}114}$ phenotype (Fig. 7). Our data therefore, suggest that Ent2 binds (via the ENTH domain) and downregulates Bem3 activity, perhaps by routing Bem3 to endocytic sites.

Discussion

This study establishes for the first time the existence of a link between the yeast endocytic adaptor epsin-2 (Ent2) and the process of cell division, and it also proves that different epsin paralogs have specific signaling functions.

Here, we used a dominant-negative approach, based on overexpression of the ENTH domain, to investigate potential signaling differences between the two yeast epsin paralogs Ent1 and Ent2. Cells overexpressing ENTH2 (but not ENTH1) displayed striking defects in cell division (Fig. 1; supplementary material Movie 1). Our data indicate that the ENTH2-specific dominant-negative effect is not a direct consequence of inhibition of Cdc42 GAP activity [common to both ENTH1 and ENTH2 (Aguilar et al., 2006)] but is rather the result of interference with signaling pathways involved in septin organization. These abnormalities in septin assembly are likely to cause aberrant septum formation (Fig. 1C,D), perhaps via Chs2 mistargeting or misregulation and a delay in entering mitosis (Fig. 4). It should be noted that although epsin deletion dramatically increased the extent of the ENTH2 phenotype, it does not phenocopy it. Thus, we hypothesize that a parallel septin regulatory pathway may exist (Fig. 8).

Based on our previous findings, one would predict that overexpression of the ENTH domain would lead to Cdc42 hyperactivation via GAP inhibition (Aguilar et al., 2006). In agreement, ENTH1-overexpressing cells show mildly enhanced Cdc42 signaling (e.g. cells with slightly elongated buds and enlarged cells with small buds; Fig. 1B, insets). Although ENTH2 overexpression may also lead to enhanced Cdc42 activation, the cell-division phenotype does not seem to be compatible with Cdc42 hypersignaling, as an ENTH-insensitive GAP domain was unable to rescue or alleviate the manifestations of the phenotype (supplementary material Table S3). Moreover, mutations that impaired ENTH GAP activity inhibition (Y100 and T104) could still induce the cell-division phenotype (Fig. 3C; supplementary material Table S3). In fact, ENTH2 overexpression phenocopies impairment (and not hyperactivation) of Cdc42 signaling (Richman et al., 1999; Richman et al., 2002). Thus, similar to ENTH2 overexpression, expression of Cdc42 mutant proteins defective for signaling [unable to bind effectors (Richman et al., 1999; Richman and Johnson, 2000; Richman et al., 2002) or cycling impaired (Gladfelter et al., 2002)] led to defects in cell division and to misorganization of septin. Interestingly, some of these mutants have been reported to interact poorly with the septin-organizing protein Bem3 (Richman and Johnson, 2000). Furthermore, septin deficiencies produced phenotypes very similar to those observed upon ENTH2 overexpression, including elongated, chained buds and aberrant septa (Versele and Thorner, 2004; Schmidt et al., 2003; Roh et al., 2002; Cid et al., 1998).

Since known critical ENTH domain functions, such as membrane bending [via helix-0 (Ford et al., 2002)] or GAP inhibition [via YT-patch (Aguilar et al., 2006)] are not required for the induction of the cell-division phenotype (Fig. 3C; supplementary material Table S3), our results suggest a novel ENTH2-specific activity. Furthermore, we mapped the ENTH2 domain determinants necessary and sufficient for this activity to loop 7 and helix-8 of ENTH2 (NSE-H8 site; Fig. 3). We propose that the NSE-H8 region constitutes an Ent2 specialization that enables high-efficiency regulation of the septin organization

