<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Suppressor Analysis Uncovers That MAPs and Microtubule Dynamics Balance with the Cut7/ Kinesin-5 Motor for Mitotic Spindle Assembly in Schizosaccharomyces pombe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Yukawa, Masashi; Yamada, Yusuke; Toda, Takashi</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>G3: Genes, Genomes, Genetics, 9 (1): 269 - 280</td>
</tr>
<tr>
<td><strong>Issue Date</strong></td>
<td>2019-01-01</td>
</tr>
<tr>
<td><strong>DOI</strong></td>
<td>10.1534/g3.118.200896</td>
</tr>
<tr>
<td><strong>Self DOI</strong></td>
<td></td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://ir.lib.hiroshima-u.ac.jp/00048609">http://ir.lib.hiroshima-u.ac.jp/00048609</a></td>
</tr>
<tr>
<td><strong>Right</strong></td>
<td>Copyright © 2019 Yukawa et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<a href="http://creativecommons.org/licenses/by/4.0/">http://creativecommons.org/licenses/by/4.0/</a>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.</td>
</tr>
<tr>
<td><strong>Relation</strong></td>
<td></td>
</tr>
</tbody>
</table>
Suppressor Analysis Uncovers That MAPs and Microtubule Dynamics Balance with the Cut7/Kinesin-5 Motor for Mitotic Spindle Assembly in Schizosaccharomyces pombe

Masashi Yukawa,*† Yusuke Yamada,† and Takashi Toda*†
*Hiroshima Research Center for Healthy Aging and †Laboratory of Molecular and Chemical Cell Biology, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan
ORCID IDs: 0000-0002-1723-890X (M.Y.); 0000-0001-7851-1757 (T.T.)

ABSTRACT The Kinesin-5 motor Cut7 in Schizosaccharomyces pombe plays essential roles in spindle pole separation, leading to the assembly of bipolar spindle. In many organisms, simultaneous inactivation of Kinesin-14s neutralizes Kinesin-5 deficiency. To uncover the molecular network that counteracts Kinesin-5, we have conducted a genetic screening for suppressors that rescue the cut7-22 temperature sensitive mutation, and identified 10 loci. Next generation sequencing analysis reveals that causative mutations are mapped in genes encoding α-, β-tubulins and the microtubule plus-end tracking protein Mal3/EB1, in addition to the components of the Pkl1/Kinesin-14 complex. Moreover, the deletion of various genes required for microtubule nucleation/polymerization also suppresses the cut7 mutant. Intriguingly, Klp2/Kinesin-14 levels on the spindles are significantly increased in cut7 mutants, whereas these increases are negated by suppressors, which may explain the suppression by these mutations/deletions. Consistent with this notion, mild overproduction of Klp2 in these double mutant cells confers temperature sensitivity. Surprisingly, treatment with a microtubule-destabilizing drug not only suppresses cut7 temperature sensitivity but also rescues the lethality resulting from the deletion of cut7, though a single klp2 deletion per se cannot compensate for the loss of Cut7. We propose that microtubule assembly and/or dynamics antagonize Cut7 functions, and that the orchestration between these two factors is crucial for bipolar spindle assembly.

KEYWORDS fission yeast kinesin microtubule dynamics mitotic spindle suppressor

In eukaryotic cells, the assembly of mitotic spindles is a crucial step in accurate chromosome segregation. The mitotic spindle, a dynamic ensemble of microtubules, microtubule-associated proteins (MAPs) and motor proteins, ensures the proceeding of mitosis by aligning sister chromatids in the equatorial region and segregating them toward opposite poles (Mitchison and Salmon 2001; Woodruff et al. 2014). In order to assemble bipolar mitotic spindles, collaborative forces exerted by multiple kinesin motors, collectively called mitotic kinesins, are necessary (Yount et al. 2015).

