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Proper timing of cytokinesis is regulated by Schizosaccharomyces pombe Etd1

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Cytokinesis must be initiated only after chromosomes have been segregated in anaphase and must be terminated once cleavage is completed. We show that the fission yeast protein Etd1 plays a central role in both of these processes. Etd1 activates the guanosine triphosphatase (GTPase) Spg1 to trigger signaling through the septum initiation network (SIN) pathway and onset of cytokinesis. Spg1 is activated in late anaphase when spindle elongation brings spindle pole body (SPB)–localized Spg1 into proximity with its activator Etd1 at cell tips, ensuring that cytokinesis is only initiated when the spindle is fully elongated. Spg1 is active at just one of the two SPBs during cytokinesis. When the actomyosin ring finishes constriction, the SIN triggers disappearance of Etd1 from the half of the cell with active Spg1, which then triggers Spg1 inactivation. Asymmetric activation of Spg1 is crucial for timely inactivation of the SIN. Together, these results suggest a mechanism whereby cell asymmetry is used to monitor cytoplasmic partitioning to turn off cytokinesis signaling.

Introduction

Coordination of cytokinesis with mitosis is required to ensure proper chromosome segregation and genomic stability. It is unclear how cells determine when to initiate and when to terminate cytokinesis. In the fission yeast Schizosaccharomyces pombe, cytokinesis is regulated by the septum initiation network (SIN). Activation of the SIN in late anaphase triggers initiation of cytokinesis. In SIN mutants, the actomyosin contractile ring disassembles prematurely, causing cells to fail cytokinesis and become multinucleate. In contrast, failure to inactivate the SIN triggers repeated rounds of cytokinesis and defective entry into the next interphase (for reviews see Balasubramanian et al., 2004; Doxsey et al., 2005; Krapp and Simanis, 2008). How SIN activity is coordinated with cell cycle progression is unclear.

SIN signaling requires activation of the GTPase Spg1 (Schmidt et al., 1997). Once activated, Spg1-GTP binds the Cdc7 kinase and recruits it to the spindle pole body (SPB; Sohrmann et al., 1998). SPB-localized Cdc7 then promotes activation of the Sid2 protein kinase (Sparks et al., 1999). Active Sid2 translocates from the SPB to the cell division site to trigger cytokinesis. Most GTPases are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Although a GAP for Spg1 is known, no GEF has been identified. The Byr4 and Cdc16 proteins form a two-component GAP, which inactivates Spg1 (Furge et al., 1998). The Byr4–Cdc16 complex inhibits cytokinesis in interphase by binding to Spg1 at the SPB and keeping it in the inactive GDP-bound state (Cerutti and Simanis, 1999). Upon entry into mitosis, Byr4–Cdc16 leaves the SPBs, allowing Spg1 to become partially active at both SPBs (Sohrmann et al., 1998; Cerutti and Simanis, 1999). However, further activation of the SIN is blocked by Cdk1 activity (Guerin et al., 2000; Li et al., 2000; Chang et al., 2001; Krapp et al., 2008). At anaphase onset, two things happen: (1) loss of Cdk1 activity allows increased SIN activity (Guerin et al., 2000; Li et al., 2000; Chang et al., 2001; Krapp et al., 2008), and (2) Spg1 is inactivated at one of the two SPBs by Byr4–Cdc16 (Cerutti and Simanis, 1999; Li et al., 2000). It is known that Spg1 remains active at the newer, or daughter, SPB (Sohrmann et al., 1998; Grallert et al., 2004), but the purpose of asymmetric activation of the SIN pathway is unknown. Although return of Byr4–Cdc16 to the SPB or SPBs is important for SIN inactivation after completion of cytokinesis, it has been unclear...
how cells sense completion of cytokinesis and turn off Spg1 and SIN signaling.

In the budding yeast *Saccharomyces cerevisiae*, the pathway analogous to the SIN is known as the mitotic exit network (MEN; for reviews see Seshan and Amon, 2004; Fraschini et al., 2008). Similar to the SIN, the MEN components display asymmetric localization patterns. In budding yeast, this asymmetry appears to be important for coordinating MEN activation and mitotic exit with other cell cycle events. It has been proposed that passage of the SPB into the bud brings the SPB-localized GTPase Tem1 (the Spg1 homologue) into contact with its putative GEF Lte1 that localizes exclusively in the bud. This mechanism ensures that mitotic exit only occurs after the spindle has oriented correctly, allowing the nucleus to move through the mother–bud neck into the bud (Bardin et al., 2000; Pereira et al., 2000). The molecular mechanism for the activation of Tem1 by Lte1 is still unknown. Although Lte1 has homology to the Ras exchange factor Cdc25, it has not been shown to directly activate Tem1 in vitro, and additional experiments showed that an Lte1 mutant lacking the Cdc25 homology domain (CHD) can still promote mitotic exit (Yoshida et al., 2003). Homologues of Lte1 have not been identified in *S. pombe*.

