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Kip3, the yeast kinesin-8, is required for clustering of kinetochores at metaphase

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In *Saccharomyces cerevisiae*, chromosome congression clusters kinetochores on either side of the spindle equator at metaphase. Many organisms require one or more kinesin-8 molecular motors to achieve chromosome alignment. The yeast kinesin-8, Kip3, has been well studied in vitro but a role in chromosome congression has not been reported. We investigated Kip3's role in this process using semi-automated, quantitative fluorescence microscopy and time-lapse imaging and found that Kip3 is required for congression. Deletion of *KIP3* increases inter-kinetochore distances and increases the variability in the position of sister kinetochores along the spindle axis during metaphase. Kip3 does not regulate spindle length and is not required for kinetochore-microtubule attachment. Instead, Kip3 clusters kinetochores on the metaphase spindle by tightly regulating kinetochore microtubule lengths.

Introduction

During mitosis, chromosomes congress to the equator of the mitotic spindle. Positioning of the chromosomes depends in part on the highly conserved kinesin-8 family of molecular motors.[1-5] The kinesin-8s are plus-end directed microtubule motors that localize to the mitotic spindle.[1,2,6-9] In *Schizosaccharomyces pombe*, Drosophila and mammalian systems, the role of the kinesin-8 motor in chromosome congression has been established.[3,10-12]

The kinesin-8 in *S. cerevisiae* is Kip3. Kip3 has been shown to regulate the plus-end dynamics of cytoplasmic microtubules in order to position the nucleus at the bud neck prior to anaphase.[6,13,14] Kip3 also localizes to microtubules of the mitotic spindle.[8,14] In budding yeast, bioriented sister kinetochores cluster on either side of the spindle equator at metaphase.[15-18] Each kinetochore is attached to the plus-end of a kinetochore microtubule; therefore plus-end dynamics affect kinetochore position. In an analysis of kinesin motor function, Tytell and Sorger[8] proposed that loss of Kip3 caused lagging chromosomes during anaphase A. Gardner and coworkers[19] reported that Kip3 regulates interpolar microtubule lengths to control mitotic spindle length. Both groups discounted the role of Kip3 in metaphase kinetochore positioning. Gardner and coworkers proposed that Cin8, the yeast kinesin-5, aligns metaphase kinetochores by regulating kinetochore microtubule lengths.[19] Therefore, the importance of Kip3 for chromosome congression remains unclear.

In this paper, we show that Kip3 is important for yeast chromosome congression, but does not control spindle length. Using semi-automated, quantitative fluorescence microscopy and time-lapse imaging, we determined that Kip3 decreases the variability in kinetochore positions along the metaphase spindle.

Results

Deletion of *KIP3* mispositions metaphase kinetochores. During metaphase, kinetochores in budding yeast form clusters on either side of the spindle equator.[15-18] We analyzed the role of Kip3 in kinetochore clustering using semi-automated, quantitative fluorescence microscopy, which measures the distribution of fluorescent kinetochore components along the spindle axis. In the absence of Kip3, kinetochore components Nuf2-GFP, Mtw1-GFP and Spc105-GFP are no longer clustered, but become irregularly distributed along the spindle axis (**Fig. 1A and S1**). The average distributions support the premise that kinetochore components scatter in the absence of Kip3, because there is no longer a large dip in fluorescence at the spindle equator in the *kip3*Δ mutant cells (**Fig. 1B–D**). The total amount of Nuf2-GFP within the spindle does not change when *KIP3* is deleted (data not shown). The three kinetochore components analyzed are found in three different subcomplexes of the kinetochore: the Ndc80 complex, the MIND complex and the Spc105 complex. The Ndc80 complex is at the outer kinetochore, bound to microtubules, whereas the other two complexes are part of the middle kinetochore. Because proteins from three different kinetochore subcomplexes are not clustered in the absence of Kip3, we conclude that Kip3 is required for positioning of kinetochores along the spindle axis.

Kinetochore mispositioning in the *kip3*Δ mutant cells could be explained by a defect in kinetochore-microtubule attachment, as proposed for kinesin-8 mutants in *S. pombe*.[10,20] In budding yeast, kinetochore-microtubule attachment defects can be detected as localization of the kinetochore away from the spindle axis.[21] Mutations in the Ndc80 protein result in detached