The plus-end directed Kinesin-5 is essential for mitosis and, therefore, influences viability in many eukaryotes from fungi to human beings (budding yeast Cin8 and Kip1, fission yeast Cut7, Aspergillus BimC, Drosophila Klp61F, Xenopus Eg5 and human Kif11) (Enos and Morris 1990; Hagan and Yanagida 1990; Le Guellec et al. 1991; Heck et al. 1993; Blangy et al. 1995). The primary role of Kinesin-5 motors has been shown to be the establishment of spindle bipolarity by driving spindle pole separation. They form homotetramers that crosslink and subsequently slide apart antiparallel microtubules, thereby generating an outward pushing force to separate the two poles (Kashina et al. 1996; Kapitein et al. 2003). The sole member of the fission yeast Kinesin-5 family, Cut7, is indispensable for mitotic progression.
Temperature-sensitive mutations in cut7 display mitotic arrest with monopolar spindles at the restrictive temperature (Hagan and Yanagida 1990; Hagan and Yanagida 1992). This phenotypic consequence is identical to what is observed in other organisms, including human cells when Kinesin-5 activity is inhibited (Sawin et al. 1992; Kapoor et al. 2000). Because its function is essential to cell proliferation, Kinesin-5 molecules have been targeted for the development of anticancer drugs (Wacker et al. 2012; Ma et al. 2014; Dumas et al. 2016). However, at present, our knowledge on the physiology and regulation of Kinesin-5 motors is still too limited to draw a complete picture of their functions in vivo. It is therefore crucial to obtain a comprehensive understanding of how Kinesin-5 molecules establish spindle bipolarity, and which molecules regulate this kinesin.

Kinesin-5 motors are localized along spindle microtubules during mitosis. However, rather than having a uniform localization pattern on the mitotic spindle, the Kinesin-5 motors are enriched in two regions: at the medial midzone which corresponds to the microtubule plus ends, and near the centrosome which is proximal to the microtubule minus ends (Yount et al. 2015). Interestingly, recent work has shown that fungal Kinesin-5 motors are bi-directional: they can move processively toward both plus- and minus-end direction on the microtubules under various conditions (Gerson-Gurwitz et al. 2011; Roostalu et al. 2011; Edamatsu 2014; Britto et al. 2016). It has been reported that individual molecules of budding yeast Cin8 and fission yeast Cut7 initially move toward the minus end, and when concentrated/crowed in clusters on the microtubule, they switch motility from a minus-end- to plus-end-directed manner, thereby generating robust outward force (Britto et al. 2016; Shapira et al. 2017). This bi-directionality and motility switch may account for the biased localizations of Kinesin-5 on the spindle, though presently, its physiological relevance and significance remain to be established.

Mitotic spindle assembly, i.e., the formation of a microtubule bipolar array, requires another Kinesin family motor, Kinesin-14, which exhibits a minus-end-directed motility (She and Yang 2017). In fission yeast, two Kinesin-14s, Pkl1 and Klp2, counteract Cut7-dependent spindle pole separation (Pidoux et al. 1996; Troxell et al. 2001; Furuta et al. 2008; Braun et al. 2009). Pkl1 is localized predominantly to the mitotic spindle pole body (SPB, the fungal equivalent of the animal centrosome), where it forms a ternary complex with Msd1 and Wdr8 (referred to as the MWP complex) (Toya et al. 2007; Ikebe et al. 2011; Syrovatkina and Tran 2015; Yukawa et al. 2015). During early mitosis when duplicated SPBs initially separate, this anchorage generates inward force at the SPB against Cut7-mediated outward force (Yukawa et al. 2018). Klp2, on the other hand, is localized to the spindles in a punctate manner and crosslinks microtubule bundles using the N-terminal-located second microtubule binding domain (Braun et al. 2009), by which it generates inward forces at the SPBs. Accordingly, the deletion of either pkl1 or klp2 suppresses the temperature sensitivity caused by the cut7 mutations (Pidoux et al. 1996; Troxell et al. 2001; Rodriguez et al. 2008). It is noteworthy that although Pkl1 and Klp2 collaboratively antagonize Cut7, their contributions are not identical; while the pkl1 deletion (pkl1Δ) can completely bypass the requirement of Cut7’s essentiality, klp2Δ is incapable of rescuing cut7Δ (Yukawa et al. 2018).