pathway most probably through a strong interaction with the septin organization protein Bem3 (and Cdc42 GAP). We hypothesize that, in contrast to Rga1/2 binding (Aguilar et al., 2006), the Ent2-Bem3 interaction has a unique signaling role distinct from inhibition of GAP activity. In fact, whereas ENTH2 binds Rga1 and Rga2 at a site next to their GAP domain (Aguilar et al., 2006) (Fig. 8), it binds Bem3 at its N-terminus [the opposite end to the GAP domain (Aguilar et al., 2006)] (Fig. 8). Although Rga1 and Rga2 were reasonably efficient at rescuing ENTH1^{Y100R} cell polarity defects, Bem3 was extremely weak (Aguilar et al., 2006). Also, ENTH domain YT patch mutants were mostly defective in their interaction with Rga1 and Rga2 but have been shown to be only slightly impaired in Bem3 binding (Aguilar et al., 2006). By contrast, the DEQ mutation greatly affected interaction of ENTH2 with Bem3 (Fig. 5E; supplementary material Fig. S8) with no discernible consequences on Rga2 binding (Fig. 5E; supplementary material Fig. S8). However, *BEM3* deletion does not completely abolish the ENTH2-induced abnormalities in cell division (supplementary material Table S3), suggesting the existence of other ENTH2 targets besides Bem3.

Our data indicate that yeast epsins localize to dynamic cortical structures (Fig. 6; supplementary material Movie 7) present all over the cell cortex, but concentrated in known sites of Cdc42 signaling (Richman et al., 2002) such as bud necks and regions of polarized growth (Fig. 6). These observations are in agreement with our hypothesis that epsins coordinate Cdc42 signaling (i.e. cell polarity and cell division pathways) with endocytosis, in time and space. The articulation of these processes is crucial for proper cell physiology; in fact, it has been proposed that endocytosis of Cdc42 is central to the consolidation of incipient cell polarity sites (Marco et al., 2007). Similarly to Cdc42, Bem3 has been proposed to undergo delivery to and endocytosis from polarized punctate structures (Ozbudak et al., 2005; Knaus et al., 2007). Genetic evidence presented in this study suggested that the physiological role of Ent2 is to downregulate Bem3 function (Fig. 7). Specifically, we speculate that Ent2 binds Bem3 via its ENTH domain and links it with endocytic structures. Supporting this idea, we verified that the ENTH2 dominant-negative approach (i.e. by interfering with the Ent2-Bem3 interaction) led to accumulation of depolarized Bem3-GFP puncta at the cortex (Fig. 5B, right). Interestingly, the constitutively activated mutant Bem3$^{\Delta1-114}$ showed an impaired intracellular localization (mainly cytosolic, compare supplementary material Fig. S7B and Fig. S6) and also triggered defects in cell division with abnormal septin structures (supplementary material Fig. S7). Moreover, epsin-deficient cells showed irregular Bem3-GFP membrane structures (supplementary material Fig. S9). Therefore, we suggest that although independently recruited to membranes, Ent2 contributes to Bem3 polarization and/or to polarity maintenance. We speculate that once at the cortex, Bem3 is engaged by Ent2 (via its ENTH domain) and concentrated in the highly polarized epsin2-rich (and Cdc42-abundant) regions. This complex would be incorporated into endocytic structures (via epsin classical adaptor function) and removed from the plasma membrane, imposing the dynamics of endocytosis on the Bem3 signaling molecule.

Whether epsin binding also affects Bem3-specific functions and activities, remains to be established. In fact, beyond Bem3 GAP activity, a comprehensive study of its biochemical and signaling properties is still needed and constitutes the focus of intense research in our laboratory.

How does this novel function of yeast epsin 2 in cell division fit within the existing body of knowledge in the field? Boucrot and Kirchhausen have recently demonstrated that endocytosis is a central mechanism for reduction of cell surface area during cell division (Boucrot and Kirchhausen, 2007). It has been also reported that knockout or knockdown of elements of the endocytic machinery can affect the process of cell division (Tang and Cai, 1996; Niswonger and O'Halloran, 1997; Boucrot and Kirchhausen, 2007). Along the same lines, our results support this emerging concept of an active role of the endocytic machinery in cell division and further point to a signaling role for epsin in the process.

Materials and Methods
Reagents
Materials were purchased from Fisher Scientific (Fairlawn, NJ) or Sigma (St Louis, MO) unless stated otherwise. Plasmids and strains used in this study are listed in supplementary material Tables S1 and S2 respectively.

