Lte1 promotes mitotic exit by controlling the localization of the spindle position checkpoint kinase Kin4

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.1107784108">http://dx.doi.org/10.1073/pnas.1107784108</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Sun Jun 18 09:32:19 EDT 2017</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/70141">http://hdl.handle.net/1721.1/70141</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Article is made available in accordance with the publisher’s policy and may be subject to US copyright law. Please refer to the publisher’s site for terms of use.</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td></td>
</tr>
</tbody>
</table>
Lte1 promotes mitotic exit by controlling the localization of the spindle position checkpoint kinase Kin4

Jill E. Falk, Leon Y. Chan, and Angelika Amon

For a daughter cell to receive a complete genomic complement, it is essential that the mitotic spindle be positioned accurately within the cell. In budding yeast, a signaling system known as the spindle position checkpoint (SPOC) monitors spindle position and regulates the activity of the mitotic exit network (MEN), a GTPase signaling pathway that promotes exit from mitosis. The protein kinase Kin4 is a central component of the spindle position checkpoint. Kin4 primarily localizes to the mother cell and associates with spindle pole bodies (SPBs) located in the mother cell to inhibit MEN signaling. In contrast, the kinase does not associate with the SPB in the bud. Thus, only when a MEN bearing SPB leaves the mother cell and the spindle is accurately positioned along the mother–bud axis can MEN signaling occur and cell division proceed. Here, we describe a mechanism ensuring that Kin4 only associates with mother cell-located SPBs. The bud-localized MEN regulator Lte1, whose molecular function has long been unclear, prevents Kin4 that escapes into the bud from associating with SPBs in the daughter cell.

Polarized cell division or the division of the cell along a pre-determined axis is critical to building higher-order biological structures. Eukaryotic cells that divide in this manner must position the mitotic spindle along this defined axis to ensure that each mitotic product receives a complete genomic copy. The spindle position checkpoint (SPOC) is a feedback mechanism that delays cell-cycle progression in response to defects in spindle position, and it has been found to be operative during the division of budding yeast, fruit fly stem cells, and cultured mammalian cells (1–3). This surveillance mechanism is best understood in budding yeast, where every cell division is polarized and requires proper positioning of the mitotic spindle along the mother–bud axis to produce euploid daughter cells. The SPOC prevents exit from mitosis when the spindle is not aligned along the mother–bud axis (1). When the SPOC is defective, cells with mispositioned spindles inappropriately exit from mitosis, giving rise to abnormal mitotic products with either zero or two nuclei (4–7).

In cells with mispositioned spindles, the SPOC delays cell-cycle progression by inhibiting the mitotic exit network (MEN). This pathway triggers exit from mitosis by bringing about the inactivation of mitotic cyclin-dependent kinases (CDKs), which are responsible for promoting entry into and progression through mitosis (reviewed in ref. 8). MEN signaling commences with the activation of the MEN GTPase Tem1. The GTPase then signals through a kinase cascade to activate the protein phosphatase, Cdc14, the major antagonist of mitotic cyclin-CDK activity.

The SPOC controls mitotic exit by regulating the activation of Tem1. When the spindle is mispositioned, the SPOC kinase, Kin4, activates the GTPase-activating protein complex (GAP) of Tem1, the Bub2-Bfa1 complex, through phosphorylation of Bfa1 on residues S150 and S180 (9). Kin4 itself is regulated at multiple levels. For example, phosphorylation by the protein kinase Elm1 is critical for kinase activity, whereas the protein phosphatase 2A bound to its targeting subunit Rts1 regulates Kin4 localization (10–12).

How does the SPOC sense spindle position and translate this spatial information into a chemical signal to regulate the MEN? The work by Adames et al. (13) proposed a model in which interactions between microtubules and the bud neck inhibit the MEN, but how this could lead to Kin4 activation, if indeed Kin4 is activated by spindle misposition, is not known. We previously proposed a model termed the zone model, which posits that the budding yeast cell is divided into a MEN inhibitory zone in the mother cell and a MEN activating zone in the daughter cell and that a sensor, the GTPase Tem1, moves between them. Tem1 as well as most other components of the MEN reside at spindle pole bodies (SPBs; yeast centrosomes). An activator of the MEN, Lte1, localizes to the bud; the MEN inhibitor, Kin4, is enriched in the mother cell and at the mother SPB (4, 7, 14, 15). The asymmetric localization of these two key Tem1 regulators allows the cell to trigger the MEN only when at least one SPB has escaped the zone of inhibition in the mother cell, where Kin4 resides, and enters the zone of activation in the bud where Lte1 resides (Fig. L). When the spindle is mispositioned, neither SPB escapes the mother cell, and the MEN remains inhibited (Fig. 1F). Importantly, in the zone model, spindle position is coupled to MEN activation during every cell cycle and is not a regulatory mechanism that is only elicited in response to spindle misposition.