In this study, we sought to identify, in an unbiased manner, genes that antagonize the outward force generated by Cut7/Kinesin-5. To this end, we screened for extragenic suppressors that are capable of rescuing the temperature sensitivity of the hypomorphic cut7 mutants. Consequently, we identified 6 suppressor genes. Subsequent analysis of these genes led us to the proposition that in addition to the MWP complex, microtubule stability and/or dynamics play a crucial role in antagonizing Cut7-mediated outward force. In fact, we have found that chemical perturbation of microtubule stability/dynamics is capable of suppressing the hypomorphic cut7 mutant phenotype. Remarkably, we have further discovered that treatment with a microtubule destabilizing drug is sufficient to bypass the essential function of Cut7. We discuss how microtubule stability and/or dynamics keep inward and outward forces in balance, thereby assembling bipolar mitotic spindles.

**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 YE5S liquid media and agar plates were used. Wild-type strain (513; Supplemental Table S1), temperature-sensitive cut7-22, and pkl1 deletion 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), and R. McIntosh (University of Colorado, Boulder, CO), 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 YE5S plates with or without a drug (thiamazole, TBZ, T8904, Sigma-Aldrich Co., St. Louis, MO). In some experiments, methyl 2-benzimidazolecarbamate (MBC/CBZ, 378674, Sigma-Aldrich Co., St. Louis, MO) was also used. Some of the YE5S plates also contained Phloxine B (P2759, Sigma-Aldrich Co., St. Louis, MO), a vital dye that accumulates in dead cells and stains the colonies dark pink (Moreno et al. 1991). The plates were incubated at various temperatures from 27° to 36° as necessary.

**Preparation and manipulation of nucleic acids**

Enzymes were used as recommended by the suppliers (New England Biolabs Inc., Ipswich, MA and Takara Bio Inc., 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 fluorescent-tagging.

**Isolation of revertants from the cut7 mutants**

Approximately 2 × 10^7 cut7-22 cells/plate were spread on YE5S plates (>20 plates) and directly incubated at 36°. After incubation for several days, revertant colonies were picked up, streaked on YE5S plates, and incubated at 36°. Revertants were backcrossed with a strain containing deletion of the same kinesin, respectively, which was confirmed by nucleotide sequencing of these genes in corresponding skf mutants.
Next generation sequencing and annotation of *skf* genes

At least 5 independent colonies of each *skf* mutant were mixed and cultured in the liquid YESS. A parental cut7-22 mutant was also cultured. Genomic DNA was purified according to a standard method (Iida et al. 2014). Whole genome sequencing was performed by BGI Next Generation Sequencing (NGS) services (Shenzhen, China). Individual sequence data were analyzed using Mudi (Mutation discovery, http://naoii.nig.ac.jp/mudi_top.html) (Iida et al. 2014).

Fluorescence microscopy and time-lapse live cell imaging

Fluorescence microscopy images were obtained by using the 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 and at either 27°C, 33°C or 36°C for the ts mutants. The latter two cultures were incubated in rich YE5S media until mid-log phase at 27°C and subsequently shifted to the restrictive temperature of either 33°C or 36°C before observation. To keep cultures at the proper temperature, a temperature-controlled chamber (Air Therm SMT; World Precision Instruments Inc., Sarasota, FL) was used. The sections of images were compressed into a 2D projection using the DeltaVision maximum intensity algorithm. Deconvolution was applied before the 2D 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 fluorescent signal intensities

For quantification of GFP-Pkl1, Msd1-GFP, Wdr8-GFP and GFP-Alp4 levels at SPBs in pre-anaphase cells (spindle length < 3.0 μm), a 4 × 4-pixel (0.43-μm square) region of interest (ROI) with maximum sum intensity was determined using the deconvolved and projected images. For quantification of Klp9-GFP located at the spindle midzone during anaphase B (spindle length > 6.0 μm), a 20 × 20-pixel (2.15-μm square) ROI with maximum sum intensity was determined. After subtracting the mean intensity of background signals outside cells, the values of the maximum sum intensities were used for statistical data analysis. For quantification of mCherry-Atb2, GFP-Klp2 and Ase1-GFP located on the metaphase spindle (spindle length < 3.0 μm), maximum sum intensity along individual spindles was determined, and after background subtraction, the values were divided by the spindle length in each cell.