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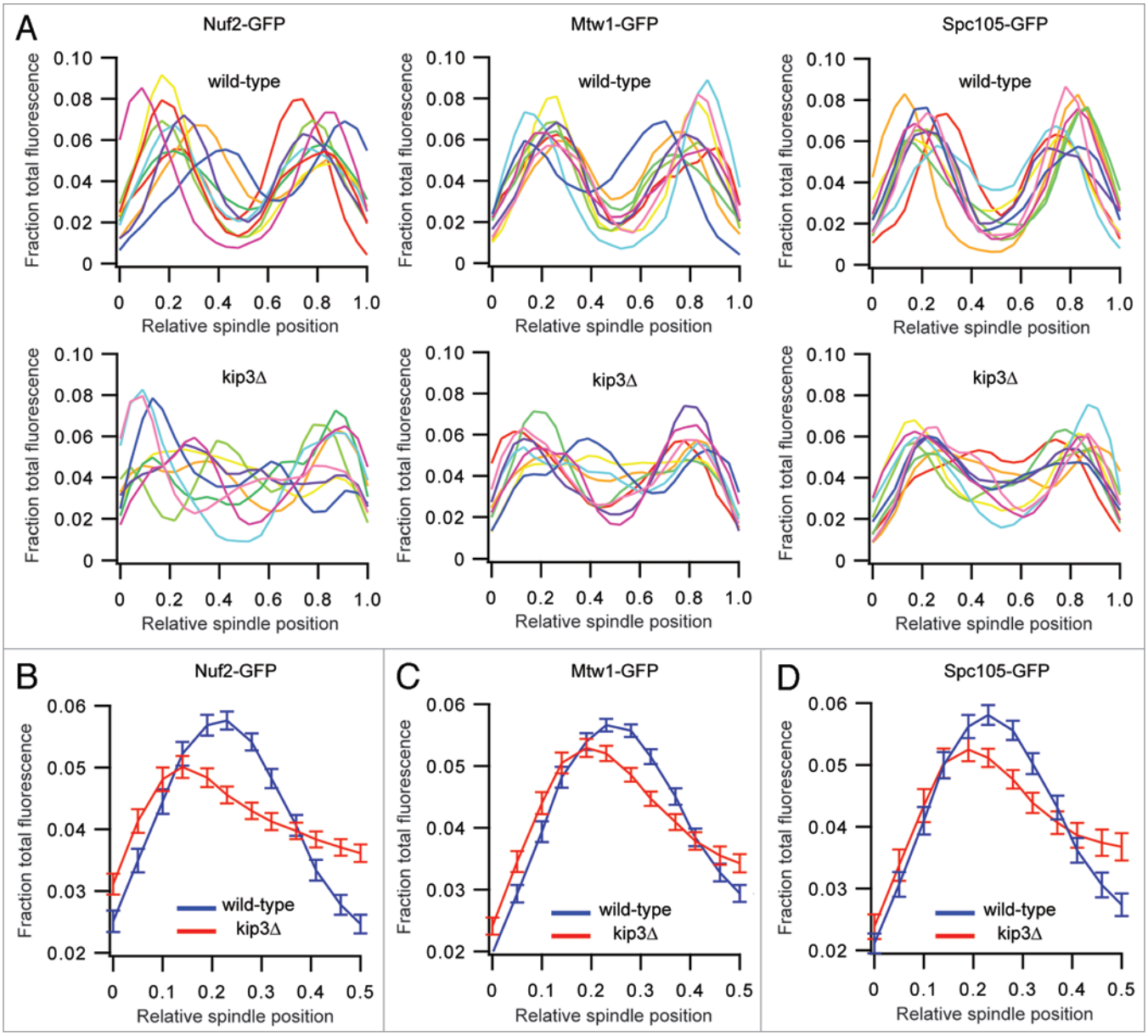

Figure 1. Kinetochore distribution is altered in the absence of Kip3. Kinetochore distribution was measured in strains labeled with Spc110-mCherry and Nuf2-GFP, Mtw1-GFP or Spc105-GFP, as described in the Materials and Methods. (A) Representative distributions of Nuf2-GFP, Mtw1-GFP and Spc105-GFP fluorescence between Spc110-mCherry foci in individual wild-type and *kip3*Δ mutant spindles. Each colored line shows the kinetochore component distribution for a single cell. The spindle poles are at the origin and position 1 on the x-axis. (B–D) Average distributions of kinetochore components. The origin of the x-axis designates the spindle pole and spindle position 0.5 denotes the spindle equator. The error bars represent the standard error of the mean. (B) Average Nuf2-GFP fluorescence distributions. Wild-type strain MSY107-33B, N = 94; *kip3*Δ mutant strain MMWY19-2A, N = 90. (C) Average Mtw1-GFP fluorescence distributions. Wild-type strain MMWY81-2B, N = 116; *kip3*Δ mutant strain MMWY81-16C, N = 88. (D) Average Spc105-GFP fluorescence distributions. Wild-type strain MSY265-1D, N = 76; *kip3*Δ mutant strain MMWY83-5A, N = 46.

kinetochores.[22,23] We scored images of wild-type, *kip3*Δ mutant and *ndc80-1* mutant cells grown at the nonpermissive temperature for foci of kinetochore fluorescence lying off the pole-to-pole axis. The percentage of mitotic cells containing unattached kinetochores was high in *ndc80-1* mutant cells (**Fig. S2**), but was low in *kip3*Δ mutant and wild-type cells (41%, N = 73, 7%, N = 113 and 4% N = 73, respectively; **Fig. S2**). Therefore, deletion of *KIP3* does not appear to cause kinetochore detachment from the microtubules.

The cytoplasmic function of Kip3 is to position the spindle at the bud neck during metaphase.[13,14,24] Consistent with this result, we observed twenty-five percent of *kip3*Δ mutant spindles positioned away from the bud neck prior to anaphase, whereas all wild-type spindles were near the bud neck by this time. The distribution of Nuf2-GFP fluorescence was similar in both classes of *kip3*Δ mutant spindles (**Fig. 2 and S3**). Therefore, deletion of *KIP3* results in declustered kinetochores regardless of the position of the spindle within the cell.

