The Nuclear Transport Factor Kap121 Is Required for Stability of the Dam1 Complex and Mitotic Kinetochore Bi-orientation

Highlights
- Kap121 is required for accurate kinetochore bi-orientation in budding yeast
- Kap121 binds components of the Dam1 complex and is required for its stability
- Tubulin and RanGTP together induce release of Kap121 from the Dam1 complex

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The Nuclear Transport Factor Kap121 Is Required for Stability of the Dam1 Complex and Mitotic Kinetochore Bi-orientation

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SUMMARY

The karyopherin (Kap) family of nuclear transport factors facilitates macromolecular transport through nuclear pore complexes (NPCs). The binding of Kaps to their cargos can also regulate, both temporally and spatially, the interactions of the cargo protein with interacting partners. Here, we show that the essential yeast Kap, Kap121, binds Dam1 and Duo1, components of the microtubule (MT)-associated Dam1 complex required for linking dynamic MT ends with kinetochores (KTs). Like mutations in the Dam1 complex, loss of Kap121 function compromises the formation of normal KT-MT attachments during mitosis. We show that the stability of the Dam1 complex in vivo is dependent on its association with Kap121. Furthermore, we show that the Kap121/Duo1 complex is maintained in the presence of RanGTP but Kap121 is released by the cooperative actions of RanGTP and tubulin. We propose that Kap121 stabilizes the Dam1 complex and participates in escorting it to spindle MTs.

INTRODUCTION

All macromolecules transit between nuclear and cytoplasmic compartments through massive macromolecular assemblies termed nuclear pore complexes (NPCs) embedded in the nuclear envelope (NE). NPCs function as selective gatekeepers, preventing many macromolecules from entering the central channel while allowing nuclear transport factors (NTFs) to pass. Many of these NTFs are members of a group of structurally related proteins termed karyopherins (Kaps, a.k.a. importins and exportins) that bind nuclear import or export signals in “cargo” molecules (often proteins), forming complexes capable of traversing NPCs. Kap movement through NPCs is thought to be mediated by a series of low-affinity interactions between the Kap and the NPC proteins (termed nucleoporins, or Nups) containing phenylalanine-glycine repeats (FG-Nups) present in the NPC central channel (reviewed in Wente and Rout, 2010; Aitchison and Rout, 2012).

Key to the spatial directionality of nuclear transport of many molecules is the small guanosine triphosphatase Ran (RanGTPase). Within the interior of the nucleus, Ran is maintained in the guanosine triphosphate (GTP)-bound state by a chromatin-bound Ran guanine nucleotide exchange factor (Ran-GEF: Rcc1 in vertebrates and Prp20 in yeast). Conversely, cytoplasmic Ran is predominately bound to GDP due to the activity of a cytoplasmic RanGTPase activating protein (RanGAP: RanGAP1 in vertebrates and Rna1 in yeast). Upon entry into the nuclear interior, incoming importin/cargo complexes encounter RanGTP, and binding of RanGTP to the importin triggers the dissociation of the cargo. Conversely, exportin/cargo complexes bind cooperatively to RanGTP, and the resulting trimeric complex is competent for export from the nucleus. Once in the cytoplasm, RanGAP binding induces the conversion of RanGTP to guanosine diphosphate Ran (RanGDP), facilitating dissociation of the exportin/cargo complex.

In addition to their roles in nucleocytoplasmic transport, Kaps are emerging as global regulators of a diverse set of cellular processes. For example, the function of Kaps during mitosis in both spindle and NPC assembly (Walther et al., 2003; Harel et al., 2003; Ryan et al., 2007; Rotem et al., 2009) has been studied in vertebrate systems. Following mitotic NE breakdown, importin-β binds proteins termed spindle assembly factors (SAFs) and temporally inhibits their function by preventing their interactions with spindle microtubules (MTs). This inhibitory activity imposed by importin-β is relieved through RanGTP binding, which induces release of SAFs in the immediate vicinity of chromatin (reviewed in Kalab and Heald, 2008). Using an analogous mechanism, importin-β also functions as a negative regulator of NPC assembly by temporally restricting Nup subcomplexes from assembling into higher-order assemblies (reviewed in Güttinger et al., 2009).

Other mitotic functions for Kaps include the ability of these proteins to function as chromatin targeting factors. At the onset of mitosis, the nuclear export factor Crm1 directs a number of regulatory proteins to kinetochores (KTs; Arnaoutov et al., 2005; Zuccolo et al., 2007; Torosantucci et al., 2008; Roscioli et al., 2012). For instance, Crm1 facilitates the targeting of the Ran-binding proteins RanGAP1 and RanBP2 to KTs, where they function in regulating KT-MT attachments (Arnaoutov et al., 2005; Zuccolo et al., 2007). Similarly, the yeast counterpart of Crm1, Xpo1, targets spindle assembly checkpoint (SAC)
regulators to detached KTs upon SAC activation (Scott et al., 2009). This intranuclear targeting event requires RanGTP.

In this study, we show that compromising Kap121 function leads to an abnormal distribution of mitotic KTs along the spindle axis. The role of Kap121 at the KT-MT interface is distinct from its conventional role in nuclear import, and instead is linked to its physical association with the MT-associated Dam1 complex. We show that Kap121 is required for the stability of components of the Dam1 complex, including Dam1 and Duo1, and conditions that restore cellular levels of these proteins, including the addition of a bulky C-terminal tag, such as GFP, rescue KT bi-orientation defects in kap121 mutants. Furthermore, the interaction of Kap121 with the Dam1 complex is maintained in the presence of RanGTP, but Kap121 is released by the cooperative actions of tubulin and RanGTP, suggesting that Kap121 deposits the Dam1 complex directly at spindle MTs. Together, these data reveal a function for Kap121 in regulating the integrity of KT-MT interactions, and they provide insight into contributions of Kaps to chromosome transmission during cell division.

RESULTS

Kap121 Is Necessary for KT Bi-orientation

Cells compromised for Kap121 function display mitotic progression defects (Makhnevych et al., 2003). For example, cultures of kap121-34 temperature-sensitive mutant cells show increased numbers of large-budded, 2N cells in the cell population at both permissive (23°C) and non-permissive (37°C) temperatures for growth (Makhnevych et al., 2003; Figure 1A). These data, and previous observations documenting altered timing of spindle elongation (Makhnevych et al., 2003), suggest that kap121-34 cells stall at the metaphase-to-anaphase transition. Two likely explanations for this phenotype are defects in anaphase spindle elongation (He et al., 2001; Severin et al., 2001) and SAC-mediated arrest stemming from defective KT-MT interactions (reviewed in McAlinsh et al., 2003; Tanaka et al., 2005; Westermann et al., 2007; Biggins, 2013). Consistent with the latter, we observed that (1) the SAC protein Mad1 is recruited to KTs in mitotic kap121-34 cells (Figure 1A), (2) kap121-34 cells lacking a functional SAC (kap121-34 mad2Δ) exhibit reduced growth, and (3) kap121-34 cells exhibit an increased sensitivity to MT-destablizing drugs (Figure 1B).