In this study, we examined the function of a recently identified component of the SIN called Etd1. A previous study identified Etd1 as an essential component of the SIN pathway that localizes to the cell cortex and division site but not to the SPB (Daga et al., 2005). Our work in this study suggests that Etd1 is a positive regulator of the Spg1 GTPase and a potential homologue of budding yeast Lte1. We show that proper regulation of Etd1 is crucial for both activation of Spg1 in anaphase and inactivation of Spg1 when cytokinesis is complete.

**Results**

*etd1*Δ-null mutants display a cold-sensitive phenotype similar to that of *lte1* mutants in budding yeast

Activation of the Spg1 GTPase is a key step for initiation of cytokinesis in fission yeast. Budding yeast Lte1 promotes activation of the Spg1 homologue in budding yeast called Tem1. However, a fission yeast counterpart to Lte1 has not been identified. The SIN component Etd1 is the only SIN component that, like Lte1, localizes to the cell cortex and not the SPB. Although conventional blast searches using Etd1 or Lte1 did not suggest that they might be homologues, comparison of the protein sequences of Etd1 and three different budding yeast Lte1 orthologues using multiple sequence alignment (Corpet, 1988) showed that the full length of Etd1 aligns specifically with the Ras GEF CHD of the Lte1 orthologues, although the total sequence similarity is low (Fig. S1 A). This weak similarity between Etd1 and Lte1 suggested that these two proteins might be homologues. The budding yeast *lte1*Δ deletion mutant is viable at high but not low temperatures (Shirayama et al., 1994). Similarly, tetrad analysis of heterozygous diploids (*etd1*Δ/*etd1*Δ:: *ura4*) showed that the *etd1*Δ deletion mutant is viable at 36°C but dead at lower temperatures, indicating that, like *lte1*Δ cells, *etd1*Δ cells are cold sensitive (Fig. S1 B and not depicted).

A previous study citing data not depicted found that *etd1*Δ was essential at 36°C (Daga et al., 2005); however, we repeatedly observed viability of *etd1*Δ spores after germination and growth at 36°C. In addition, we found that a strain in which *etd1*Δ transcription was shut off using a regulatable promoter was also viable at 36°C (Fig. S1 B). Thus, we are unable to explain the earlier observations. Although *etd1*Δ cells grew at 36°C (Fig. S1 B), 21% (193/907) of cells failed cytokinesis with a SIN phenotype (Fig. S1 C, arrows). Upon shift to 25°C, *etd1*Δ cells died with a SIN phenotype, failing cytokinesis and becoming multinucleate (Fig. S1, C and D). Thus, like budding yeast *lte1*Δ mutants, *etd1*Δ cells are sick but viable at high temperatures.

Etd1 interacts genetically and physically with Spg1

To test whether Etd1 functions like Lte1, we examined the relationship between Etd1 and the Spg1 GTPase. Several lines of evidence suggest that Etd1 functions upstream of Spg1. First, ectopic expression of *spg1*Δ in wild-type cells induces multiple rounds of actomyosin ring assembly and septum formation without cell separation (Schmidt et al., 1997). Similarly, *etd1*Δ cells also showed multiple septa after moderate or strong overproduction of Spg1 (Fig. S2 A). Second, moderate overproduction of Spg1 partially rescued the growth defect of *etd1*Δ cells at low temperatures (Fig. S2 B). Third, we found that the *etd1*Δ growth defect could be suppressed by a partially activated allele of Spg1 (Fig. 1 A). In these experiments, partial activation of Spg1 was achieved by fusing GFP to its C terminus, which results in occasional interphase septa, suggesting a gain of Spg1 function (Fig. 1 B, arrows; and not depicted). In addition to the growth defect, *spg1-GFP* also suppressed the *etd1*Δ cytokinesis defect (Fig. 1 B). In contrast, the *spg1-GFP* allele was unable to rescue mutations in SIN pathway components downstream of Spg1 (Fig. S2 C). Together, these results suggest that Etd1 functions upstream of Spg1.

These observations prompted us to test whether Etd1 binds directly to Spg1. Spg1 was expressed in bacteria as a GST fusion. Lysates from cells expressing GST-Spg1 or GST alone were incubated with lysates from bacteria expressing Etd1 fused with maltose-binding protein (MBP) or MBP alone, and complexes were pulled down using glutathione beads. Analysis of the bound material showed that MBP–Etd1 (but not MBP) was pulled down, indicating the interaction is either transitory or relatively weak.
to just one SPB in anaphase and telophase (Fig. 2 A, left; Sohrmann et al., 1998; Cerutti and Simanis, 1999; Li et al., 2000). Interestingly, quantitative analysis of Cdc7-GFP signal showed that when Cdc7-GFP localizes to a single SPB in late anaphase the fluorescence increased ~2.5-fold in 21 of 23 cells imaged (Fig. 2, A and B, left). In etd1Δ cells, Cdc7-GFP initially localized similarly to wild-type cells (Fig. 2 A, right), but the signal did not increase at the SPB during anaphase B (n = 13; Fig. 2 B, right). Our results suggest that the key function of Etd1 is to cause the hyperactivation of Spg1 that occurs in late anaphase. This interpretation differs from an earlier study, which suggested that Etd1 is required for maintaining Spg1 activity (Daga et al., 2005). However, our revised interpretation is consistent with their data.

