Yeast GSK-3 kinase regulates astral microtubule function via phosphorylation of the microtubule-stabilizing kinesin Kip2

Hauke Drechsler\textsuperscript{1,2}, Ann Na Tan\textsuperscript{1} and Dimitris Liakopoulos\textsuperscript{1,3}

\textsuperscript{1}Biochemistry Centre Heidelberg (BZH), INF 328, 69120 Heidelberg, Germany

\textsuperscript{2}present address: Mechanochemical Cell Biology Building, Warwick Medical School, The University of Warwick, Coventry, CV4 7AL, United Kingdom

\textsuperscript{3}present address: Centre de Recherche de Biochimie Macromoléculaire (CRBM), CNRS UMR 5237, 1919, route de Mende, 34293 Montpellier Cedex 05, France

Correspondence should be addressed to D.L.

(Email: dimitris.liakopoulos@crbm.cnrs.fr)

Phone: +33 (0) 434359567

Keywords: GSK-3, Kip2, EB1, CLIP-170, yeast

Subject category: cytoskeleton
Abstract

The *S. cerevisiae* kinesin Kip2 stabilises astral microtubules and facilitates spindle positioning through transport of microtubule-associated proteins, such as the yeast CLIP-170 homologue Bik1, dynein and the Adenomatous Polyposis Coli-related protein Kar9 to the plus ends of astral microtubules.

Here, we show that Kip2 associates physically with its processivity factor Bim1, the yeast homologue of the EB1 plus end-tracking protein. This interaction requires an EB1-binding motif in the N-terminal extension of Kip2 and is negatively regulated by phosphorylation through Mck1, the yeast Glycogen Synthase Kinase 3. In addition, Mck1-dependent phosphorylation decreases the intrinsic microtubule affinity of Kip2. Reduction in Kip2 phosphorylation leads to stabilisation of astral microtubules and accumulation of Kip2, dynein and Kar9 at microtubule plus ends, while loss of Mck1 function leads to defects in spindle positioning. Furthermore, we provide evidence that a subpopulation of Mck1 at the bud-cortex phosphorylates Kip2. We propose that yeast GSK-3 spatially controls astral microtubule dynamics and the loading of dynein and Kar9 on astral microtubule plus ends by regulating Kip2 interactions with Bim1 and microtubules.
Introduction

The microtubule plus end is a site of remarkable versatility: due to its dynamicity it easily explores cellular space, but it can also mediate and maintain stable interactions with other cytoskeletal factors. Functionality of microtubule plus ends can be modified by a set of specialised proteins termed +TIPs that are able to stably associate with the growing end (Akhmanova and Steinmetz, 2008; Perez et al., 1999). Among +TIPs, the members of the EB1 and CLIP-170 families play a central role: EB1 proteins are required for the plus end tracking of other proteins (Akhmanova and Steinmetz, 2008; Honnappa et al., 2009; Vaughan, 2005), while members of the CLIP-170 family are positive regulators of MT growth (Brunner and Nurse, 2000; Komarova et al., 2002; Steinmetz and Akhmanova, 2008).

Yeast homologues of EB1 (Bim1) and CLIP170 (Bik1) are both involved in spindle positioning during mitosis of *S. cerevisiae*. Spindle positioning in budding yeast ensures that the spindle elongates along the mother-bud axis during anaphase and depends on two redundant pathways, the dynein- and the Kar9 pathway. The plus end-directed kinesin motor Kip2 participates in both pathways by transporting +TIPs to the plus ends of astral microtubules (aMTs). In the Kar9 pathway, Kip2 is required for efficient accumulation of the +TIP Kar9 at aMT plus ends (Maekawa et al., 2003).

Similar to Adenomatous Polyposis Coli (APC), a tumour suppressor that links microtubules to actin, Kar9 mediates interactions of aMTs with cortical actin, that are required for pre-anaphase spindle positioning and nuclear migration close to the bud (Bienz, 2001; Miller et al., 2000; Miller and Rose, 1998). As part of the dynein pathway, Kip2 transports Bik1 and cytoplasmic dynein from the spindle poles to the plus ends of astral microtubules (Sheeman et al, 2003; (Carvalho et al., 2004; Roberts et al., 2014). Dynein is subsequently offloaded from aMTs and immobilised at the cell
cortex, where it pulls on aMTs facilitating correct positioning of the mitotic spindle in anaphase (Moore et al., 2009).

Besides its role in spindle positioning, Kip2 has an intriguing property in budding yeast: it mediates microtubule stabilisation (Carvalho et al., 2004; Cottingham and Hoyt, 1997; Huyett et al., 1998). Deletion of KIP2 results in extremely short aMTs, while KIP2 overexpression leads to cells with abnormally long aMTs. Stabilisation of aMTs by Kip2 seems to be coupled to the transport of Bik1 to aMT plus ends (Carvalho et al., 2004).

Glycogen Synthase Kinase 3 (GSK-3) is a highly conserved kinase with a key role in signalling during development (Doble and Woodgett, 2003; Doble and Woodgett, 2007; Kim et al., 2009; Wu and Pan, 2010) as well as in regulation of microtubule function and chromosome segregation (Wakefield et al, 2003; Tighe et al., 2007; Buttrick and Wakefield, 2008). In migrating cells and developing neurons, GSK-3 regulates cell polarisation by phosphorylating several +TIPs including APC and CLASP2 (Etienne-Manneville and Hall, 2003; Watanabe et al., 2009). However, the role of GSK-3 in microtubule regulation in other systems, including yeast cells, is poorly defined.

Here we show that Kip2 physically interacts with Bim1 via its N-terminal extension that precedes the kinesin motor domain. This extension is heavily phosphorylated by yeast GSK-3 kinase homologue Mck1 in a cell-cycle dependent manner, which probably requires a priming phosphorylation by the LATS-related kinase Dbf2. We provide evidence that the N-terminal extension is a regulatory hot-spot, as phosphorylation not only interferes with Bim1 binding, but also reduces the microtubule affinity of Kip2. We propose that Mck1 and possibly Dbf2 control
spindle positioning by spatial regulation of aMT dynamics and the deposition of dynein and Kar9 at aMT plus ends through phosphorylation of the kinesin Kip2.

Results

Kip2 is phosphorylated by budding yeast GSK-3/Mck1

Mitotic Cdc28 (the budding yeast Cdk1) phosphorylates Kip2 in vitro (Ubersax et al., 2003). In line with this, we identified two potential phosphorylation sites at S63 and T275 within Kip2 that fit the Cdc28 consensus (S/T-P-X-R) (Fig. 1A). And indeed, in western blot analysis of cell extracts Kip2 C-terminally tagged with 13 myc epitopes (Kip2\textsubscript{13myc}) displayed a complex migration pattern that collapsed after treatment with alkaline phosphatase (Fig. S1A). This suggested that part of Kip2 is present in cells in a number of phosphoisoforms that display different electrophoretic mobilities on SDS PAGE. Furthermore, substitution of S63 by alanine (Kip2-AT\textsubscript{13myc}) largely abrogated Kip2 phosphorylation (Figs. 1B, S1A). The single T275A substitution did not show any significant effect in vivo (Fig. S1A), while combination of S63- and T275- to alanine mutations (Kip2-AA\textsubscript{13myc}) displayed similar reduction to Kip2-AT\textsubscript{13myc} (Figs 1B, S1A). We next tested whether Cdc28 phosphorylates Kip2 in vivo and inhibited Cdc28 over time using the cdc28-as1 strain (Ubersax et al., 2003). In this experiment, the bona fide Cdc28 substrate Kar9 served as an internal positive control (Liakopoulos et al., 2003). While phosphorylation of Kar9\textsubscript{TAP} decreased rapidly within 20 minutes after Cdc28 inhibition, Kip2\textsubscript{13myc} phosphorylation remained fairly stable over 50 minutes (Fig. S1B). In addition, pairwise deletion of the yeast cyclins Clb1-6 that are essential for Cdc28 activity did
not significantly decrease Kip2 phosphorylation (Fig. S1C). Hence, we conclude that Cdc28 is not the major kinase involved in Kip2 phosphorylation.

We took a systematic approach to identify the major kinase responsible for Kip2 phosphorylation. We did not detect any reduction in Kip2\textsuperscript{13myc} phosphorylation in cells with mutations in the yeast Polo kinase (cdc5-2; Charles at al., 1998), the Mitotic Exit Network kinase Cdc15 (cdc15-1; Shirayama et al., 1998), or the yeast Aurora kinase (ipl1-321; Biggins et al., 1999; data not shown). Next, we examined Kip2 phosphorylation in 60 strains each deficient for a non-essential kinase. We observed the most prominent reduction in Kip2 phosphorylation in cells deleted for MCK1, which encodes one of four homologues of the GSK-3 kinase in budding yeast (Fig. 1B, S1D; the other three GSK-3 homologues are Rim11, Mrk1 and Ygk3). Phosphorylation of Kip2 in rim11Δ cells is only marginally reduced (Fig. 1B) while deletion of Mrk1 or Ygk3 had no significant negative effect on Kip2 phosphorylation levels (Fig. S1D). Moreover, phosphorylation of Kip2 \textit{in vivo} decreased upon down-regulation of MCK1 (expressed under the regulatable \textit{P}_{GAL1-10} promoter) and hyper-phosphorylation was restored upon overexpression of the kinase, while this effect was absent upon introduction of the S63A mutation (Fig. 1C). Thus, we conclude that Mck1 is a major kinase responsible for the S63-dependent Kip2 phosphorylation.

\textbf{Mck1 phosphorylates the unstructured N-terminus of Kip2}

We next searched for regions of Kip2 that are targeted by Mck1. GSK-3 is known to sequentially phosphorylate multiple residues within a continuous S/TxxxS/T pattern upstream of a first phosphorylation site, that is often (but not always) phosphorylated by a priming kinase (Roach, 1991). Such a pattern extends throughout the entire S/T-rich N-terminal region of Kip2 (32 S/T within the first 80 aa) and is predicted to be heavily phosphorylated by GSK-3 at three S/T clusters
according to several algorithms (clusters A, B and C; Fig. 1A; for site prediction see Methods). Furthermore, T275 is also part of a GSK3-consensus. Thus, mutations at S63 and T275 disrupt not only the Cdc28, but also the GSK-3 consensus and may abrogate phosphorylation either by inhibiting priming phosphorylation or by stopping the sequential phosphorylation cascade. In line with this, overexpression of MCKI failed to induce hyper-phosphorylation of Kip213myc variants with mutations in S63 or in the N-terminal GSK-3 clusters (Fig. 1C). Since a single substitution at S63 has the same effect as substituting the S/Ts in the entire N-terminal clusters with aspartates, we conclude that S63 is indeed critical for Mck1-dependent Kip2 phosphorylation.

In order to investigate differences in phosphorylation of the different N-terminal variants of Kip2 in vitro (Fig. 1A), we labelled living cells with 32P-orthophosphate and examined phosphorylation by autoradiography after immunoprecipitation (Figs 1D,E). Consistent with our previous results, phosphorylation of Kip213myc in mck1Δ cells was severely reduced as was also the case for Kip2-AT13myc (and to a similar extent for Kip2-AA13myc). Deletion of the clusters A and B (Δ37-Kip213myc) severely reduced phosphorylation, while Kip2 deleted for the whole N-terminus before aminoacid 70 (Δ70-Kip213myc) did not exhibit any detectable phosphorylation (Fig. 1D), even considering that Δ70-Kip213myc protein levels were clearly lower compared to wild-type Kip2. These data suggest that the S/T-rich terminus of Kip2 bears the major phosphorylation sites and that phosphorylation extends through all three GSK-3 consensus clusters.

We next tested phosphorylation of Kip2 by Mck1 in vitro. We thus purified from bacteria an MBP-fusion of Kip2 lacking the C-terminal tail (Kip2 1-560) followed by GFP (MBP Kip2ΔC GFP; this fusion was more stable than full-length Kip2 isolated from yeast and from insect cells) and Mck1TAP from yeast cells. In line with
the requirement for a priming-phosphorylation by GSK-3 kinases, Mck1\textsuperscript{TAP} alone was not able to phosphorylate \textsuperscript{MBP}Kip2ΔC (Fig. 1F). However, pre-incubation with recombinant Cdk1/Clb2 allowed \textsuperscript{MBP}Kip2ΔC to become efficiently phosphorylated after Mck1\textsuperscript{TAP} addition. Introducing a S63A mutation reduced phosphorylation, while a double S63A T275A mutation nearly abrogated \textit{in vitro} phosphorylation. These results suggested that priming phosphorylation by Cdk1/Clb2 at S63 can support sequential phosphorylation by Mck1 of sites further upstream. This predicted that mutation of T59 to an unphosphorylatable residue should prevent phosphorylation of the upstream cluster and phenocopy the S63A mutation. To test this, we constructed \textit{GST}Kip2\textsuperscript{1-80} fusions bearing the S63A or the T59A substitution and examined their phosphorylation \textit{in vitro}. As predicted, both constructs were only poorly phosphorylated compared to the wild type control. (Fig. 1G). Taken together, our results suggest that Mck1 sequentially phosphorylates Kip2 at the GSK-3 clusters at its N-terminus \textit{in vivo} and \textit{in vitro}. It is also likely that the GSK-3 consensus at T275 is phosphorylated with the T275 acting as a priming site. However, the data cannot exclude that T275A is a priming mutation only in the presence of S63A.

**Dbf2 may act as priming kinase for Mck1**

Although our \textit{in vitro} experiments suggest that Cdc28 is a priming kinase for Mck1, inhibition of Cdc28 did not affect Kip2 phosphorylation \textit{in vivo} nor did deactivation of Cdc28 decrease Kip2 phosphorylation in absence of Mck1 (Fig. S1B, C,D). Furthermore, S63 is part of cluster C, but not the most downstream serine in that cluster. We therefore reasoned that S69 or S72 might act as priming sites \textit{in vivo}.

Indeed, we observed a clear reduction of Kip2\textsuperscript{1\textsuperscript{3myc}} phosphorylation upon introduction of S69A and mostly in S72A mutations, suggesting that these sites indeed control phosphorylation of the upstream cluster \textit{in vivo} (Fig. 2A). Serine 69
matches the consensus for protein kinase C, while the sites 69-75 are also predicted CKI sites (ELM; http://elm.eu.org/). However, we did not observe any significant reduction in Kip2\textsuperscript{13myc} phosphorylation in yeast \textit{stt1-1} (PKC) or \textit{yck1Δ yck2-2ts} (CKI) mutants (Fig. S1E). Therefore, the (priming) kinases phosphorylating these sites remain unknown.