DNA manipulations
DNA constructs were made using standard techniques. Site directed mutagenesis was done using the QuikChange kit (Stratagene, La Jolla, CA).

Preparation of a mouse polyclonal anti-yeast ENTH domain antibody
Three-month-old BALB/c mice were inoculated with 20 μg of bacterially produced and purified yeast ENTH1 and/or 2 in complete Freund's adjuvant (CFA; Sigma, St Louis, MO) in phosphate-buffered saline (PBS). The animals were injected intraperitoneally at days 0 and 15 and bled at day 30. The titer of anti-ENTH antibodies was monitored by ELISA with peroxidase-labeled donkey IgG anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Although detection of endogenous yeast epsin in whole cell lysates by western blotting was very weak, the polyclonal serum was able to generate a detectable signal for overexpressed ENTH domains at a dilution 1:1000.

Yeast culture conditions and transformation procedures
Yeast strains were grown in standard yeast extract-peptone-dextrose (YPD) or synthetic medium with dextrose, lacking appropriate amino acids for plasmid maintenance at 30°C for 3-4 days, unless indicated otherwise. For cell-cycle arrest, cells were pelleted, resuspended in YPD and arrested by adding 15 μg/ml nocodazole for 4 hours. The percentage of cells arrested was morphologically determined using bright-field microscopy. For expression of ENTH1 or ENTH2, arrested cells were then washed twice with sterile water and resuspended in synthetic medium without methionine for 3 hours. For liquid culture assays, ~10^5 cells were added to 10 ml selective medium and incubated at 30°C for 36 hours in the absence of methionine. The optical density (OD) at 600 nm was measured. Yeast was transformed by the lithium acetate method according to the Clontech yeast handbook.

Western blotting
Cell lysates expressing wild-type and mutant ENTH domains were prepared by harvesting cells grown for 4 hours in selective medium lacking methionine, vortexing with glass beads and boiling in Laemmli's protein sample buffer. Lysates were resolved by SDS-PAGE and the proteins of interest were detected by western blotting. The presence of ENTH domains was revealed by using a mouse polyclonal anti-yeast ENTH domain serum at 1:1000 dilution or a mouse monoclonal anti-HA antibody (Covance, Princeton, NJ) at 1:2000 dilution. The loading control, VDAC1 (porin) was detected by using a specific mouse monoclonal antibody (Mitosciences, Eugene, OR) at 1:1000 dilution.

Microscopy
Images were acquired using a Zeiss Axiovert 200M in white-field mode and an Olympus IX-71 inverted microscope equipped with a variable angle epifluorescence illumination system. Image processing was performed with Adobe Photoshop and ImageJ software. For calcofluor white (CFW) staining yeast cells were incubated with 1 mg/ml CFW solution in PBS for 10 minutes at room temperature, followed by three washes with PBS. Cell viability was ascertained by pelleting 0.5 OD$_{600nm}$ cells, resuspending in 100 μl YPD containing 30% (v/v) Methylene blue solution (1 mg/ml in sterile water) and imaging with a bright-field microscope. For actin and DNA staining, cells were fixed with 4% formaldehyde, washed three times with PBS, permeabilized with 0.1% Triton X-100 and labeled with Rhodamine-phalloidin and DAPI, respectively. For live-cell imaging, 2-5 OD$_{600nm}$ of cells were pelleted and resuspended in 100 μl of medium (pH 7.5). 10 μl of cells were spotted on selective media-embedded agarose beds and imaged at 100× at regular intervals with appropriate optics.

Electron microscopy

Cells were processed according to the method of Collins et al. (Collins et al., 2000) to preserve the cell wall integrity. Briefly, cells were fixed with 3% glutaraldehyde and embedded in 2% ultralow-temperature agarose. The cells were post-fixed in 3% KMnO$_4$ and embedded in Spurr's resin. Sections were cut and observed on a Phillips EM410 microscope.

