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<tr>
<td><strong>Citation</strong></td>
<td>Molecular Biology of the Cell , 28 (25) : 3647 – 3659</td>
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<td><strong>Issue Date</strong></td>
<td>2017-12-01</td>
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<tr>
<td><strong>DOI</strong></td>
<td>10.1091/mbc.e17-08-0497</td>
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<td><strong>Self DOI</strong></td>
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A microtubule polymerase cooperates with the kinesin-6 motor and a microtubule cross-linker to promote bipolar spindle assembly in the absence of kinesin-5 and kinesin-14 in fission yeast

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\textbf{ABSTRACT} Accurate chromosome segregation relies on the bipolar mitotic spindle. In many eukaryotes, spindle formation is driven by the plus-end-directed motor kinesin-5 that generates outward force to establish spindle bipolarity. Its inhibition leads to the emergence of monopolar spindles with mitotic arrest. Intriguingly, simultaneous inactivation of the minus-end-directed motor kinesin-14 restores spindle bipolarity in many systems. Here we show that in fission yeast, three independent pathways contribute to spindle bipolarity in the absence of kinesin-5/Cut7 and kinesin-14/Pkl1. One is kinesin-6/Klp9 that engages with spindle elongation once short bipolar spindles assemble. Klp9 also ensures the medial positioning of anaphase spindles to prevent unequal chromosome segregation. Another is the Alp7/TACC-Alp14/TOG microtubule polymerase complex. Temperature-sensitive \textit{alp7cut7pkl1} mutants are arrested with either monopolar or very short spindles. Forced targeting of Alp14 to the spindle pole body is sufficient to render \textit{alp7cut7pkl1} triply deleted cells viable and promote spindle assembly, indicating that Alp14-mediated microtubule polymerization from the nuclear face of the spindle pole body could generate outward force in place of Cut7 during early mitosis. The third pathway involves the Ase1/PRC1 microtubule cross-linker that stabilizes antiparallel microtubules. Our study, therefore, unveils multifaceted interplay among kinesin-independent and -independent pathways leading to mitotic bipolar spindle assembly.

\textbf{INTRODUCTION} The microtubule plays a myriad of roles in many biological processes. During mitosis, this biopolymer forms the bipolar spindle that aligns sister chromatids in the middle of the cell before segregating them to opposite poles. Perturbation of this mitotic apparatus can lead to aneuploidy, a major risk factor for cancer development, miscarriage, birth defects, and other human diseases (Wittmann \textit{et al.}, 2001; Godinho and Pellman, 2014). In addition to microtubules, the mitotic spindle employs a dynamic ensemble of various microtubule-associated proteins (MAPs) and motor proteins. Not only do these proteins play a crucial role as structural components in establishing spindle bipolarity, they also regulate the dynamic properties of the spindle microtubule during distinct mitotic stages (Desai and Mitchison, 1997; Jiang and Akhmanova, 2011; Heald and Khodjakov, 2015).

One of the key MAPs regulating spindle bipolarity is the \textit{Dis1/XMAP215/TOG} family, which possesses microtubule polymerase activity (Brouhard \textit{et al.}, 2008; Al-Bassam and Chang, 2011). Although TOG family proteins are thought to regulate microtubule
plus-end dynamics, they localize to the centrosome or spindle pole body (SPB, the fungal equivalent of the centrosome), from which microtubules are nucleated (Charrasse et al., 1998; Matthews et al., 1998; Cullen et al., 1999). In fission yeast, two TOG family proteins, Alp14/Mtc1 and Dis1, have been identified and found to share an essential function in cell division (Garcia et al., 2002). Alp14/TOG forms a complex with Alp7/Mia1/TACC, where Alp7 acts as an SPB-targeting factor for this complex during mitosis (Sato et al., 2004; Sato and Toda, 2007); localization of Alp14 to the mitotic spindle is an initial, essential step for this protein to act as a microtubule polymerase for mitotic spindle assembly (Sato et al., 2004; Tang et al., 2014).

Kinesin motor proteins comprise a large protein family (kinesin-1 to kinesin-14) (Lawrence et al., 2004; Hirokawa et al., 2009) and play diverse roles in microtubule-mediated cellular activities. This family can be classified into three subgroups called N-kinesins, M-kinesins, and C-kinesins, where the motor domain is located in the N-terminal region, in the middle region or in the C-terminal region, respectively. In general, N-kinesins and C-kinesins drive microtubule plus-end- and minus-end-directed motilities respectively, while M-kinesins depolymerize microtubules.

In most eukaryotes, kinesin-5 (budding yeast Cin8 and Kip1, fission yeast Cut7, Aspergillus BimC, Drosophila Klp61F, Xenopus Eg5 and human Kif11) plays an essential role in bipolar spindle formation and successful cell division. Kinesin-5 molecules form homotetramers, allowing them to cross-link and subsequently slide anti-parallel microtubules apart, thereby generating outward pushing force onto spindle poles (Kashina et al., 1996; Kapitein et al., 2005). In line with this notion, mutation, depletion, or chemical inhibition of kinesin-5 results in the appearance of monopolar spindles with duplicated, yet unseparated, centrosomes/SPBs (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt et al., 1992; Roof et al., 1992; Heck et al., 1993; Mayer et al., 1999). Interestingly, it is thought that outward force generated by kinesin-5 is antagonized by the inward force exerted by minus-end-directed motors such as kinesin-14 (budding yeast Kar3, fission yeast Pkl1 and Klp2, Aspergillus KlpA, Drosophila Ncd, Xenopus XCTK2 and human HSET/KIF11) and dynein (O’Connell et al., 1991). To study the impact of cut7 ts mutations on mitotic progression in detail, we observed the dynamic behavior of spindle microtubules and SPBs in wild-type and cut7Δpkl1Δ cells expressing mCherry-Atb2 (a microtubule marker) (Toda et al., 1984) and Cut12-GFP (an SPB marker) (Bridge et al., 1998) (Figure 1, B–F). We found that cut7Δpkl1Δ cells could assemble bipolar spindles (Figure 1, B and C) but stayed in a preanaphase stage for a much longer period of time (21 ± 5 min vs. 11 ± 3 min for wild type; Figure 1D) and that their spindle length at the onset of anaphase B was significantly shorter (1.19 ± 0.51 μm vs. 2.48 ± 0.46 μm for wild type; Figure 1E). Furthermore, spindle elongation rate during anaphase B was considerably slower in cut7Δpkl1Δ cells compared with wild-type cells (0.38 ± 0.11 μm/min vs. 0.53 ± 0.06 μm/min for wild type; Figure 1F). These results are consistent with those shown in a recent publication (Rincon et al., 2017), which appeared during the preparation of this article.