Although the role of Kin4 in MEN regulation is well-defined, the role of Lte1 is not clear. The protein is essential for exit from mitosis at low temperatures and has homology to guanine nucleotide exchange factors (GEFs). Despite this homology, Lte1 GEF activity has, however, not been detected in vitro (16, 17). Here, prompted by the observation that when Kin4 and Lte1 reside in the same compartment, Lte1 activation of the MEN prevails over Kin4 inhibition (4, 17–19), we investigate the possibility that Lte1 inhibits Kin4. Indeed, we find that Lte1 prevents Kin4 from associating with the SPB in the daughter cell during anaphase. Our results indicate that a key aspect of spindle position surveillance is to allow Kin4 to inhibit MEN signaling from SPBs that reside in the mother cell but not from SPBs that reside in the daughter cell. At least two control mechanisms ensure that this finding is the case: (i) Kin4 is restricted to the mother cell, and (ii) bud-restricted Lte1 inhibits any Kin4 that enters the bud from loading onto the SPBs in the bud. By these mechanisms, inappropriate inhibition of mitotic exit by Kin4 is prevented.

Results

Lte1 Prevents Kin4 from Associating with SPBs in the Bud. Genetic studies have placed LTE1 upstream of or in parallel to KIN4 in regulating the MEN. The mitotic exit defect of lte1Δ cells is

Author contributions: J.E.F., L.Y.C., and A.A. designed research; J.E.F. and L.Y.C. performed research; J.E.F., L.Y.C., and A.A. analyzed data; and J.E.F., L.Y.C., and A.A. wrote the paper.

The authors declare no conflict of interest.

1J.E.F. and L.Y.C. contributed equally to this work.

2Present address: Department of Molecular and Cell Biology and QB3, University of California, Berkeley, CA 94720-3200.

3To whom correspondence should be addressed. E-mail: angelika@mit.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1107784108/-/DCSupplemental.
Suppressed by deletion of *KIN4* (14, 15, 19). Owing to the homology of Lte1 with GEFs, we previously proposed that Lte1 promotes mitotic exit by functioning as a GEF for Tem1 and thus, in parallel to Kin4. However, Lte1 does not harbor GEF activity in vitro (17), indicating that Lte1 might function in mitotic exit through a different mechanism. Insight into a potential function for Lte1 in promoting mitotic exit came from the observation that when Lte1 and Kin4 are in the same compartment—either by targeting Lte1 to the mother cell or by targeting Kin4 to the bud—Lte1 activation of the MEN prevails over Kin4 inhibition of this signaling pathway. These findings raise the possibility that Lte1 is an inhibitor of Kin4. Because Kin4 inhibits MEN signaling at SPBs (9), we first tested this hypothesis by examining the effects of deleting *LTE1* on Kin4 localization.

Kin4 localization is dynamic. In G1 cells, the protein localizes to the cell cortex. When cells enter the cell cycle, the protein transiently localizes to the emerging bud. Shortly thereafter, Kin4 predominantly localizes to the mother cell cortex. During late anaphase, the protein also binds the SPB in the mother cell (mSPB). The fact that Kin4 associates with the mSPB only during late and not during early anaphase is reflected in the quantification of Kin4 localization in anaphase cells. Only 40–50% of anaphase cells harbor Kin4 at the mSPB (15) (Figs. 1 C and D). Kin4 loading on the dSPB in *lte1*Δ mutants was not caused by the cell-cycle delay displayed by cells lacking *LTE1* (19, 20). Cells that lack both *LTE1* and *BUB2* exit mitosis with WT kinetics (4, 13, 15, 20, 21), and in these cells, Kin4 was still observed to load onto dSPBs, albeit with reduced efficiency (Fig. 2 A and B). Taken together, these data indicate that *LTE1* prevents Kin4 from loading onto the dSPB in the bud, thereby preventing spurious inhibition of the MEN.