Endocytosis assays

Cells were allowed to express ENTH1and ENTH2 for 6 hours and then were stained with Methylene blue just prior to microscopy to analyze viable cells alone. FM4-64 and Lucifer yellow uptake assays were performed according to the method of Baggett et al. (Baggett et al., 2003).

For receptor-mediated endocytosis, cells expressing Ste3-GFP were transformed with *pMET25::ENTH1/2* or empty vector. Transformants were grown overnight in selective medium, pelleted and resuspended in medium lacking methionine for ENTH1/2 expression. After 6 hours, Ste3-GFP localization was visualized using a GFP filter.

Quantification of the cell-division phenotype

The frequency of failed and successful cell division events, respectively, were determined in unsynchronized cultures using the following calculations: number of failed division attempts = Σ[(number of nuclei counted/multinucleated cell) −1] (we considered a cell as multinucleated when containing at least three nuclei); number of successful division attempts = [number of budding (bi-nucleated) cells] + (number of mono-nucleated cells/2). The percentage of failed division attempts = 100 × number of failed division attempts/total number (successful+failed) of division attempts.

References

Adams, A. E. and Pringle, J. R. (1984). Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant Saccharomyces cerevisiae. *J. Cell Biol.* **98**, 934-945.

Aguilar, R. C., Watson, H. A. and Wendland, B. (2003). The yeast Epsin Ent1 is recruited to membranes through multiple independent interactions. *J. Biol. Chem.* **278**, 10737-10743.

Aguilar, R. C., Longhi, S. A., Shaw, J. D., Yeh, L. Y., Kim, S., Schon, A., Freire, E., Hsu, A., McCormick, W. K., Watson, H. A. et al. (2006). Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. *Proc. Natl. Acad. Sci. USA* **103**, 4116-4121.

Baggett, J. J., Shaw, J. D., Sciambi, C. J., Watson, H. A. and Wendland, B. (2003). Fluorescent labeling of yeast. *Curr. Protoc. Cell Biol.* Chapter 4, Unit 4 13.

Bi, E., Maddox, P., Lew, D. J., Salmon, E. D., McMillan, J. N., Yeh, E. and Pringle, J. R. (1998). Involvement of an actomyosin contractile ring in Saccharomyces cerevisiae cytokinesis. *J. Cell Biol.* **142**, 1301-1312.

Boucrot, E. and Kirchhausen, T. (2007). Endosomal recycling controls plasma membrane area during mitosis. *Proc. Natl. Acad. Sci. USA* **104**, 7939-7944.

Bouquin, N., Barral, Y., Courbeyrette, R., Blondel, M., Snyder, M. and Mann, C. (2000). Regulation of cytokinesis by the Elm1 protein kinase in *Saccharomyces cerevisiae*. *J. Cell Sci.* **113**, 1435-1445.

Cabib, E., Roh, D. H., Schmidt, M., Crotti, L. B. and Varma, A. (2001). The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J. Biol. Chem.* **276**, 19679-19682.

Caviston, J. P., Longtine, M., Pringle, J. R. and Bi, E. (2003). The role of Cdc42p GTPase-activating proteins in assembly of the septin ring in yeast. *Mol. Biol. Cell* **14**, 4051-4066.

Cid, V. J., Adamikova, L., Cenamor, R., Molina, M., Sanchez, M. and Nombela, C. (1998). Cell integrity and morphogenesis in a budding yeast septin mutant. *Microbiology* **144**, 3463-3474.

Collins, C. S., Kalish, J. E., Morrell, J. C., McCaffery, J. M. and Gould, S. J. (2000). The peroxisome biogenesis factors pex4p, pex22p, pex1p, and pex6p act in the terminal steps of peroxisomal matrix protein import. *Mol. Cell. Biol.* **20**, 7516-7526.

Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F. and Wrana, J. L. (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat. Cell Biol.* **5**, 410-421.

Ford, M. G., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R. and McMahon, H. T. (2002). Curvature of clathrin-coated pits driven by epsin. *Nature* **419**, 361-366.

Gladfelter, A. S., Bose, I., Zyla, T. R., Bardes, E. S. and Lew, D. J. (2002). Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *J. Cell Biol.* **156**, 315-326.