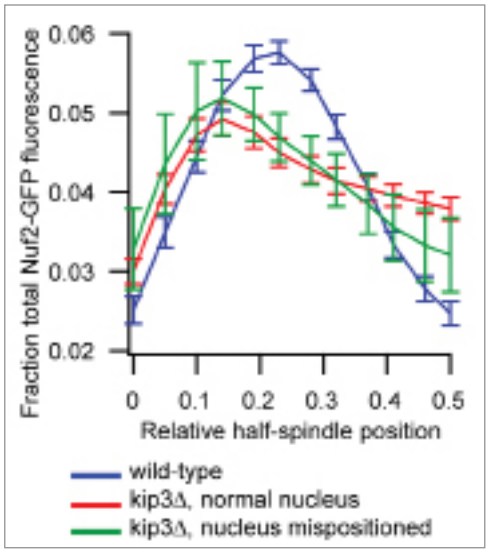

Figure 2. Kinetochore declustering is independent of nuclear position. The distribution of kinetochore component fluorescence was measured in metaphase spindles of wild-type strain MSY107-33B and *kip3*Δ mutant strain MMWY19-2A, labeled with Spc110-mCherry and Nuf2-GFP, as described in Materials and Methods. On the x-axis, the origin and 0.5 mark the spindle pole and spindle equator, respectively. Wild type in blue, N = 94; *kip3*Δ mutant with nucleus normally positioned near bud neck in red, N = 60; *kip3*Δ mutant with nucleus mispositioned in the middle of mother in green, N = 18. The error bars represent the standard error of the mean.

Cin8 is required for bipolar spindle assembly. As Cin8 was recently reported to congress chromosomes,[8,19] we examined the effect of *CIN8* deletion on kinetochore positioning compared to the effect of *KIP3* deletion. Consistent with published results,[8,19] we found that deletion of *CIN8* mispositions kinetochores, though this kinetochore positioning defect is different from the defect observed in the *kip3*Δ mutant. Deletion of *CIN8* moves kinetochores closer to the spindle equator, on average, whereas deletion of *KIP3* moves kinetochores closer to the poles (**Fig. 3A and B**). Moreover, in the absence of Cin8, efficiency of spindle formation is low (**Fig. 3C**). Therefore, Cin8 is important for assembling or maintaining a bipolar spindle,[25-28] and may work with Kip3 to properly position metaphase kinetochores.[19]

Microtubule distribution. Given the effect of *KIP3* deletion on kinetochore positioning, we expected to find a change in tubulin distribution. Instead, loss of Kip3 had little effect on the distribution of microtubules, visualized using CFP-tubulin (**Fig. 4A**), which confirms the previously reported result.[19] Surprisingly, when we analyzed the distribution of kinetochores in the CFP-tubulin strain, we discovered that merely tagging tubulin alters kinetochore distribution, broadening the peak (**Fig. 4B**). In addition, the effect of *KIP3* deletion is not as dramatic in the CFP-tubulin strain, likely because kinetochore positions have already been altered (**Fig. 4C**). We conclude that the distribution of kinetochores across the spindle can be influenced not only by the Kip3 motor, but also by changes to the surface of the microtubule. Therefore, strains with tagged tubulin are not ideal for studying the localization of microtubules and kinetochores.