In addition to preventing Kin4 from binding to SPBs, Lte1 could also inhibit Kin4 kinase activity. To test this theory, we examined the consequences of deleting *LTE1* on Kin4 kinase activity. Kin4 is a highly insoluble protein (Fig. S1), but small...
amounts of Kin4 can be immunoprecipitated; additionally, the ability of immunoprecipitated Kin4 to phosphorylate recombinant Bfa1 can be determined (9). In this assay, deletion of LTE1 did not affect Kin4-associated kinase activity (Fig. 1E). However, possible effects of Lte1 on the kinase activity of insoluble Kin4 cannot be excluded (Discussion).

Lte1 is essential at low temperature (at and below 18 °C). lte1Δ mutant cells grown at low temperature arrest in anaphase (16). If inhibiting Kin4 association with dSPBs is the critical function of LTE1 in promoting exit from mitosis, we hypothesized that Kin4 association with dSPBs would be increased at low temperature. This hypothesis seems to be the case. Kin4 was found on dSPBs in 69.1 ± 4.9% (SEM) of lte1Δ cells (Fig. 1F). This result suggests that the ability of LTE1 to prevent Kin4 association with the dSPB is especially important at low temperatures.

**Lte1 Functions Together with Mother Cell Restriction to Prevent Kin4 dSPB Binding.** Kin4 predominantly localizes to the mother cell (14, 15). This mechanism likely contributes to preventing Kin4 from associating with dSPBs. To test this possibility, we examined the consequences of losing mother cortex restriction of Kin4 in lte1Δ cells. The Kin4-S508A mutant protein localizes to both the mother cell and bud cortices. Because this allele causes lethality when expressed in cells lacking LTE1 (19), we examined Kin4-S508A localization in lte1Δ cells that also lack BUB2, which allows the lte1Δ KIN4-S508A mutant to grow (19). The fraction of budΔ lte1Δ cells harboring Kin4-S508A on dSPBs was significantly higher than the fraction of bud2Δ lte1Δ cells with WT Kin4 on dSPBs [55.2 ± 4.1 vs. 20.8 ± 5.2% (SEM), respectively] (Fig. 2B and D). In fact, Kin4-S508A was detected on dSPBs in the same percentage of anaphase bub2Δ lte1Δ cells as on the mSPB in bub2Δ cells (Fig. 2D). Similar results were obtained in cells where Lte1 was depleted but BUB2 was functional (Fig. S2). Our data indicate that Lte1 and the restriction of Kin4 to the mother cell represent two parallel mechanisms that prevent Kin4 from associating with dSPBs.

**Lte1 Can Prevent Kin4 from Binding SPBs in the Mother Cell.** Is the ability of Lte1 to inhibit Kin4 SPB loading restricted to the bud,
or could this function also be operative in the mother cell? To test this possibility, we examined the consequences of targeting Lte1 to the mother cell on Kin4 localization. The Lte1-8N mutant protein associates with both the mother cell and bud cortices, because the mutant protein binds more tightly to its cell cortex anchor, Ras (22). However, Lte1-8N is also found in the cytoplasm of both the mother cell and bud (Fig. S3A). In WT cells, Kin4 was observed to localize at mSPBs in 50.0 ± 4.0% (SEM) of anaphase cells, whereas in LTE1-8N cells, Kin4 binding to mSPBs was significantly reduced [5.6 ± 2.2% (SEM)] (Fig. 3B). We conclude that Lte1 prevents Kin4 from associating with SPBs. In WT cells, this activity is directed only to the dSPB, because Lte1 is restricted to the bud.

Cells expressing the LTE1-8N allele are defective in the SPOC but not the spindle assembly checkpoint (17) (Fig. S3 B and C). Could this defect be caused by an inability to localize Kin4 to SPBs in cells with mispositioned spindles? Lte1 binds to both SPBs in cells where spindle misposition is triggered by the inactivation of dynein (δyn1Δ), but binding was greatly reduced in δyn1Δ cells expressing the LTE1-8N allele (Fig. 3C). We conclude that the LTE1-8N allele causes SPOC defects at least in part by preventing Kin4 from loading onto SPBs in cells with mispositioned spindles.

In both DYN1 and δyn1Δ cells, LTE1-8N affected the localization of Kin4 not only at SPBs but also at the cell cortex. Kin4 was found at both the mother and bud cortices and also in the cytoplasm in LTE1-8N cells. Overall, the localization of Kin4 in the LTE1-8N mutant strongly resembled the localization of Lte1-8N itself (compare Fig. 3A with Fig. S3A). How the LTE1-8N allele affects Kin4 at the cortex is not yet clear. The protein could bind Kin4 tightly and thus, could determine Kin4 localization. Alternatively, the localization defect of Kin4 in the LTE1-8N mutant could be caused by the effects of Lte1 on overall cell polarity (δyn1Δ mutant display defects in polarity cap localization) (7). Based on the observation that overexpressed Lte1 determines the localization of Kin4 (see below), we favor the aforementioned binding model.

**Lte1 Physically Interacts with Kin4.** To begin to determine the mechanism used by Lte1 to prevent the association of Kin4 with SPBs, we examined the consequences of overexpressing LTE1 from the methionine repressible MET25 promoter on Kin4 localization. Overexpressed Lte1 mostly associates with the bud cortex, but some protein is also found in the cytoplasm and at the mother cell cortex (23) (Fig. S5). Like the LTE1-8N allele, overproduced Lte1 prevented Kin4 association with the mSPB (Fig. 4 A and B). Interestingly, overproduced Lte1 also affected cortical Kin4. Whereas Kin4 displayed the expected enrichment at the mother cell cortex in WT cells, a fraction of Kin4 associated with the bud cortex in cells that overexpress LTE1 (Fig. 4 A and C). The ability of Lte1 to recruit a mother cortex-associated protein to the bud cortex was specific to Kin4. Overexpression of Lte1 showed little to no effect on the localization of the mother cortex-associated proteins Num1 and Sfk1 (23, 24) (Fig. S5 A and B) or the localization of the bud cortex-associated proteins Kex1 and Spa2 (23, 26) (Fig. S5 C and D). This finding indicates that Lte1, when overproduced, can determine the localization of Kin4 but not other mother cell cortex-associated proteins. Overexpression of LTE1 allows cells with mispositioned spindles to inappropriately exit from mitosis (4). The ability of overexpressed Lte1 to prevent Kin4 from associating with SPBs in the mother cell provides an explanation for this phenotype.

The observation that overexpressed Lte1 or Lte1-8N can recruit Kin4 to sites in the cell where they localize raises the possibility that Lte1, directly or indirectly, interacts with Kin4. To test this possibility, we asked whether Lte1 can coimmunoprecipitate with Kin4. This possibility seems to be the case; the two proteins are found in a complex (Fig. 4D). Thus, although Kin4 and Lte1 are primarily located in different cellular compartments, the proteins can interact with each other when this compartmentalization is disrupted in cell extracts. Consistent with this idea is the observation that the interaction between Kin4-S508A and Lte1 was not increased compared with the interaction between WT Kin4 and Lte1 (Fig. 4D). We conclude that Lte1 can bind to Kin4 in a direct or indirect manner and when overexpressed, determines the localization of Kin4. This finding seems to be critical for preventing the association of Kin4 with dSPBs.

**The N Terminus of Kin4 Mediates Regulation by Lte1.** We next wished to identify the region of Kin4 that mediates its interaction with Lte1. Kin4 consists of an N-terminal kinase domain (amino acids 1–341) and a regulatory C-terminal domain (amino acids 342–800), which harbors a cortex-targeting domain in the extreme C terminus and a mother cell-targeting region in the middle of the protein (amino acids 503–511) (Fig. 5A) (19). When the C-terminal regulatory domain is deleted, the N-terminal kinase domain localizes to the bud cortex from S-phase to late anaphase (19). This observation raises the possibility that an Lte1 interaction domain is located in this part of the protein. Indeed, whereas kin4Δ (1–341) localized to the bud cortex in WT cells, the protein no longer associated with the bud cortex in cells lacking LTE1 (Fig. 5 B and C). Furthermore, Lte1 is sufficient to direct the localization of this Kin4 fragment to other sites in the cell. In cells expressing the LTE1-8N allele, kin4Δ (1–341) was now no longer restricted to the bud, but it was, as the Lte1-8N protein, capable of localizing to the mother and bud cortices (Fig. 5 D and E). Coimmunoprecipitation analyses also confirmed an interaction between kin4Δ (1–341) and Lte1 (Fig. S6A).

