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

Megan M. Wargacki,1 Jessica C. Tay,1 Eric G. Muller,1 Charles L. Asbury2 and Trisha N. Davis1,*

1Department of Biochemistry and 2Department of Physiology and Biophysics; University of Washington; Seattle, WA USA

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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.15,16 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 Sorger8 proposed that loss of Kip3 caused lagging chromosomes during anaphase A. Gardner and coworkers19 reported that Kip3 regulates inter-polar 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 kip3Δ 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 kip3Δ 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
The cytoplasmic function of Kip3 is to position the spindle at the bud neck during metaphase.\textsuperscript{13,14,24} Consistent with this result, we observed twenty-five percent of kip3\textsuperscript{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\textsuperscript{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.

We scored images of wild-type, kip3\textsuperscript{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\textsuperscript{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.\textsuperscript{13,14,24} Consistent with this result, we observed twenty-five percent of kip3\textsuperscript{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\textsuperscript{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.
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

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 kip3Δ 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.
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. The mammalian kinesin-8 forms a motor-dependent gradient along most kinetochore microtubules. 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∆ 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∆ 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.

**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. Moreover, yeast kinetochores form two clusters, each containing one sister kinetochore from each pair of replicated chromatids. 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 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.
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 Sorger8 showed that kip3Δ 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).14 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 cin8Δ 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 kinetochoore microtubule is slightly longer in the presence of Kip3 (i.e., deletion of KIP3 decreases kinetochoore 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 colleagues9 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 kinetochoore 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-hMX3, 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 pAFS91,18 with CFP. The CFP-TUB1 fusion was integrated at the URA3 locus after linearization by StuI digestion. pLKL60Y (LacOrarray::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 microscope system (Aplied Precision, Issaquah, WA) containing an Olympus IX70 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 These images were converted to 8- and 16-bit TIFF files using R3D converter.95 Custom Matlab programs, CalcMate and Fluorocal, were used to select regions in the image to quantify. Fluorocal 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 axis.

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 fluorophor 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, Group Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam, University Medical Center, Rotterdam).
Table 1. Yeast strains

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

Table 2. Plasmids

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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 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 kip3Δ 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 between 1.28–1.53 µm. kip3Δ 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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