**Spindle elongation allows Etd1-dependent SIN activation in late anaphase**

One explanation for the previous results could be that Spg1 only becomes fully activated when the elongating spindle brings the SPB into closer proximity to the cell tips, which are enriched in Etd1 (Daga et al., 2005). Therefore, we tested whether spindle elongation was required for full activation of the SIN by disrupting spindle assembly with the microtubule-depolymerizing drug methyl-2-benzimidazole-carbamate (MBC). To avoid metaphase arrest, we used mad2Δ cells, which continue to cycle without any microtubules present (He et al., 1997). In MBC-treated cultures, we observed two different patterns of Cdc7-GFP localization. Cells that had already completed spindle elongation when placed in MBC showed bright Cdc7-GFP signal at one SPB near the cell tip (n = 43; Fig. 3 A, type 1), similar to untreated cells. In cells that entered mitosis after being put in MBC, SPB-localized Cdc7-GFP appeared faintly but never increased in brightness before disappearing (n = 34; Fig. 3 A, type 2).

One explanation for these results is that activation of the SIN at late anaphase requires the elongation of the spindle during anaphase B, which could bring SPB-localized Spg1 closer to cortical Etd1 at cell tips.

Previous studies have shown that SIN activity is required for maintenance and assembly of the actomyosin ring in late mitosis (Fankhauser et al., 1995; Balasubramanian et al., 1998; Hachet and Simanis, 2008). Because the SIN activity seems to...
MBC showed that 17 out of 23 cells failed to complete cytokinesis as a result of actomyosin ring disassembly before the completion of ring constriction. Of these 17 cells, 4 showed no ring constriction, and 13 showed very slight to partial constriction. To be reduced in the absence of the anaphase spindle, we examined whether this had any effect on actomyosin ring maintenance in cells treated with MBC. Examination of mad2Δ cdc7-GFP rlc1-GFP cells by time-lapse microscopy in the presence of MBC showed that 17 out of 23 cells failed to complete cytokinesis as a result of actomyosin ring disassembly before the completion of ring constriction. Of these 17 cells, 4 showed no ring constriction, and 13 showed very slight to partial constriction.

Figure 2. etd1Δ cells do not fully activate Spg1 in late anaphase. (A) Cdc7-GFP was imaged by time-lapse microscopy in wild-type (left) and etd1Δ cells (right) at 25°C. The dotted lines show the edge of each cell. Time is indicated in minutes. (B) Quantification of Cdc7-GFP fluorescence for each SPB (lSPB, left SPB; rSPB, right SPB) using arbitrary units (A.U.) is shown.
and displayed incomplete septa as visualized by differential interference contrast (DIC; Fig. 3 B and Fig. S3, A and B). These results suggest that anaphase spindle elongation is essential for full activation of the SIN, which in turn is required for maintenance of the actomyosin ring and cytokinesis.

If failure to bring SPB-localized Spg1 into proximity with Etd1 at cell tips is the reason for the cytokinesis defects that occur when spindle elongation is blocked, artificially increasing the cellular concentration of Etd1 would be predicted to rescue these defects. To test this hypothesis, we examined the effect of moderate overexpression of Etd1 on actomyosin ring maintenance and cytokinesis in the presence of MBC. We observed that 92% (n = 13) of cells moderately overexpressing GFP-Etd1 in the presence of MBC maintained the actomyosin ring until it finished constriction and thus completed cytokinesis like unperturbed wild-type cells (Fig. 3 C and Fig. S3 C). These results suggest that full activation of the SIN at late anaphase requires elongation of the spindle during anaphase B, which could bring SPB-localized Spg1 closer to cortical Etd1 at cell tips.

Among known SIN components, Etd1 is the only one that does not localize to the SPB. Localization of Etd1 to cell tips might be a way to ensure that the SIN is only activated at the correct point in the cell cycle. We wondered whether selective targeting of Etd1 to the SPB might cause defects in the timing of SIN activation. To test this idea, we targeted Etd1 to the SPB by fusing it to the SPB-binding domain of Ppc89. Ppc89 is a structural component of the SPB that links the proteins of the SIN to the SPB (Rosenberg et al., 2006). GFP-etd1-ppc89 was
stronger at 36°C than at 25°C (unpublished data). This result could be explained if Spg1 is intrinsically more active at 36°C than at 25°C, which could also explain why Etd1 is not essential at 36°C (see Discussion). Overall, these results suggest that localization of Etd1 to the cells tips is important to ensure that the SIN is not fully activated until spindle elongation brings SPB-localized Spg1 close to the cell tips. Either Etd1 overexpression or forced localization of Etd1 to the SPB disrupts the dependence of SIN activation on spindle elongation.

Etd1 and SIN inactivation at the end of cytokinesis

Our aforementioned experiments show that Etd1 is required for SIN activation and may need to be inactivated to terminate SIN signaling. We wondered whether inhibition of Etd1 might be important for the eventual inactivation of Spg1 at the end of cytokinesis.
cytokinesis. First, we examined precisely when Spg1 becomes inactivated in wild-type cells using the Cdc7-GFP reporter. Observation of wild-type cells expressing Cdc7-GFP by time-lapse analysis showed that, in all cells observed \((n=28)\), Spg1 is completely inactivated as soon as cells complete actomyosin ring constriction and septum formation (Fig. 4 A), indicating that SIN inactivation is coincident with the partitioning of the cytoplasm between the two daughter cells. We then examined whether Etd1 underwent any changes in localization at the time of Spg1 inactivation. Because endogenous Etd1-GFP is difficult to observe (Daga et al., 2005), we examined Etd1 localization using a strain that expressed increased GFP-Etd1 levels from a heterologous promoter. As reported previously (Daga et al., 2005), we observed that GFP-Etd1 localized to the cell cortex and was enriched at cell tips during interphase (Fig. 4 B and not depicted). However, we also noticed that in late anaphase and telophase, GFP-Etd1 left the cell cortex and was present in the cytoplasm (Fig. 4 B and not depicted). In cells that had completed cytokinesis and septum formation, we found that GFP-Etd1 localized asymmetrically, with one of the resulting daughter cells having a much brighter signal in both the cell cortex and cytoplasm (Fig. 4 B and not depicted). Examination of 530 cells that had completed septum formation showed that in 86.80% of the cells, the GFP-Etd1 signal was brighter in the cell with inactive SIN, as indicated by the absence of Cdc7-GFP fluorescence at the SPB (Fig. 4 B and not depicted). Using either GFP-Etd1 to visualize the septum (Fig. 4 C) or the actomyosin ring marker Rlc1-mCherry (Fig. 4 D) to monitor progression through cytokinesis, it was clear that Etd1 localization became asymmetric at about the time that the ring finished constriction and septum formation completed. Quantification of total GFP-Etd1 fluorescence in each half of the cell showed that the asymmetry was generated by a combination of an increase in fluorescence in one half of the cell and a decrease in the other half. The fluorescence intensity only became asymmetric when cytokinesis was completed (Fig. 4 C). These results show that Etd1 is lost from the daughter cell with the SPB with active SIN. Because Etd1 is required for full Spg1 activation, loss of Etd1 at this time could promote inactivation of Spg1 and the SIN.

If loss of Etd1 at the end of cytokinesis is important for turning off the SIN, elevated Etd1 levels should prevent SIN inactivation. However, a previous study found that strong overproduction of Etd1 resulted in a SIN loss of function phenotype (Daga et al., 2005). When we characterized this phenotype further, we found that strong overexpression of Etd1 caused Spg1 to become displaced from SPBs in the majority of cells (Fig. 5 A). Western blot analysis showed that these phenotypes were not caused by changes in Spg1 levels (Fig. 5 B), and the SPB localization of other SIN components was unperturbed by Etd1 overproduction (Fig. 5 C), suggesting a specific effect on Spg1. These results suggest that Etd1 may bind to Spg1 in a way that blocks the ability of Spg1 to localize to the SPB, so that when Etd1 is...
strongly overexpressed, it titrates Spg1 away from the SPB and thus blocks SIN activity.

When more moderate overexpression was used via an attenuated promoter such as the one used to analyze GFP-Etd1 localization (Fig. 4), Spg1 remained active longer than in wild-type cells, as judged by Cdc7-GFP localization at the SPB. Wild type and cells moderately overexpressing GFP-Etd1 were fixed and analyzed both for the presence of Cdc7-GFP at the SPB and for whether they had completed actomyosin ring constriction using Rlc1-mCherry to mark the actomyosin ring. Examination of wild-type cells showed that, of cells displaying Cdc7-GFP signal at the SPB, only 3% (n = 116) appeared to have completed ring constriction. This is in contrast to cells moderately overexpressing GFP-Etd1, where 48% (n = 143) of cells with Cdc7-GFP at the SPB had completed ring constriction. Similar results were obtained using time-lapse analysis, which showed that 100% of wild-type cells (n = 28) inactivated Spg1 as soon as the ring finished constriction (Fig. 4 A). In contrast, 91% (n = 55) of cells moderately overexpressing GFP-Etd1 kept Spg1 active after ring constriction (Fig. 4 D) and sometimes even after cells separated (Fig. 4 B and Fig. S4 A). Although these cells were viable, they often underwent an additional round of actin ring assembly in the daughter cell with lingering SIN activity, as determined by SPB-bound Cdc7, followed by septum formation (Fig. S4, A and B). Examination of asynchronous cells overexpressing GFP-Etd1 showed that 31% of septated cells (n = 109; Fig. S4 B) contained ectopic septa. Thus, increased levels of Etd1 interfere with Spg1 inactivation at the end of cytokinesis.

The SIN antagonizes Etd1 cortical localization and promotes Etd1 asymmetry

Both because Etd1 leaves the cell cortex when the SIN becomes active in late anaphase and because Etd1 is lost from the daughter cell that has the SPB with active SIN, we wondered whether SIN signaling was responsible for these changes in Etd1 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization. To test whether SIN activity affected Etd1 localization, we reduced SIN activity using the sid2 localization.
actomyosin ring in the cell center, and then each nucleus undergoes spindle elongation, causing each daughter cell to inherit two nuclei. During spindle elongation, Cdc7 localizes to just one SPB on each spindle, resulting mostly with each daughter cell getting one SPB with active SIN, but occasionally both Cdc7-containing SPBs go to the same cell. Through this approach, we created cells that had either symmetric or asymmetric SIN signaling and then monitored whether these cells inactivated Spg1 normally when actomyosin ring constriction completed. Dikaryon cells expressing Cdc7-GFP and Rlc1-mCherry were generated by transient inactivation of the cdc11-123 mutation and then analyzed by time-lapse microscopy. As in the earlier study (Okazaki and Niwa, 2008), we observed four different types of dikaryons based on the patterns of Cdc7-GFP localization to the SPBs (Fig. 7 C). The type I, II, and III dikaryons displayed symmetric Cdc7-GFP localization, with one Cdc7-GFP–containing SPB in each future daughter cell. Interestingly, 92% (n = 37) of these cells maintained Cdc7-GFP at one or both SPBs after completion of actomyosin ring constriction and often formed additional rings and septa (Fig. 7 A). In contrast, 100% (n = 5) of the dikaryons that displayed Cdc7-GFP at both SPBs in one of the future daughter cells had lost Cdc7-GFP from both SPBs upon completion of actomyosin ring constriction and septum formation, as is observed in wild-type cells (Fig. 7, B and C [type IV]). Consistent with our earlier results in wild-type cells, in type IV cells, GFP-Etd1 localization remained symmetric after cell division in type I, II, and III cells, which had symmetric SIN signaling (Fig. 8 B and not depicted). Similar to normal haploid cells (Fig. 4 D), moderate overexpression of GFP-Etd1 produced a prolonged activation...
Figure 7. Analysis of dikaryons shows that asymmetric SIN signaling is important for SIN inactivation. *cdc11-123 cdc7-GFP rlc1-mCherry* cells were grown in YE at 25°C, shifted to 36°C for 2.5 h to allow binucleate cells to form, and then returned to 25°C for an additional 2 h and imaged by time-lapse microscopy at 25°C. (A and B) Cells with symmetric (A) or asymmetric (B) SIN activation are shown. Asterisks indicate completion of actomyosin ring contraction. Time is indicated in minutes. (C) Distribution patterns of Cdc7-GFP (green circles) during late anaphase and telophase in *cdc11-123* dikaryons. Percentages of cells with a prolonged SIN activity are shown. *n* indicates the total number of cells of each type imaged.
GAP. Etd1 was first described as an essential component of the SIN required to connect SIN activity with the actomyosin ring (Daga et al., 2005). Our work suggests that Etd1 is a functional homologue of Lte1. Both Etd1 and Lte1 are cortical proteins that promote activation of their respective GTPases (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000; Molk et al., 2004; Daga et al., 2005; this study). Etd1 has limited sequence similarity to the Ras GEF CHD of Lte1. Additionally, Etd1 and Spg1 bind to each other in vitro. Similar to \textit{lte1Δ} mutants, \textit{etd1Δ} cells show cell division arrest at lower temperatures but are able to grow at 36°C. Overall, these data are consistent with Etd1 being the \textit{S. pombe} homologue of Lte1.

It is intriguing that Etd1 and Lte1 are not essential at 36°C. Although \textit{etd1Δ} cells are viable at 36°C, they have a high rate of cytokinesis failure, suggesting that they barely have enough SIN signaling for viability. Thus, the viability of \textit{etd1Δ} cells at 36°C could be the result of a slight enhancement of the activity of other pathways that regulate the SIN at 36°C. The enhanced expression of \textit{spg1+} observed in response to heat shock and other environmental stresses could also increase SIN activity at higher temperatures (Chen et al., 2003). Alternatively, high temperatures could enhance the spontaneous nucleotide exchange rates for Spg1 and GAP. Etd1 was first described as an essential component of the SIN required to connect SIN activity with the actomyosin ring (Daga et al., 2005). Our work suggests that Etd1 is a functional homologue of Lte1. Both Etd1 and Lte1 are cortical proteins that promote activation of their respective GTPases (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000; Molk et al., 2004; Daga et al., 2005; this study). Etd1 has limited sequence similarity to the Ras GEF CHD of Lte1. Additionally, Etd1 and Spg1 bind to each other in vitro. Similar to \textit{lte1Δ} mutants, \textit{etd1Δ} cells show cell division arrest at lower temperatures but are able to grow at 36°C. Overall, these data are consistent with Etd1 being the \textit{S. pombe} homologue of Lte1.

**Discussion**

**Is Etd1 a functional orthologue of budding yeast Lte1?**

The Spg1 and Tem1 small GTPases act at the top of the SIN and MEN pathways, respectively, to regulate cytokinesis and mitotic exit. Both GTPases are negatively regulated by a two-component GAP and formed by Byr4–Cdc16 in fission yeast and Bub2–Byr4/Bfa1 in budding yeast (for reviews see Bardin and Amon, 2001; McCollum and Gould, 2001; Krapp and Simanis, 2008). It has been unclear whether Spg1 is regulated by a GEF. In budding yeast, the putative GEF Lte1 positively regulates Tem1 at the end of mitosis (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000). However, obvious homologues of Lte1 have not been apparent in the fission yeast genome, and therefore, it was thought that Spg1 might be regulated simply through its GAP. Etd1 was first described as an essential component of the SIN required to connect SIN activity with the actomyosin ring (Daga et al., 2005). Our work suggests that Etd1 is a functional homologue of Lte1. Both Etd1 and Lte1 are cortical proteins that promote activation of their respective GTPases (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000; Molk et al., 2004; Daga et al., 2005; this study). Etd1 has limited sequence similarity to the Ras GEF CHD of Lte1. Additionally, Etd1 and Spg1 bind to each other in vitro. Similar to \textit{lte1Δ} mutants, \textit{etd1Δ} cells show cell division arrest at lower temperatures but are able to grow at 36°C. Overall, these data are consistent with Etd1 being the \textit{S. pombe} homologue of Lte1.

It is intriguing that Etd1 and Lte1 are not essential at 36°C. Although \textit{etd1Δ} cells are viable at 36°C, they have a high rate of cytokinesis failure, suggesting that they barely have enough SIN signaling for viability. Thus, the viability of \textit{etd1Δ} cells at 36°C could be the result of a slight enhancement of the activity of other pathways that regulate the SIN at 36°C. The enhanced expression of \textit{spg1+} observed in response to heat shock and other environmental stresses could also increase SIN activity at higher temperatures (Chen et al., 2003). Alternatively, high temperatures could enhance the spontaneous nucleotide exchange rates for Spg1 and GAP.
Activation of the SIN in anaphase

Cytokinesis should not be initiated until anaphase spindle elongation has moved the chromosomes away from the cell division site. Anaphase SIN activation appears to be governed at least in part by loss of Cdk1 activity, which occurs when cyclin B is degraded in anaphase (Sparks et al., 1999; Guertin et al., 2000; Chang et al., 2001; Krapp et al., 2008). In this study, we show that Etd1 also acts in anaphase to promote SIN signaling by increasing Spg1-GTP levels. One explanation for how Etd1 promotes anaphase SIN activation could be that spindle elongation brings SPB-localized Spg1 closer to cortical Etd1 at cell tips. In this way, cells would ensure that the SIN does not become fully activated until chromosomes have been segregated away from the midzone. Consistent with this, we showed that a block in spindle elongation caused a defect in anaphase Spg1 activation similar to that in etd1Δ mutant cells, producing cells in which the contractile actomyosin ring is prematurely disassembled, leading to defective cytokinesis. Importantly, moderate overexpression of Etd1 rescues the defects observed when spindle elongation is blocked. These results can be collectively encapsulated in a model shown in Fig. 9. It is interesting that SIN signaling causes the release of Etd1 from the cell cortex in late anaphase when the SIN becomes active. It is unclear how the release of Etd1 from the cortex affects its function, but if Etd1 interacts with Spg1 in the cytoplasm, release of Etd1 from the cortex could potentially be a mechanism for the SIN to promote its own activation through positive feedback.

Asymmetry and SIN inactivation

The cell must ensure that cytokinesis occurs only once each cell cycle. We showed that SIN signaling is normally inactivated as soon as cells complete actomyosin ring constriction and septation formation, which partitions the cytoplasm between the two daughter cells. This suggests that cells can somehow sense that cytokinesis is complete and inactivate Spg1 to turn off the SIN. Inhibition of Etd1 function may be one mechanism for turning off SIN signaling. Models to explain the timely termination of SIN signaling must explain/incorporate three types of observations revealed in this study: (1) Etd1 stimulates SIN signaling, and yet, paradoxically, SIN signaling in turn appears to inactivate Etd1, (2) SIN activity and the consequent disappearance of Etd1 are asymmetric in the cell, and (3) inactivation of SIN signaling is coincident with the partitioning of cellular cytoplasm into two compartments.

Etd1 activates SIN signaling, which then promotes asymmetric disappearance of Etd1 after cytokinesis. This situation, in which the activity of one cell cycle component triggers its own eventual inactivation, is analogous to that of Cdk1–cyclin B and the anaphase-promoting complex (APC), where Cdk1 activity is required for the APC to become active (Félix et al., 1990; Hershko et al., 1994; Minshull et al., 1994; Shteinberg and Hershko, 1999) and then the APC inactivates Cdk1 by degrading cyclin B. In both cases, there are additional events that must take place before the second step inactivates the first. For example, the chromosomes must be properly attached to the mitotic spindle before the APC can become active, and for the SIN pathway, Etd1 is only lost from the cell compartment with the active SIN after septum formation is complete.

It is unclear how completion of septum formation is monitored and promotes the disappearance of Etd1. Our results show that asymmetric SIN signaling is important for timely
inactivation of cytokinesis of the SIN. Therefore, it seems likely that completion of cytokinesis is monitored indirectly by a mechanism that uses the asymmetry in SIN signaling to sense partitioning of the cytoplasm. One explanation for these results could be that during cytokinesis, SIN signaling promotes degradation of Etd1, but this degradation is limited because too much Etd1 degradation would reduce SIN activity, resulting in an equilibrium state during cytokinesis. At this stage, Etd1 is present throughout the cell, and the SIN is active at just one SPB. When cytokinesis partitions the cell, the ratio of SIN signaling to Etd1 increases twofold in the cell with active SIN, which could conceivably tip the balance toward Etd1 degradation and eventual loss of SIN signaling. Although the relative change in Etd1 and SIN activities is only twofold, there are a wide variety of ways that the system could work, including positive feedback (Ferrell, 2008), which would make it sensitive to twofold changes. Our data using dikaryon cells are consistent with this type of model. It is important to note that the dikaryon cells were generated by blocking cytokinesis and then allowing them to proceed through another growth cycle as binucleates before they entered the next round of mitosis. This results in the cells being twice the size of a normal wild-type cell when they attempt cytokinesis (unpublished data). Thus, during cytokinesis, these cells should have the same ratio of SIN activity to Etd1 as in wild-type cells because although SIN signaling comes from two SPBs, these cells have twice as much Etd1 as the result of being twice the size of normal wild-type cells. In the situation in which each cell inherits an SPB with active SIN, upon completion of cytokinesis, nothing changes about the ratio of SIN activity to Etd1 in each daughter cell because although each cell has half as much Etd1, they have half as much SIN signaling. Therefore, SIN signaling is maintained after cell division because cytoplasmic partitioning does not alter the SIN to Etd1 ratio. In contrast, when one daughter cell inherits both SPBs with active SIN, the ratio of SIN to Etd1 doubles as in wild-type cells, which could then tip the balance toward Etd1 degradation and SIN inactivation. In the future, a combination of molecular modeling and quantitative in vivo measurements will be necessary to work out the exact mechanism for terminating SIN signaling. In conclusion, this work provides an example of how asymmetric localization of cellular factors may be important for cell division even in symmetrically dividing cells, and it raises the possibility that mechanisms for asymmetric cell division in stem cells in metazoans may have their origins in systems for regulating cell division in simple unicellular ancestors.

Materials and methods

Strains and culture conditions

Fission yeast strains used in this study are listed in Table S1. Strains not created in this study were provided by J. Jimenez (Centro Andaluz de Biología del Desarrollo, Sevilla, Spain) and M. Balasubramanian (The National University of Singapore, Singapore, Republic of Singapore). Genetic crosses and general yeast techniques were performed as described previously (Moreno et al., 1991). S. pombe strains were grown in rich medium (yeast extract [YE]) or Edinburgh minimal medium (EMM) with appropriate supplements (Moreno et al., 1991). EMM with 5 µg/ml thiamine or YE was used to repress expression from the nmt1 promoter. For expression of GFP-Etd1, cells were grown for 2 d in EMM solid without thiamine at 25°C and then grown in EMM liquid without thiamine for an additional 20 h. YE containing 100 mg Genetin (G-418, Sigma-Aldrich) per liter was used for selecting KanR cells. For serial dilution drop tests for growth, four serial 10-fold dilutions were made, and 5 µl of each dilution was spotted on plates with the starting cell number of 10⁴. Cells were grown in liquid YE or EMM at 25°C and then spotted onto YE or EMM plates at the indicated temperatures and incubated for 3–5 d before photography. All strains with an etd1Δ genotype were built by genetic crosses with the strain DM3547. After tetrad dissection, the YE plates containing single spores were incubated at 36°C, and the final round clones were screened for the etd1Δ phenotype at 25°C and for the absence or presence of the plasmid-containing etd1Δ gene through the leucine marker. GFP fluorescence or G4-18 resistance was used to screen for other markers in the strains of interest. For treatment with MBC (Sigma-Aldrich), log-phase cells were treated for 10 min with MBC at the final concentration of 25 µg/ml from a 10 mM stock solution and then centrifuged in a microfuge (model 5415C, Eppendorf) to concentrate the cells and imaged in the presence of 25 µg/ml MBC.

Preparation of dikaryons using the cdc11-123 mutant

S. pombe dikaryon cells were obtained as previously described (Okazaki and Niwo, 2008). In brief, early log-phase cdc11-123 cells were shifted to 36°C for 2.5 h. The formation of binucleate cells was checked before returning to permissive temperature at 25°C. The cells were returned to 25°C for 2 h. The binucleate cells were then imaged by time-lapse microscopy at 25°C (see Microscopy).

Epitope tagging and plasmids

For fission yeast expression, the etd1Δ gene was amplified by PCR from wild-type fission yeast genomic DNA and cloned between the Xho1 and Smal sites of the pREP2X and pREP41X vectors (Forsburg, 1993). The pREP41-EGFP-Etd1-1Pcap91–273 plasmid was created by a three-piece ligation. An Nde1–Xho1 fragment containing the etd1Δ gene and an Xho1–Smal fragment containing the last 1,566 nucleotides of ppc89Δ were generated by PCR and cloned between the Nde1 and Smal sites of the pREP41-EGFP vector (Craven et al., 1998). For bacterial expression, the etd1Δ gene was amplified by PCR from a CDNA library (Two-hybrid; Clontech Laboratories, Inc.) and cloned between the EcoRI and PstI sites of the MBP tag vector pMAL-C2X (New England Biolabs, Inc.). All resulting plasmids were sequenced to confirm the absence of nucleotide changes. K. Gould (Vanderbilt University, Nashville, TN) provided plasmids for over-expression of Spg1.

Immunoblot analysis

Cell pellets were obtained and processed as described previously (Matsuo et al., 2006). In brief, 10⁵ (5 OD units at 595 nm) exponentially growing cells were collected and lysed by incubation with 0.6 M NaOH for 10 min. Next, the cells were collected, and the supernatant was removed. 70 µl of SDS sample buffer (60 mM Tris-HCl, pH 6.8, 4% β-mercaptoethanol, 4% SDS, 0.01% bromophenol blue, and 5% glycerol) was added to the pellet, and samples were boiled for 3 min. Proteins were subjected to 10% SDS-PAGE, blotted onto Immobilon-P (Millipore), and probed with monoclonal anti-GFP (1:250 dilution; Invitrogen) or anti-α-tubulin antibody TAT1 (1:500 dilution; a gift from K. Gull, University of Oxford, Oxford, England, UK).

Binding assay

Escherichia coli containing expression plasmids for MBP, MBP-Etd1, GST, and GST-Spg1 was induced and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 µg/ml PMSF, 1% NP-40, and 20% glycerol). For binding assays, the lysates were mixed and incubated at 4°C for 2 h, followed by the addition of glutathione beads and another 1 h incubation at 4°C. Next, glutathione beads with immobilized GST or GST-Spg1 were washed three times with lysis buffer and suspended in Western blotting with anti-MBP antibodies (New England Biolabs, Inc.).

Microscopy

GFP fusion proteins were observed in cells after fixation with 20% methanol or in live cells. DNA and cell wall material were visualized with DAPI (Sigma-Aldrich) at 2 µg/ml and calcofluor white [CW; Sigma-Aldrich] at 50 µg/ml, respectively. Images were captured using a microscope (Eclipse E600, Nikon) with a digital camera (ORCA-ER; Hamamatsu Photonics) and IPLab Spectrum software (Signal Analytics). For time-lapse experiments, exponentially growing cells were concentrated, and 1.8 µl of the cell suspension was placed on a microscope slide with a 2% YE agar solution. Alternatively, 1.5 µl of log-phase cells was concentrated by centrifugation and suspended in 100 µl of medium (YE or EMM without thiamine for cells expressing GFP-Etd1), and 30 µl of the cell suspension was mixed with...
30 µl of 1 mg/ml soybean lecini (L1395; Sigma-Aldrich), placed in 35-mm glass-bottomed culture dishes (P35-1-5-10-C; MatTek), and immersed in 3 ml of medium (YE or EMW without thiamine for cells expressing GFP-Etd1) for time-lapse imaging. Time-lapse experiments were made at 25°C by acquiring epifluorescence images in z planes and 2 x 2 binning. Cells were then viewed on a microscope (Axiovert 200; Carl Zeiss, Inc.) with an argon ion laser system (Melles Griot). Images were captured using an IEEE 1394 digital charge-coupled device camera (C4742-80-12AG; Hamamatsu Photonics) and UltraView RS confocal imaging system software (PerkinElmer). Fluorescence intensity measurements for SPB-localized Cdc7-GFP were made using IMag software (Bak) by placing a circle around SPB-localized Cdc7-GFP and measuring the maximal fluorescence. A nearby cytosolic region was used as background. GFP-Etd1 fluorescence was measured using ImageJ software [Bethesda Institutes of Health] by drawing the outline of the two daughter cells, excluding the medial region, and measuring the mean of total fluorescence. A nearby region outside of the cell was used as background.

Online supplemental material

Fig. S1 shows a sequence alignment comparing Et1 with Tal1 family members and the phenotype of etd1Δ cells. Fig. S2 shows epistasis analysis supporting the conclusion that Etd1 functions upstream of Sgg1. Fig. S3 shows additional cells of time-lapse experiments of actomyosin ring dynamics in the absence of microtubules. Fig. S4 shows that moderate overexpression of Etd1 causes prolonged SIN activation and regulated rounds of cytokinesis. Table S1 lists strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200902116/DC1.

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