To address the involvement of the spindle assembly checkpoint (SAC) in the mitotic delay observed in cut7Δpkl1Δ cells, we combined the deletion of genes encoding the core SAC components Mad1, Mad2, and Bub1 (Musacchio, 2015) with the cut7Δpkl1Δ mutant. Tetrad analysis showed that authentic SAC signaling is required for optimal growth or even viability of cut7Δpkl1Δ cells (Figure 1G, and Supplemental Figure S1B). These results indicate that cut7Δpkl1Δ cells manage to survive due to a SAC-mediated delay in mitotic progression, thereby providing enough time for the correction of erroneous kinetochore-microtubule attachments.

Fission yeast kinesin-6/Klp9 motor activity is essential for the viability in the absence of Cut7 and Pkl1

Although not completely normal, cut7Δpkl1Δ mutant cells assemble bipolar spindles and continue to proliferate. How is this possible in the absence of Cut7ΔKlp9 and Pkl1Klp9, two major plus- and minus-end-directed motors? One possibility is that other kinesin motors establish and maintain spindle bipolarity in place of Cut7...
and Pkl1. To investigate this possibility, we examined synthetic lethality between each deletion of other kinesin-encoding genes and cut7Δpkl1Δ. Fission yeast contains seven kinesin motors (Tea2, Klp2, Klp3, Klp5, Klp6, Klp8, and Klp9) besides Cut7\(^{\text{Kin-5}}\) and Pkl1\(^{\text{Kln-14}}\); none of them is essential for cell viability on their own. Genetic crosses indicated that among them, only one kinesin, Klp9, becomes essential when combined with cut7Δpkl1Δ (Figure 2A and Supplemental Figure S2A). Klp9 belongs to the plus-end-directed kinesin-6 family and accumulates at the spindle midzone at the onset of anaphase B to promote spindle elongation during late mitosis (Fu et al., 2009).

Recently, it was reported that Klp9 plays two key roles (Meadows et al., 2017); one involves the N-terminal motor region, thereby accelerating anaphase B spindle elongation as previously shown (Fu et al., 2009), while the other functions to control proper timing of anaphase onset and chromosome segregation (Choi and McCollum, 2012). Interestingly, the latter function is executed by the C-terminal nonmotor region independently of motor activity (Meadows et al., 2017). Given the complexity of Klp9 functions, we next addressed which activity within Klp9 makes a contribution to the viability of cut7Δpkl1Δ cells. To this end, we implemented two different types of Klp9 mutants; one is Klp9-rigor (G296A, a substitution within its motor domain), which is defective in plus-end motility and the other is Klp9-Δ38C (lacking the C-terminal 38 amino acids), which abolishes the temporal role in anaphase onset (Meadows et al., 2017). Tetrads analysis clearly indicated that Klp9-rigor, but not Klp9-Δ38C, is lethal in the absence of Cut7 and Pkl1 (Figure 2, B and C). This demonstrates that the plus-end motility of Klp9\(^{\text{Kin-6}}\) renders cut7Δpkl1Δ cells viable.

Klp9-dependent spindle elongation and positioning during anaphase B are indispensable for proper chromosome segregation in the absence of Cut7 and Pkl1

Next, we observed the timing of Klp9 accumulation on spindle microtubules in cut7Δpkl1Δ cells. As aforementioned, in wild-type cells, Klp9 is always localized to the spindle midzone only after sister chromatids separate and reach each SPB, which coincides with anaphase B onset (Fu et al., 2009; Meadows et al., 2017) (Figure 2D, top row). This was also the case in the pkl1 single mutant (middle row). Interestingly, the timing of Klp9 localization did not alter even in cut7Δpkl1Δ double mutant cells (bottom row). These results suggest that Klp9 localization to the spindle midzone during anaphase B is essential for cell viability in the absence of Cut7 and Pkl1; Klp9 motor does not function prior to anaphase B in cut7Δpkl1Δ as in wild-type cells.

To explore the defective phenotypes of klp9cut7Δpkl1Δ triple mutants, we used the cut7-22 ts mutant (Hagan and Yanagida, 1990, 1992). Deletion of klp9 enhanced temperature sensitivity of the cut7-22 single mutant and conferred the ts phenotype to the cut7-22pkl1Δ double mutants (Supplemental Figure S2B). To characterize the terminal phenotype of cut7-22pkl1Δklp9A cells, we observed spindle microtubule behavior by time-lapse live imaging at 36°C. Intriguingly, triple mutant cells appeared to assemble bipolar spindles (Figure 2, E and F), albeit shorter in length. However, spindle elongation rate during anaphase B was substantially decreased compared with the cut7Δpkl1Δ cells (0.15 ± 0.06 μm/min vs. 0.53 ± 0.14 μm/min) (Figure 2G). Notably, these anaphase B spindles did not reach cell tips (Hagan and Petersen, 2000) and instead were displaced from the cell middle (34%, n = 80, see frames after 16 min time point in Figure 2E). Moreover, the septum started to form during spindle displacement (shown by arrowheads in Figure 2E). This asymmetric positioning of shorter spindles and

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**FIGURE 1:** The cut7Δpkl1Δ double deletion results in SAC-dependent mitotic delay with a short preanaphase spindle. (A) Indicated strains were streaked on a rich YESS agar plate containing Phloxine B and incubated at 33°C for 2 d. (B) Kymographic images of mitotic wild-type (top) and cut7Δpkl1Δ cells (bottom) expressing a tubulin marker (mCherry-Atb2, red) and an SPB marker (Cut12-GFP, green). Pictures were taken at 2 min intervals. Scale bar, 10 μm. (C) Profiles of mitotic progression in wild-type (gray line, n = 27) and cut7Δpkl1Δ cells (red line, n = 24). Changes of the inter-SPB distance were plotted against time. (D) The time between the initiation of SPB separation and onset of anaphase B. (E) Spindle length at anaphase onset. (F) Spindle growth rate during anaphase B. Results are given as means ± SD. Data sets were compared with a two-tailed unpaired Student’s t tests (**** p < 0.0001). (G) Tetrads analysis on crosses between mad1Δpkl1Δ and cut7Δpkl1Δ strains. Individual spores (a–d) in each ascus (1–4) were dissected on YESS plates and incubated at 27°C for 3 d. Representative tetrad patterns are shown. Circles, triangles and squares with green lines indicate pkl1Δ single mutants, cut7Δpkl1Δ double mutants and mad1Δpkl1Δ double mutants, respectively. Assuming 2:2 segregation of individual markers allows the identification of mad1Δcut7Δpkl1Δ triple mutants (indicated by dashed magenta circles).
FIGURE 2: Motor activity of Kinesin-6/Klp9 is essential for the viability in the absence of Cut7 and Pkl1. (A, B, C) Tetrad analysis. Spores were dissected on crosses between klp9Δpkl1Δ and cut7Δpkl1Δ strains (A), between klp9Δpkl1Δ and cut7Δpkl1Δ strains (B), or between klp9Δpkl1Δ and cut7Δpkl1Δ strains (C) respectively. Replication was performed as in Figure 1A. Circles and triangles with green lines indicate pkl1Δ single mutants and cut7Δpkl1Δ double mutants, respectively. Squares with green lines indicate klp9Δpkl1Δ double mutants (A), klp9Δpkl1Δ double mutants (B), or klp9Δpkl1Δ double mutants (C). Assuming 2:2 segregation of individual markers allows the identification of triple mutants in each cross (indicated by dashed magenta circles). (D) Kymographic images of a wild-type, pkl1Δ, or cut7Δpkl1Δ cell expressing Klp9-GFP (green) along with a kinetochore marker (Mis6-2mRFP, red) (Flory et al., 2002; Fong et al., 2008). Images were taken at 2-min intervals. The timing of SPB separation, anaphase onset, and Klp9-GFP accumulation at the spindle midzone is indicated with white, blue, and yellow arrowheads, respectively. Scale bar, 10 μm. (E) Time-lapse images of a mitotic cut7Δpkl1Δ cell. Spindle microtubules (mCherry-Atb2, red) and SPB (Cut12-GFP, green) were visualized (top). Images were taken at 2-min intervals on shifting the temperature from 27°C to 36°C for 2 h. The septation sites are indicated with yellow arrowheads in the DIC images (bottom). The cell peripheries are outlined with dotted lines (top). Scale bar, 10 μm. (F, G) Profiles of mitotic progression in cut7Δpkl1Δ (gray, n = 30) and cut7-22pkl1Δ (yellow, n = 21) under the same condition as in E. Changes of the inter-SPB distance were plotted against time (F). Spindle growth rate after the inter-SPB distance reached 1.5 μm was calculated (G). Data are given as means ± SD; ****p < 0.0001 (two-tailed unpaired Student’s t test).

Together, these results indicate that Klp9 plays an essential role in the absence of Cut7 and Pkl1 and that its motor activity is required for spindle elongation and proper positioning during anaphase B, thereby averting the emergence of lethal chromosome segregation errors.

Temperature-sensitive klp9 mutants also lead to defective anaphase and chromosome missegregation in the absence of Cut7 and Pkl1

To recapitulate the terminal phenotypes of the cut7-22pkl1Δklp9Δ triple mutants and confirm the role of Klp9 in proper spindle formation, we isolated three independent klp9 mutant alleles (klp9-1, -2, -3) by PCR-based error-prone mutagenesis (see Materials and Methods). These mutants displayed ts growth in the cut7Δpkl1Δ deletion background (Figure 3, A and B); however, none of these alleles was ts in the wild-type background (Supplemental Figure S3A). While klp9-2 contained a single point mutation within the motor domain (K333M, Figure 3A), klp9-3 contained two mutations in the motor domain and the C-terminal tail region (I198T and P586S, respectively). Subsequently, we used klp9-3M and klp9-3T, which each contained one of two mutations (klp9-3M, I198T and klp9-3T, P586S, Figure 3A). The klp9-2 and klp9-3M mutants showed more severe temperature sensitivity than klp9-1 (Figure 3B). Hereafter, we sought to characterize the klp9-2 allele in more detail.

To define the cell-cycle stage for which Klp9 function is essential, we performed synchronous culture analysis with centrifugal elutriation using the klp9-2cut7Δpkl1Δ mutants. Small early-G2 cells were harvested by elutriation at 27°C, shifted up to 36°C, and cell viability was monitored as cells proceeded through the cell cycle. Triple mutant cells exhibited a decline in cell viability at two time points, 90 and 165 min (black line in the right panel of Figure 3C). This viability drop paralleled the emergence of the chromosomal missegregation phenotype (blue line in the right panel of Figure 3C, representative images of mitotic cells with missegregating chromosomes are shown in Supplemental Figure S3B). Moreover, time-lapse live imaging analysis showed that klp9-2cut7Δpkl1Δ triple mutants formed shorter bipolar spindles and displayed much slower spindle elongation during anaphase B (24 min and afterward in Figure 3D). Overall, these

septation might account for the terminal chromosome “cut” phenotype in cut7Δpkl1Δ cells, in which klp9 transcription was shut off as recently described (cut7Δpkl1Δklp9Δ) (Rincon et al., 2017). Taken together, these results indicate that Klp9 plays an essential role in the absence of Cut7 and Pkl1 and that its motor activity is required for spindle elongation and proper positioning during anaphase B, thereby averting the emergence of lethal chromosome segregation errors.
defective phenotypes are very similar, if not identical, to those observed in cut7-22pkl1Δklp9Δ cells described earlier (Figure 2, E–G). Taking these results together, we conclude that Klp9\(^{Kin-6}\) and Cut7\(^{Kin-6}\) coordinate to promote anaphase B spindle elongation and are also required for proper positioning of the spindle apparatus, thereby escaping from the lethal cut phenotype imposed by the cytokinetic machinery.

Several nonmotor microtubule-associated proteins are required for cell viability of cut7Δpkl1Δ mutants

As Klp9 appears to execute its essential function in the absence of Cut7 and Pkl1 only during anaphase B, another question arises: how can preanaphase bipolar spindles, albeit shorter in length, assemble in the absence of these kinesin motors? Very recent work (Rincon et al., 2017) ascribed this role to the microtubule cross-linking/bundling factor Ase1/PRC1 (Loidioce et al., 2005; Yamashita et al., 2005; Janson et al., 2007) and the microtubule stabilizer Cls1/Peg1/CLASP (Grallert et al., 2006; Bateman and Chang, 2007). However, as these two MAPs are not required for either microtubule nucleation or polymerization per se (Janson et al., 2007; Al-Bassam et al., 2010; Al-Bassam and Chang, 2011), we presumed that some other factors/MAPs must be functional in early spindle assembly, in particular at the SPB, the microtubule-organizing center (MTOC). To identify such factors, we systematically examined synthetic lethality between each deletion of a cohort of MAPs and cut7Δpkl1Δ. Random spore analysis and tetrad dissection uncovered that at least five MAPs (Alp7/Mia1, Alp14/Mtc1, Dis1, Csi1, Csi2) (Nabeshima et al., 2001; Nakaseko et al., 2001; Oliferenko and Balasubramanian, 2002; Sato et al., 2004; Hou et al., 2012; Costa et al., 2014), in addition to Ase1, were required for the viability of cut7Δpkl1Δ cells (Figure 4A and Supplemental Figure S4). Notably, Alp14 and Dis1 belong to the Dis1/XMAP215/TOG family of microtubule polymerases (Al-Bassam and Chang, 2011; Al-Bassam et al., 2012; Hussmann et al., 2016; Matsuo et al., 2016).

Alp7/TACC is of our particular interest, as this protein forms a stable complex with Alp14/TOG (Sato et al., 2004; Al-Bassam et al., 2012; Hussmann et al., 2016) and recruits Alp14 to the mitotic SPB, thereby ensuring spindle assembly/elongation from this MTOC (Ling et al., 2009; Sato et al., 2009; Tang et al., 2014). Given these characteristics, we pursued the
molecular details by which Alp7 plays an essential role in cut7Δpkl1Δ cells.

Localization of Alp7/TACC to the mitotic SPB is a prerequisite for proper spindle assembly in the cut7Δpkl1Δ mutants

Alp7/TACC plays multiple roles in cell cycle-dependent microtubule organization that are executed through interactions with several other proteins in a spatiotemporal manner (Sato et al., 2004; Tang et al., 2013, 2014; Tang and Toda, 2015; Okada et al., 2014; Zheng et al., 2014). A number of previous studies mapped functional domains within Alp7 that are required for each of its distinct roles, and hence several Alp7 mutants with defects in specific functions are available. To address which function and localization of Alp7 are important, we crossed individual alp7 mutants with a cut7Δpkl1Δ strain. This genetic analysis unequivocally showed that the regions/residues within Alp7 that are responsible for localization to the mitotic SPB are crucial for the viability of cut7Δpkl1Δ cells (Figure 4B and Supplemental Figure SSA).

To create alp7 ts mutants in the cut7Δpkl1Δ background, we implemented Alp7-5A, which contains alanine substitutions at five amino acid residues (K341, Y344, R346, K347, and Y348) (Tang et al., 2014), which is lethal in the cut7Δpkl1Δ background (Figure 4B). We introduced random mutations into these five residues by PCR-based, site-directed mutagenesis (Masuda et al., 2013) and screened for ts mutants in a cut7Δpkl1Δ strain (Materials and Methods). Three ts alleles (alp7-13, -17, and -20) were isolated, and nucleotide sequencing indicated that all three isolates indeed contained missense mutations within the five residues (alp7-13: R346I, K347R, Y348L; alp7-17: Y344T; alp7-20: Y344R) (Supplemental Figure S5B). As expected, these mutants displayed temperature sensitivity only when combined with cut7Δpkl1Δ mutations (Figure 4C). Alp7-5A is specifically defective in SPB localization during mitosis (Tang et al., 2014) and, consistent with this, Alp7-GFP intensities at the mitotic SPBs in the alp7-13 and alp7-20 mutants at the restrictive temperature showed a substantial reduction of fluorescence signals by 70% and 96%, respectively (Figure 4D and Supplemental Figure SSC). We performed immunoblotting to examine the total protein content of Alp7-GFP in these mutants and found that the levels were indistinguishable from that in wild-type cells (Supplemental Figure SSD). This indicates that Alp7-13 and Alp7-20 mutant proteins are specifically defective in localizing to the mitotic SPBs. Therefore, Alp7 localization to the mitotic SPB is essential for the viability of cut7Δpkl1Δ cells.

To examine the impact of these alp7 mutations on spindle assembly in the cut7Δpkl1Δ background, we observed the terminal phenotype of alp7-13cut7Δpkl1Δ or alp7-20cut7Δpkl1Δ cells expressing mCherry-Atb2 and Cut12-GFP. Notably, in both triple mutants, the frequency of cells containing monopolar or very short spindles (<0.5 μm) was dramatically increased on incubation at 36°C for 2 h compared with that of the cut7Δpkl1Δ double mutant (Figure 5, A and B). The alp7-20cut7Δpkl1Δ triple mutants showed the monopolar spindle phenotype to some extent even at 27°C (6.9% vs. <1.0% for cut7Δpkl1Δ; Figure 5B). To capture mitotic progression in a single cell, we performed time-lapse live imaging. The triple mutant cells spent much longer preanaphase with monopolar or very short spindles (Figure 5C). A majority of the mutant cells (95%, n = 26) maintained a short spindle (<1.5 μm) at least for 30 min, and >70% cells (19 of 26) remained in this stage for more than 40 min (Figure 5, D and E). This result indicates that cells have the capability to assemble short bipolar spindles in the absence of Cut7K5-3 and Pkl1K14 through the SPB-localizing activity of Alp7.

![Figure 4](image-url)
Alp7 is required for bipolar spindle assembly during early mitosis of the cut7Δpkl1Δ cells. (A) Representative spindle morphologies of the alp7-13cut7Δpkl1Δ triple mutants. Cells containing a bipolar (top), a monopolar (middle), or a very short spindle (the inter-SPB distance is <0.5 μm, bottom) are shown (Cut12-GFP, green, and mCherry-Atb2, red). Enlarged images of spindle areas (squares) are shown on the left panels. Positions of the SPB are indicated with arrowheads. The cell peripheries are outlined with dotted lines. Scale bars, 10 μm (main images) and 1 μm (enlarged images of the boxed regions). (B) The percentage of cells containing a bipolar (top), a monopolar (middle), or a very short spindle (light blue). Each strain was grown at 27°C and shifted to 36°C for 2 h. In each experiment, more than 25 mitotic cells were observed. (C) Time-lapse images of a mitotic alp7-13cut7Δpkl1Δ cell. Spindle microtubules (mCherry-Atb2; red) and SPBs (Cut12-GFP; green) were visualized. Images were taken at 2-min intervals after 2 h incubation at 36°C. The positions of cell ends are indicated with dotted curved lines (top), and enlarged images of the boxed regions are presented at the bottom. Scale bars, 10 μm (top) and 1 μm (bottom). (D, E) Profiles of mitotic progression in alp7-13cut7Δpkl1Δ (light blue, n = 26) cells under the same condition as in C. Changes in the inter-SPB distance were plotted against time (D). The percentage of cells retaining a short spindle (the inter-SPB distance is <1.5 μm) for >30 min after SPB separation was counted (E). Data for cut7Δpkl1Δ are shown for comparison; this is the same as that presented in Figure 2, F and G. Data are given as means ± SD; **p < 0.01; ***p < 0.001; ****p < 0.0001 (two-tailed unpaired Student’s t test).

**FIGURE 5:**

Having obtained viable cells that contain SPB-tethered Alp14NLGFP in the alp7Δcut7Δpkl1Δ background, we next followed the profiles of mitotic progression and spindle assembly in this strain. It was found that these cells could assemble short bipolar spindles with delayed anaphase onset, which were very similar, if not identical, to those of cut7Δpkl1Δ cells (Figure 6D and Supplementary Figure S7, A–C). Collectively, our data indicate that in fission yeast, at least two distinct pathways (see below), one kinesin-6 mediated and the other microtubule polymerase dependent, ensure spindle assembly at different stages of mitosis in the absence of Cut7K15 and Pkl1K114.

**DISCUSSION**

In this study, we have explored the pathways that lead to bipolar spindle assembly in the absence of Cut7K15 and Pkl1K114 in fission yeast. Previous studies in many organisms have shown that the critical task performed by kinesin-5 in bipolar spindle assembly becomes dispensable by the depletion or inhibition of kinesin-14 (Saunders and Hoyt, 1992; O’Connell et al., 1993; Pidoux et al., 1996; Mountain et al., 1999; Sharp et al., 1999; Tanenbaum and Medema, 2010; Salemi et al., 2013). Here we have unearthed several factors that promote bipolar spindle assembly in cut7Δpkl1Δ cells. Our results show that otherwise nonessential Klp9K16 and
various MAPs including the Alp14/TOG microtubule polymerase are absolutely required to render these double mutant cells viable.

Roles of kinesin-6 in mitotic spindle formation and integrity

Kinesin-6 family members (fission yeast Klp9, Caenorhabditis elegans ZEN-4, Drosophila Pavarotti and Subito, and human MKLP1/CHO1/Kif23, MKLP2/Kif20A/Rab6-KIFL, and MPP1/Kif20B) are localized to the spindle midzone at anaphase (Nislow et al., 1992; Adams et al., 1998; Raich et al., 1998; Hill et al., 2000; Abaza et al., 2003; Cesario et al., 2006; Fu et al., 2009). In general, loss of this kinesin results in a disorganized midzone at anaphase and subsequent cytokinesis defects. Some of the kinesin-6 members (e.g., human MKLP1) act to directly bundle antiparallel microtubules and slide them apart, while others (e.g., human MKLP2) do so indirectly through loading the chromosome passenger complex (CPC) containing Aurora B and other regulators to the spindle midzone. As shown previously (Fu et al., 2009; Meadows et al., 2017; Rincon et al., 2017) and in this study (Figure 7), fission yeast Klp9/Kin-6 plays both roles in late mitosis, that is, spindle elongation and CPC recruitment. Klp9/Kin-6 may, therefore, retain features of an ancestral dual-function motor that are executed by different kinesin-6 members in human cells. It would be of great interest to test in human cells whether the depletion or inhibition of kinesin-6s leads to the lethality by simultaneous inactivation of kinesin-5 and kinesin-14 (and/or dynein).

Drosophila Subito was the first kinesin-6 member to be reported with a function in spindle assembly during the early stages of mitosis; Subito mutants improperly assemble metaphase microtubules, resulting in SAC activation and the emergence of lagging shown. The positions of SPBs are indicated with orange arrowheads. The cell peripheries are outlined with dotted lines. Some Alp14\textsuperscript{NL5-GFP} signals are detected in the nucleus and along the microtubules in addition to SPBs. Scale bar, 10 μm.

(D) Profiles of mitotic progression in alp7\textsuperscript{Δ}cut7\textsuperscript{Δ}pkl1\textsuperscript{Δ} cells containing SPB-tethered Alp14\textsuperscript{NL5-GFP}. Changes of the inter-SPB distance are plotted against time (green lines; n = 14). Data for wild-type (thin gray lines) and cut7\textsuperscript{Δ}pkl1\textsuperscript{Δ} (thin red lines) are the same as those shown in Figure 1C. See Supplemental Figure S7, A–C, for quantitative data.
chromosomes (Cesario et al., 2006). As the midzone localization behavior of Klp9\textsuperscript{Kin-6} on anaphase onset is not altered in cut7\textsuperscript{Δpkl1}\textsuperscript{Δ} cells, we envisage that it is unlikely that Klp9\textsuperscript{Kin-6} takes part in spindle formation during early mitosis, though we cannot formally exclude this possibility at the moment. Yet, it is noteworthy that in either cut7\textsuperscript{Δpkl1}\textsuperscript{Δ}klp9\textsuperscript{Δ} or klp9\textsuperscript{Δ}cut7\textsuperscript{Δpkl1}\textsuperscript{Δ} mutant cells, spindles are frequently displaced away from the cell equator, resulting in chromosome missegregation including the cut phenotype on septation. As previously reported (Syrovatkina and Tran, 2015), it is possible that long protruding microtubules induced by the loss of Pkl1 contact one cell tip, which subsequently pushes back the nucleus toward the opposite cell end, resulting in nuclear displacement and the cut phenotype. However, no obvious microtubule protrusions were detected in the triple mutants. Klp9\textsuperscript{Kin-6} (and Cut7\textsuperscript{Kin-5}) may, therefore, play a role in centering the spindle microtubule in late mitosis to prevent chromosome missegregation. An alternative explanation is that the constricting cytokinesis ring/septum pushes the nuclear envelope/spindle to one side of the cell, which is not Klp9-dependent. Future research should elucidate how bipolar spindles are positioned at the cell equator during anaphase B.

Similarities and differences of kinesin functions in mitotic spindle formation between fission yeast and humans

In human cells, it has been shown that kinesin-12/Kif15/Hklp2 functions redundantly with Kif11/Eg5\textsuperscript{Kin-5} to promote spindle bipolarity (Tanenbaum et al., 2009; Vanneste et al., 2009). Although Kif15\textsuperscript{Kin-12} is not required for bipolar spindle assembly under normal conditions, overproduction of Kif15\textsuperscript{Kin-12} can drive bipolar spindle assembly even when Eg5\textsuperscript{Kin-5} is fully inhibited, indicating that Kif15\textsuperscript{Kin-12} has the potential to take over all essential functions of Eg5\textsuperscript{Kin-5}. As Klp9\textsuperscript{Kin-6} on its own is not capable of replacing the essentiality for Cut7\textsuperscript{Kin-5} under any conditions so far tested (Rincon et al., 2017), functions of fission yeast Klp9\textsuperscript{Kin-6} and human Kif15\textsuperscript{Kin-12} are likely to be different. Intriguingly, however, the simultaneous inhibition of three motors, Eg5\textsuperscript{Kin-5}, Kif15\textsuperscript{Kin-12}, and dynein, does prevent bipolar spindle formation (van Heesbeen et al., 2014). This situation echoes the lethality of cut7\textsuperscript{Δpkl1}\textsuperscript{Δ}klp9\textsuperscript{Δ} triple mutants and illuminates the conservation, diversification, and adaptability through evolution in the molecular pathways leading to kinesin-dependent bipolar spindle formation.

Strict dependence of initial spindle assembly on microtubule polymerization in the absence of kinesin-5/Cut7 and kinesin-14/Pkl1

Given the involvement of the Klp9\textsuperscript{Kin-6} motor in late mitosis and the dispensability of all other kinesin molecules, we wondered as to how cut7\textsuperscript{Δ-22pkl1}\textsuperscript{Δ}klp9\textsuperscript{Δ} and klp9\textsuperscript{Δ}cut7\textsuperscript{Δpkl1}\textsuperscript{Δ} (and a klp9 shut-off strain recently reported [Rincon et al., 2017]) could assemble bipolar spindles although shorter in length. Our subsequent analysis has uncovered the requirement of six MAPs in cut7\textsuperscript{Δpkl1}\textsuperscript{Δ} cells. Alp14 and Dis1 are microtubule polymerases, belonging to the Dis1/XMAP215/TOG family (Al-Bassam and Chang, 2011; Al-Bassam et al., 2012; Hussmann et al., 2016; Matsumo et al., 2016). We show that Alp7-mediated recruitment of Alp14 to the mitotic SPB is a critical step for this function, as forced targeting of Alp14 to this MTOC bypasses the necessity of Alp7/TACC, a loading factor for this polymerase. Dis1 primarily functions at the microtubule plus ends by physically interacting with the plus-end tracking protein Mal3/EB1 and the outer kinetochore component Ndc80 (Hsu and Toda, 2011; Matsuo et al., 2016). We previously showed that Dis1 becomes essential in the absence of either Alp7 or Alp14 (Garcia et al., 2002), implying that in cut7\textsuperscript{Δpkl1}\textsuperscript{Δ} cells, more microtubule polymerase activities provided by either Alp14 or Dis1 are needed than in wild-type cells for bipolar spindle assembly.

We surmise that the polymerizing microtubule plus ends push the opposite SPB, by which they provide outward force during early mitosis and that this force is sufficient to separate SPBs and promote assembly of short bipolar spindles (Figure 7). We propose that this alternative pushing force generated by Alp14-mediated microtubule polymerization could compensate for the loss of Cut7\textsuperscript{Kin-5} if antagonizing Pkl1\textsuperscript{Kin-14} is absent.

Other MAPs essential for cell viability in the absence of kinesin-5/Cut7 and kinesin-14/Pkl1

Other MAPs identified in this study include Ase1. The role of this microtubule-bundling factor was recently reported (Rincon et al., 2017), and this MAP is likely to function throughout mitosis at the antiparallel microtubule overlapping zones as a cross-linker in concert with Cls1/CLASP (Loidice et al., 2005; Yamashita et al., 2005; Bratman and Chang, 2007; Meadows and Miller, 2008; Syrovatkina et al., 2013). We consider that during early mitosis Ase1 (and Cls1) functions in concert with Alp7 and Alp14 in SPB separation and spindle elongation and, during later stages, acts together with Klp9\textsuperscript{Kin-6} to elongate and stabilize anaphase B spindles (Figure 7).
Besides Ase1, two other proteins Csi1 and Csi2 have been identified. Previous work showed that Csi1 is important for Alp7 localization to the mitotic SPB (Zheng et al., 2014), and this localization of Csi1 in turn requires Csi2 (Costa et al., 2014). Hence we envisage that the requirement of Csi1 and Csi2 in cut7Δpkl1Δ cells is through Alp7 localized to the SPB.

Concluding remarks

In total, our study has uncovered interplay among individual kinesins and between kinesin-dependent and -independent pathways that are required for mitotic bipolar spindle assembly. These include three mitotic kinesins, one essential (Cut7^{Δ6-9}) and two nonessential motors (Klp9^{Glu-6} and Pkl1^{K14-14}), and six nonmotor MAPs. These MAPs are further classified into two functional groups. One includes microtubule polymersases and their regulators that promote microtubule elongation from the SPB/MTOC, while the other is a microtubule-bundling factor that stabilizes antiparallel microtubules (Rincon et al., 2017). Kinesin-5 is deemed to be a promising target of cancer chemotherapeutics; however, drug-resistant cell lines often appear, which hampers the clinical usage of these inhibitors (Wacker et al., 2012; Ma et al., 2014; Dumas et al., 2016). Development of small molecule modulators against human homologues identified in this study and their combined usage with kinesin-5 inhibitors would be a complementing strategy for future tumor treatment.

MATERIALS AND METHODS

Strains, media, and genetic methods

Fission yeast strains used in this study are listed in Supplemental Table S1. Media, growth conditions, and manipulations were carried out as previously described (Moreno et al., 1991; Bähler et al., 1998; Sato et al., 2005). For most of the experiments, rich YESS liquid media and agar plates were used. Wild-type strain (S13; Supplemental Table S1), temperature-sensitive cut7-22, pkl1 deletion, klp9^{Ggr} and klp9^{MiC} strains were provided by P. Nurse (The Francis Crick Institute, London, England, UK), I. Hagan (Cancer Research UK Manchester Institute, University of Manchester, Manchester, England, UK), R. McIntosh (University of Colorado, Boulder, CO), and J. B. Millar (University of Warwick, Coventry, England, UK), respectively. Spot tests were performed by spotting 5–10 μl of cells at a concentration of 2 × 10^7 cells/ml after 10-fold serial dilutions onto rich YESS plates with or without a drug (TB2). Some of the YESS plates also contained Phloxine B, a vital dye that accumulates in dead or dying cells and stains the colonies dark pink due to a reduced ability to pump out the dye. Plates were incubated at various temperatures from 27°C to 36°C as necessary.

Preparation and manipulation of nucleic acids

Enzymes were used as recommended by the suppliers (New England Biolabs, Ipswich, MA, and Takara Bio, Shiga, Japan).

Strain construction, gene disruption, and the N-terminal and C-terminal epitope tagging

A PCR-based gene-targeting method (Bähler et al., 1998; Sato et al., 2005) was used for complete gene disruption and epitope tagging (e.g., GFP, 2mRFP, CFP, and mCherry) in the C terminus, by which all motors (e.g., GFP, 2mRFP, CFP, and mCherry) in the C terminus, by which all Alp14 (e.g., GFP, 2mRFP, CFP, and mCherry) in the C terminus, by which all

Isolation of klp9 or alp7 temperature-sensitive mutants in the cut7Δpkl1Δ background

The klp9 ts mutants were constructed by PCR-based random mutagenesis (Tang et al., 2017). The G418-resistance marker gene cassette (kanR) was inserted in the 3′ flanking region of the klp9 gene (klp9^{kan}). The klp9-kanR fragment purified from this strain was amplified with PCR using Takara Ex Taq polymerase (Takara Bio, Shiga, Japan). The alp7 ts mutants were constructed by site-directed mutagenesis using a two-step PCR amplification method (Masuda et al., 2013), by which random mutations (S′-mn3′) were introduced into the five amino acid residues corresponding to K341, Y344, R346, K347, and Y348 within Alp7. To isolate klp9 or alp7 mutants, pools of mutagenized PCR fragments were ethanol precipitated and transformed into a cut7Δpkl1Δ strain (MY990, Supplemental Table S1). Alp7-13myc was originally used for ts mutant isolation and later 13myc was replaced with GFP. G418-resistant transformants were screened for temperature sensitivity at 36°C. These mutants were crossed with a cut7Δpkl1Δ strain and random spore analysis was performed. In all segregants, the ts phenotype cosegregated with G418 resistance. Subsequently, nucleotide sequencing was performed to determine the mutated sites in the klp9 or alp7 gene of these mutants.

Fluorescence microscopy and time-lapse live cell imaging

Fluorescence microscopy images were obtained by using a DeltaVision microscope system (DeltaVision Elite; GE Healthcare, Chicago, IL) comprising a wide-field inverted epifluorescence microscope (IX71; Olympus, Tokyo, Japan) and a Plan Apochromat 60×, NA 1.42, oil immersion objective (PLAPON 60 × O; Olympus Tokyo, Japan). DeltaVision image acquisition software (softWoRx 6.5.2; GE Healthcare, Chicago, IL) equipped with a charge-coupled device camera (CoolSNAP HQ2; Photometrics, Tucson, AZ) was used. Live cells were imaged in a glass-bottomed culture dish (MatTek Corporation, Ashland, MA) coated with soybean lectin and incubated at 27°C for most of the strains or at 36°C for the ts mutants. The latter were cultured in rich YESS media until mid-log phase at 27°C and subsequently shifted to the restrictive temperature of 36°C before microscopic observation. To keep cultures at the proper temperature, a temperature-controlled chamber (Air Therm SMT; World Precision Instruments, Sarasota, FL) was used. Time-lapse imaging was continued for further 40 min at 36°C. The sections of images acquired at each time point were compressed into a two-dimensional projection using the DeltaVision maximum intensity algorithm. Deconvolution was applied before the two-dimensional projection. Images were taken as 14–16 sections along the z-axis at 0.2 μm intervals; they were then deconvolved and merged into a single projection. Captured images were processed with Photoshop CS6 (version 13.0; Adobe, San Jose, CA).

Quantification of fluorescence signal intensities

For quantification of signal intensities of the fluorescent marker-tagged protein located at the SPB (Alp7-GFP, Figure 4D), 14 sections were taken along the z-axis at 0.2-μm intervals. Projection images of maximum intensity were obtained after deconvolution, and, on subtracting background intensities, only values of maximum fluorescence intensities covering the whole GFP signals within the z-sections were used for statistical data analysis.

Immunohistochemistry

Protein extracts were prepared by mechanical disruption of cells in an extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.05% NP-40, 10% glycerol, 1.5 mM p-nitrophenyl phosphate, 0.04% sodium deoxycholate) at 4°C. Cell debris was removed by centrifugation (10,000 × g, 4°C, 10 min). Protein concentrations were determined by the bicinchoninic acid method (Pierce). Western blots were probed with primary antibodies as indicated in the figures.
1 mM phenylmethylsulfonyl fluoride [PMSF], 1x protease inhibitor cocktail from Sigma-Aldrich Co., St. Louis, MO) with acid-washed glass beads in the FastPrep FP120 apparatus (2 × 25 s, power 5.5; BIO-101, Vista, CA). Protein extracts were loaded and resolved on denaturing 4–12% gradient gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 10% skim milk and then blotted with either anti-GFP (rabbit polyclonal, TP401; Torrey Pines Biologabs, CA) or anti-γ-tubulin antibody (mouse monoclonal, produced GTU-88, Sigma-Aldrich Co., St. Louis, MO). After having been washed, the blots were incubated with anti-rabbit or anti-mouse horseradish peroxidase–conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK) in 1% skim milk at a dilution of 1:2000. The ECL chemiluminescence kit (GE Healthcare) was used for detection.

Statistical data analysis

We used the two-tailed unpaired Student’s t test to evaluate the significance of differences in different strains. All the experiments were performed at least twice. Experiment sample numbers used for statistical testing are given in the corresponding figures and/or legends. We used this key for asterisk placeholders to indicate p values in the figures, for example, ****< 0.0001.

ACKNOWLEDGMENTS

We are grateful to Heinrich Leonhardt, Paul Nurse, Iain Hagan, Jonathan Miller, J. Richard McIntosh, and Gislene Pereira for providing us with strains used in this study. We thank Ken’ya Furuta for thoughtful discussion and Corinne Pinder for critical reading of the manuscript and useful suggestions. This work was supported by the Japan Society for the Promotion of Science (JSPS) (KAKENHI Scientific Research [A] 16H02503 to T.T., a Challenging Exploratory Research grant 16K14672 to T.T., Scientific Research [C] 16K07694 to M.Y.), the Naito Foundation (T.T.), and the Uehara Memorial Foundation (T.T.).

REFERENCES


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Vardy L, Toda T (2000). The fission yeast γ-tubulin complex is required in G1 phase and is a component of the spindle assembly checkpoint. EMBO J 19, 6098–6111.