To determine whether additional Lte1-interacting domains are present in Kin4, we examined the ability of overproduced Lte1 to recruit the C-terminal Kin4 fragment kin4Δ (342–800) into the bud. kin4Δ (342–800) remained evenly associated with both the mother and bud cortices in cells overexpressing LTE1 (Fig. S6 B and C). We conclude that the 341 N-terminal amino acids of Kin4 harbor an Lte1 interaction domain.

**Lte1-interacting domain of Kin4 contains the Kin4 kinase domain and a short 40-amino acid N-terminal extension.** Deletion of the first 40 amino acids abolished the bud cortex association of the Kin4 kinase domain (Fig. 5F). This region of the protein is, however, not the only Lte1-interacting domain. Kin4 lacking the first 40 amino acids [kin4Δ (41–800)] was still targeted to the bud cortex by high levels of LTE1; however, the targeting was not as efficient, because some Kin4 remained at the mother cell cortex as well (Fig. 5G, symmetrical category). This finding indicates that the first 40 amino acids of Kin4 represent an Lte1-interacting domain by itself; that additional domains exist in the context of full-length Kin4.

Could Lte1 be an integral part of the dynamic localization pattern of Kin4? Kin4 transiently localizes to the nascent bud early in the cell cycle (19). We were not able to reliably address whether LTE1 was required for this transient localization, but we were able to examine the role of Lte1 in the localization of Kin4-S508A. Localization of this Kin4 mutant protein at the cortex of small buds is more pronounced (presumably because its subsequent enrichment at the mother cell cortex is impaired), and Kin4 localization to small buds can be assessed more easily. Kin4-S508A localizes to the bud cortex in small-budded WT cells (19) (Fig. S7). This enrichment in small buds was dramatically reduced in lte1Δ mutants (Fig. S7). We conclude that LTE1 is necessary for early bud cortex localization of Kin4-S508A. This result, furthermore, raises the possibility that Lte1 is an integral part of Kin4 localization control during early stages of the cell cycle.

**Discussion**

Keeping Kin4 off the daughter spindle pole is essential for cell-cycle progression. This finding is evident from the analysis of a present allele (kin4Δ–S508A) in cells that lack LTE1. In such cells, Kin4 localizes to the daughter spindle pole, and cells arrest in anaphase. It is, thus, not surprising that multiple mechanisms are in place that prevent Kin4 from associating with dSPBs (19). Throughout most of the cell cycle, most of Kin4 is restricted to the mother cell (14, 15). Furthermore, Lte1 prevents any Kin4...
that has escaped the mother cell from binding to the dSPB. The escape of Kin4 from the mother cell compartment happens every cell cycle. Although Kin4 is greatly enriched in the mother cell, low levels of Kin4 are also observed in the bud (19). Loss of LTE1 leads to these low levels of Kin4 associating with the dSPB, and exit from mitosis is delayed. Importantly, this cell-cycle delay is suppressed in its entirety by deleting KIN4 (14, 15, 19), indicating that the main function of Lte1 in promoting mitotic exit is to prevent Kin4 from associating with the dSPB.

The ability of Kin4 to associate with dSPBs in cells lacking LTE1 is more pronounced at low temperature, providing an explanation for why LTE1 is essential at low temperatures. However, the basis for this observation is not clear. Mother cortex enrichment of Kin4 is not obviously altered at low temperature, and loading of Kin4 is not only increased at dSPBs, but it is also modestly increased at mSPBs at low temperatures (Fig. 1). This finding raises the interesting possibility that Kin4 associates more stably with SPBs at low temperatures, and therefore, Lte1 is especially needed to prevent Kin4 from binding to the dSPB. We also do not yet know whether preventing Kin4 from associating with dSPBs is the sole function of Lte1 in mitotic exit. The observations that (i) the mitotic exit defect of lte1Δ mutants is completely suppressed by the deletion of KIN4 and (ii) Kin4 binding to dSPBs is increased at low temperature when LTE1 is essential suggest that this is the case. However, Lte1 plays an important role in regulating aspects of cell polarity, and cell polarity factors have been implicated in MEN regulation (22, 27). Thus, additional MEN regulatory roles cannot be excluded. Clearly, it will be necessary to compare lte1Δ mutants with KIN4 mutants that have lost their ability to interact with Lte1.

Our data provide insights into the mechanism used by Lte1 to prevent Kin4 from associating with dSPBs. Lte1 and Kin4 are found in a complex, and Lte1 seems to determine the localization of Kin4. When Lte1 is overproduced and high levels of Lte1 are present at the bud cortex, a fraction of Kin4 is redirected from the mother cell cortex to the bud cortex. A Kin4 truncation that retains its Lte1 interaction domain, kin4(1–341), communoprecipitates with Lte1 and is partly redirected from the bud cortex to the mother cell cortex by an Lte1 mutant protein (Lte1–8N) that associates symmetrically localized to both cortices. Cells were then induced to express Lte1 in synthetic complete medium lacking methionine for 4.25 h. Cortex association was assessed using the following categories: bud cortex, asymmetrically localized to bud cortex; mother cortex, asymmetrically localized to mother; symmetric, symmetrically localized to both cortices. n > 50 cells, and error bars represent the SEM.
fect Kin4 activity assayed by immunoprecipitation kinase assays, it may be premature to conclude that LTE1 does not affect Kin4 activity. Cortical Kin4, which presumably is a significant constituent of the insoluble Kin4 pool, could very well be inhibited by Lte1. Although it is not yet known whether Lte1 binding inhibits Kin4 kinase activity, it is clear that Lte1 binding to Kin4 affects the ability of Kin4 to associate with SPBs and the phosphorylation state of Kin4 (Fig. 58). Kin4 is hyperphosphorylated during late stages of mitosis but is hypophosphorylated during S-phase and early mitosis. Based on the correlation between phosphorylation status and presumptive time of KIN4 function during the cell cycle, we previously suggested that dephosphorylated Kin4 was active (12). We find that Kin4 is hypophosphorylated in cells lacking LTE1. Therefore, it is consistent with the idea that Lte1 regulates Kin4 by controlling its phosphorylation status. Whether it does so by regulating Kin4 activity itself, the Kin4 phosphatase PP2A-Rts1, the Kin4 kinase Elm1, other Kin4 kinases, or the availability of Kin4 itself to be phosphorylated remains to be determined.

Our data not only show that LTE1 plays a key role in preventing Kin4 that escapes the mother cell from associating with the dSPB but also raise the possibility that Lte1 regulates aspects of the normal localization pattern of Kin4. Kin4 very transiently localizes to the bud early in the cell cycle. This localization is enhanced in the KIN4-S508A mutant that lacks mother cell-targeting signals. Interestingly, the transient enrichment of this Kin4 mutant protein at the bud cortex depends on LTE1. Based on this observation and previous studies, we propose the following model for how Kin4 localization is controlled. As the bud emerges and all vescicular transport is directed to the growing bud, Kin4 is transported into the bud and transiently anchored there by Lte1. This initial association is disrupted by the mechanisms that direct Kin4 to the mother cell cortex, which is mediated by the C-terminal regulatory domain of Kin4, and could involve selective degradation of bud-anchored Kin4 or a retrograde transport mechanism along the cell cortex into the mother cell. After Kin4 reaches the mother cell cortex, the protein is no longer able to associate with the dSPB. However, this spatial segregation mechanism is not completely effective. Some Kin4 either remains in the bud or escapes from the mother cell into the bud. Lte1 together with other mechanisms, such as Cdc4–CDK–controlled SPB loading mechanisms, prevent this pool of Kin4 from binding to SPBs (19). The importance of Lte1 in this control mechanism is illustrated by the fact that deletion of LTE1 causes a cell-cycle arrest/delay in anaphase that depends on KIN4.

Finally, our data have important implications for our understanding of SPOC function. The isolation of a genetic condition—KIN4-S508A lte1Δ—that inhibits exit from mitosis even in cells with correctly positioned spindles indicates that spindle misposition is not a prerequisite for the ability of Kin4 to inhibit the MEN, and thus, Kin4 is not activated by spindle misposition. Rather, our results indicate that SPOC function is based on the spatial restriction of the MEN regulators Kin4 and Lte1 and inhibition of Kin4 by Lte1. In fission yeast, a signaling pathway homologous to the MEN, known as the septation initiation network (SIN), governs the final stages of cell division. Fission yeast contains a functional homolog of Lte1 (Etd1), and the protein kinase Pkp1 is similar in sequence to Kin4 (28). It will be interesting to determine whether a similar regulatory relationship exists between these two proteins in fission yeast.

Materials and Methods

Yeast Strains and Growth Conditions. All strains are derivatives of W303 (A2587) and are listed in Table S1. LTE1-8N was generated with a two-step gene replacement strategy using the URA3 gene from Kluyveromyces lactis and a PCR product containing the 8N mutation derived from plasmid pA1843 [Yplac128-LTE1-8N–GFP (pGM180); gift from Marco Geymonat, Gurdon Institute, University of Cambridge, Cambridge, United Kingdom]. Plasmid pA1946 was digested with Swal replacing the LEU2 locus to generate the pMET25-LTE1 allele. KEL1-GFP, SPA2-GFP, NUM1-GFP, SFX1-GFP, LTE1-GFP, and LTE1-8N-GFP were generated by standard PCR-based methods (29). 2HA-Kin4(342–800)-GFP, 3HA-Kin4(41–341)-GFP, and 3HA-KIN4(41–800)-GFP were generated by similar PCR-based methods using plasmid pA1517 and standard PCR-based methods (29). Growth conditions are detailed in Figs. 1–5.

Plasmid Construction. pA1946 was generated by digesting pNH605-pCYC7 (gift from the Lim laboratory, University of California, San Francisco, CA) with PspOMI and BamHI, a PCR fragment containing pMET25 with PspOMI and XhoI, and a PCR fragment containing the coding sequence of LTE1 with XhoI and BamHI and ligating all three fragments together. pA1517 (pFA6a-KanMx6-pKIN4-3HA) was generated by amplifying the promoter of KIN4 (1,000 bp upstream of the KIN4 ORF), digesting this fragment with BstY1 and PacI, digesting pFA6a-KanMx6-pGAL1-10–3HA (29) with BglII and PacI, and ligating these two fragments together.

Differential Centrifugation and Solubility. Differential centrifugation and differential solubility analyses were performed as described (30). Cells were grown to midexponential phase, harvested, washed with 10 mM Tris Cl, pH 7.5, and 10 mM sodium azide, and resuspended in 10 mM Tris CI, pH 7.5, 100 mM EDTA, and 0.5% ␤-mercaptoethanol. Cells were incubated for 20 min at 30 °C and pelleted by gentle centrifugation followed by spheroplasting in 40 mM Heps-KOH, pH 7.5, 1.2 M sorbitol, 0.5 mM MgCl2, and 8 μg/ml oxalylate at 30 °C. Cells were then washed with the spheroplasting buffer without oxalylate, lysed with P100 Kin4, and resuspended in 50 mM Tris Cl, pH 7.5, 1.5 mM EDTA, and 0.2 M sorbitol, and lysed by dounce homogenization. The lysate was centrifuged for 5 min at 500 × g. The supernatant was then subjected to a 13,000 or 100,000 × g centrifugation for 10 min or 1 h, respectively. To determine if various denaturing or solubilizing treatments could solubilize P100 Kin4, the clarified cell lysates were treated with 500 mM NaCl, 1% Triton X-100, 100 mM sodium carbonate to change the pH to 11.5 or 2 M urea for 1 h at 4 °C before centrifugation. Samples were centrifuged for 1 h at 100,000 × g to separate soluble and pelletable fractions.

Other Methods. Indirect in situ immunofluorescence methods to stain Tub1 were as described (31). Live cell imaging of Kin4-GFP, Lte1-GFP, and mCherry-Tub1 was as previously described (12). Num1-GFP, Sfx1-GFP, KEL1-GFP, and Spa2-GFP were imaged as Kin4-GFP. Immunoprecipitations were performed as previously described (12). Immunoblot analysis of Kin4-3HA, Kin4-GFP, Lte1-13MyC, and Kar2 was as previously described (12, 14, 19, 32). Kin4 kinase assays were performed as previously described (12).

ACKNOWLEDGMENTS. We thank Gislen Pereira for communicating results before publication and Frank Solomon, Fernando Monje-Casas, and members of the A.A. laboratory for comments on the manuscript. This work was supported by a National Science Foundation Predoctoral Fellowship (to L.Y.C.) and National Institutes of Health Grant GM056800 (to A.A.), and A.A. is also an investigator of the Howard Hughes Medical Institute.