Statistical data analysis

We used the two-tailed unpaired Student’s *t*-test to evaluate the significance of differences between the mean fluorescence signal intensities derived from different strains (Figures 2B, 3C and 4B-D and Supplemental Figures S2C-D, S3B-D, S4D, S5 and S6). For testing the significance of differences between frequencies of mitotic cells displaying abnormally extended microtubules in individual strains, we performed the two-tailed χ² test (Figure 1F and Supplemental Figure S8). All the experiments were performed at least twice. Sample numbers used for statistical testing were given in the corresponding figures and/or legends. We used this key for asterisk placeholders to indicate *p*-values in the figures: e.g., ****, *P* < 0.0001.

Data Availability Statement

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at Figshare: https://doi.org/10.25387/g3.7351985.

RESULTS

Isolation of revertants from the cut7-22 temperature sensitive mutants

In order to identify genes counteracting the Cut7/Kinesin-5 function, we isolated spontaneous mutants capable of suppressing the *cut7-22* temperature-sensitive (ts) growth defect at 36°C. This screening yielded in total 25 revertants. Subsequent genetic analysis, including backcrossing with a wild-type *cut7* strain, defined that 3 isolates are intragenic, while the remaining 22 revertants contain extragenic suppressors. Nucleotide sequencing of the *cut7* gene in the 3 intragenic suppressors showed that each revertant contains one additional point mutation within the *cut7* ORF besides the original mutation (P1021S) (Olmsted et al. 2014; Yukawa et al. 2018); two mutations were located at the stalk domain of Cut7 (R433Q and L687F), while the third mutation resided at the tail domain (S1027L) (Supplemental Figure S1A).

Pair-wise crosses between each of the 22 strains carrying extragenic suppressors indicated that these mutations consist of 10 linkage groups, designated *skf1*-*skf10* (*skf*: suppressor of *kinesin five*). To identify causative mutations within extragenic suppressors, we performed next generation sequencing and analyzed whole-genome sequence data using the Mudi (Mutation discovery), a web tool for identifying mutations based on bioinformatics approaches (Iida et al. 2014). This analysis successfully identified individual genes of *skf1*-*skf6* (Figure 1A and B and Supplemental Figure S1B). These 6 genes could be categorized into two functional groups: Group I (*skf1*-*skf3*) comprises of genes encoding the components of the MWP complex (Skf1/Pkl1, Skf2/Wdr8 and Skf3/Msd1) as we expected (Yukawa et al. 2015), while Group II (*skf4*-*skf6*) includes those encoding microtubule constituents such as β-tubulin (Skf4/ Nda3) (Hiraoka et al. 1984), α2-tubulin (Skf5/Atb2) (Toda et al. 1984) and the microtubule plus-end tracking protein EB1 homolog (Skf6/Mal3) (Beinhauer et al. 1997; Carvalho et al. 2003; Asakawa et al. 2005). A single *skf4*-1/nda3-G56D strain displayed hypersensitivity to a microtubule-destabilizing drug, thiaobendazole (TBZ), while an *skf5*-1/atb2-G410A strain showed modest resistance instead (Supplemental Figure S1C). The reason for this difference was currently unknown. The mutation sites within α2- and β-tubulin molecules correspond to the highly conserved amino acids that are located at the outer surface of the α/β-tubulin heterodimer (Supplemental Figure S1D and E). Consistent with these assignments, each deletion of *skf1*-*skf6* genes suppressed the *cut7*-22 ts growth defect at 36°C (Figure 1C).