Gonzalez-Gaitan, M. (2003). Signal dispersal and transduction through the endocytic pathway. *Nat. Rev. Mol. Cell. Biol.* **4**, 213-224.

Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S. and Takenawa, T. (2001). Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* **291**, 1047-1051.

Iwase, M. and Toh-e, A. (2001). Nis1 encoded by YNL078W: a new neck protein of Saccharomyces cerevisiae. *Genes Genet. Syst.* **76**, 335-343.

Iwase, M., Luo, J., Nagaraj, S., Longtine, M., Kim, H. B., Haarer, B. K., Caruso, C., Tong, Z., Pringle, J. R. and Bi, E. (2006). Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. *Mol. Biol. Cell* **17**, 1110-1125.

Jenna, S., Hussain, N. K., Danek, E. I., Triki, I., Wasiak, S., McPherson, P. S. and Lamarche-Vane, N. (2002). The activity of the GTPase-activating protein CdGAP is regulated by the endocytic protein intersectin. *J. Biol. Chem.* **277**, 6366-6373.

Kadota, J., Yamamoto, T., Yoshiuchi, S., Bi, E. and Tanaka, K. (2004). Septin ring assembly requires concerted action of polarisome components, a PAK kinase Cla4p, and the actin cytoskeleton in Saccharomyces cerevisiae. *Mol. Biol. Cell* **15**, 5329-5345.

Kaksonen, M., Toret, C. P. and Drubin, D. G. (2003). A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell* **123**, 305-320.

Knaus, M., Pelli-Gulli, M. P., van Drogen, F., Springer, S., Jaquenoud, M. and Peter, M. (2007). Phosphorylation of Bem2p and Bem3p may contribute to local activation of Cdc42p at bud emergence. *EMBO J.* **26**, 4501-4513.

Longtine, M. S. and Bi, E. (2003). Regulation of septin organization and function in yeast. *Trends Cell Biol.* **13**, 403-409.

Longtine, M. S., Theesfeld, C. L., McMillan, J. N., Weaver, E., Pringle, J. R. and Lew, D. J. (2000). Septin-dependent assembly of a cell cycle-regulatory module in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **20**, 4049-4061.

Luo, J. Y., Vallen, E. A., Dravis, C., Tcheperegine, S. E., Drees, B. and Bi, E. F. (2004). Identification and functional analysis of the essential and regulatory light chains of the only type II myosin Myo1p in Saccharomyces cerevisiae. *J. Cell Biol.* **165**, 843-855.

Maldonado-Baez, L., Dores, M. R., Perkins, E. M., Drivas, T. G., Hicke, L. and Wendland, B. (2008). Interaction between Epsin/Yap180 adaptors and the scaffolds Ede1/Pan1 is required for endocytosis. *Mol. Biol. Cell* **19**, 2936-2948.

Marco, E., Wedlich-Soldner, R., Li, R., Altschuler, S. J. and Wu, L. F. (2007). Endocytosis optimizes the dynamic localization of membrane proteins that regulate cortical polarity. *Cell* **129**, 411-422.

Nichols, J. T., Miyamoto, A. and Weinmaster, G. (2007). Notch signaling-constantly on the move. *Traffic* **8**, 959-969.

Niswonger, M. L. and O'Halloran, T. J. (1997). A novel role for clathrin in cytokinesis. *Proc. Natl. Acad. Sci. USA* **94**, 8575-8578.

Okuzaki, D., Tanaka, S., Kanazawa, H. and Nojima, H. (1997). Gin4 of S-cerevisiae is a bud neck protein that interacts with the Cdc28 complex. *Genes Cells* **2**, 753-770.

Ozbudak, E. M., Becskei, A. and van Oudenaarden, A. (2005). A system of counteracting feedback loops regulates Cdc42p activity during spontaneous cell polarization. *Dev. Cell* **9**, 565-571.

Richman, T. J. and Johnson, D. I. (2000). Saccharomyces cerevisiae cdc42p GTPase is involved in preventing the recurrence of bud emergence during the cell cycle. *Mol. Cell. Biol.* **20**, 8548-8559.