To assess mitotic defects associated with the kap121-34 mutant, we examined the distribution of KTs, using the central KT component Mtw1 tagged with GFP, and their positioning relative to spindle pole bodies (SPBs), visualized by Spc42-mCherry. As wild-type (WT) cells progress toward the metaphase-to-anaphase transition, KTs align on the mitotic spindle and adopt a bi-lobed configuration in which two separate KT foci (~1 μm apart) are positioned along the spindle axis between separated sister SPBs (Figure 1C, WT; Goshima and Yanagida, 2000; Tanaka et al., 2000; He et al., 2001; McAlinsh et al., 2003; De Wulf et al., 2003; Tanaka et al., 2005). While each of these lobes presumably contains a mixture of syntelic and bi-ori-ented sisters KT pairs (Marco et al., 2013), those incorrect mon-orientated attachments are eventually resolved just before sister-chromatid separation and anaphase onset.

However, in kap121-34 cells (grown at 23°C), the distribution pattern of Mtw1-GFP in metaphase cells suggested a defect in KT alignment and potentially KT bi-orientation. Most kap121-34 cells contained multiple, individual Mtw1-GFP foci distributed along the spindle axis, between separated sister SPBs (Figure 1C), and overlapping with GFP-Tub1 (Figure S1A). In addition, a subpopulation of cells contained KTs collapsed into a single focus between SPBs. These phenotypes were also detected in kap121-34 cells arrested in metaphase by depletion of the anaphase promoting complex/cyclosome (APC/C) co-activator Cdc20 (Figure 1D, P<sub>MET3</sub>-HA-CDC20, +Met). Furthermore, time-lapse imaging of mitotic kap121-34 cells revealed that KTs remain largely unclustered during this cell-cycle stage (Figure S1B). To assess whether these phenotypes reflect defects of KT bi-orientation, we monitored the distribution of GFP-labeled CEN11 in the kap121-34 mutant. In an actively growing cell population, we observed that <10% of large-budded kap121-34 cells displayed separated sister chromosomes and thus a bi-lobed configuration of the GFP-labeled CEN11 loci. In contrast, ~60% of WT cells contained visible bi-lobed CEN11 signals (Figure 1E). These data are consistent with a KT bi-orientation defect in the kap121-34 mutant.

Furthermore, we investigated whether defects in mitotic KT bi-orientation were specific for the kap121-34 allele. To test this, we examined KT distribution in another temperature sensitive (Ts) allele (kap121-41; Leslie et al., 2002) and cells depleted of WT Kap121 (P<sub>MET3</sub>-HA-KAP121; Figures S1C and S1D). As with the kap121-34 mutant, both kap121-41 mutant cells and cells depleted of Kap121 failed to properly bi-orient their sister KTs on the mitotic spindle (Figure S1C). Kap121-depleted cells showed the most severe KT defects, because Mtw1-GFP foci appeared off the spindle axis and distributed throughout the nucleus, consistent with the detachment of KTs from spindle MTs (Figure S1D). Quantification of the percentage of metaphase cells presenting KTs in the bi-lobed configuration revealed that all three alleles that altered Kap121 function (kap121-34, kap121-41, and P<sub>MET3</sub>-KAP121) displayed a low percentage of cells with bi-orientated KTs (Figure 1F). In contrast, mutants lacking Kap123, the Kap family member most closely related to Kap121 (Wozniak et al., 1998), showed no defects in KT distribution (Figure S1E). Taken together, these results imply that Kap121 function is required for KT bi-orientation during mitosis.

Inhibition of Kap121-Mediated Import Does Not Affect KT Bi-orientation

Because yeast cells undergo a closed mitosis, it was possible the KT bi-orientation defect in kap121 mutants arose as a consequence of failed Kap121-mediated nuclear import. If true, we would predict a direct correlation between conditions that inhibited Kap121-mediated import and defects in KT distribution. To test this, we examined KT alignment in cells overproducing Nup53 (P<sub>GAL</sub>-HA-NUP53). In these cells, Kap121-mediated import is specifically inhibited despite the presence of WT Kap121 (Marelli et al., 2001). In a P<sub>GAL</sub>-HA-NUP53 strain background, we examined Kap121-mediated transport (using an NLS<sub>mon</sub>-GFP reporter) and KT positioning (Mtw1-mCherry) following induction of NUP53 overexpression. Before induction, NLS<sub>mon</sub>-GFP accumulates in the nucleus and KT bi-orientation...
Figure 1. Cells Compromised for Kap121 Function Exhibit KT Bi-orientation Defects

(A) kap121-34 mutant cells exhibit a SAC-mediated mitotic delay. Daniel Finley 5 (DF5) (WT) and kap121-34 cultures were grown at 23°C and then shifted to 37°C for 4.5 hr. Quantification of the percentage of large-budded cells was determined under the indicated conditions (left; n ≥ 100 cells). kap121-34 cells synthesizing Mad1-mCherry and Mtw1-GFP (KT marker) were imaged following growth at 23°C and 3 hr after shifting to 37°C using an epifluorescence microscope (right). Scale bar, 2 μm.

(B) Cultures of the indicated strains were serially diluted, spotted onto YPD plates (top) or YPD plates containing benomyl (0, 5, or 15 μg/ml; bottom), and incubated at the indicated temperatures for 2–3 days.

(C) DF5 (WT) and kap121-34 cells producing Mtw1-GFP and Spc42-mCherry (SPB marker) were imaged using epifluorescence microscopy. Arrows point to KT clusters. Scale bar, 2 μm.

(D) P_{MET3}-HA-CDC20 and kap121-34 P_{MET3}-HA-CDC20 cells synthesizing Mtw1-GFP were grown overnight in medium lacking methionine (−Met). Methionine was added to cultures for 2.5 hr to deplete Cdc20 and induce metaphase arrest (+Met). A bar graph shows the percentage of large-budded cells (n ≥ 100 cells) presenting KT foci in a bi-lobed or in an unclustered or collapsed configuration under the indicated growth conditions.

(E) WT and kap121-34 cells containing a 1.7-kb lacO marker integrated ~1.1-kb away from the CEN11 loci and producing LacI-GFP were examined using an epifluorescence microscope. Images of cell populations were acquired from cells grown at 23°C or following growth at 37°C for 3 hr. A bar graph displays the percentage of large-budded cells (n ≥ 100) in the population with a visible bi-lobed LacI-GFP signal. Scale bar, 5 μm.

(F) A bar graph shows the percentage of large-budded cells in WT, kap121-34, kap121-41, or P_{MET3}-HA-KAP121 cell populations containing bi-lobed KTs or unclustered or collapsed KT foci (n ≥ 100 cells) at the indicated temperatures or expressing (−Met) or repressing (+Met) HA-KAP121.
in mitotic cells appeared normal (Figures 2A and 2C, uninduced, NLS\textsuperscript{Pro} \textsuperscript{GFP}). In contrast, overproduction of Nup53 prevents nuclear accumulation of NLS\textsuperscript{Pro} \textsuperscript{GFP} (Figures 2A and 2C, induced, NLS\textsuperscript{Pro} \textsuperscript{GFP}). However, despite the inhibition of Kap121-mediated import in these cells, mitotic chromosome alignment appeared normal, as indicated by the consistent presence of bi-lobed, bi-oriented KTs in cells transiting through metaphase (Figure 2A, induced, Mtw1-mCherry).

Our conclusion that compromising Kap121-mediated nuclear import alone does not give rise to KT bi-orientation defects is supported by two additional observations. First, we have detected that the addition of a protein A tag to the C terminus of Kap121 (Kap121-pA) inhibited nuclear import of the NLS\textsuperscript{Pro} \textsuperscript{GFP} reporter (Figures 2B and 2D), potentially by altering cargo binding to sites near the C terminus of Kap121 (Kobayashi and Matsuura, 2013). However, cells synthesizing the Kap121-pA fusion showed no defects in KT bi-orientation (Figure 2B). Second, ectopic expression of WT KAP121 in kap121-34 cells leads to partial restoration of import (Figure 2C; Leslie et al., 2002). Despite this restored nuclear import, ectopic expression of KAP121 in kap121-34 cells does not rescue WT growth rates (data not shown), and significantly, KT bi-orientation remained defective in these cells (Figure 2C). On the basis of these data, we conclude that the KT bi-orientation defects observed in the various kap121 mutant alleles cannot be explained strictly by the inhibition of cargo import.

Kap121 Binds the Dam1 Complex

Previous studies have reported various proteins detected in association with Kap121, with many established or potential import cargos (Gavin et al., 2002; Leslie et al., 2004; Krogan et al., 2006). Most of these have no obvious functional link to KTs, with the exception of Duo1, a protein identified in association with Kap121 in a high-throughput protein-protein interaction screen (Krogan et al., 2006). Duo1 is a structural component of the Dam1 complex, an essential MT-bound assembly required for physically linking dynamic MT tips with the outer KT (Hofmann et al., 1998; Cheeseman et al., 2001; Janke et al., 2002; Westermann et al., 2005; Miranda et al., 2005; Wang et al., 2007; Lampert et al., 2010, 2013; Tien et al., 2010, 2013; Umbreit et al., 2014). Mutations in DUO1 (duo1-2) exhibit KT alignment defects phenotypically similar to the kap121 mutants (Figure S2; Janke et al., 2002; Scharfenberger et al., 2003; Gillett et al., 2004; Umbreit et al., 2014). On the basis of these observations, we examined the interactions of Kap121 with the Dam1 complex. Kap121-pA was purified from cells also producing Duo1-13Myc or Dam1-13Myc. Western blot analysis of Kap121-pA bound proteins revealed both Duo1-13Myc and Dam1-13Myc co-purified.
C-Terminal Tagging of Duo1 Leads to the Suppression of KT Bi-orientation Defects in kap121-34 Cells

Our data lead us to conclude that the KT bi-orientation defect in the kap121-34 mutant is likely linked to the association of Kap121 with the Dam1 complex but potentially not with an import event mediated by Kap121. Consistent with this latter conclusion, an examination of GFP-tagged Dam1, Duo1, or Ipl1 in the kap121-34 mutant revealed that each GFP fusion protein was nucleoplasmic (Figure 4A). In WT cells, Duo1-GFP was visible at KTs, with mitotic cells exhibiting a linear, bi-lobed distribution of KT-associated Duo1-GFP. Surprisingly, the localization pattern of Duo1-GFP in kap121-34 cells appeared largely indistinguishable from WTs at both 23°C and 37°C (Figure 4A), suggesting the Duo1-GFP fusion may suppress the KT bi-orientation defect of the kap121-34 mutant. A phenotypic suppression effect was also observed in cells synthesizing Dam1-GFP (Figures 4A and S3A). We more closely examine the effects that Duo1-GFP had on KT bi-orientation. As in WT cells, Duo1-GFP in the kap121-34 mutant co-localized with the KT marker Mtw1-mCherry (Figure 4B) and was positioned between separated SPBs (labeled with Spc42-mCherry, Figure 4C). These results implied that the Duo1-GFP fusion protein bound to KTs and was capable of suppressing the KT bi-orientation defect of the kap121-34 mutant (Figure 4C). Similarly, the expression of Duo1-GFP fusion suppressed the KT bi-orientation defect in cells depleted of Kap121 (Figure S3B). This rescue effect is not limited to GFP fusions, because Duo1 constructs containing a C-terminal protein A or mCherry epitope were also capable of suppressing the KT bi-orientation defect in the kap121-34 mutant (Figures S3C and S3D). However, the addition of smaller peptide tags (3x hemagglutinin, or 3HA) to Duo1 failed to restore KT alignment, suggesting a bulky C-terminal tag contributes to the phenotypic suppression effect (Figure S3C). Although the presence of a bulky C-terminal tag on Duo1 rescued sister KT bi-orientation, it did not rescue the import defect of kap121-34 cells (Figure S3D) or its temperature-sensitive growth phenotype (data not shown). In kap121-34 cells expressing Duo1-mCherry, the NLS^NOC-GFP reporter failed to accumulate in nuclei (Figure S3D).

Loss of Kap121 Binding Destabilizes the Dam1 Complex

The ability of a bulky C-terminal tag, such as GFP, on Duo1 to restore normal KT bi-orientation in the kap121-34 mutant led us to hypothesize that mitotic defects associated with this mutant reflect changes in interactions of Kap121 with the Dam1 complex. To further evaluate this idea, we examined whether these interactions were altered in the kap121-34 mutant. In contrast to the binding of WT Kap121 to the Dam1 complex members (Figure 4), no Dam1-13Myc, Duo1-13Myc, or Ipl1-13Myc was detected in association with purified Kap121 (Figure S4B). Similarly, experiments performed with Duo1-pA, Dam1-pA, or Ipl1-pA failed to detect the kap121-34 mutant protein (Figure S4B).

Because both the assembly and the overall integrity of the Dam1 complex is compromised upon the loss of individual complex members (Li et al., 2002), we examined whether the decreased association of Kap121 with the Dam1 complex altered cellular levels of Duo1 and Dam1. Using western blotting analysis, we examined cellular levels of Duo1-3HA and Dam1-3HA in kap121-34 cells at both permissive and non-permissive temperatures for growth. Strikingly, the levels of both HA-tagged proteins were lower in kap121-34 cells compared to WT cells in
cultures grown at 23°C (Figure 5A). After a shift to 37°C for 3 hr, both proteins showed a further reduction to barely detectable levels (Figure 5A). Similarly, in cells depleted of Kap121, we observed decreased levels of Duo1-3HA (Figure S4C, P<sub>MET3</sub> HA-KAP121, +Met). Taken together, these results imply that the physical association of Kap121 with the Dam1 complex contributes to maintaining normal cellular levels of the complex.

The reduction in the Dam1 complex members in the kap121-34 mutant and cells depleted of Kap121 offers a likely explanation for its observed KT bi-orientation defects. Suppression of these defects by production of Duo1-GFP or Dam1-GFP (each driven by their endogenous gene promoters) led us to examine the cellular levels of the GFP fusions in kap121-34 mutant cells. As shown in Figure 5B, levels of GFP-tagged Duo1 and Dam1 in kap121-34 cells at 23°C and 37°C were comparable to WT cells. Similarly, unlike the HA-tagged version of Duo1, we observed that cellular levels of Duo1-GFP appeared unchanged following depletion of Kap121 (Figure S4C). These data imply that the addition of a bulky C-terminal tag stabilizes Duo1 and Dam1 in cells compromised for Kap121 function.

To further assess the functional relationship between KT-MT defects in kap121-34 cells and reduced levels of Duo1 detected in these cells, we analyzed whether overproducing Duo1 could suppress the KT bi-orientation defects in kap121-34 cells. For these experiments, the endogenous DUO1 promoter was replaced with the inducible MET3 promoter. In the P<sub>MET3</sub>-HA-DUO1 strain background, induction of the MET3 promoter (−Met) leads to increased cellular levels of Duo1 in kap121-34 cells (Figure 5C). Overexpression of HA-Duo1 in kap121-34 cells leads to an increase in the number of mitotic cells with bi-oriented KTs as visualized with Mtw1-GFP (Figure 5C). These data further support the conclusion that the KT bi-orientation defect detected in kap121 mutant cells is linked to the loss of Dam1 complex stability.

Kap121 Is Released from the Dam1 Complex by RanGTP and Tubulin
The binding of Kaps with their transport cargos is modulated by RanGTP. Nuclear RanGTP binds to importins and stimulates the release of cargo, leading to the accumulation of the cargo in the nucleoplasm and thus the directionality of nuclear import (reviewed in Wozniak et al., 1998). Given the nuclear localization of the Dam1 complex and its interactions with Kap121, we examined whether this complex was sensitive to RanGTP. We affinity purified Duo1-pA or, as a positive control for RanGTP-induced release, Nup53-pA from cell extracts containing Kap121-13Myc. Isolated complexes were then incubated with either RanGTP or RanGDP. Consistent with previous observations (Marelli et al., 1998), we observed that incubation of the Kap121/Nup53 complex with RanGTP, but not RanGDP, led to Kap121-13Myc release from Nup53-pA (Figure 6A, lanes 2–5). However, the Duo1-pA/Kap121-13Myc complex was unaffected by the addition of RanGDP or RanGTP (Figure 6B, lanes 2–5). On the basis of these data, we conclude that the interactions of examined. WT and kap121-34 mutant cell populations synthesizing Mtw1-GFP are shown for comparison.
Kap121 with Duo1 are atypical of importin/cargo complexes and that RanGTP alone is insufficient to stimulate the release of Duo1 from Kap121.

Precedents exist for RanGTP-resistant interactions between importins and their binding partners. For example, Kap121 binds the nuclear protein Ulp1, and their association is resistant to RanGTP (Panse et al., 2003). Instances have also been documented in which RanGTP-resistant importin-cargo interactions can be specifically dissociated by additional factors. For example, the RanGTP-resistant interactions of Kap104 and the RNA binding proteins Nab2 and Nab4 are disrupted in the presence of both RanGTP and mRNA (Lee and Aitchison, 1999). On the basis of interactions between the dynamic ends of MTs and the Dam1 complex (reviewed in Westermann et al., 2007), we examined whether the Kap121/Dam1 complex was sensitive to a combination of RanGTP and tubulin. Affinity-purified, bead-bound Duo1-pA/Kap121-13Myc and Nup53-pA/Kap121-13Myc complexes were incubated with purified tubulin and either RanGDP or RanGTP. The inclusion of tubulin with RanGDP or RanGTP did not alter the observable effects of RanGDP or RanGTP alone on the Nup53-pA/Kap121-13Myc interaction (Figure 6A, lanes 6 and 7). In contrast, the addition of tubulin, together with RanGDP, to the Duo1-pA/Kap121-13Myc complex induced a slight increase in the release of Kap121-13Myc from Duo1-pA (Figure 6B, lanes 6 and 7). However, most strikingly, a combination of tubulin and RanGTP led to the release of most Kap121-13Myc from Duo1-pA (Figure 6B, lanes 8 and 9). On the basis of these results, we conclude that the cooperative interactions of RanGTP and tubulin with the Kap121/Dam1 complex induce the dissociation of Kap121.

DISCUSSION

We have shown that Kap121 binds the Dam1 complex and their association is required for normal cellular levels of the Dam1 complex components. In various kap121 mutant backgrounds, cellular levels of the Dam1 complex are reduced and defects in mitotic KT alignment and chromosome segregation are detected. While the interactions of Kap121 with Dam1 complex components appear to protect the Dam1 complex, Kap121 is not essential for its nuclear import and this function is likely redundant with other importins. However, once in the nucleus, our results lead us to propose that Kap121 plays a role in the intranuclear targeting of the Dam1 complex to spindle MTs. This idea is based on the observation that the Kap121/Duo1 complex is resistant to RanGTP-mediated dissociation but Kap121 is released by a combination of tubulin and RanGTP.

Analysis of kap121 Ts strains revealed that these mutants display defects in mitotic progression and aneuploidy (Makhnevych et al., 2003) and reduced fitness in the absence of the SAC (Figure 1). Consistent with these results, our analysis of KT distribution in kap121 Ts strains revealed striking defects in the ability of chromosomes to bi-orient on the mitotic spindle. Specifically, we observed that most mitotic kap121-34 and kap121-41 cells display KT s that are either unclustered along the spindle axis or collapsed into a single focus (Figures 1 and S1). Similar, but even more severe, phenotypes were associated with depletion of Kap121 (PMET4-HA-KAP121, +MET; Figure S1). Moreover, analysis of the positioning of GFP-labeled CEN11 in kap121-34 cells revealed that these cells were largely unable to establish...
normal, separated sister chromosomes and thus a bi-lobed configuration of the CEN11 loci. These results support the conclusion that Kap121 Ts strains possess KT bi-orientation defects (Figure 1E).

The defects in KT bi-orientation observed in the Kap121 mutants appear unlinked to the nuclear import functions of Kap121, because various conditions that similarly inhibited the import of Kap121 cargos, including overproducing Nup53 and replacing endogenous Kap121 with Kap121-pA, did not induce mitotic KT bi-orientation defects (Figure 2). Furthermore, we found that although ectopic expression of WT Kap121 could partially suppress the import defect of kap121-34 cells, it only nominally improved growth of the mutant (data not shown) and it failed to suppress the mitotic KT bi-orientation defect (Figure 2). We hypothesize that these observations reflect a dominant-negative function for the kap121-34 protein that specifically manifests itself in the context of Kap121’s role in KT bi-orientation. Defining the molecular basis for this phenomenon will require further study. However, what is clear from these data is that the failure of chromosomes to bi-orient on the spindle in cells compromised for Kap121 function is unlikely to be a consequence of a general defect in Kap121-mediated protein import. Instead, the function of Kap121 in KT bi-orientation appears linked to its physical interaction with the Dam1 complex. This complex is an essential, MT-bound assembly that forms a ring-like structure that encircles the MT lattice and loads onto MT plus-end tips, where it functions in both the establishment and the maintenance of stable bi-polar KT-MT interactions (reviewed in Westermann et al., 2007). The Dam1 complex is a proposed regulator of plus-end MT dynamics, and it functions as a coupling device linking KTs to dynamic MTs. Positioned at the KT-MT interface, this complex allows KTs to maintain attachments to MT plus ends during KT bi-orientation when significant forces are applied to sister KTs from opposing spindle MTs. Because this complex is required for maintaining stable, load-bearing KT-MT interactions, scenarios that lead to the loss of Dam1 complex functionality give rise to errors in mitotic KT bi-orientation (Janke et al., 2002; Schareffberger et al., 2003; Gillett et al., 2004; Umbreit et al., 2014), as illustrated in duo1-2 and dam1-1 mutant cells (Figure S2; Gillett et al., 2004; Umbreit et al., 2014). These same mutant phenotypes are seen in the kap121 mutants (Figures 1 and S1).

We show that Kap121 physically interacts with the Dam1 complex (Figure 3). Purification of separate components of the complex, including Dam1 and Duo1, revealed bound Kap121. It remains to be determined, however, which member or members of the complex directly interact with Kap121. Binding of Kap121 to the Dam1 complex may contribute to the nuclear import of the complex; however, analysis of the kap121 Ts mutants leads us to conclude that Kap121 is not essential for nuclear import of Dam1 complex members (Figure 4) and that other Kaps can support nuclear import of components of this complex, either functioning alone or redundantly with Kap121. Our data lead us to conclude that the interaction of Kap121 with the Dam1 complex reflects distinct, non-transport related functions for this Kap. First, we show that Kap121 is required for the stability of the Dam1 complex (Figures 5 and S4). The kap121-34 mutant protein, which itself appears stable and present at WT levels in the mutant (Leslie et al., 2002; data not shown), is not detected in physical association with the Dam1 complex, and in the kap121-34 mutant, cellular levels of Duo1 and Dam1 are dramatically decreased (Figures 5 and S4). Conditions that stabilize Duo1 or Dam1 (i.e., the addition of a large C-terminal tag) or overproduction of Duo1 suppress the KT bi-orientation defects seen in kap121 mutant cells (Figures 4 and S5). Cumulatively, these data lead us to conclude that the loss of Duo1 or Dam1 stability gives rise to the observed mitotic KT bi-orientation defects detected in cells lacking Kap121 function.

The molecular basis for the role of Kap121 in maintaining the stability of the Dam1 complex remains to be determined. Kap121 could contribute to the assembly of the Dam1 complex or, through its binding to the complex, could mask domains of complex components that would render the complex susceptible to degradation. The latter scenario is plausible, because both Dam1 and Duo1 are highly basic proteins with isoelectric points of 9.97 and 10.76 (Westermann et al., 2005). The charged nature of these proteins would likely render them susceptible to non-specific, electrostatic interactions, making these proteins...
potentially prone to aggregation or non-specific interactions. Because Kap121 binding sites on various interacting proteins are rich in basic residues (Lusk et al., 2002), Kap121 could interact with basic residue clusters present within Duo1 and/or Dam1. The binding of Kap121 could mask positively charged residues until the appropriate binding partners are encountered within the nucleoplasm. This could include MTs, because the C-termini of both Dam1 and Duo1 contain stretches of basic amino-acid residues that are thought to comprise the binding domains for the highly acidic surface of MTs (Westermann et al., 2005; Wang et al., 2007; Ramey et al., 2011a, 2011b). Conceptually similar functions for Kap-β/importin-β have been proposed in other contexts. In a manner analogous to a chaperone, Kap-β/importin-β binding to positively charged proteins in the cytoplasm, including ribosomal proteins and histones, suppresses their aggregation and eventual degradation (Jäkel et al., 2002). In addition, Kap-β/importin-β binds the SAF HURP blocking recognition of a destruction box motif in HURP and APC/C-mediated proteasomal degradation (Song et al., 2014).

We envisage that Kap121 is not a constitutive component of the Dam1 complex and that the binding of Kap121 to the Dam1 complex likely occurs at stages before the association of the Dam1 complex with dynamic MTs at the KT-MT interface. In our model, the Kap121/Dam1 complex would be stable in the cytoplasm and after entering the nucleoplasm. Unlike typical Kap/cargo complexes that are dissociated by RanGTP in the RanGTP-rich environment of the nucleoplasm, the Kap121/Dam1 complex is resistant to RanGTP-induced dissociation (Figure 6). A similar RanGTP-resistant complex has been previously observed between Kap121 and the SUMO isopeptidase, Ulp1. In this case, Kap121 appears to contribute to the tethering of Ulp1 to the nucleoplasmic face of the NPC (Panse et al., 2003).

When importin/cargo complexes have been shown to be resistant to RanGTP dissociation in vitro, it has been suggested that the Kap plays a secondary role of escorting the cargo to distinct locations within the nucleoplasm, where an additional factor or factors can function, in conjunction with RanGTP, to release cargo from the Kap. For instance, Kap114 interacts with TATA-binding protein, Tbp1, in a manner that is resistant to dissociation by RanGTP (Pemberton et al., 1999). This RanGTP-resistant interaction enables Kap114 to target Tbp1 to chromatin, specifically to TATA-containing promoter regions (Pemberton et al., 1999). Kap114 binding to TATA-containing DNA sequences, in combination with RanGTP binding, triggers dissociation of Kap114 from Tbp1 and association of the latter with chromatin. Also, as discussed in the Results, release the Nab2 from its importin Kap104 is dependent upon a cooperative interaction with RNA and RanGTP (Lee and Aitchison, 1999).

Here we show that a combination of tubulin and RanGTP work cooperatively to release Kap121 from Duo1 in vitro (Figure 6). The RanGTP-resistant interactions of Kap121 with the Dam1 complex would allow Kap121 to escort the complex to its destination on KT-associated MT ends. On the basis of these observations, we propose that the in vivo release of Kap121 from the Dam1 complex in the nucleus (i.e., an environment rich in RanGTP) would be facilitated by tubulin, potentially free or MT associated, at the dynamic plus ends of MTs adjacent to KTs. In the model we propose, Kap121 would not be concentrated with Dam1 complexes at the KT-MT interface. Consistent with this idea, we detect Dam1-GFP and Duo1-GFP at KTs, but we do not observe a similar accumulation of Kap121-GFP (Figure S3). Kap121-GFP is functional, and the cells producing this fusion exhibit no defects in KT bi-orientation (Figure S5).

In conclusion, through our characterization of its function, we have uncovered a previously undetected and essential role for Kap121 in KT bi-orientation during mitosis. Our data point to a mechanistically more complex function for Kap121 than previously anticipated for this NTF, including a role for Kap121 in the stability of the Dam1 complex and its localization to spindle MTs.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media
All yeast strains used in this study are listed in Table S1, and growth conditions used are described in the Supplemental Experimental Procedures.

Plasmids and DNA Manipulations
All plasmids used in this study are listed in Table S2. See the Supplemental Experimental Procedures.

Fluorescence Microscopy
Acquisition of images of GFP- or mCherry-tagged proteins was performed using an epifluorescence microscope. For more information, see the Supplemental Experimental Procedures.

Image Analysis and Signal Quantification
Image processing (cropping and linear brightness and contrast adjustment) and calculation of NLS-nuc-GFP nuclear-to-cytoplasmic fluorescence intensity ratios were performed as previously described (Cairo et al., 2013). Average nuclear-to-cytoplasmic fluorescence intensity ratios were plotted, and cell-to-cell variability is displayed as SE.

Immunopurification of Protein A-Tagged Proteins
Cells producing protein A-tagged fusion proteins were flash frozen and dispersed with a planetary ball mill (Retsch). Protein A-tagged proteins were purified from yeast cell lysates using immunoglobulin G-coupled magnetic beads as previously described (Alber et al., 2007). Refer to the Supplemental Experimental Procedures.

Western Blotting
Western blotting analysis was performed as previously described (Cairo et al., 2013). For additional details, see the Supplemental Experimental Procedures.

RanGTP- and Tubulin-Induced Release Experiments
Immunopurified protein complexes isolated from yeast lysates were incubated with purified Ran alone or in combination with purified tubulin. Bound and unbound fractions were separated using a magnetic stand, and bound fractions were eluted with SDS-PAGE sample buffer. For more information, refer to the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.041.

AUTHOR CONTRIBUTIONS
L.V.C. conducted the experiments. L.V.C. and R.W.W. designed the experiments and wrote the manuscript.
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Arnaoutov, A., Azuma, Y., Ribbeck, K., Joseph, J., Boyarchuk, Y., Karpova, T., McNaughton, J., and Dasso, M. (2005). Crm1 is a mitotic effector of Ran-GTP in so-
Supplemental Information

The Nuclear Transport Factor Kap121 Is Required for Stability of the Dam1 Complex and Mitotic Kinetochore Bi-orientation

Lucas V. Cairo and Richard W. Wozniak
Figure S2

Mtw1-GFP

WT

kap121-34

duo1-2

dam1-1

23°C  37°C

% Large-Budded Cells

- bi-lobed KT
- unclustered or collapsed KT

23°C  37°C  23°C  37°C  23°C  37°C  23°C  37°C

WT  kap121-34  duo1-2  dam1-1
Figure S3

A. Bar chart showing the percentage of large-budded cells at different temperatures and conditions:
- MTW1-GFP
- DAM1-GFP

B. Imaging analysis of Duo1-GFP and Spc42-mCherry in the presence of mCherry with +Met (3.5h):
- GFP
- mCherry
- Merge

C. Imaging analysis of Duo1-GFP and Spc42-mCherry with DUO1, DUO1-PA, DUO1-3HA, DAM1-3HA under 23°C and 37°C conditions.

D. Imaging analysis of Duo1-mCherry with kap121-34, NLS\textsuperscript{isofluoride}-GFP, and DUO1-mCherry at 23°C and 37°C.
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figure 1. The distribution and dynamics of KTs in *kap121-34* cells. The positioning of KTs alone (Mtw1-GFP) or in combination with either spindle-MTs (GFP-Tub1) or SPBs (Spc42-mCherry) was documented in the indicated strain backgrounds using epifluorescence microscopy. (A) Displayed are representative images of WT and *kap121-34* cells producing Mtw1-mCherry and GFP-Tub1. Arrows indicate the location of KT clusters. Bar, 2 µm. (B) Two separate mitotic *kap121-34 MTW1-GFP* cells (Cell 1 and Cell 2) were imaged at 2-min intervals for a total of 10-min. Bar, 5 µm. (C) Presented are images of WT and *kap121-41* cells synthesizing Mtw1-GFP and Spc42-mCherry. Arrows indicate KT positioning. Bar, 2 µm. (D) *P_{MET3}-HA-KAP121* cells expressing Mtw1-GFP and Spc42-mCherry were grown in medium lacking methionine (-Met). Methionine was added (+Met) to cultures and they were incubated for the indicated times to suppress *MET3*-mediated expression of *HA-KAP121*. Whole cell lysates were collected and HA-Kap121 levels were analyzed by western blotting (WB) using an anti-HA (HA-Kap121) antibody (left panel). An anti-Gsp1 antibody was also used to assess the total amount of protein loaded per lane. Cells were also examined 3.5 h after addition of methionine using epifluorescence microscopy. Arrows point to KT clusters (Mtw1-GFP) and SPBs are visualized (Spc42-mCherry). Bar, 2 µm. (E) Representative images exhibit the distribution of KTs (Mtw1-GFP) in WT, *kap123Δ*, and *kap121-34* cells. The bar graph to the right shows the percentage of large-budded cells in each population that exhibit either a bi-lobed or unclustered/collapsed KT orientation (n ≥
100 cells). Bar, 5 µm.

**Figure S2. Related to Figure 1. Dam1 complex mutants produce similar KT-alignment defects as *kap121-34* cells.** The localization of KTs (Mtw1-GFP) was examined in WT, *duo1-2*, *dam1-1*, and *kap121-34* cells using an epifluorescence microscope. Cells were imaged at 23°C and 3 h following a shift to 37°C. Bar, 5 µm. Each cell population analyzed was scored for the presence of large-budded cells presenting either bi-lobed or unclustered/collapsed KT foci. The bar graph shows the percent representation of both categories of KT morphology in the indicated strains (n ≥ 100 cells).

**Figure S3. Related to Figure 4. Duo1-GFP suppresses KT bi-orientation defects in cells lacking Kap121 function but does not restore Kap121-mediated import.** (A) Large-budded cells within a culture of WT and *kap121-34* cells producing either Dam1-GFP or Mtw1-GFP were scored for the presence of bi-lobed KTs or unclustered/collapsed KT foci. Bar graphs display the percentage of both classes of KT distribution within each cell population (n ≥ 100 cells). (B, C, and D) Localization of the indicated -GFP- or -mCherry-tagged proteins was acquired using epifluorescence microscopy. (B) *P_{MET3}^{HA}-KAP121* cells producing Duo1-GFP in combination with either Mtw1-mCherry (KTs) or Spc42-mCherry (SPBs) were examined following transfer to medium containing methionine (+Met) for 3.5 h to deplete Kap121. Arrows indicate the location of KTs. Bar, 2 µm. Large-budded *P_{MET3}^{HA}-KAP121* cells
synthesizing Duo1-GFP were tallied for the presence of either bi-lobed or unclustered/collapsed KT morphologies. The bar graph shows the percent representation of both categories of KT morphology in the $\text{P}_{\text{MET3-HA-KAP121}}$ cells synthesizing Duo1-GFP in the presence and absence of methionine. In parallel, $\text{P}_{\text{MET3-HA-KAP121}}$ cells producing Mtw1-GFP, grown under the same conditions, are displayed for comparison. (n $\geq$ 100 cells). (C) Presented are the images of $\text{kap121-34}$, $\text{kap121-34 DUO1-3HA}$, $\text{kap121-34 DAM1-3HA}$, and $\text{kap121-34 DUO1-PA}$ strains producing Mtw1-GFP (KTs) that were grown at 23°C and 3 h after shifting cultures to 37°C. Bar, 5 μm. Each cell populations analyzed was scored for the presence of large-budded cells presenting either bi-lobed or unclustered/collapsed KT foci. The bar graph shows the percent representation of both categories of KT morphology in the indicated strains (n $\geq$ 100 cells). (D) Kap121-mediated import (detected using NLS$_{\text{Pho4}}$-GFP) was followed in $\text{kap121-34}$ cells producing Duo1-mCherry grown as described in (C). Bar, 5 μm. Large-budded cells within the analyzed cell populations were scored for the presence of bi-lobed or unclustered/collapsed KT foci and the bar graph reveals the percent representation of either KT distribution (n $\geq$ 100 cells). For comparison, analysis of a $\text{kap121-34 NLS}_{\text{Pho4}}$-GFP $\text{MTW1-mCHERRY}$ strain grown under the same conditions is shown.

**Figure S4.** Related to Figure 5. The kap121-34 mutant protein does not interact with the Dam1 complex or Ipl1. (A and B) The indicated protein-A-tagged fusion proteins
were affinity purified from cell lysates containing the specified Myc-tagged fusion proteins as described in the Figure 3 legend. Bound complexes were released with three concentrations of MgCl$_2$ (0.05 M, 0.5 M, and 2 M) and a final 0.5 M acetic acid (AA) elution. Eluted fractions were analyzed by western blotting (WB) analysis using anti-protein-A, anti-Myc, anti-Nup53 (a binding partner of Kap121), and anti-Kap121 antibodies. Samples of the cell lysates (Input) and the last wash (LW) are shown. (C) $P_{\text{MET3}}$-HA-KAP121 cells synthesizing either an HA- or GFP-tagged version of Duo1 were shifted into methionine containing medium (+Met) to suppress expression of HA-KAP121, and whole cell lysates were collected at the indicated times. Kap121 and Duo1 levels were analyzed by western blotting (WB) using either an anti-HA (for HA-Kap121 and Duo1-3HA) or anti-GFP (for Duo1-GFP) antibody. Additionally, each blot was probed with an anti-Gsp1 antibody to evaluate protein loading.

**Figure S5. Related to Figure 4. Localization of Kap121-GFP and KT.** The localization of Kap121-GFP and the KT marker Mtw1-mCherry in cells grown at 23°C was evaluated using epifluorescence microscopy. Bar, 5 µm.
EXTENDED EXPERIMENTAL PROCEDURES

Yeast strains and media

All yeast strains used in this study are listed in Table S1. Yeast strains were grown in either YPD (1% yeast extract, 2% bactopeptone, 2% glucose,) or synthetic media containing 0.17% yeast nitrogen base (lacking amino acids and ammonium sulfate), 0.5% ammonium sulfate, 2% glucose, and the necessary supplements. Cell cultures were grown at 23°C unless otherwise indicated. To overproduce Nup53, a strain containing the $P_{GAL1}$-$NUP53$ allele, where the endogenous $NUP53$ promoter was replaced with the inducible $GAL1/10$ promoter, was grown to an $OD_{600}$ of ~0.5 in YPD containing 2% raffinose. Cultures were diluted to an $OD_{600}$ of ~0.3 and galactose was added at a final concentration of 3% for a total of 10 h. For temperature shift of Ts mutants ($kap121$-$34$, $kap121$-$41$, $dam1$-$1$, and $duo1$-$2$), cells were grown in YPD to an $OD_{600}$ of ~0.5 at 23°C and then shifted to 37°C for 3 h. To deplete Kap121 or Cdc20, strains harboring either the $P_{MET3}$-$KAP121$ or $P_{MET3}$-$CDC20$ alleles were grown to an $OD_{600}$ of ~0.5 in synthetic media lacking methionine (CM-Met) at 23°C. Cells were then collected by centrifugation and resuspended in YPD supplemented with methionine (20 mg/ml) and were grown for either 2.5 h (Cdc20 depletion) or 4 h (Kap121 depletion) at 23°C. Cellular depletion of either protein was monitored by western blot analysis. For classification of large-budded cells for quantification, dividing cells with a daughter > 0.5 times the diameter of the attached mother was categorized as large-budded. To evaluate growth rates of mutants on YPD plates alone, or YPD plates containing benomyl, cells
were grown in YPD at 23°C to an OD$_{600}$ of 0.8-1.2, diluted, then spotted onto YPD plates and incubated at 25°C and 37°C, or 30°C for growth on benomyl, for 2-3 days.

**Plasmids and DNA manipulations**

Yeast cell transformations involving autonomously replicating plasmids or PCR derived DNA cassettes for genomic integration was performed using a lithium acetate/polyethylene glycol (PEG)-based method previously described (Schiestl and Gietz, 1989). DNA cassettes encoding fusion proteins with carboxy-terminal GFP+, mRFP, mCherry, protein-A, HA$_3$, and Myc$_{13}$ were constructed using PCR (Longtine et al., 1998). DNA cassettes for genomic integration encoding NLS$^{\text{Pho4}}$-GFP were made as previously described (Cairo et al., 2013) using the plasmids pEBO836 (NLS$^{\text{Pho4}}$-GFP) as template DNA for targeted integration at the URA3 locus. All genomic integration events were confirmed by a variety of techniques including PCR, western blotting analysis, and fluorescence microscopy.

**Fluorescence microscopy**

Images of GFP- or mCherry-tagged fusion proteins were acquired using an epifluorescence microscope. Image acquisition was achieved using an Axio Observer.Z1 outfitted with an UPlanS-Apochromat 100×/1.40 NA oil immersion objective lens (Carl Zeiss, Inc.) and an AxioCam MRm digital camera (Carl Zeiss, Inc.). Axiovision software
was used to store all acquired images (Carl Zeiss, Inc.) and ImageJ (National Institutes for Health) was utilized for post-acquisition image analysis.

**Image analysis and signal quantification**

Image processing (cropping and linear brightness/contrast adjustment) and quantification of nuclear/cytoplasmic NLS\textsuperscript{Pho4}-GFP fluorescence intensity ratios were carried out using ImageJ software. Calculation of NLS\textsuperscript{Pho4}-GFP nuclear/cytoplasmic fluorescence intensity ratios was performed as previously described (Cairo et al., 2013). Briefly, the nuclear/cytoplasmic fluorescence intensity ratio for a given cell was calculated by measuring the mean fluorescence intensity of two 0.5 x 0.5 \( \mu \text{m} \) regions, one within the nucleus and the other in the cytoplasm. Background fluorescence intensity was adjusted for by subtracting the calculated background signal (a region outside of the cell) from both nuclear and cytoplasmic values. The average nuclear/cytoplasmic fluorescence intensity ratio was determined using a total of > 50 cells total under the indicated conditions. Cell to cell variability is expressed as standard error.

**Immunopurification of protein-A tagged proteins**

Kap121-pA, kap121-34-pA, Nup53-pA, Duo1-pA, Dam1-pA, and Ipl1-pA fusion proteins were purified from yeast cell extracts as previously described (Alber et al., 2007). Yeast cells producing pA-tagged fusion proteins were grown in 1 L of YPD to an OD\textsubscript{600} of 1.0 at either 23\textdegree C \((kap121-34)\) or 30\textdegree C. Cells were harvested, washed twice in
250 ml of water, and once with 250 ml of IP wash buffer (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium chloride). Next, cryogenic lysis was performed utilizing a planetary ball mill (PM100; Retsch) yielding ~1.0-2.0 g of yeast cell lysate in powder form (Alber et al., 2007). Lysates were then placed on ice and ice-cold IP buffer containing complete protease inhibitor cocktail tablets (1 pellet per 50 mL of buffer; Roche Applied Science) was added at a 1:2 weight-to-volume ratio. The resulting slurry was kept on ice for ~30 min with vigorous mixing at 5-10 min intervals. Lysates were cleared by centrifugation (1500 x g for 10 min at 4°C) (Alber et al., 2007). The supernatants were transferred to 2 mL tubes and IgG-conjugated magnetic beads (prepared as described below; Sigma-Aldrich) were added at a ratio of 3 mg of beads to 2 mL of lysate. Samples were incubated at 4°C with end-over-end rotation for a total of 1 h. Following binding, beads were collected on the tube wall using a magnetic stand for ~2.5 min and the remaining lysate was aspirated off. Beads were then washed with ice-cold IP buffer 10 times prior to elution. Elution of bead-bound protein complexes was performed at 4°C using a step gradient of MgCl₂ (50, 500, and 2000 mM; 500 µL volumes) as well as a final 0.5 M acetic acid elution for a total of 3 min per elution (Lusk et al., 2007; Makhnevych et al., 2007; Van de Vosse et al., 2013). Eluates were then precipitated overnight at 4°C using a 10% trichloroacetic acid (TCA), 0.015% sodium deoxycholate. The following day, the protein precipitates were collected by centrifugation at 4°C. The pellet was then washed with 1 mL of ice-cold acetone, dried, resuspended in sample buffer, and analyzed by SDS-PAGE and western blotting analysis.
RanGTP- and tubulin-induced release experiments

Purified human recombinant Ran was loaded with either GDP or GTP nucleotide as follows. Aliquots of recombinant Ran (~10 mg/mL; kind gift from Yuh Min Chook, U of Texas, Southwestern; see Chook and Blobel, 1999 for isolation procedure) in Ran buffer (20 mM Tris pH 8.0, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 10% glycerol, 5 mM beta-mercaptoethanol) were supplemented with concentrated stocks to a final concentration of 25 mM EDTA, 2 mM dithiothreitol, and 0.5 mM of either GTP or GDP nucleotide (Sigma-Aldrich) and incubated on ice for 90 min. Nucleotide loading of Ran was initiated by the addition of magnesium acetate to 30 mM and a final Ran concentration of 9 mg/mL. Samples were incubated on ice for 30 min and then flash frozen in liquid N₂ and stored at -80°C.

To perform the Ran and tubulin release experiments, Nup53-pA and Duo1-pA fusion proteins were affinity purified from cells producing Kap121-13Myc as described above. Following binding of protein-A fusions to the magnetic beads, the bead bound complexes were washed 5 times with cold IP buffer containing a protease inhibitor cocktail. Next, IP buffer containing 0.5 mM ATP was used to wash the beads for 30 min at 23°C. The beads were collected using a magnetic stand and then resuspended in IP buffer containing 1% glycerol and 0.5 mM dithiothreitol (1 mL). To the bead suspensions, RanGDP (5 µg), RanGTP (5 µg), or purified bovine tubulin (5 µg; Cytoskeleton, Inc.) was added individually, or in the indicated paired combinations, and incubated for 30 min at 23°C. Bovine tubulin was used in our studies as it was shown in other in vitro studies to bind the yeast Dam1 complex (Westermann et al., 2005; Wang et
al., 2007). Beads were separated from released proteins using the magnetic stand. Those proteins in solution represent the released fraction. The released proteins and those remaining bound to the beads were analyzed by SDS-PAGE and western blotting analysis.

**IgG conjugation to epoxy-coated magnetic beads**

IgG from rabbit serum (Sigma-Aldrich) was conjugated to Epoxy M-270 Dynabeads (Invitrogen) as previously described (Alber et al., 2007). Beads were resuspended in 2 mL of PBS (30 mg/ml) containing sodium azide (0.02%) and stored at 4°C.

**Western blotting**

Procedures for preparing whole cell lysates was conducted as previously described (Lusk et al., 2007). Briefly, cells were grown to an OD$_{600}$ of 1.0 in the appropriate medium, harvested by centrifugation, resuspended in SDS-PAGE sample buffer, vigorously sonicated, and heated in boiling water for 10 min.

To perform western blotting analysis on proteins contained in whole cell lysates and eluted IP fractions, samples were loaded onto SDS-PAGE gels to separate protein. Following gel-electrophoresis, resolved protein was transferred to nitrocellulose membranes. Post-transfer membranes were blocked with lyophilized milk powder (5%) resuspended in PBS containing Tween-20 (0.1%). Rabbit polyclonal antibodies were used to detect Kap121 (Leslie et al., 2002) and Nup53 (Marelli et al., 1998) as well as
GFP- (Maknevych et al., 2003) and protein-A-tagged (Sigma-Aldrich) fusion proteins. Also, mouse monoclonal antibodies were used to probe for proteins fused to Myc (9E10; Roche), and HA (sc-7392; Santa Cruz Biotechnology Inc.) epitope tags. Primary antibodies were detected with HRP-conjugated donkey anti-rabbit (BioRad) or sheep anti-mouse (BioRad) secondary antibodies.
**SUPPLEMENTAL TABLES.**

**Yeast strains.**

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SUPPLEMENTAL REFERENCES