Given that mutations/deletions of genes encoding tubulins or the EB1 homolog suppressed the growth defect caused by *cut7-22*, we next examined whether deletions of other genes involved in microtubule assembly and/or dynamics also display suppressing activity toward *cut7-22*. For this purpose, we examined gene deletions of *alp16* (encoding an ortholog of GCP6, a component of the γ-tubulin complex, γ-TuC) (Fujita et al. 2002; Anders et al. 2006; Masuda and Toda 2016), *alp7* (encoding an ortholog of the transforming acidic coiled-coil
protein TACC) (Sato et al. 2004), and alp14 and dis1 (encoding XMAP215/TOG microtubule polymerases) (Nabeshima et al. 1995; Garcia et al. 2001; Al-Bassam et al. 2012; Hussmann et al. 2016; Matsuo et al. 2016), all of which play crucial roles in promoting proper mitotic spindle assembly. Intriguingly, these deletions individually rescued the cut7-22 ts mutants (Figure 1D). In line with these results, the emergence of monopolar spindles was substantially suppressed in all the double mutants compared to a single cut7-22 mutant strain (Figure 1E and F). These results indicate that in addition to the impairment of the Pkl1 minus-end directed motor, the perturbation of spindle microtubule assembly including nucleation, polymerization and stabilization, also rescues the cut7 mutant.

Figure 1 Identification of suppressor genes that rescue the cut7-22 temperature sensitive mutant. (A) Spot test. Indicated strains were spotted onto rich YE5S agar plates and incubated at 27°C, 33°C or 36°C for 3 d. 10-fold serial dilutions were performed in each spot. cell conc., cell concentration, temp., temperature. (B) A summary table of skf genes. Group I includes genes encoding the MWP complex, while those in Group II encode either tubulins or the microtubule plus-end tracking protein Mal3/EB1. n.d.: not determined. (C, D) Spot tests. The same procedures were followed as in (A). (E) Representative images of spindle microtubules in indicated strains. Each strain contains mCherry-Atb2 (MTs) and GFP-Alp4 (SPBs). (F) The percentage of mitotic cells containing bipolar (green) or monopolar spindles (magenta). Individual cells were grown at 33°C and spindle morphology was observed. All P-values were obtained from the two-tailed x² test. Data are presented as the means ± SE (≥24 cells). ***, P < 0.01, ****, P < 0.001, ***** P < 0.0001. n.s., not significant.
Klp2/Kinesin-14 levels on the spindles is an important determinant of temperature sensitivity of the cut7 mutant

We previously showed that two Kinesin-14s, Pkl1 and Klp2, generate collaborative inward forces against the outward force exerted by Cut7/Kinesin-5; the deletion of either of these two genes suppresses the cut7ts mutations (Yukawa et al. 2018). Indeed, Group I suppressors include genes encoding the Pkl1-containing MWP complex (Figure 1B). Hence, one plausible scenario of rescuing the cut7ts mutants is the downregulation of Kinesin-14 levels on the spindles is an important determinant of temperature sensitivity of the cut7 mutant. To investigate this possibility, we measured the levels of Kinesin-14s, Pkl1 and Klp2, in various strains, at mitotic SPBs and on spindle microtubules, respectively. Interestingly, we found that Klp2 levels were substantially lessened by either mal3Δ or alp16Δ, whereas conversely, those in the cut7-22 single mutant were increased (Figure 2A and B).

Intriguingly, in the double mutants (cut7-22mal3Δ or cut7-22alp16Δ), Klp2 levels were significantly lower than those in cut7-22 (Figure 2B). The reduction of Klp2 intensities on the spindle microtubules could not be attributed to the gross defects in spindle microtubule structures, as markers for the spindle midzone and antiparallel microtubules (Klp9/Kinesin-6 and Ase1/PRC1), the ana-phase B kinesin and the microtubule antiparallel crosslinker, respectively (Leiódice et al. 2005; Yamashita et al. 2005; Fu et al. 2009) were properly localized to this region in mal3Δ or alp16Δ cells (Supplemental Figure S2A–D). It is of note that the requirement of Mal3 for the microtubule localization of Klp2 has been previously reported (Manca-Capelli et al. 2012), which is in line with our current result. In contrast, Pkl1 levels at mitotic SPBs remained largely unaltered or even became higher in all the strains examined (Supplemental Figure S3A and B). In addition, the levels of the other two components of the MWP complex, Msd1 or Wdr8, were not decreased in alp16Δ cells (Supplemental Figure S3C and D).

If the reduced levels of Klp2 on the spindle microtubules were responsible for the suppression of the ts phenotype of cut7-22 by mal3Δ or alp16Δ, increased dosage of Klp2 in the double mutants might confer temperature sensitivity again. This was indeed the case; mild overexpression of Klp2 conferred ts growth of cut7-22mal3Δ or cut7-22alp16Δ double mutants (Figure 2C). These observations imply that the reduced localization of Klp2 at the spindle microtubules, which results from mutations in mal3 or alp16, is the main, if not the sole, reason for suppression of the cut7 mutants. We envision that in the cut7 mutants,
the increased accumulation of Klp2 on the spindle microtubules contributes to the generation of excessive inward force.

Intensities of spindle microtubules are increased in the cut7-22 mutant but reduced in the absence of Mal3/EB1 or Alp16/GCP6

In the cut7-22 mutant, more Klp2 proteins accumulate on the spindles. Moreover, we previously showed that this mutant displays hyper-resistance to TBZ (Yukawa et al. 2015). These findings raised the possibility that microtubule numbers are increased, by which spindle microtubules become more stable in the cut7 mutant than in wild-type cells, leading to Klp2 accumulation and TBZ resistance. To interrogate this point, we directly measured fluorescence signal intensities of mCherry-Atb2 on the spindle microtubule. Intriguingly, the levels of preanaphase spindle microtubules (< 3 μm) were higher in the cut7 mutant cells than those in wild-type cells at both the permissive and restrictive temperature, 27°C and 36°C respectively (Figure 3A-C and Supplemental Figure S4A and B).

We further found that the spindle intensities remained higher in the cut7-22/pkl1Δ or cut7-22/klp2Δ double mutant, but they were significantly reduced in either of cut7-22/alp16Δ or cut7-22/mal3Δ cells (Figure 3C). These results suggest that Klp2 accumulation on the spindles in the cut7 mutant cells might be attributable to increased spindle levels and that its reduced loading in cut7-22/alp16Δ or cut7-22/mal3Δ leads to compromised inward force, resulting in the suppression of temperature sensitivity. It is also noted that the activity of spindle microtubule nucleation/assembly is potentiated when Cut7/Kinesin-5 function is compromised independently of Klp2. However, the levels of Alp4/GCP2, a core component of the γ-TuC (Vardy and Toda 2000) per se, were not increased in the cut7 mutants (Supplemental Figure S4C and D). This result indicates that the potentiation of spindle assembly/ nucleation in the cut7 mutants, if this is the case, does not result from the accelerated recruitment of the microtubule nucleator γ-TuC to the mitotic SPBs.

We further measured the levels of spindle microtubules and Klp2 in Pkl1-overproduced cells. Previous work showed that Pkl1 overproduction recapitulates the phenotype of cut7 mutants, e.g., the emergence of monopolar spindles, because of the generation of excessive inward force (Pidoux et al. 1996; Yukawa et al. 2018). We found that the signal intensities of Klp2 were not significantly altered (Supplemental Figure S5A), while those of spindle microtubules were increased as in cut7-22 cells (Supplemental Figure S5B). Currently, we do not know the underlying reason for this difference between the cut7-22 mutation and Pkl1 overproduction with regards to Klp2 levels. It is possible that as Pkl1 is localized to the mitotic spindles as well as the SPBs, Pkl1 on the spindles may structurally interfere with additional recruitment of Klp2 to these locations. In any case, the higher levels of the Klp2 protein on the mitotic spindle appear specific to the cut7-22 mutant.

Drug-induced microtubule destabilization rescues the cut7 temperature sensitive mutation with reduced Klp2 levels at the spindle microtubule

As the temperature sensitivity of cut7-22 mutants is suppressed by the deletion of various genes that promote microtubule nucleation, assembly and organization, we presumed that simple microtubule destabilization by treatment with anti-microtubule drugs might also effectively rescue the growth defect of the cut7 ts mutant cells. Therefore, we assessed the impact of TBZ treatment on cut7 ts mutant strains. As shown in Figure 4A, growth properties of not only cut7-22 but also all the other cut7 ts alleles examined were ameliorated on TBZ-containing plates (Figure 4A).

Figure 3 Intensities of the spindle microtubule are increased or conversely reduced in cut7-22 or mutations that suppress cut7-22, respectively. (A) Representative images showing preanaphase spindle microtubules. While wild-type cells contain mCherry-Atb2 (MTs), GFP-Alp4 (SPBs) and Cut11-GFP (a marker for the nuclear membrane and mitotic SPBs) (West et al. 1998), cut7-22 cells contain mCherry-Atb2 and GFP-Alp4. Both strains were incubated at 27°C and preanaphase cells (spindle length is < 3 μm) were imaged. Scale bar, 10 μm. (B) Distribution of spindle microtubule intensities in wild-type (gray circles) and cut7-22 cells (red circles). Spindle microtubule levels of individual mitotic cells shown in (A) were plotted against the spindle length. A vertical dotted line represents the spindle length (3 μm) at metaphase. (C) Quantification of the spindle microtubule fluorescence intensities of mCherry-Atb2 were measured in indicated strains that were incubated at either 27°C or 36°C. Most (>80%) of cut7-22 mutant cells arrested with monopolar spindles; therefore, spindle intensities of mutant cells displaying short spindles were measured. The values of wild-type cells incubated at 27°C or 36°C were set as 100%, and compared to those from other strains incubated at 27°C or 36°C, respectively, for 2 h. All p-values were obtained from the two-tailed unpaired Student’s t-test. Data are presented as the means ± SE (≥11 cells). ***, P < 0.001. n.s., not significant.

Given that the intensities of spindle-localizing Klp2 and those of the spindles themselves are proportionate and more importantly, that their levels correlate with whether cut7-22 displays temperature sensitivity (higher) or not (lower) (see Figures 2B and 3C), we reasoned that TBZ treatment would decrease Klp2 levels on mitotic spindles. Indeed, we found that in the liquid culture, the Klp2 levels on the spindles as well as spindle microtubule intensities were reduced (Figure 4B and C). In clear contrast, the Pkl1 levels at the mitotic
SPBs were not decreased or even became higher with TBZ treatment (Figure 4D). This was also the case for Msd1 and Wdr8 (Supplementary Figure S6A and B). Intriguingly, mild overproduction of either Klp2 or Pkl1 in cut7-22 mutants led to the impairment of TBZ-mediated rescuing activity; the ts phenotype of cut7-22 cells reappeared in the presence of TBZ upon higher dosage of Klp2 or Pkl1 (Figure 4E). This result indicates that microtubule destabilization by either mutations in genes encoding certain MAPs or a chemical reagent could rescue the cut7 ts phenotype, which is derived from compromised inward force.

**Microtubule destabilization by a drug even bypasses the essentiality of Cut7/Kinesin-5 for viability**

We then performed tetrad dissection of diploids heterozygous for cut7 and pkl1 (cut7+/Δ/pkl1+), and allowed spores to germinate on plates containing TBZ. Remarkably, this procedure rendered otherwise lethal cut7Δ haploid segregants viable; cut7Δ cells formed colonies, despite the fact that their size was much smaller than that of wild-type or cut7Δ pkl1Δ doubly deleted segregants (Figure 5A) (Olmsted et al. 2014; