Lte1 contributes to Bfa1 localization rather than stimulating nucleotide exchange by Tem1

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Lte1 is a mitotic regulator long envisaged as a guanosine nucleotide exchange factor (GEF) for Tem1, the small guanosine triphosphatase governing activity of the Saccharomyces cerevisiae mitotic exit network. We demonstrate that this model requires reevaluation. No GEF activity was detectable in vitro, and mutational analysis of Lte1’s putative GEF domain indicated that Lte1 activity relies on interaction with Ras for localization at the bud cortex rather than providing nucleotide exchange. Instead, we found that Lte1 can determine the subcellular localization of Bfa1 at spindle pole bodies (SPBs). Under conditions in which Lte1 is essential, Lte1 promoted the loss of Bfa1 from the maternal SPB. Moreover, in cells with a misaligned spindle, mislocalization of Lte1 in the mother cell promoted loss of Bfa1 from one SPB and allowed by-pass of the spindle position checkpoint. We observed that lte1 mutants display aberrant localization of the polarity cap, which is the organizer of the actin cytoskeleton. We propose that Lte1’s role in cell polarization underlies its contribution to mitotic regulation.

Introduction

In the eukaryotic cell cycle, mitotic exit and cytokinesis must be coordinated with the partition of the duplicated chromosomes to opposite ends of the extended mitotic spindle. In budding yeast, the site of cytokinesis is established well before mitosis, so the mitotic spindle has to be correctly aligned along the longitudinal axis of the dividing cell so that mother and bud compartments each receive a complement of DNA. Similarly, in asymmetric stem cell divisions of more advanced eukaryotes, the axis of the mitotic spindle has to be correctly aligned with the axis of polarized growth and the plane of cell division (Yamashita et al., 2003; Bosl and Li, 2005; D’Aquino et al., 2005; Pereira and Schiebel, 2005; Seshan and Amon, 2005). Lte1 was seen as a positive mitotic regulator because lte1 mutants undergo a telophase arrest at low temperature (Shirayama et al., 1994a). Lte1 shares homology with the guanosine nucleotide exchange domain of the Ras–guanosine nucleotide exchange factor (GEF) Cdc25 (Shirayama et al., 1994a). Thus, when TEM1 was isolated as a high copy number suppressor of the cold sensitivity of an lte1 mutant, it was proposed that Lte1 might be a GEF for (MEN), which governs the transition from late M to G1. The key switch element in the MEN is a small, Ras-like GTPase called Tem1. Activated Tem1, presumably Tem1-GTP, triggers a signaling cascade of Cdc15 and Dbf2-Mob1 kinases to activate Cdc14 phosphatase. Cdc14 has multiple targets whose dephosphorylation drives the cell cycle out of mitosis (for review see Stegmeier and Amon, 2004).

Regulation of the MEN is complex, with multiple, partially redundant pathways. The most studied regulators are Lte1 and the Bfa1–Bub2 complex, although Cdc5, Kin4, Kel1/2, Gic1 and -2, Ste20, Cla4, Cdc42, and Ras2 are also implicated (Hu et al., 2001; Höffken and Schiebel, 2002, 2004; Jensen et al., 2002a; Seshan et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2003; Bosl and Li, 2005; D’Aquino et al., 2005; Pereira and Schiebel, 2005; Seshan and Amon, 2005). Lte1 was seen as a positive mitotic regulator because lte1 mutants undergo a telophase arrest at low temperature (Shirayama et al., 1994a). Lte1 shares homology with the guanosine nucleotide exchange domain of the Ras–guanosine nucleotide exchange factor (GEF) Cdc25 (Shirayama et al., 1994a). Thus, when TEM1 was isolated as a high copy number suppressor of the cold sensitivity of an lte1 mutant, it was proposed that Lte1 might be a GEF for...
in vivo. We report that neither approach gave any support for effects of precise changes in the GEF domain in full-length Lte1.

A point mutation analysis was also conducted to reveal the effects of purified Lte1 could provide GEF activity for Tem1 in vitro. Lte1 could impinge on Tem1 located elsewhere in the cell. It is difficult to understand how the putative GEF activity of cortical tubules with the daughter cell cortex (Adames et al., 2001; Molk et al., 2004; Pereira and Schiebel, 2005; Fraschini et al., 2006; Maekawa et al., 2007). If so, it is somewhat surprising that Tem1 alone showed a high intrinsic exchange activity (Geymonat et al., 2002). The functionality of Tem1 with and without additional Lte1 (Fig. 1 A). As reported, the purified 6His-Lte1 protein was functional, as it could interact Tem1 was tested directly in an in vitro assay. For this purpose, the bacterially produced Tem1 has been confirmed in an earlier study of GTPase activity (Geymonat et al., 2002). Using a filter-based assay, we monitored the loss of tritiated GDP from purified Tem1 alone showed a high intrinsic exchange activity (Geymonat et al., 2002), although a more complex pattern of negative regulatory activity in vivo may also operate (Ro et al., 2002; Fraschini et al., 2006; Kim et al., 2008).

**Results**

**Tem1 GDP to GTP turnover is not stimulated by Lte1 in vitro**

The putative guanosine nucleotide exchange activity of Lte1 on Tem1 was tested directly in an in vitro assay. For this purpose, 6His-Lte1 was expressed and purified from its native yeast source (Geymonat et al., 2007), whereas Tem1 protein was produced in Escherichia coli as described previously (Geymonat et al., 2002). The purified 6His-Lte1 protein was functional, as it could interact in vitro with GST-Ras2V19 but not with control GST (Fig. 1 D) as previously reported (Yoshida et al., 2003). The functionality of the bacterially produced Tem1 has been confirmed in an earlier study of GTPase activity (Geymonat et al., 2002). Using a filter-based assay, we monitored the loss of tritiated GDP from purified Tem1 with and without additional Lte1 (Fig. 1 A). As reported, Tem1 alone showed a high intrinsic exchange activity (Geymonat et al., 2002).
As an alternative test of the putative GEF activity of Lte1, we determined the in vivo effects of site-specific mutations in the GEF-like domain of Lte1. Lte1 interacts with Ras via its GEF domain (Yoshida et al., 2003; Seshan and Amon, 2005), which shows homology with hSos and scCdc25, two known GEFs for Ras proteins (for reviews see Cherfils and Chardin, 1999; Quilliam et al., 2002). Therefore, previous mutational analyses of scCdc25 and hSos (Park et al., 1994; Hall et al., 2001) served as models for site-specific mutagenesis of Lte1 (Fig. S1). The idea that Lte1 is a GEF for Tem1 is complicated by the additional requirement of the Lte1 GEF domain for interaction with Ras (Yoshida et al., 2003). The putative interaction with Tem1 would facilitate nucleotide exchange, whereas the established interaction with Ras might activate Lte1 exchange activity and, coupled with Lte1 phosphorylation, allow localization of Lte1 to the bud cortex (Yoshida et al., 2003; Seshan and Amon, 2005).

Site-specific mutagenesis of the GEF domain of Lte1

As an alternative test of the putative GEF activity of Lte1, we determined the in vivo effects of site-specific mutations in the GEF-like domain of Lte1. Lte1 interacts with Ras via its GEF domain (Yoshida et al., 2003; Seshan and Amon, 2005), which shows homology with hSos and scCdc25, two known GEFs for Ras proteins (for reviews see Cherfils and Chardin, 1999; Quilliam et al., 2002). Therefore, previous mutational analyses of scCdc25 and hSos (Park et al., 1994; Hall et al., 2001) served as models for site-specific mutagenesis of Lte1 (Fig. S1). The idea that Lte1 is a GEF for Tem1 is complicated by the additional requirement of the Lte1 GEF domain for interaction with Ras (Yoshida et al., 2003). The putative interaction with Tem1 would facilitate nucleotide exchange, whereas the established interaction with Ras might activate Lte1 exchange activity and, coupled with Lte1 phosphorylation, allow localization of Lte1 to the bud cortex (Yoshida et al., 2003; Seshan and Amon, 2005).
Mutations that are predicted to block interaction of Lte1 with small G proteins were modeled on scCdc25 glutamic acid substitutions at conserved R1374 and R1444 residues. These mutations block interaction with Ras, thereby preventing complementation of cdc25 mutants (Park et al., 1994). The equivalent K1273E and R1343E mutations in the GEF region of LTE1 genes were expressed from native promoters on integrative plasmids. Neither mutated form of LTE1 rescued the cold sensitivity of a deletion mutant lacking the entire LTE1 ORF, indicating that the GEF region of Lte1 is required for activity of the full-length protein in vivo (Fig. 2 A).

Lte1-F1387E is a second type of mutational change that was modeled on Cdc25-R1489E, which prevents complementation of a cdc25 mutant but, in contrast to R1374E and R1444E, retains its interaction with Ras (Park et al., 1994). Similarly, Lte1-V1382E was modeled on hSos-I956E, which has reduced exchange activity in vitro but again retains almost full interaction with Ras (Hall et al., 2001). Importantly, both Lte1-F1387E and -V1382E complemented the cold sensitivity of lte1Δ mutants at 12°C (Fig. 2 A). Thus, two independently selected forms of Lte1 that are expected to lose exchange activity but retain interaction with Ras complemented the cold sensitivity of lte1Δ cells.

Cortical localization is important for Lte1 activity and requires coordinated interaction with Ras and phosphorylation (Yoshida et al., 2003; Seshan and Amon, 2005). Thus, Lte1-F1387E, which is predicted to retain some Ras interaction and rescued lte1Δ cold sensitivity, did show some cortical localization (clear in all small to medium buds) and some phosphorylation, albeit less than wild type (Fig. 2, B and C). In contrast, Lte1-K1273E and -R1343E showed no cortical localization (Fig. 2 B and not depicted), no phosphorylation (Fig. 2 C and not depicted), and no complementation activity, which is consistent with its predicted and experimental lack of interaction with Ras2 in vivo (Fig. 2 D). Therefore, the putative GEF domain of Lte1 is required for cortical localization through interactions with Ras rather than providing GEF activity per se.

**Multiple domains of Lte1 are required for its complete activity in vivo**

As well as causing cold sensitivity, lte1 mutations are synthetically lethal with deletions of the Cdc14 early anaphase release (FEAR) genes SPO12 and SLK19 and of several genes involved in microtubule dynamics (Heil-Chapdelaine et al., 2000). To test whether Lte1’s GEF-like domains alone are required for complementation of synthetic lethality, we examined complementation by lte1Δ-EcoRI. Lte1Δ-EcoRI has an internal deletion of residues 327–892 but retains its N- and C-terminal GEF domains. Lte1Δ-EcoRI localizes at the bud cortex–like full-length Lte1 (Fig. 3 A) and complements the cold sensitivity of lte1Δ mutants as wild-type Lte1 (Fig. 3 B). However, Lte1Δ-EcoRI was unable to complement the inviability of lte1Δ spo12Δ or lte1Δ slk19Δ, even if overexpressed (Fig. 3 C and not depicted), nor was it able to suppress the SpoC in dyn1Δ cells when overexpressed. Equally, overexpression of Lte1-K1273E and -F1387E was also unable to bypass the SpoC in dyn1Δ cells with mispositioned spindles (Fig. S2). Therefore, differing Lte1 activities may complement the different physiological challenges posed by low temperature, synthetic lethality, and mispositioned spindles. Thus, activity of the GEF-like domain of Lte1 alone is insufficient to explain all of Lte1’s in vivo roles. Rather, the complex pattern of complementation by different alleles of Lte1 points to multiple activities of the molecule.

**Lte1 contributes to the correct localization of Bfa1 at the SPBs**

If Lte1 is not a GEF for Tem1, how does it promote mitotic progression? Recently, a new aspect of MEN regulation has been described. Before MEN activation, Bfa1–Bub2 shifts from having a similar, low affinity localization at both SPBs in early anaphase to displaying an asymmetric, higher affinity localization at the dSPBs of correctly elongated anaphase spindles (Kaydasi and Pereira, 2009; Monje-Casas and Amon, 2009). As Lte1 has such a striking spatial distribution, we considered whether Lte1 affects the MEN through influencing the distribution of Bfa1 on the SPBs.

Surprisingly, we were unable to construct an lte1Δ BFA1-GFP strain to test whether loss of Lte1 activity affected Bfa1 localization because this genetic combination proved lethal. However, Bfa1-GFP with wild-type activity in vivo has been
However, it was unable to form colonies on medium containing glucose, in which the partially functional Lte1-F1387E permitted an effective depletion of Lte1 activity (Fig. 4 A). After 4–6 h in glucose-containing medium, there was a uniform cell cycle arrest with large buds, elongated spindles, and separated nuclei symptomatic of a MEN arrest. A MEN arrest was also indicated by the rescue of viability by a hyperactive (HyA) form of Dbf2 (Dbf2 HyA; Fig. 4 B). The constitutively active Dbf2 HyA was independent from upstream elements of the MEN pathway, having been isolated by its ability to complement a deletion of TEM1 (Fig. S3). Similarly, deletion of the Bfa1 regulator KIN4 restored the viability of BFA1-GFP lte1 cells (Fig. 4 B) just as it rescued the cold sensitivity of lte1 mutants (Pereira and Schiebel, 2005) or lte1 synthetic lethality with FEAR mutations (D’Aquino et al., 2005).

The conditional expression of Lte1 allowed us to monitor the localization of Bfa1-GFP at anaphase in the presence or absence of Lte1. When Lte1-F1387E was expressed, Bfa1 localized, as expected, on both SPBs on short spindles. With spindle elongation, the dSPB approached the bud neck, and Bfa1 at the previously described (Pereira et al., 2001), and BFA1-GFP has been successfully combined with mutant lte1 by others (Kim et al., 2008). Synthetic lethality with our BFA1-GFP construct was specific to lte1Δ and did not occur with the MEN mutations cdc15-1, mob1-77, or cdc14-1 nor with deleted FEAR genes SPO12 and SLK19. Moreover, in wild-type cells, our Bfa1-GFP displayed the expected pattern of SPB association and retained spindle assembly checkpoint activity (Fig. 4 C and not depicted). Thus, the reason for the inviability of BFA1-GFP lte1Δ encountered in this study is unclear but may stem from subtle differences in our construction of the fusion between BFA1 and GFP and/or features of the genetic background of S. cerevisiae 15D.

Whatever the cause of BFA1-GFP inviability, it importantly provided an experimental system in which Bfa1 localization at SPBs could be monitored at 30°C in cells with an essential requirement for Lte1.

The terminal phenotype of BFA1-GFP lte1Δ was studied in cells carrying an integrative plasmid expressing Lte1-F1387E from a GAL1 promoter. This strain was viable on galactose-containing medium, in which Lte1-F1387E was expressed. However, it was unable to form colonies on medium containing glucose, in which the partially functional Lte1-F1387E permitted an effective depletion of Lte1 activity (Fig. 4 A). After 4–6 h in glucose-containing medium, there was a uniform cell cycle arrest with large buds, elongated spindles, and separated nuclei symptomatic of a MEN arrest. A MEN arrest was also indicated by the rescue of viability by a hyperactive (HyA) form of Dbf2 (Dbf2 HyA; Fig. 4 B). The constitutively active Dbf2 HyA was independent from upstream elements of the MEN pathway, having been isolated by its ability to complement a deletion of TEM1 (Fig. S3). Similarly, deletion of the Bfa1 regulator KIN4 restored the viability of BFA1-GFP lte1Δ cells (Fig. 4 B) just as it rescued the cold sensitivity of lte1 mutants (Pereira and Schiebel, 2005) or lte1 synthetic lethality with FEAR mutations (D’Aquino et al., 2005).

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mSPB disappeared, whereas the Bfa1-GFP signal intensified at the dSPB until spindle disassembly and cytokinesis occurred (Fig. 4, C and E). In contrast, most cells depleted for Lte1 arrested in late anaphase with elongated spindles and Bfa1-GFP visible on both SPBs. This symmetrical distribution of Bfa1-GFP was not caused by the late anaphase arrest per se because the Bfa1-GFP was only seen at the dSPBs in mob1-77, cdc15-1, and cdc14-1 mutants undergoing similar anaphase arrests (Fig. 4 D and not depicted).

As mentioned in a previous paragraph, the growth arrest of \textit{lte1Δ BFA1-GFP} cells could be bypassed with Dbf2 HyA, allowing the effects of Lte1 loss to be observed in cycling \textit{lte1Δ BFA1-GFP DBF2 HyA} cells. In this situation, Bfa1-GFP was found at both SPBs, even when anaphase spindles were correctly orientated. This again contrasts to the preferential localization of Bfa1-GFP to the dSPBs of cells expressing Lte1 (Fig. 4, C and E) and demonstrates that Lte1 can contribute to the distribution of Bfa1 at SPBs in anaphase cells independently of MEN activity.

Finally, the relationship between the synthetic lethality of \textit{lte1Δ BFA1-GFP} and Bfa1 localization was examined after deletion of KIN4. Loss of KIN4 in cells with a misoriented spindle was found to reduce the turnover of Bfa1 at SPBs so that Bfa1-GFP accumulated asymmetrically at one SPB (Caydasi and Pereira, 2009). Similarly, the localization of Bfa1 at both SPBs in arrested \textit{lte1Δ BFA1-GFP} cells was switched to the dSPB when viability was restored through deletion of KIN4 (Fig. 4, C and E).

Although these different approaches consistently show a need for Lte1 activity in driving the asymmetric distribution of Bfa1 onto the dSPB, it remained important to exclude any possible artifacts arising from the altered properties of the tagged Bfa1-GFP. Therefore, the localization of native, untagged Bfa1 in wild type and \textit{lte1Δ} mutants was also monitored by indirect immunofluorescence with anti-Bfa1 antibody. Wild-type and \textit{lte1Δ} cells were cultured at 30 or 12°C to reveal the \textit{lte1Δ} mutant defect in mitotic progression. Extended anaphase spindles of wild-type cells at both temperatures and of \textit{lte1Δ} mutants at 30°C showed, as expected, a predominantly asymmetric distribution of Bfa1 on dSPBs. However, Bfa1 was seen at both SPBs on \textgreater90% of the extended mitotic spindles of \textit{lte1Δ} mutants undergoing anaphase arrest at 12°C (Fig. 5). Thus, a role of Lte1 in generating the asymmetric localization of Bfa1 to the dSPB has been confirmed using untagged Bfa1.

Mislocalization of Lte1 in mother cells increases asymmetric distribution of Bfa1 on SPBs of misoriented spindles

Localization of Lte1 at the mother bud cortex inactivates the SpOc and allows cell cycle progression even if nuclei are not partitioned between mother and daughter cells (Bardin et al., 2000; Castillon et al., 2003). In this study, we have used the novel \textit{lte1-8N} allele of \textit{LTE1} to investigate how mislocalization of Lte1 at the mother cortex affects Bfa1 distribution at the SPBs of misaligned spindles. \textit{Lte1-8N} was initially isolated as a serendipitous insertion of eight residues (APDLSFCAA) at position 212 during a routine point mutagenesis protocol. This position is outside both putative N- and C-terminal GEF domains. The phenotype of \textit{lte1-8N} was recognized because of its effects on Lte1 localization: \textit{Lte1-8N} is present at both mother and daughter cortices throughout the whole cell cycle, whereas wild-type Lte1 is restricted to the bud cortex from bud emergence to late M (Fig. 6 A). \textit{Lte1-8N} is active, as judged by complementation of the cold sensitivity of an \textit{lte1Δ} strain (Fig. 6 B). Also, the predicted abrogation of the SpOc by \textit{Lte1-8N} was confirmed by monitoring the Spb marker Spc29-CFP in \textit{dyn1Δ} mutants cultivated at low temperature (Yeh et al., 1995).

In control \textit{LTE1 dyn1Δ} mutants, binaural mother cells containing only two SPBs accumulated, indicating that spindle misalignment had triggered the SpOc (Fig. 6 C). However, in \textit{lte1-8N dyn1Δ} mutants, multinucleate mother cells with more than two SPBs appeared, such as the tetranucleate example with multiple empty buds shown in Fig. 6 C. A similar result was seen when the SpOc was impaired by the deletion of \textit{BFA1} (Fig. 6 D). \textit{Lte1-8N} also abrogated the SpOc response to spindle misalignment triggered by a \textit{kar9} mutation at normal temperatures (Fig. 6 E). Thus, \textit{Lte1-8N} impairs the activity of the SpOc regardless of culture temperature or the genetic defect causing spindle misorientation.

The effect of the maternally localized Lte1 on Bfa1 distribution at the SPBs was then investigated by indirect immunofluorescence of Bfa1 and tubulin in \textit{LTE1 dyn1Δ} and \textit{lte1-8N dyn1Δ} cells cultivated at 14°C (Fig. 7 A). The intensities of Bfa1 foci were quantified in binaural mother cells in which the nuclei had entirely separated on extended but intact mitotic spindles. The ratio of Bfa1 signal intensities at the two SPBs indicates the degree of symmetry, with a value of 1 indicating completely symmetrical localization. In \textit{LTE1 dyn1Δ} cells, the intensities of Bfa1 at the two SPBs were within a factor of two of each other in >65% of cells, indicating a largely symmetrical distribution of Bfa1 on both SPBs of the cells undergoing a SpOc arrest. In contrast, 64% of \textit{dyn1Δ lte1-8N} mutants had relative intensities of Bfa1 signal that differed by more than threefold, indicating increased asymmetric localization of Bfa1 on one SPB (Fig. 7 B). Thus, in cells with a misoriented spindle, \textit{lte1-8N} abrogates the SpOc and does so with an increase in the asymmetrical distribution of Bfa1 at the SPBs.

\textbf{Bfa1 localization at SPBs is independent of Tem1}

Fraschini et al. (2006) proposed that the mechanism driving the asymmetrical accumulation of the Bfa1–Bub2 complex at the dSPB depended on Bub2 GAP activity acting on Tem1 at the mSPB. To test this idea, we investigated the distribution of Bfa1-GFP in the absence of Tem1 using a \textit{tem1Δ} strain kept alive by the expression of HyA Dbf2. We analyzed Bfa1 distribution in cells with short, correctly oriented spindles (early anaphase), in cells with a correctly elongated spindle (late anaphase), and in cells in which the spindle was elongated in the mother (mispositioned). The large majority of cells in early anaphase or with a mispositioned spindle showed a symmetrical Bfa1 distribution with signal ratios within a factor of two (Fig. 8). On the contrary, in late anaphase cells, Bfa1 was clearly asymmetrical with a signal ratio always >4, which is in accordance with a previous study (Pereira et al., 2000). These results indicate that neither the switch from symmetrical to asymmetrical localization of Bfa1 during a normal cell cycle nor the persistent symmetrical localization at both SPBs on misaligned spindles is regulated by Tem1 activity.
**Lte1 affects polarity cap behavior**

Whatever the mechanism that alters the affinity of the Bfa1–Bub2 complex to the SPBs after correct alignment of the mitotic spindle, it seems that the actin and microtubule networks are responsible for communicating this spindle position information to the SPBs (Pereira et al., 2001; Maekawa et al., 2007; Caydasi and Pereira, 2009; Monje-Casas and Amon, 2009). Therefore, the effect of Lte1 on Bfa1 localization at SPBs may stem from perturbations in the actin and/or microtubule networks, from elements that control these networks, or from the signal generation...
Importantly, Kel1 interacts with Lte1, and Kel1 deletion is able to rescue the cold sensitivity of an lte1Δ strain (Höfken and Schiebel, 2002). During the early stages of bud development in wild-type cells, Spa2 and Kel1 occupy a crescent-shaped zone at the bud tip. They then disperse and reappear at the bud neck before cytokinesis (Video 1). In lte1Δ mutants, the Spa2 and Kel1 signals did not always remain at the tip of the bud as it emerged from the mother but instead veered to one side of the bud (Video 2). This behavior could be observed and quantified in still images in which Spa2 and Kel1 were found at the sides of the buds in more than half of the lte1Δ mutants observed, whereas in wild-type cells, the polarity cap remained apical (Fig. 9, A and B). This phenotype was complemented by expression of wild-type or 8N forms of Lte1 (unpublished data). If lte1Δ cells were incubated at the restrictive temperature of 12°C, the polarizome accumulated at the side of the bud in >80% of arrested cells, and no apical signals were detected (Fig. 9 B). In addition, a faint process itself. Previously, no alterations were noted in the actin distribution of lte1 mutants (Höfken and Schiebel, 2002). Similarly, we were unable to see any gross changes in microtubules in wild type and lte1 mutants, although submicroscopic events cannot be excluded. However, we did see a marked difference between polarity cap behavior of wild-type and lte1Δ cells. The polarity cap coordinates polarization of the actin cytoskeleton with exocytosis and endocytosis to focus growth at the bud tip (for review see Moseley and Goode, 2006). It also influences microtubule behavior through transport of tubulin along actin filaments (for review see Pruyne et al., 2004). The polarity cap was visualized for live imaging using Spa2- and Kel1-GFP together. Although similar results were obtained using either tagged protein alone (unpublished data), the combined tags were easier to monitor. Spa2 is a core polarizome subunit of the polarity cap (for review see Moseley and Goode, 2006), whereas Kel1 affects cell morphology and cell fusion (Philips and Herskowitz, 1998).
seen in \textit{lte1}$^{-}$ cells cultivated at the same temperature (Fig. 5),\textit{lte1}$^{-}$\textit{kel1}$^{-}$ mutants displayed an asymmetrical distribution of Bfa1 at one SPB in the majority of the late anaphase cells observed (Fig. 9 C).

Although deletion of \textit{KEL1} restored the asymmetric localization of Bfa1 at SPBs, only 27% regained apical polarization localization in anaphase cells cultivated at 12°C. 63% of cells continued to have their polarizome mislocalized at the side of the bud, whereas in the remaining 10% of cells, a dispersed signal was seen. The interpretation of this result is somewhat complicated by the morphological perturbations caused by the deletion of \textit{KEL1} (Philips and Herskowitz, 1998). Nevertheless, deletion of \textit{KEL1} in an \textit{lte1}$^{-}$ mutant clearly did not fully restore normal localization of Spa2-GFP at 12°C.

signal could sometimes be observed at the bud neck. However, deletion of \textit{BFA1}, which rescues the MEN defect of \textit{lte1} mutants, did not restore apical localization of the polarity cap (Fig. 9, A and B). Thus, Lte1 has a hitherto unknown, MEN-independent role in maintaining the apical axis of the polarity cap.

To test further the proposal that polarity cap behavior influences the distribution of Bfa1 at SPBs, we made a deletion of the polarity cap gene \textit{KEL1} in an \textit{lte1}$^{-}$ mutant. As deletion of \textit{KEL1} rescues the cold sensitivity of \textit{lte1}$^{-}$ mutants (Höfken and Schiebel, 2002; unpublished data), we asked whether this was accompanied with a change in the distribution of Bfa1 at the SPBs. \textit{lte1}$^{-}\textit{kel1}$ cells were cultivated overnight at 12°C, and the localization of Bfa1 and tubulin were observed. In contrast to the mainly symmetrical localization of Bfa1 at SPBs seen in \textit{lte1}$^{-}$ cells cultivated at the same temperature (Fig. 5), \textit{lte1}$^{-}\textit{kel1}$ mutants displayed an asymmetrical distribution of Bfa1 at one SPB in the majority of the late anaphase cells observed (Fig. 9 C).

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that Tem1 does not require a GEF for its activity is also supported by observations of the septation initiation network (SIN) pathway in *S. pombe*, in which most MEN-like homologues are conserved but no GEF for Spg1 has been described (Bardin and...
Amon, 2001). Furthermore, Tem1 and Spg1 have atypically high intrinsic nucleotide exchange activities (Furgle et al., 1998; Geymonat et al., 2002) that question the need for additional specific exchange factors.

Instead of regulation of the MEN by GEF activity, we show by several experimental approaches that Lte1 can contribute to the asymmetric localization of Bfa1 at dSPBs. This pattern of localization has already been linked to mitotic progression (Pereira et al., 2001; Molk et al., 2004; Pereira and Schiebel, 2005; Piatti et al., 2006; Maekawa et al., 2007; for review see Pereira and Schiebel, 2001), and one proposal is that it allows any Tem1 remaining in the maternal cell to activate the MEN (Fraschini et al., 2006; Piatti et al., 2006). Lte1’s influence on the association of Bfa1 at the SPBs was revealed in two different situations under which LTE1 is essential for viability, namely growth at 12°C and in combination with a form of BFA1-GFP. In both instances, deletion of LTE1 caused late anaphase arrest, leading to the appearance of Bfa1 on both SPBs, which is in contrast to the accumulation of Bfa1 at the dSPBs of cells expressing Lte1 (Pereira et al., 2001).

The synthetic lethality of an lte1Δ BFA1-GFP strain could be complemented by the expression of a HyA form of Dbf2 or by deleting KIN4, but the localization pattern of Bfa1 in these two conditions was different. With Dbf2 HyA, Bfa1 remained on both SPBs in late anaphase cells, as might be expected by bypass of the regulatory aspects of the MEN by a constitutively active form of the pathway’s effector kinase. On the contrary, deletion of KIN4 restored the asymmetric distribution of Bfa1 observed in wild-type cells, confirming the role of Kin4 in Bfa1 localization (Caydasi and Pereira, 2009).

The role of Lte1 in Bfa1 localization was further demonstrated by the properties of Lte1-8N, which localizes to both mother and bud cortices. As expected from earlier work in which Lte1 was present in mother cells, the LTE1-8N allele abrogated the Spo4 defect in cells with a misaligned spindle (Bardin et al., 2000; Castillon et al., 2003), and this coincided with a switch of Bfa1 localization to one SPB. Collectively, these observations confirm the link between the localization pattern of Bfa1 at the SPBs with cell cycle progression and, for the first time, demonstrate the involvement of Lte1 in this process.

How might Lte1 at the bud cortex affect events at the SPBs? First, whatever the mechanism, it does not act through Tem1 because deletion of TEM1 neither alters the accumulation of Bfa1 at the dSPB in normal cells nor does it prevent the continued localization of Bfa1 at both SPBs in cells with misaligned spindles (Fig. 8; Pereira et al., 2000). Nevertheless, this result cannot exclude that, when present, Tem1 can still contribute to Bfa1 distribution at the SPBs. In considering how Lte1 might influence Bfa1 binding at SPBs, it was shown recently that the formin Bni1 is required for the asymmetric association of Bfa1 to one SPB (Monje-Casas and Amon, 2009). Bni1 nucleates the formation of polarized actin filaments that extend along the mother–bud cell axis from the bud tip. Localization of Bni1 at the bud tip occurs through interactions with polarity cap components, including Spa2 (for review see Park and Bi, 2007). In this study, we found that the polarity cap behaved abnormally in lte1Δ mutants and often took up nonapical positions in the developing bud. Moreover, we showed that deletion of the polarity cap component KEL1 rescues the cold sensitivity of an lte1Δ strain with restoration of the asymmetrical distribution of Bfa1 to one SPB in late anaphase. This demonstrates a connection between the polarity cap and Bfa1 localization. Thus, collectively, Lte1 and Bni1 both affect aspects of polarity cap activity: Lte1 affects polarity cap localization, whereas Bni1 is needed for actin filament formation from the polarity cap. Therefore, it is particularly striking that, under certain conditions, loss of either Lte1 or Bni1 activity can prevent accumulation of Bfa1 at the dSPB. Therefore, we suggest that Bni1 and Lte1 exert their effects on Bfa1 localization at SPBs by similar means. In the Bni1 study, the signal linking the polarity cap to the SPBs was not described (Monje-Casas and Amon, 2009). However, it was suggested that cues from the asymmetry of the actin cytoskeleton were transmitted to the SPBs via cytoplasmic microtubules. Indeed, other work also identified microtubules as the signaling conduits between the bud cortex and SPBs (Pereira et al., 2001; Maekawa et al., 2007; Caydasi and Pereira, 2009). We propose that the apparent requirement for a polarized actin cytoskeleton might stem from the role of actin filaments in directing the ends of cytoplasmic microtubules in their exploration of the bud neck and cortex (for reviews see Bretscher, 2003; Pryne et al., 2004). This process aligns the axis of the mitotic spindle with that of mother and bud and so would prime the cell for activation of the MEN.

What then happens at 12°C that makes Lte1 essential? A well-described consequence of low temperature is a change in the kinetics of microtubule polymerization and depolymerization (Hamel et al., 1984). Therefore, we envisage that the alteration in polarization in the absence of Lte1 combined with low temperature may be sufficient to disturb the signaling cascade that appears to exist between polarity, microtubule behavior, and the dynamics of Bfa1 association with the SPBs. It is especially notable that lte1 mutants are sensitive to drugs that depolymerize microtubules (Shirayama et al., 1994a) and that lte1Δ is synthetically lethal or sick with defects in several genes involved in microtubule metabolism, including BIM1, CIN8, CIK1, DYN1, JNM1, and KAR3 and -9 (summarized at Saccharomyces Genome Database http://www.yeastgenome.org/). Intriguingly, many of these same mutations are also synthetically lethal with deletions of BFA1 or BUB2, and bfa1Δ/bub2Δ strains are also sensitive to microtubule-depolymerizing drugs (Hoyt et al., 1991; Li, 1999). These phenotypic similarities are difficult to reconcile with a model of Tem1 regulation by antagonistic Lte1 GEF and Bfa1–Bub2 GAP activities. Instead, they are consistent with different altered responses to microtubule perturbation. The essential activity of Lte1 is not just limited to growth at 12°C because at higher temperatures, the continued activity of Lte1 is indicated by the multiple examples of synthetic lethality and by the aberrant polarity cap behavior of lte1Δ mutants.

To summarize, Lte1’s role in controlling mitotic exit requires reevaluation. Rather than providing guanosine exchange activity for Tem1, as previously supposed (Shirayama et al., 1994a; Bardin et al., 2000; Pereira et al., 2000), Lte1 can influence the localization of Bfa1 at SPBs, which in turn appears to be an important factor in mitotic progression. The possible linkage between Lte1’s role in cell polarization and SPB behavior clearly offers an exciting new area for study that is being actively pursued.
After submission of this manuscript, the *S. pombe* ETD1 gene was characterized as a functional homologue of budding yeast LTE1 (García-Cortés and McCollum, 2009). The Etd1 protein influences activity of the SIN pathway, and it displays cortical asymmetry, which may be coordinated with differential SIN activity at the SPBs. Interestingly, the authors also question whether the Etd1 protein acts as a GEF for Spg1, the *S. pombe* homologue of Tem1.

### Materials and methods

#### Strains and culture

Most strains are derivatives of BF264-15DU: a ura3Δns ade1 his2 leu2-3,112 trpl-1 (Table I; Richardson et al., 1989). Yeast extract peptone (YPE) complete medium or yeast nitrogen base selective medium was used. For cold sensitivity assays, 10-fold serial dilutions of stationary phase cultures were spotted on YEP agar plates and incubated at 30°C for 2 d or at 12°C for 10–14 d.

#### Plasmids

Plasmids are listed in Table II. Yiplac128-based plasmids expressed *Lte1* from its own promoter with C-terminal 3HA or GFP epitope tags and were integrated at the *LEU2* locus (Jensen et al., 2002a). Derivatives were C-terminally tagged with GFP using a fusion between the last 875 bp of *BFA1* and *GFP* cloned, respectively, into a pRS304 vector containing the *ADH1* terminator. Integrated trans- formants were verified by visual screening. Endogenous Spa2 and Kel1 were C-terminally tagged with GFP using a fusion between the last 948 bp of *SPA2* or the last 674 bp of *KEL1* and GFP cloned, respectively, into a pYH501 derivative. Integrants were verified by visual screening. Endogenous Spa2 and Kel1 were C-terminally tagged with GFP using a fusion between the last 948 bp of *SPA2* or the last 674 bp of *KEL1* and GFP cloned, respectively, into a pYH501 derivative. Integrants were verified by visual screening.

### Table I. Yeast strains

<table>
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<th>Strain name</th>
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<th>Source or reference</th>
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<tbody>
<tr>
<td>15D</td>
<td>MATα, ura3Δns ade1 his2 leu2-3,112 trpl-1-1</td>
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<td>MATα le1::KAN∞</td>
<td>Jensen et al., 2002a</td>
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<td>Geymonat et al., 2007</td>
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<td>MATα ura3-1 trpl-1-28 leu2-0 lys2Δ his7 mob1::kanMX4 pep4::LEU2 + pMH903::RAS219</td>
<td>Geymonat et al., 2007</td>
</tr>
</tbody>
</table>

Evolva A/S, Frederiksberg, Denmark.

For conditional expression of *Lte1*, a 5’ 1,058-bp fragment of *LTE1* in frame with a 6His tag under the control of the *GAL1* promoter was amplified by PCR using pMH19-1Ule1 (Geymonat et al., 2007) as template and was cloned into Yiplac128-based plasmids. Endogenous *Bfa1* was C-terminally tagged with GFP using a fusion between *BFA1*’s final 875 bp and GFP in pRS304 containing the *ADH1* terminator. Integrants at the *BFA1* locus were verified by PCR and visual screening. Endogenous *Spc29* was C-terminally tagged using a fusion between *Spc29*’s final 418 bp and GFP in pRS304 vector containing the *ADH1* terminator. Integrated transformants were verified by visual screening. Endogenous *Spa2* and *Kel1* were C-terminally tagged with GFP using a fusion between the last 948 bp of *SPA2* or the last 674 bp of *KEL1* and GFP cloned, respectively, into pRS306 or pGKFP (Jensen et al., 2002b). Integrants were verified by visual screening.

#### Protein purification

6His::*Lte1* and GST::Ras219 were expressed and purified from yeast (Geymonat et al., 2007). Maltose-binding protein (MBP)–Bfa1 and MBP::Tem1 were expressed in *E. coli* BL21 (Geymonat et al., 2002). GST::Bfa1 and MBP::Tem1 were expressed in *E. coli* (Geymonat et al., 2002). GST::Bfa1 comprises the N-terminal 230 residues of Bfa1 and was cloned into pRS306 or pKGFP (Jensen et al., 2002b). Integrants were verified by visual screening.

#### Lte1 and Ras19 binding assay

Glutathione agarose beads (GE Healthcare) carrying GST and GST::Ras219 were mixed at 4°C for 2 h with approximately equal amounts of 6His::*Lte1*, as described previously (Yoshida et al., 2003). Bound proteins were eluted with 20 mM of reduced glutathione, and *Lte1* was detected by Western blotting with anti-6His antibody (EMD).

**Lte1-3HA–Ras2 coimmunoprecipitation**

Logarithmically growing cultures were arrested with 10 mM hydroxyurea for 2 h. Anti-HA antibody (12CA5; S. Ley, National Institute for Medical
Table II. Plasmids

<table>
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<th>Name</th>
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Guanosine nucleotide exchange assay

Nucleotide exchange was assayed by loss of [3H]GDP from Tem1 as described previously (Geymonat et al., 2002). In brief, for [3H]GDP loading, purified Tem1 or the Tem1–Bfa1 complex was incubated with 0.1 Mbq [3H]GDP at 25°C for 10 min in loading buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 0.1 mM DTT). Then, for GDP release, 10 µl [3H]GDP at 25°C for 10 min in loading buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 0.1 mM DTT). The mixture was passed through nitrocellulose filters, and the filter-bound radioactive nucleotide was determined by scintillation counting.

Antibody purification

Rabbit antibodies were raised to GST-N-terminal Bfa1 (Harlan Lanes Lab Ltd). IgG from 10 ml of serum was purified with a HiTrap protein A column (GE Healthcare). Anti-Bfa1 antibodies were obtained by affinity purification with MBP-Bfa1 immobilized on Protran nitrocellulose transfer membrane (Whatman Ltd; Pringle et al., 1991). Antibody specificity was confirmed in Western blots with cell extracts of wild-type and lte1 mutant. Online supplemental material is available at http://www.embj.org/cgi/content/full/jcb.200905114/DC1.

Online supplemental material

Fig. S1 shows the alignment of the GEF-like domain of Lte1 with other known GEFs. Fig. S2 is an assay for the bypass of the SPoC by over-expression of different alleles of LTE1. Fig. S3 describes the isolation and characterization of the dbf2 HyA allele. Fig. S4 demonstrates the specificity of the anti-Bfa1 antibody. Video 1 shows the polarity cap behavior in wild-type cells. Video 2 shows how polarity cap behavior is affected in an lte1Δ mutant. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200905114/DC1.

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