Richman, T. J., Sawyer, M. M. and Johnson, D. I. (1999). The Cdc42p GTPase is involved in a G2/M morphogenetic checkpoint regulating the apical-isotropic switch and nuclear division in yeast. *J. Biol. Chem.* **274**, 16861-16870.

Richman, T. J., Sawyer, M. M. and Johnson, D. I. (2002). Saccharomyces cerevisiae Cdc42p localizes to cellular membranes and clusters at sites of polarized growth. *Eukaryot. Cell* **1**, 458-468.

Roh, D. H., Bowers, B., Schmidt, M. and Cabib, E. (2002). The septation apparatus, an autonomous system in budding yeast. *Mol. Biol. Cell* **13**, 2747-2759.

Rönicke, V., Graulich, W., Mumberg, D., Müller, R. and Funk, M. (1997). Use of conditional promoters for expression of heterologous proteins in Saccharomyces cerevisiae. *Methods Enzymol.* **283**, 313-322.

Rosenthal, J. A., Chen, H., Slepnev, V. I., Pellegrini, L., Salcini, A. E., Di Fiore, P. P. and De Camilli, P. (1999). The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. *J. Biol. Chem.* **274**, 33959-33965.

Schmidt, M., Varma, A., Drgon, T., Bowers, B. and Cabib, E. (2003). Septins, under Cla4p regulation, and the chitin ring are required for neck integrity in budding yeast. *Mol. Biol. Cell* **14**, 2128-2141.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27.

Smith, G. R., Givan, S. A., Cullen, P. and Sprague, G. F., Jr (2002). GTPase-activating proteins for Cdc42. *Eukaryot. Cell* **1**, 469-480.

Takahashi, S. and Pryciak, P. M. (2007). Identification of novel membrane-binding domains in multiple yeast Cdc42 effectors. *Mol. Biol. Cell* **18**, 4945-4956.

Tang, H. Y. and Cai, M. (1996). The EH-domain-containing protein Pan1 is required for normal organization of the actin cytoskeleton in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **16**, 4897-4914.

Tolliday, N., Pitcher, M. and Li, R. (2003). Direct evidence for a critical role of myosin II in budding yeast cytokinesis and the evolvability of new cytokinetic mechanisms in the absence of myosin II. *Mol. Biol. Cell* **14**, 798-809.

Toret, C. P., Lee, L., Sekiya-Kawasaki, M. and Drubin, D. G. (2008). Multiple pathways regulate endocytic coat disassembly in Saccharomyces cerevisiae for optimal downstream trafficking. *Traffic* **9**, 848-859.

Urbanowski, J. L. and Piper, R. C. (2001). Ubiquitin sorts proteins into the intralumenal degradative compartment of the late-endosome/vacuole. *Traffic* **2**, 622-630.

Versele, M. and Thorner, J. (2004). Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J. Cell Biol.* **164**, 701-715.

Versele, M. and Thorner, J. (2005). Some assembly required: yeast septins provide the instruction manual. *Trends Cell Biol.* **15**, 414-424.

von Zastrow, M. and Sorkin, A. (2007). Signaling on the endocytic pathway. *Curr. Opin. Cell Biol.* **19**, 436-445.

Watson, H. A., Cope, M. J., Groen, A. C., Drubin, D. G. and Wendland, B. (2001). *In vivo* role for actin-regulating kinases in endocytosis and yeast epsin phosphorylation. *Mol. Biol. Cell* **12**, 3668-3679.

Wendland, B. (2002). Epsins: adaptors in endocytosis? *Nat. Rev. Mol. Cell. Biol.* **3**, 971-977.

Ziman, M., O'Brien, J. M., Ouellette, L. A., Church, W. R. and Johnson, D. I. (1991). Mutational analysis of CDC42Sc, a Saccharomyces cerevisiae gene that encodes a putative GTP-binding protein involved in the control of cell polarity. *Mol. Cell. Biol.* **11**, 3537-3544.