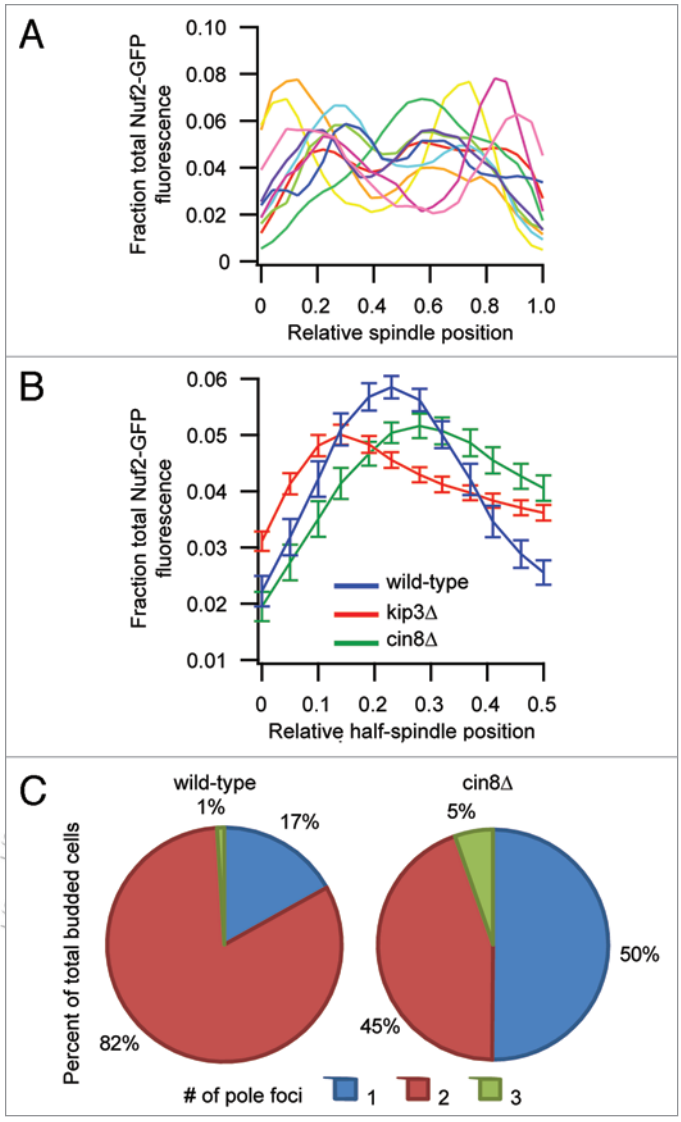

Figure 3. Phenotype of *cin8*Δ (kinesin-5) mutant. (A) Representative distributions of Nuf2-GFP fluorescence between Spc110-mCherry foci in individual *cin8*Δ mutant spindles. Each colored line shows the kinetochore component distribution for a single cell. The x-axis origin and spindle position 1 denote the two spindle poles. (B) Average Nuf2-GFP fluorescence distributions. Wild-type strain MMWY72-22D in blue, N = 58; *kip3*Δ mutant strain MMWY19-2A in red, N = 90; *cin8*Δ mutant strain MMWY72-25B in green, N = 52. The error bars represent the standard error of the mean. (C) Spc110-mCherry foci per budded cell. 1 focus in blue, 2 foci in red and 3 foci in green. Wild-type strain MMWY72-22D, N = 371; *cin8*Δ strain MMWY72-25B, N = 296.

Spindle length does not change in the absence of Kip3. By measuring the distance between fluorescently labeled spindle poles in still images (**Fig. 5A and B**) and 3D stacks of time-lapse images (data not shown), we determined that deletion of *KIP3* does not alter pre-anaphase spindle length. This result differs from the findings of Straight and coworkers, who reported that deletion of *KIP3* produces longer pre-anaphase spindles.[29] They measured the length of a bar of GFP-tubulin in 2D projections of a stack of images,[29] but because microtubules radiate off both sides of the spindle pole, fluorescent tubulin is not a definitive

marker of spindle ends. It is now possible to measure the distance between fluorescently labeled spindle poles and, using this method, we find that Kip3 does not regulate spindle length.

Kip3 localizes near kinetochores and along the spindle. Kip3 has been reported to localize in a bi-lobed pattern along the metaphase spindle, similar to kinetochore localization, as well as to the spindle mid-zone.[8] The mammalian kinesin-8 forms a motor-dependent gradient along most kinetochore microtubules.[3] Because individual kinetochore microtubules cannot be resolved in budding yeast, we cannot determine whether Kip3 also forms a gradient along kinetochore microtubules. As an alternative, we examined and categorized the distribution of Kip3-GFP in 86 metaphase cells. Although the spindles selected for analysis contained two peaks of Nuf2-Cherry, Kip3-GFP could be categorized as either two peaks (47%), high at the equator (28%) or asymmetric (26%) (**Fig. 6 and S4**). The cells with the sharpest Nuf2-Cherry peaks were often found to localize Kip3-GFP into two peaks as well, though this correlation was not statistically significant (data not shown). These results are consistent with localization of Kip3 to kinetochore microtubules and interpolar microtubules.

Interkinetochore and pole-kinetochore distances are altered in the absence of Kip3. Our bulk kinetochore analysis indicates that kinetochores are mispositioned in the absence of Kip3. We next monitored the position of a single pair of centromeres (*CEN3*) tagged with GFP. Deletion of *KIP3* increases the distance between sister centromeres and decreases the average distance from *CEN3* to the spindle pole body (**Fig. 7 and S5**). Moreover, *CEN3* positions along the spindle are more varied and *CEN3* crosses into the wrong half-spindle more often in *kip3*Δ mutant spindles than in wild-type spindles (**Fig. 7A**). These results show that individual kinetochores are not maintained in their normal position along the metaphase spindle in the absence of Kip3.

In addition, the movies of *CEN3* position over time do not reveal any kinetochore-microtubule attachment defects. Kinetochores in both wild-type and *kip3*Δ mutant cells remain, for the most part, on opposite sides of the spindle and their motion is always along the pole to pole axis (**Fig. S5**). These results are further evidence that deletion of *KIP3* does not result in kinetochore detachment from the microtubule.

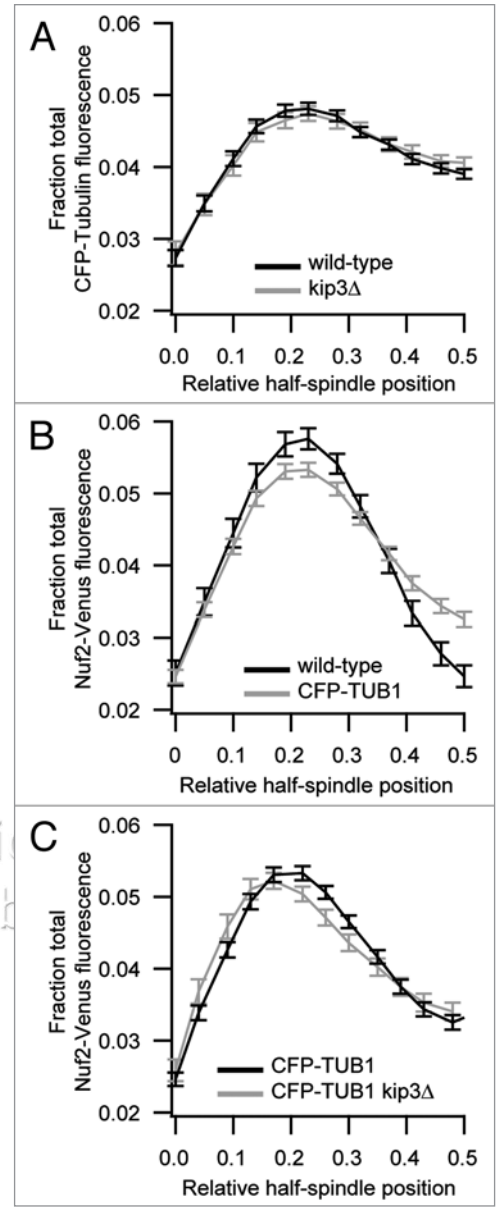

Figure 4. Fluorescently tagged tubulin alters kinetochore distribution. CFP-tubulin and Nuf2-Venus distributions were measured as described in Materials and Methods. The origin of the x-axis designates the spindle pole and spindle position 0.5 denotes the spindle equator. The error bars represent the standard error of the mean. (A) Average distributions of CFP-tubulin fluorescence. Wild-type strain MMWY113-13B in black, N = 112; *kip3*Δ strain MMWY113-18A, in grey, N = 154. (B and C) Average distributions of Nuf2-Venus fluorescence. (B) Wild-type strain MSY107-33B in black, N = 94; CFP-*TUB1* strain MMWY113-13B in grey, N = 112. (C) CFP-*TUB1* strain MMWY113-13B in black, N = 112; *kip3*Δ CFP-*TUB1* strain MMWY113-18A in grey, N = 154.

Discussion

During metaphase, sister kinetochores are attached to opposite spindle poles via dynamic microtubule linkages. A balance of forces (microtubule dynamics pulling the sister chromatids apart and cohesive tension holding the arms of the sisters together) positions sister kinetochores on either side of the spindle equator.[15,16] Moreover, yeast kinetochores form two clusters, each containing one sister kinetochore from each pair of replicated chromatids.[15] Here, we find that Kip3 plays an important role in kinetochore clustering. Deletion of *KIP3* slightly decreases the length of kinetochore microtubules while slightly increasing the average distance between kinetochores (as expected, since spindle length is not altered). However, the largest effects of *KIP3* deletion are not in these average distances, but in distance variability. In the absence of Kip3, the distance between sister kinetochores is more variable and the variability in kinetochore microtubule length is also increased. Given that spindle length is not increased in the mutant, the changes we detect in interkinetochore and pole-kinetochore distances are not an indirect consequence of spindle elongation. Instead, as kinetochores are attached to the ends of dynamic microtubules, we conclude that Kip3 is required for tight

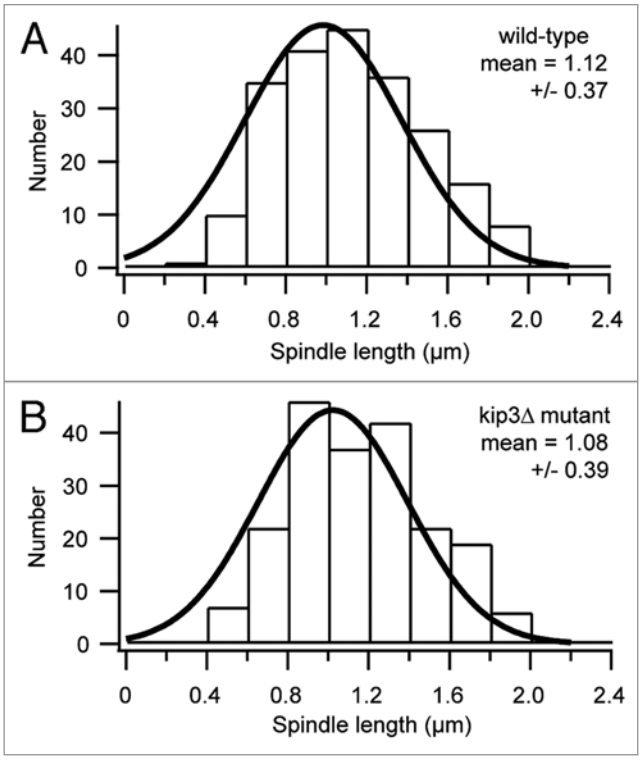

Figure 5. Spindle length is not altered in the *kip3*Δ mutant strain. Histograms of Spc110-mCherry foci separations in live asynchronous cells. Solid curves are single Gaussian fits. (A) Wild-type strain MSY107-33B, N = 201. (B) *kip3*Δ mutant strain MMWY19-2A, N = 218.

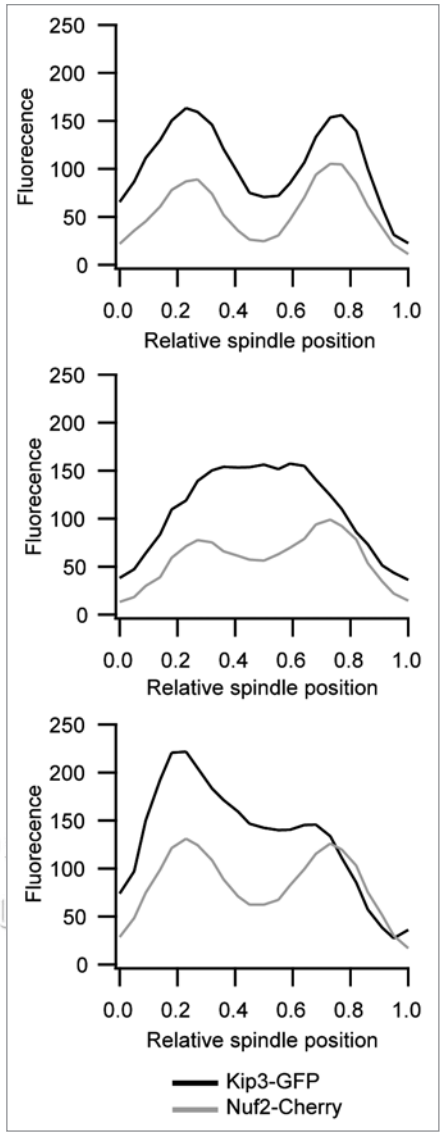

Figure 6. Kip3 Localization. The distribution of Kip3-GFP relative to Nuf2-Cherry in 86 spindles of MSY263-23B was categorized as Two Peaks (47%), High at Equator (28%) or Asymetric (26%). Representative traces show Kip3-GFP in black and Nuf2-Cherry in grey.

control of kinetochore microtubule lengths. This is consistent with a published results that Kip3 and Kif18A, Kip3's homolog in mammalian cells, control microtubule length by increasing microtubule rescue and catastrophe frequencies.[6,30]

Tytell and Sorger[8] showed that *kip3*Δ mutant cells have prolonged DNA hyperstretching between kinetochores and more instances of lagging chromosomes, compared with wild-type cells. The authors concluded that Kip3 must synchronize anaphase chromosome movements and/or coordinate the dissolution of sister chromatid cohesion. Our results suggest a different interpretation. We observe that kinetochore positions are more varied at metaphase in the absence of Kip3. When these cells enter anaphase, the kinetochores start from different places along the spindle and reach the pole at different times, thus some will appear to lag. Moreover, sister kinetochores with longer inter-kinetochore distances will have more hyperstretched DNA.

None of the six yeast kinesins are individually essential, and cells can survive with only two kinesin motors: Cin8 plus Kip3 or Cin8 plus Kar3 (kinesin-14).[31] Cin8 plays a critical role in spindle assembly, a role not shared with Kip3. Loss of Cin8 alone (even in the presence of the five other kinesin motors), blocks bipolar spindle formation in 60% of cells.[27,32] Similarly, deletion or knock down of homologous, but essential, kinesin-5 motors in higher eukaryotes results in the formation of monopolar spindles.[5,27,33,34] Although only a subset of cells is amenable to an analysis of Cin8's role in kinetochore clustering, consistent with previous results,[19] we found that kinetochores are often declustered in the bipolar spindles that manage to form in *cin8*Δ mutants. Although both Cin8 and Kip3 are required to position the kinetochores, their roles are not identical, as the deletion of *CIN8* moves kinetochores closer to the spindle equator, on average, whereas deletion of *KIP3* moves kinetochores closer to the poles.

The fission yeast kinesin-8 homolog positions kinetochores by acting as a coupler between the kinetochore and depolymerizing kinetochore microtubules.[10,20,35] There is no evidence to support a similar role for Kip3 in budding yeast. Kip3 is not essential and although cells grow with a slight metaphase delay, chromosome loss rates in the absence of Kip3 are normal.[13] In addition, chromosome detachment is not increased by deletion of *KIP3*.[8]

In vitro, Kip3 is a length-dependent depolymerase.[6,36] The simplest model predicts kinetochore microtubule length would

be shorter in the presence of Kip3 than in its absence. This is not the case. Instead, we find that the average metaphase kinetochore microtubule is slightly longer in the presence of Kip3 (i.e., deletion of *KIP3* decreases kinetochore microtubule length). Consistent with the idea that Kip3 increases microtubule length in vivo, the shortening rate of cytoplasmic microtubules is slower when Kip3 is present.[6] In addition, a mathematical model that quantitatively reproduces the in vitro results of Varga and collegues[36] shows that lowering the concentration of a processively depolymerizing microtubule motor would increase the standard deviation in microtubule lengths. How the depolymerase activity observed in vitro is regulated to yield the functions observed in vivo will be interesting to discover.

In conclusion, we find that Kip3 does not regulate spindle length and is not required to attach chromosomes to microtubules. Instead, Kip3 clusters kinetochores on the metaphase spindle by tightly regulating kinetochore microtubule length.

Materials and Methods

Media. YPD medium and SD medium are as described (Sherman et al. 1986). YC media contains 2% agar, 2% glucose, 1.7 g/l yeast nitrogen base without amino acids and without ammonium sulfate (BD Difco Cat #233520), 5 g/l ammonium sulfate, 0.1 g/l arginine, leucine, lysine, threonine, tryptophan, 0.05 g/l aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine and 0.01 g/l adenine.

Plasmids and strains. The yeast strains used in this study are listed in **Table 1**. All strains were derived from W303. Plasmids are listed in **Table 2**. C-terminal mCherry, GFP (green fluorescent protein), CFP (cyan fluorescent protein) and Venus protein fusions were created by amplifying the mCherry-hphMX3, GFP-HIS3MX6, GFP-kanMX, CFP-kanMX6, Venus-kanMX6 cassettes from plasmids pBS35, pFA6-GFP (S65T)::HIS3MX6 or pFA6-GFP (S65T)::kanMX, pBS4 and pBS7 plasmids, respectively (all gifts from Yeast Resource Center, University of Washington, Seattle, WA). The above cassettes were integrated in frame at the 3' end of the target ORFs. pBES7 was created by replacing the GFP encoding BglII-XMaI fragment in pAFS91,[38] with CFP from pBS5. The CFP-TUB1 fusion was integrated at the URA3 locus after linearization by StuI digestion. pLKL60Y (LacOarray::Kan, gift from Kerry Bloom) was sequenced and found to contain 33 LacO repeats. Integration of the 33 LacOs, 1 kb 3' of CEN3, was performed after amplifying the LacO array with 40 bp of homology to the insertion site flanking each 5' and 3' primer to the plasmid pLKL60Y.

Fluorescence microscopy and image analysis. All images were acquired with a DeltaVision microcope system (Aplied Precision, Issaquah, WA) containing an Olympus IL70 microscope, 100X, 1.35 numerical aperture oil objective, CoolSNAP HQ digital camera (Photometrics, Tucson, AZ) and optical filter sets (Omega Optical, Semrock and Chroma Technology).

Still images of live cells were acquired by mounting cells on a 1% agarose pad supplemented with SD-complete medium and exposing for 0.4 s at a single focal plane, with 1 x 1 binning.[39]

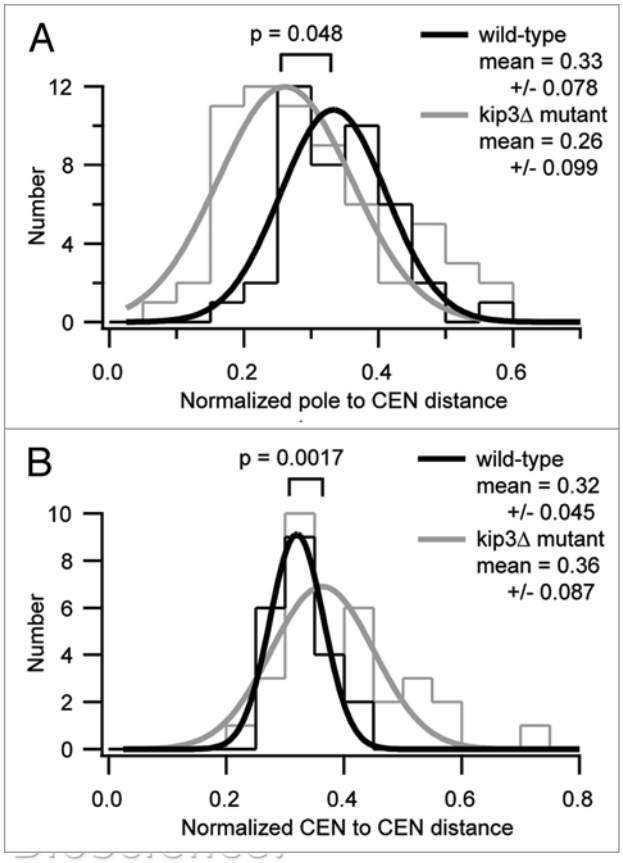

Figure 7. Interkinetochore and pole-kinetochore distances are altered in the *kip3*Δ mutant. Distribution of distances measured between pairs of *CEN3* tagged with GFP and Spc110-mCherry, marking the spindle poles, in live movies of metaphase cells. Solid bold curves are Gaussian fits. Wild-type strain MMWY61n2 in black is 23 cells from 5 experiments; kip3Δ mutant strain MMWY67n14 in grey is 32 cells from 6 experiments. (A) Comparison of normalized distance from pole to nearest *CEN3*-GFP focus. The origin of the x-axis marks the spindle pole. (B) Comparison of normalized distance between *CEN3*-GFP foci. The x-axis shows the distance between *CEN3*-GFP foci pairs.

These images were converted to 8- and 16-bit TIFF files using R3D converter.[23] Custom Matlab programs, Calcmate and Fluorcal, were used to select regions in the image to quantify. Fluorcal is based on FretSCal.[40] Next, the quantification of fluorescence across the metaphase length spindles was performed by measuring the fluorescence at each pixel along the spindle axis and subtracting the background from above and below the spindle as described.[41]

For time-lapse imaging, after cells were grown on YC medium, the cells were mounted for microscopy on 1% agarose and the coverslip affixed to the slide with Valap (1:1:1 vaseline:lanolin:paraffin). Single plane images of GFP and Cherry fluorescence (0.1 sec) were acquired at 1.0-second intervals. Spindles were imaged until they went out of focus or until the flourophor was significantly bleached. The positions of Spc110-Cherry and CEN3-LacO/LacI-GFP foci were tracked using the MTrackJ plugin (Meijering E, Biomedical Imaging Group Rotterdam, University Medical Center, Rotterdam,

Table 1. Yeast strains

Strain	Genotype	Source or reference
CRY1	Mat**a**	R. Fuller
CRY2	Matα	R. Fuller
MMWY19-2A	Mat**a** ade3Δ DAM1-myc::KANMX kip3Δ::HIS3MX6 NUF2-GFP::HISMX SPC110-Cherry::hphMX	This Study
MMWY61n2	Mat**a** ade3Δ pCUP1-GFP 12LacI12::HIS3 CEN3-LacO33rray::Kan SPC110-Cherry::hphMX	This Study
MMWY67n14	Mata ade3Δ pCUP1-GFP 12LacI12::HIS3 CEN3-LacO33array::Kan kip3Δ::HIS3MX6 SPC110-Cherry::hphMX	This Study
MMWY72-22D	Matα ade3Δ NUF2-GFP::HISMX SPC110-Cherry::hphMX	This Study
MMWY72-25B	Matα ade3Δ cin8Δ::KanMX NUF2-GFP::HISMX SPC110-Cherry::hphMX	This Study
MMWY81-2B	Mat**a** ade3Δ MTW1-GFP::HIS3MX6 SPC110-Cherry::hphMX	This Study
MMWY81-16C	Mat**a** ade3Δ kip3Δ::HIS3MX6 MTW1-GFP::HIS3MX6 SPC110-Cherry::hphMX	This Study
MMWY83-5A	Matα ade3Δ kip3Δ::HIS3MX6 SPC105-GFP::HISMX SPC110-Cherry::hphMX	This Study
MMWY113-13B	Mat**a** ade3Δ CFP-TUB1::URA3 NUF2-Venus::KanMX SPC110-Cherry::hphMX	This Study
MMWY113-18A	Mat**a** ade3Δ CFP-TUB1::URA3 kip3Δ::HIS3MX6 NUF2-Venus::KanMX SPC110-Cherry::hphMX	This Study
MMWY133-3C	Mat**a** ade3Δ MTW1-GFP::HIS3MX6 ndc80-1 SPC110-Cherry::hphMX	
MSY107-33B	Mat**a** ade3Δ DAM1-myc::KANMX NUF2-GFP::HISMX SPC110-Cherry::hphMX	This Study
MSY265-1D	Matα ade3Δ SPC105-GFP::HISMX SPC110-Cherry::hphMX	This Study
MSY263-23B	Mat**a** ade3Δ KIP3-GFP::HIS3MX6 NUF2-Cherry::hphMX	This Study
W303	ade2-1oc can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1	

*All strains were derived from W303 and have the same markers except as indicated.

Table 2. Plasmids

Plasmid	Relavant markers	Source or reference
pBES7	CFP-TUB1::URA3	Davis lab
pBS4	CFP-kanMX6	YRC
pBS5	CFP-Kan	YRC
pBS7	Venus-kanMX6	YRC
pFA6-GFP(S65T)::HIS3MX6	GFP(S65T)::HIS3MX6	YRC, Wach et al. 1997
pFA6-GFP(S65T)::kanMX	GFP-kanMX	YRC
pLKL60Y	LacO33array:KAN	Pearson et al. 2001
pPB35	mCherry::hphMX3	YRC

Netherlands) for ImageJ (Rasband WS, Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij, 1997–2009). Distances between CEN3 pairs and between CEN3 and the nearest spindle pole were quantified from tracking data using Igor Pro 6.0 software (Wavemetrics). For each cell, we determined three averages: the average distance between the CEN3 pairs and the average distance between each CEN3 and its nearest pole. These averages are plotted on the histograms shown in **Figure 7**.

Spindle length determination. The pole-to-pole distance for wild type and *kip3*Δ mutant spindles was determined by measuring the distance between the centers of Spc110-mCherry fluorescence in single plane images. Spindles that were in-focus were defined as those in which the Spc110-mCherry fluorescence signal to noise ratio was greater than 1.2 and the fluorescent intensity of the dimmer pole was at least 80% that of the brighter pole.

Wild-type metaphase spindles have clustered kinetochores and length betweens 1.28–1.53 μm. *kip3*Δ mutant strains had declustered kinetochores at all spindle lengths, so spindle lengths of 1.28–1.53 μm were compared to wild-type metaphase spindles.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/WargackiCC9-13-sup.pdf

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