Nevertheless, during the analysis of the yeast kinase deletion strains we observed that Kip2 phosphorylation is diminished in \textit{dbf20Δ} null mutants as well as in \textit{dbf2Δ} strains (Fig. S1F). Dbf20 is a close homologue of the LATS-related Dbf2 kinase in yeast. The Dbf2 kinase is a part of the mitotic exit network which is activated following anaphase to promote exit from mitosis (Bardin and Amon, 2001). Upon analysis of the \textit{dbf2-2 dbf20Δ} double mutant, we observed that Kip2\textsuperscript{13myc} phosphorylation was indeed clearly reduced (Fig. 2B). Furthermore, we examined phosphorylation of Kip2 during the cell cycle (Fig. 2C, S1G). Phosphorylation levels of Kip2\textsuperscript{13myc} were lowest during metaphase, followed by increase in Kip2\textsuperscript{3myc} phosphorylation immediately thereafter. Therefore, the pattern of Kip2 phosphorylation fits well with the timing of major Dbf2 activation. Although some phosphorylation was still evident, the increase of phosphorylation of Kip2\textsuperscript{13myc} upon anaphase entry was less pronounced in the \textit{dbf2-2 dbf20Δ} mutant (as well as in the \textit{mck1Δ} mutant (Fig. S1G)) compared to wild type, in agreement with the hypothesis that Dbf2 may act on Kip2 as a priming kinase for Mck1.

Upon closer inspection of the Kip2 sequence, we noticed that serines S13, S63 and S69 within the N-terminal clusters are preceded by an R and thus match the preferred sequence for the Dbf2 kinases [RXXS/T] (Mah et al., 2005; Fig. 1A). Furthermore T14, S18 and S33 are part of such consensus sites though not part of the GSK-3 [SXXXS] pattern. Intriguingly however, neither a single R60A nor a R66A
mutation (Figs S1H and 2A, respectively) caused significant difference in the Kip2<sup>13myc</sup> phosphorylation pattern. We refrained from mutating more arginines in the N-terminal Dbf2 consensus sites because this would drastically change the pI of this domain and could perturb Kip2-microtubule interactions (see below). Our interpretation of this data so far is that priming for Mck1 phosphorylation is mediated by Dbf2/Dbf20 within the Kip2 N-terminus and possibly by additional kinases acting at S69 and S72.

**Regulation of microtubule stability by Kip2 phosphorylation**

GSK-3 is a major regulator of microtubule function in mammalian cells (Buttrick and Wakefield, 2008), but its role in MT regulation in budding yeast is unknown. Kip2 localises exclusively on aMTs and had been reported to promote aMT stability by transporting Bik1 to aMTs plus ends (Carvalho et al., 2004). This prompted us to examine the aMT phenotypes of hypo-phosphorylated Kip2 variants bearing mutations in the GSK-3 consensus in G2/M cells. For this, we created *kip2Δ* cells that genomically express different Kip2 variants under the control of the native *KIP2* promoter. Interestingly, cells expressing Kip2 variants defective in Mck1 phosphorylation (Kip2-AT) displayed and increased number and abnormally long astral microtubules (Figs 3A, B, C). This phenotype is exacerbated in Kip2-AA-expressing cells, in which the numbers of long aMTs were increased. In addition, these cells were growth resistant to the MT-destabilising drug benomyl (Fig. 3D). Cells expressing Kip2 with substitutions in the GSK-3 consensus at T275 alone did not display a significant aMT phenotype (Fig. S2A). Consistently, reduction of Kip2 phosphorylation through deletion of *MCK1* also results in aMT stabilisation and increase in aMT number (Fig. 3A, B, C), while these aMTs phenotypes is even more pronounced in *mck1Δ rim11Δ* mutant cells, but not after combining the *mck1Δ*
deletion with mrk1Δ or ygk3Δ (Fig. S2B). Importantly, we also observed the same aMT phenotypes in dbf2-2 dbf20Δ cells in all phases of the cell cycle (Figs 3A,B,C), consistent with the role of Dbf2 as a priming kinase for Mck1. In conclusion, these experiments show that expression of the hypo-phosphorylated form of Kip2 stabilizes microtubules.

**Phosphorylation inhibits the binding of Kip2 to microtubules**

We next set out to elucidate the molecular mechanism through which Mck1-dependent Kip2 phosphorylation might affect aMT stability. We first reasoned that phosphorylation simply might control the stability of Kip2. In line with this idea, cells expressing a Kip2-AT-YFP variant display a significantly higher plus end accumulation at aMTs when compared to cells expressing wild type Kip2-YFP (Fig. 4A). This effect was not specific only for the plus ends, but is rather a result of the overall increased amounts of Kip2-AT-YFP on aMTs. Consistently, depletion of cells of Mck1 also results in increased aMT- and plus end accumulation of wild type Kip2-YFP, while overexpression of Mck1 had the opposite effect (Fig. 4A). We therefore set up a cycloheximide-chase experiment to follow degradation kinetics of different Kip2 variants and co-expressed Kip213myc-AA and wild-type Kip2TAP as an internal control (Fig. 4B). Both proteins however displayed indistinguishable turnover kinetics, suggesting that Mck1-dependent Kip2 phosphorylation does not regulate Kip2 stability and the increased plus end accumulation of the hypo-phosphorylated Kip2 must have a different cause.

Increase in affinity for microtubules or in processivity of hypo-phosphorylated Kip2 isoforms might also account for increased accumulation at aMT plus ends. In the first case, more Kip2 would be loaded onto aMTs, in the second case loaded Kip2
would be more likely to reach the plus end. We first addressed the possibility that phosphorylation of the Kip2 N-terminus increases the microtubule affinity of the motor. This unstructured, S/T-rich region is highly basic with pI=13 and is reminiscent of the Ndc80 N-terminal extension that contributes to binding of Ndc80 to microtubules (Ciferri et al, 2008). Interestingly, introducing negative charges by phosphorylation of the N-terminal extension in Ndc80 weakens its interaction with the microtubule lattice (Ciferri et al., 2008). We therefore asked whether phosphorylation of the N-terminal sequence of Kip2 would have an analogous effect on microtubule binding. Recombinant GFPKip2 purified from insect cells is hyper-phosphorylated in an S63-dependent manner in western blots, similar to yeast cells, and we verified that S63 is indeed phosphorylated using mass-spectrometry (Figs S2C,D; see also Roberts et al., 2014). We thus asked which forms of purified GFPKip2 would stronger interact with microtubules. In microtubule sedimentation assays, the affinity of the hyper-phosphorylated GFPKip2 isoforms was reduced compared to hypo-phosphorylated GFPKip2 (Fig. S2D). Furthermore, to mimic a constitutively phosphorylated Kip2, we substituted all S/T within the N-terminal cluster of our MBPKip2ΔC GFP construct by aspartates (allD-MBPKip2ΔC GFP) and repeated the co-sedimentation experiments. Again, wild type MBPKip2ΔC GFP co-sedimented with increasing tubulin concentrations, the majority of allD-MBPKip2ΔC GFP remained in the supernatant (Fig. 4C). Consistent with these findings, introduction of the same substitutions in the Kip2-YFP construct (allD-Kip2-YFP) reduced Kip2 microtubule load by app. 50% in vivo (Fig. 4A). Therefore, we propose that Mck1 controls aMT stability by phosphorylating the N-terminus of Kip2 and regulating the affinity of Kip2 for microtubules.
Mck1 controls interaction of Kip2 with the yeast EB1

During sequence analysis of the N-terminal extension we also identified a putative EB1/Bim1-binding (SxIP) motif (Honnappa et al., 2009) at aa 21-24 with S21 being part of the GSK-3 cluster A (SNIP¹; Fig. 5A; see also (Roberts et al., 2014). The site may be conserved in the proposed fission yeast Kip2 homologue Tea2 (Fig. 5A). A second motif could be identified starting at aa 415 (SNIP²). The SNIP¹ Bim1 binding site at aa 21-24 is of particular interest, since Bim1 (and also Bik1) acts as a processivity factor for Kip2 (Bieling et al., 2007; Roberts et al., 2014). Phosphorylation next to EB1 binding motifs has been shown to disrupt interactions with EB1 (Honnappa et al., 2009; Roberts et al., 2014; Zumbrunn et al., 2001). In this case, the SNIP¹ motif is flanked by the Mck1 cluster A and B, the first of which includes S21 of SNIP¹. This suggested that Mck1-dependent phosphorylation might interfere with the Kip2-Bim1 interaction. Therefore, Mck1-dependent Kip2 phosphorylation might control plus end accumulation of Kip2 by regulating Kip2 interaction to its processivity factor(s), in addition to its interaction with microtubules.

We thus first tested whether the identified motifs were required for Kip2 interaction with Bim1 in vivo. Indeed, HA-Bim1 co-immunoprecipitated Kip2¹³myc from cell extracts (Fig. 5B), while Bik1 was dispensable for this interaction in vivo (Bim1 and Bik1 physically interact with each other (Blake-Hodek et al., 2010; Wolyniak et al., 2006). The truncated Kip2 variants lacking either the first 37aa (Δ37-Kip2¹³myc, lacking also SNIP¹) or the entire N-terminal Kip2 extension (Δ70-Kip2¹³myc) lost interaction with HA-Bim1 almost completely (Fig. 5B), although the effect was difficult to assess because also the cellular levels of these Kip2 variants were decreased. However, mutation of the N-terminal SNIP¹ to SNSS abrogated the
interaction between Kip2 and Bim1 (Fig. 5C, for localisation of the Kip2-variants see Fig. 5D).

*In vitro*, recombinant $^{\text{MBP}}\text{Kip2}\Delta\text{C}^{\text{GFP}}$ protein interacted with the EB1 domain of Bim1 (GST-EB1Bim1) and this interaction was abrogated after deletion of the N-terminal sequences that included the SxIP$^1$ motif (Fig. 5E). Mutation of the N-terminal SxIP$^1$ motif reduced, yet did not abolish, the interaction with GST-EB1Bim1, while additional mutation of the second SxIP$^2$ motif weakened the interaction further (Fig. 5E). Importantly, deletion of the first 70 N-terminal aminoacids of Kip2 essentially abrogated binding. Hence, we concluded that the N-terminal SNIP$^1$ is indeed a Bim1-binding site in Kip2.

Does Mck1 regulate the interaction between Kip2 and Bim1? In support of this notion, we found that only the hypo-phosphorylated form of Kip2 co-immunoprecipitated with $^\text{HA}\text{Bim1 in vivo}$ (Fig. 5C). Importantly, the recombinant allD-$^{\text{MBP}}\text{Kip2}\Delta\text{C}^{\text{GFP}}$ variant failed to interact with the EB1 binding domain of Bim1 *in vitro* (Fig. 5E). Therefore, this data suggest that the N-terminal Kip2 domain contains a functional EB1-interaction motif that mediates interaction of Kip2 with Bim1 and that phosphorylation of Kip2 by Mck1 close to the EB1-binding motif inhibits the Kip2-Bim1 interaction. Thus, Mck1-dependent phosphorylation interferes with the cellular activity of Kip2 by two different mechanisms; first, by reducing its overall affinity to microtubules and additionally, by inhibiting Kip2 binding to its processivity factor Bim1.

**Kip2 phosphorylation controls the amount of Kar9 and dynein on astral microtubule plus ends**

We examined the implications of Mck1-dependent Kip2 regulation in spindle positioning. As mentioned, Kip2 is required for deployment of dynein and Kar9 to
aMT-plus ends (Maekawa et al., 2003; Sheeman et al., 2003). Thus, we first analysed genetic interactions of *MCK1* with the dynein and the Kar9 pathway. Both pathways require aMTs for their function and their simultaneous genetic deactivation is lethal for yeast cells. Depletion of Mck1 resulted in synthetic growth defects of cells deleted either for *DYN1* (the dynein heavy chain) or *KAR9* (Fig. 6A), suggesting that Mck1 is indeed required for proper function of both pathways, and consistent with the role of Mck1 in regulation of aMT function by Kip2 phosphorylation. In agreement with this idea, we found that a fraction of spindles were mispositioned in *mck1Δ* cells as well as in cells expressing the hypo-phosphorylated Kip2-AT variant (Fig. 6B), mainly due to an increase of the distance of the spindle from the cleavage apparatus at the bud neck.

As Kip2 is able to transport dynein to the microtubule plus end (Roberts et al., 2014), we next examined whether lack of Kip2 phosphorylation by Mck1 would affect deployment of dynein to aMT plus ends. For this, we quantified localisation of dynein on aMT plus ends in cells expressing the hypo-phosphorylated Kip2-AT variant. Dynein displayed a significant accumulation at aMT ends in these cells (Fig. 6C). Depletion of Mck1 (which was expressed under the repressible *P*\_GAL1-10 promoter) also resulted in increase of the amount of dynein at aMT ends, while Mck1 overexpression led to the opposite effect (Fig. 6C). Thus, we propose that Mck1 controls plus end localisation of dynein by adjusting the microtubule affinity and processivity of Kip2.

The protein Kar9 requires Bim1 for aMT localisation (Miller et al., 2000) and also depends on Kip2 for transport to the plus ends of aMTs (Carvalho et al., 2004; Maekawa et al., 2003). We therefore examined as well whether the interaction between Kip2 and Bim1 is required for localisation of Kar9 complexes to MT plus
ends. In agreement with this hypothesis, intensity of Kar9 at aMT plus ends was clearly reduced in cells expressing Kip2-SS as a sole Kip2 source (Fig. 6C). Intriguingly, expression of the hypo-phosphorylated Kip2-AT variant did not increase the amount of Kar9 at bud MT plus ends significantly. Since Mck1 phosphorylation inhibits binding of Kip2 to Bim1, and the Kip2-SS variant showed reduced targeting of Kar9 to aMT plus ends, we nevertheless think that that Mck1 may control the plus end deployment of Kar9, besides dynein.

The phosphorylation of Kip2 requires microtubules and may take place at cortical sites

Mammalian GSK-3 is spatially regulated at cortical sites (Etienne-Manneville and Hall, 2003; Wittmann and Waterman-Storer, 2005). We thus asked whether phosphorylation of Kip2 by Mck1 takes place at specific cellular sites and examined closer the localisation Mck1 and Rim11 using spinning disc confocal microscopy and subsequent deconvolution. Mck1-GFP localises throughout the cytoplasm and the nucleus, as previously reported (Huh et al., 2003). In addition, Mck1-GFP accumulates at the cortex of emerging and small buds, at the bud neck, as well as at the spindle poles, either at the spindle pole bodies (SPBs, the yeast microtubule organising centres) or at kinetochores (Fig. 6D). Rim11-GFP displays similar localisation, but does not localise to the spindle poles (data not shown). The localisation of Mck1 suggests that Kip2 phosphorylation could take place either in the cytoplasm, at the cortex or at the spindle poles, since Kip2 localises exclusively on aMTs.

To further clarify this issue, we asked whether aMTs would be required for Kip2 phosphorylation. After depolymerisation of aMTs with nocodazole,
phosphorylation of Kip2 was lost within 20 min (Fig. 6E). Loss of phosphorylation is independent of the nocodazole-induced cell cycle arrest, since it still occurred in mad2Δ cells. In view of the cortical localisation of Mck1 and Rim11, this data suggest that Mck1 does not phosphorylate Kip2 in the cytoplasm or at the SPB, but possibly as aMTs reach the cell cortex. It is therefore possible that Mck1 controls aMT function by phosphorylating Kip2, thereby regulating dynein- and Kar9- plus end turnover at cortical sites (see discussion).

Discussion

In this work we show that Mck1 phosphorylates Kip2 regulating aMT length and the load of dynein and Kar9 complexes at aMT plus ends. We demonstrate that phosphorylation occurs mainly at the entire S/T-rich N-terminal part of Kip2. Phosphorylation of Kip2 by Mck1 occurs in a sequential manner and requires S63. Mck1 has been shown to prefer substrates that are pre-phosphorylated close to the GSK-3 consensus (Tan et al., 2014), similar to its mammalian counterpart (Fiol et al., 1988). Substitution of GSK-3 sites by D or E residues supports phosphorylation of S/T residues lying further upstream in vitro (Roach, 1991). However, substitution of S63 in Kip2 by D or E failed to mimic and rather abrogated phosphorylation (data not shown).

Two lines of evidence suggests that phosphorylation at T275 in a GSK-3 consensus found in the motor domain of Kip2 contributes to Kip2 regulation. First, the S63A T275A substitutions displayed the strongest effects regarding aMT stabilisation and resistance of cells to the MT destabilising effect of benomyl (Fig. 3D). Second, phosphorylation by Mck1 was reduced after introduction of the T275A mutation in vitro (Fig. 1F). We thus think that phosphorylation of Kip2 at T275
contributes to Kip2 regulation by Mck1, but how exactly T275 phosphorylation affects Kip2 function remains unclear.

It is also not clear which kinases prime Kip2 for phosphorylation downstream of the GSK-3 consensus sites. Our in vivo experimental data does not support the in vitro data suggesting that Cdc28 acts as a priming kinase at S63 (Ubersax et al., 2003). In fact, later proteomic studies did not identify Kip2 among the in vivo Cdc28 substrates (Holt et al., 2009). In contrast, Dbf2/Dbf20 is a strong candidate for a priming kinase: phosphorylation of Kip2 was reduced in dbf2-2dbf20Δ cells and deactivation of Dbf2 kinases led to aMT stabilization. Moreover, the N-terminal extension of Kip2 contains 9 sites that match the Dbf2 consensus. In addition, Kip2 phosphorylation is cell cycle regulated and increases in anaphase, at the time of Dbf2 activation, while this regulation is lost upon Dbf2 deactivation. Further experiments will be required to address this issue, which is complicated by the fact that other kinases seem to act on the Kip2 N-terminus, mainly at positions 69 and 72.

Importantly, lack of Kip2 phosphorylation by Mck1 stabilizes aMTs similar to the overexpression of Kip2 (Carvalho et al., 2004). Therefore, aMT stabilisation in cells with reduced Kip2 phosphorylation could be due to increased cellular amounts of Kip2. Several lines of evidence argue against this idea: first, cellular steady-state levels of the aMT-stabilising Kip2-AT and Kip2-AA variants did not significantly differ from wild-type (for example Figs 1B or S1A). Second, cellular Kip2 levels did not increase upon Mck1 down-regulation or decrease upon Mck1 overexpression (Fig. 1C). And finally, turnover of Kip2-AA was indistinguishable from wild type Kip2 (Fig. 4B). In contrast, the stabilising effect of Kip2-AT on aMTs can be adequately explained by the finding that loss of Kip2 phosphorylation increases the amount of the Kip2 that is able to bind aMTs, a prediction that we
confirmed in vivo (Fig. 4A) and in vitro (Fig. 4C). This in turn suggests that aMT stabilisation in Kip2-AT expressing cells would be due to increased transport of the Kip2 cargo Bik1 to aMT plus ends. Intriguingly however, we did not detect any significant increase of Bik1 at aMT plus ends in Kip2-AT expressing cells, thus the molecular mechanisms that cause aMT stabilisation in these cells remain to be determined.

We found that Kip2 interacts with Bim1 via an EB1-interaction motif present inside the Kip2 N-terminal domain, close to the Mck1 and Dbf2 phosphorylation sites. Although we can efficiently detect the Kip2-Bim1 interaction in pull downs, the complex is probably not very stable, as it cannot withstand a gel filtration (Roberts et al., 2014). Furthermore, we propose that a similar N-terminal extension of Tea2 (the fission yeast Kip2 homologue) contains two EB1 binding sites as well. Fission yeast EB1, Mal3, binds to the Tea2 N-terminus and acts as a processivity factor, since Tea2 does not efficiently track MTs in the absence of Mal3 (Bieling et al., 2007; Browning and Hackney, 2005). Similarly, Bim1 and Bik1 act as processivity factors for Kip2 and are required for Kip2 to counteract the minus-end force when it transports dynein in vitro (Roberts et al., 2014). This evidence is consistent with our observation that abrogation of Kip2 phosphorylation leads to Kip2- and dynein accumulation at aMT plus ends and that the Bim1-Kip2 association is required for efficient transport of Kar9 to the aMT plus ends. However, it is not clear how Kip2 transports Kar9 to the ends of MTs. Acting as a processivity factor for Kip2, Bim1 may indirectly affect Kar9 transport to aMT plus ends (Browning and Hackney, 2005). Alternatively, the Bim1-Kar9 complex may be transported “hitchhiking” on Kip2, although it is difficult to envisage how Bim1 would bind simultaneously to both proteins. A third
possibility is that Kip2 indirectly alters plus end localisation of Kar9 by affecting deployment of Bim1 to aMT ends.

Our data suggests that Kip2 phosphorylation by Mck1 weakens the association of Kip2 with Bim1 and aMTs. In this manner, Mck1 and Dbf2 could control aMT dynamics at the cell cortex (Fig. 7). According to this model, Kip2 becomes phosphorylated by Mck1 as it reaches the cortex. Phosphorylated Kip2 dissociates from aMTs, causing aMT destabilisation (Figs 7A,B). Dissociation of the Kip2-Bik1 complex from aMTs upon phosphorylation by Mck1/Dbf2 may be also part of the mechanism utilised for offloading of dynein at the cell cortex (Fig. 7B). This idea is also supported by the finding that overexpression of Mck1 results in reduction of dynein and depletion of Mck1 in increase of dynein at aMT plus ends. Conversely, when Mck1/Dbf2 phosphorylates Kip2, it disrupts not only the interaction between Kip2 and aMTs, but also the interaction between Kip2 and Bim1. This would lead to dissociation of Bim1 (and possibly also Kar9) from Kip2 and its deployment to aMT plus ends (Fig. 7C). Our model predicts that both dynein- and Kar9-dependent spindle positioning should be defective in cells lacking Mck1, as also supported by the genetic interactions of mck1Δ with both dyn1Δ and kar9Δ. In vitro assays are certainly required to test these ideas in detail.

To this date, the role of yeast GSK-3 in control of aMT dynamics was largely unknown. This study fills a gap between yeast and mammalian cells. Kip2 stabilises aMTs, binds to Bim1 and promotes transport of APC-related Kar9 to aMT plus ends in budding yeast. This resembles kinesin KIF17, which was recently found to stabilise microtubules, interact with EB1 and localise APC to MT plus ends in mammalian cells (Jaulin and Kreitzer, 2010). We found that Mck1 accumulates at cortical sites of polarised growth in yeast cells and that it regulates aMT dynamics and the amount of
Kip2 and dynein at aMT plus ends. Moreover, Mck1 regulates the interaction between Kip2 and Bim1. We have also shown that the Kip2-Bim1 interaction is required for efficient localisation of Kar9 to aMT plus ends. In mammalian cells, GSK-3 phosphorylates +TIPs such as CLASP2 or APC, and weakens their interaction with MTs or other +TIPs (Rubinfeld et al., 1996; Zumbrunn et al., 2001), while local inhibition of GSK-3 stabilises microtubules and promotes their interaction with the cortical cytoskeleton during cell migration (Akhmanova et al., 2001; Etienne-Manneville and Hall, 2003; Kumar et al., 2009; Watanabe et al., 2009). Regulation of Kip2 by Mck1 in budding yeast may be another example for a GSK-3-dependent mechanism utilised to couple regulation of microtubule dynamics with control of aMT-cortical interactions.

Materials and methods

Yeast growth and media

All strains were derivates of S288C (ura3-52 lys2-801 ade2-101 trp1Δ63 his1Δ200 leu2Δ1) or BY4743 (for details see Table S1 in supplementary material) and grown on standard media.

Sequence analysis and generation of Kip2 variants

Sequence analysis to identify phosphorylation sites was performed by NetPhosK (Technical University of Denmark, http://www.cbs.dtu.dk/services/NetPhosK/) and the Eukaryotic Linear Motif resource ((Gould et al., 2010), ELM; http://elm.eu.org/). All Kip2 variants were generated by site directed mutagenesis. Untagged versions as well as Venus-YFP tagged Kip2 variants were cloned into the integrative pRS305 plasmid under control of the endogenous KIP2 promoter and integrated into the LEU2 locus. 13xmyc-tagged Kip2 variants expressed
under control of the \textit{KIP2} promoter were sub-cloned into the pRS314 plasmid. For full plasmid list see Table S2 in supplementary material.

**Microscopy**

Microscopy was carried out with an Olympus IX81 Microscope operated by the Cell R software (Olympus Germany, Hamburg). Microtubules were visualised by integrating a GFP-Tub1 or CFP-Tub1 encoding plasmid into the \textit{URA3}, \textit{LYS2} or \textit{TRP1} locus and analyzed in small-budded cells with spindles no longer than 2 µm using ImageJ. Length measurements were made by ImageJ. \textit{Fluorescence intensities} were measured with ImageJ in an area of 0.3 µm (a circle of 0.32 µm radius) in the relevant focal plane of a 9-image z-stack after background subtraction. Only bud aMTs of metaphase cells were examined. This restriction takes account for the temporal and spatial localisation pattern of Mck1 and Rim11 (see Figs 5E and S3E). Mck1-GFP and Rim11-GFP imaging was performed using a Perkin Elmer spinning disc on a Nikon TE2000 inverted microscope. Deconvolution was performed with Huygens Essential 3.4 (Scientific Volume Imaging, Hilversum, The Netherlands).

**Statistical analysis**

Statistical comparison of datasets was made using a 2-tailed t-test of unequal variance. The number of measurements was large enough to allow generation of histograms. aMT lengths are stochastic and expected to follow a normal distribution.

**Protein expression and purification**

\textit{His}_{6}eGFP-Kip2, \textit{His}_{6}eGFP-Kip2-AA, \textit{His}_{6}mCherry-Bik1:} All proteins were expressed in SF9 cells from a pFastBacM13 vector integrated in its corresponding Bacmid system carried by DH10Bac cells (BAC-TO-BAC\textsuperscript{TM} expression system; Invitrogen, Paisley, UK) according to (Wasilko et al., 2009). Cells were lysed in 50
mM HEPES pH 7.5, 150 mM NaCl, 3 mM EGTA, 1.5 mM MgCl, 0.5 mM ATP, 5% glycerol, 0.1% Tween-20, 15 mM sodium pyrophosphate plus protease inhibitors. Purification was carried out in a two-steps using Blue Sepharose (6 Fast Flow) and Ni²⁺-Sepharose (HisTrap HP, both GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

_Mck1-CBP_: Yeast cells expressing Mck1-TAP expressed from its genomic locus were lysed in 50 mM Tris pH 7.5, 300 mM NaCl, 1.5 mM MgCl, 0.5 mM DTT, 0.1 mM ATP, 10% Glycerol, 0.01% NP-40, complete protease inhibitor and 1 mM β-phospho-glycerate. Bait protein was allowed to bind to IgG-Sepharose (6 Fast Flow, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and bound protein was eluted by incubating with TEV-protease.

_His₆Bim1, GST-Kip2¹⁻⁸⁰, GST-EB1Bim1_, and_MBP Kip2ΔC_ constructs: Cells expressing the corresponding proteins from the pET28 vector in Rosetta (Merck, Darmstadt, Germany) were lysed in 50 mM Tris pH 7.5, 200 mM NaCl, 1 mM MgCl, 1 mM EGTA, 0.01% NP-40 plus protease inhibitors (Complete™, Boehringer) and purified using standard conditions After elution, the proteins were dialysed against the lysis buffer, aliquotted and snap-frozen in liquid nitrogen.

**Western blots, CHX-chase, immunoprecipitation and -staining, metabolic labelling**

_Western blots_: Protein extraction was performed as in (Knop et al., 1999) or with glass beads in 50 mM Tris pH 7.6, 150 mM NaCl, 1 mM MgCl, protease- and phosphatase-inhibitors and 0.1% NP-40 (better resolution of phosphospecies). For visualisation of Kip2 phosphoisoforms cell lysates were analyzed by SDS-PAGE on 6% polyacrylamide gels and western blot. Antibodies used for western blot: _primary_:
anti-c-myc (1:2000, Rabbit polyclonal, Sigma) anti-HA (1:2000, 12CA5, ABGENT, San Diego, USA), anti-GFP (Rabbit polyclonal, gift from J. Lechner), anti-Arc1 (1:40000, Rabbit polyclonal, gift from E. Hurt), anti-His antibodies (1:1000, 6-His, Covance, Emeryville, USA), rabbit PAP, polyclonal (1:1000, DakoCytomation) and anti-tubulin antibody (1:1000, DM1A, Sigma-Aldrich, St.-Louis, USA). secondary: anti-mouse IgG Peroxidase (Fc-part) and anti-rabbit IgG Peroxidase (whole molecule) (both 1:10000, goat, Sigma-Aldrich, St.-Louis, USA). For cycloheximide-chase cells were grown in selective media, protein translation was stopped by addition of 500µg/ml cycloheximide and samples were analysed by western blot. For CIP-treatment lysates were treated with 0.5 u/µg Protein CIP or phosphatase inhibitor cocktail II (Sigma-Aldrich, St.-Louis, USA). The reaction-mix was incubated for 30 minutes at 37°C.

*Immunoprecipitations of HA-Bim1 and HA-Bik1: P_{GAL1}-dependent expression* was induced at OD 0.5 for 4 hours by adding galactose to 2% final concentration. Cells were lysed in 50 mM Tris pH 7.6, 150 mM NaCl, 1 mM MgCl₂, protease- and phosphatase-inhibitors and 0,1% NP-40 and proteins precipitated overnight with 2 µg anti HA-antibody (clone 7HA, Sigma-Aldrich, St.-Louis, USA) and protein A-Sepharose.

*Immunostaining:* Infected SF9 cells were harvested washed twice with PBS and fixed with 4% paraformaldehyde. After washing with PBS, permeabilisation with 1% Triton in PBS for 10 min and additional washing with PBS, cells were blocked in PBS + 0.1% Tween + 1% BSA for 1h at 4°C. Cells were stained over night at 4°C with anti-tubulin antibody (1:250 in blocking buffer, DM1A, Sigma-Aldrich, St.-Louis, USA), washed 3 times 10 min with PBS and incubated for 1 hour at 4°C with secondary antibody (anti-mouse Alexa680, 1:1000 in blocking buffer (Molecular
Probes/Invitrogen, Invitrogen, Paisley, UK). Cells were again washed 3 times with PBS and mounted for microscopy.

**Metabolic labelling:** Cells were grown to OD 0.5 in low phosphate YPD. 25 ODs were harvested and re-suspended in 1ml low phosphate YPD with 4% Glucose. Cells were grown for an additional hour in presence of 500µCi/ml inorganic $^{32}$P phosphate. After cell lysis Kip2-13myc was immunoprecipitated (see above) by anti-c-myc (1:2000, Rabbit polyclonal) antibodies for 2 hours at 4°C and analyzed by western blot.

**In vitro kinase assay and in vitro binding**

The His$_6$ tag of eGFP-Kip2 was cleaved of by His$_6$-TEV protease. Uncleaved His$_6$-eGFP as well as the TEV-protease itself was removed with metal affinity beads (Talon IMAC, Clonetech Laboratories, Mountain View, USA).

6 pmol of purified eGFP-Kip2 were incubated with 2.5 pmol purified Mck1-CBP for 30 minutes at 30°C in kinase-buffer (50 mM Tris pH7.0, 10 mM MgCl$_2$, 0.1 mM EGTA, 1 mM DTT, 100 µM unlabeled ATP, 0.3-0.4 µM $^{32}$P γ-ATP). For the subsequent *in vitro* binding assay, an excess of His$_6$mCherryBik1 (120 pmol) or His$_6$Bim1 (300pmol) was added and the buffer adjusted to 150 mM NaCl (plus complete protease inhibitor). Proteins were allowed to bind to metal affinity beads for 1h at 4°C and beads were washed five times with IP-Buffer (see above). Supernatants and beads were analyzed by SDS-Page followed by western-blot or autoradiography.

**Microtubule sedimentation assay**

Microtubule polymerisation of 200mM tubulin was induced by 5% glycerol and 1mM GTP for 30 minutes at 37°C. Tubulin was diluted to 10mM final concentration in 50mM Tris pH 7.0, 150mM KCl supplemented with 10mM Taxol or
30 µM nocodazole. Polymerised and non-polymerised tubulin was separated by centrifugation at 67000g at 28°C through a 40% glycerol cushion. His$_6$eGFP-Kip2 was cleared for 15 minutes at 67000g and 28°C. 7pmol Kip2 and ~ 40nmol tubulin were incubated for 20 min at 28°C. Bound Kip2 was again separated by centrifugation through a glycerol cushion. Supernatants and microtubule-containing pellets were analyzed by SDS-PAGE and western blotting as described above.

**Acknowledgements**

We are grateful to R. Lemaitre for help with protein expression; L. Stevermann for the Bim1 protein and critical reading of the manuscript; to J. Reichert for conducting the mass-spectrometry, Y. Barral, J. Ortis and J. Lechner for sharing information, strains, plasmids and antibodies; to E. Hurt, and T. Söllner for technical support and to U. Engel, P. Bankhead and the Nikon Imaging Center for support with microscopy and deconvolution. The Nikon Imaging Center is supported by CellNetworks-Cluster of Excellence (EXC81). H.D. is supported by the Boehringer Ingelheim Foundation, A.N.T. and D.L. were supported by the German Research Foundation (DFG).
Figures

Fig. 1. Mck1 phosphorylates Kip2 in vivo and in vitro

(A) Details of Kip2 variants described in this paper and abbreviations used in labelling as well as graphical representation of Kip2, the position of the GSK-3, the Cdc28 and the Dbf2 consensus sites, S63 and T275 in red. The SxIP Bim1/EB1 binding motif is marked in yellow.

(B) Comparison of phosphorylation and expression levels between unphosphorylatable Kip2$^{13\text{myc}}$-variants and Kip2$^{13\text{myc}}$ in mck1Δ and rim11Δ cells. Phosphorylation of Kip2$^{13\text{myc}}$ is reduced upon introduction of S63-to-A mutation, in mck1Δ cells and marginally in rim11Δ cells. Shown is an western blot against the myc
epitope of whole cell extracts. Variant labelling see (A), AA corresponds to Kip2^{13myc}-S63A T275A. Arc1 serves as loading control.

(C) Depletion of Mck1 leads to decrease in Kip2 phosphorylation. Overexpression of Mck1 does not increase phosphorylation of hypo-phosphorylated Kip2^{13myc} variants mutated in the GSK-3 consensus site(s). HA\-Mck1 expression from the GAL1-10 promoter (P_{GAL}) was induced by addition of galactose (+), depletion (-) by addition of glucose to the media. Western blot shows Kip2^{13myc} phosphorylation and HA\-Mck1 expression levels. Arc1 serves as loading control.

(D), (E) In vivo $^{32}$P-phosphate labelling of Kip2^{13myc} variants and Kip2^{13myc} in different kinase mutants. Kip2 loading: western blot of the Kip2^{13myc} variants after immunoprecipitation from cells labelled with $^{32}$P-phosphate, the same blot was used for the autoradiography shown.

(F) Mck1 phosphorylates Kip2 in vitro. MBP\-Kip2ΔC\-GFP and displayed variants were pre-incubated with Cdk1/Cyclin B and/or ATP followed by incubation with Mck1^{TAP} and γ$^{32}$P-ATP as indicated. Autoradiography of subsequent SDS-PAGE is shown. Mck1^{TAP} phosphorylates Kip2 only after pre-incubation with Cdk1 and phosphorylation is abolished after introduction of the S63A and S275A mutations.

(G) Sequential phosphorylation of Kip2 N-terminus by Mck1. A fusion of the 80 N-terminal aminoacids of Kip2 to GST (GST\-Kip2^{1-80}) is phosphorylated only after pre-incubation with Cdk1. Mutation of S63 but also of the upstream T59 abrogated phosphorylation.
Fig. 2. Dbf2 and additional kinases act on Kip2 as a priming kinases for GSK-3

(A) The sites S69 and S72 downstream of S63 act presumably as phosphorylation priming sites for Mck1. Western blots of the indicated Kip2\textsuperscript{13myc} variants. Kip2\textsuperscript{13myc} phosphorylation is reduced upon mutation of the indicated sites.

(B) Kip2\textsuperscript{13myc} phosphorylation is clearly reduced in \textit{dbf2-2 dbf20Δ} mutants

(C) Kip2 phosphorylation is cell cycle regulated and increases in anaphase (western blot against Kip2\textsuperscript{13myc} of cell extracts from cells synchronized with α–factor for 3h and released in fresh medium lacking the pheromone, at 30°C).
Fig. 3. Mck1 and Dbf2 regulate aMT stability through Kip2 phosphorylation

(A) Lack of Mck1- and Dbf2-dependent Kip2 phosphorylation stabilises aMTs. Images of microtubules from kip2Δ cells expressing unphosphorylated Kip2 variants and from depicted kinase mutants. The MTs shown here do not represent the mean MT length but are characteristic for each strain.

(B) Length distribution and corresponding statistics for bud aMTs in G2/M cells of depicted strains. Number of counted aMTs shown in brackets, p is the p value
for the statistical significance of the difference between the distribution of the strain on the left of the table and the MT distribution of the strain denoted in brackets. Imaging of all strains was performed in the same experiment. See Fig. S2B for additional aMT length measurements.

(C) The number of aMTs is increased upon lowering Kip2 phosphorylation. The number of aMTs growing from either spindle pole was counted, N>100 cells for each strain.

(D) *kip2Δ* cells show growth sensitivity, and Kip2-AA-expressing cells resistance to the microtubule-destabilising drug benomyl. See Fig. S2A for images of Kip2-SA and SE (T275-to-E substitution) variants.
Fig. 4. Phosphorylation regulates association of Kip2 with microtubules

(A) Mck1-dependent phosphorylation regulates Kip2 load on aMTs. Fluorescence intensity of Kip2-YFP, Kip2-AT-YFP and allD-Kip2-YFP in wild type-cells and of Kip2-YFP in \(^{HA}\)Mck1 overexpressing or -depleted cells. Examples of quantified images are given above each graph. Numbers of counted aMTs in brackets, arbitrary intensity units ±sem are shown. Bar: 3 μm

(B) Cycloheximide chase of plasmid-borne Kip2\(^{13}\text{myc}\) AA under control of the \(KIP2\) promoter in cells expressing Kip2\(^{TAP}\) from the \(KIP2\) locus as an internal control. Kip2 phosphoisoforms were not resolved here in order to facilitate comparison between different Kip2 variants.

(C) A variant of Kip2 with a phospho-mimicking N-terminus (allD-MBP\(^{\text{Kip2∆C\text{GFP}}}\)) binds less efficiently to microtubules \textit{in vitro}, compared to the unchanged variant. Shown are coomassie stained gels of microtubule sedimentation assays, after incubation of \textit{in vitro} polymerised, taxol-stabilized MTs with recombinant and MBP\(^{\text{Kip2∆C\text{GFP}}}\) or allD-MBP\(^{\text{Kip2∆C\text{GFP}}}\). S: supernatant, P: pellet. Refer also to Fig. S2D.
Fig. 5. Kip2 interacts with Bim1 over its N-terminal extension in phosphorylation-dependent manner

(A) Alignment of the N-terminal extensions of Kip2 and fission yeast Tea2. The proposed EB1-interacting motifs are shown in green, positively charged residues in yellow. The GSK-3 consensus at S63 is shown in bold.

(B), (C) Bim1 interacts with the EB1-binding motif at the Kip2 N-terminus, independently of Bik1. Immunoprecipitations of HA-Bim1 from kip2Δ or kip2Δ bik1Δ cells expressing depicted plasmid-borne Kip213myc variants. Note that only un- (or hypo)-phosphorylated Kip2 isoforms co-precipitate with HA-Bim1 (seen in C). P_{GAL3}HA-BIM1 expression was induced for 3h prior to immunoprecipitation. Immunoprecipitated proteins (IP) and extracts after the immunoprecipitation (input) were probed in western blots with anti-HA and anti-myc antibodies. Asterisk indicates antibody heavy chain. SS refers to the Kip213myc variant with the SNIP motif mutated to SNSS.
(D) Localisation of the Kip2-YFP variants which show decreased interaction with Bim1 (C and D). Representative images of cells expressing CFP-Tub1 and integrated Kip2-YFP variants from the KIP2 promoter as the sole Kip2 source.

(E) The two EB1 binding motifs are required for Kip2-Bim1 interaction in vitro. Shown are coomassie stained gels INPUT: amounts of the recombinant proteins used in the pulldown assay, the EB1 domain of Bim1 (EB1Bim1) as a GST-fusion was bound to beads (bait). S^{IP1} and S^{IP2} denote the recombinant Kip2 variants with respective mutations (SNIP to SNNN) in the two EB1 binding motifs of Kip2 (see text). Mutation of these motifs does not abrogate but weakens the Kip2-interaction in an additive manner. The Kip2-Bim1 interaction is nearly lost upon deletion of the N-terminus (Δ70) or upon mutation of the N-terminal GSK-3 clusters to aspartates (allD, refer also Fig. 1A).
Fig. 6. Kip2 phosphorylation controls the amount of Kar9 and dynein on aMT plus ends and spindle positioning

(A) Genetic interactions of MCK1 with genes required for spindle positioning. 

$P_{GAL}^{HA}$MCK1 kar9Δ and $P_{GAL}^{HA}$MCK1 dyn1Δ cells display slow growth upon deactivation of MCK1 expression on glucose-containing plates.

(B) Spindles are mispositioned in mck1Δ cells and cells expressing Kip2-AT. Plotted is the angle of the spindle to the mother-bud axis (a, y-axis), against the distance from the bud neck (d, x axis), the vertical line denotes the bud neck. Numbers of spindles counted in brackets.

(C) Fluorescence intensity of Dyn1-YFP and Kar9-YFP on aMT plus ends in cells expressing the Kip2 variants indicated or in cells expressing wild type Kip2 in
HA Mck1 overexpressing or depleted cells. Numbers of counted aMTs in brackets, arbitrary intensity units ±sem are shown. Bar: 3 µm

(D) Localisation of Mck1-GFP. A Mck1 sub-pool localises to the incipient bud, to the bud cortex until G2/M and the bud neck. Mck1 also localises to the spindle poles (possibly SPBs or kinetochores) from S phase until anaphase. Bar: 2.5 µm

(E) Phosphorylation of Kip2 requires microtubules. Time course showing Kip2\textsuperscript{13myc} phosphorylation in extracts of wild type- and spindle assembly checkpoint-defective \textit{mad2Δ} cells, after MT depolymerisation. Nocodazole was added at 40 min (arrow). Equal amount of cells were used to generate the extracts for each time point.
Fig. 7. Model for regulation of astral microtubule functions by Mck1

Left: Mck1 phosphorylates Kip2 at the N-terminal extension, including the S63-containing GSK-3 consensus, close to the EB1-binding motif (SNIP). Phosphorylation disrupts interaction between Kip2 and Bim1. In addition, phosphorylation (involving possibly also T275) causes dissociation of Kip2 from aMTs. Dbf2 and unknown kinase(s) act as priming kinase for Mck1. Right: Mck1 may regulate aMT function through Kip2 phosphorylation in three ways. A: Unphosphorylated Kip2 binds and stabilises aMTs. B: When aMTs reach the cortex, Mck1 phosphorylates Kip2, leading to Kip2 dissociation from aMTs and offloading of dynein at cortical sites. The process may be enhanced upon anaphase onset, the time of Dbf2 activation. C: Phosphorylation by Mck1 also disrupts interaction between Kip2 and Bim1, leading to release of transported Bim1-Kar9 complexes at aMT plus ends. Dephosphorylation of Kip2 by a phosphatase restarts the cycle.
References


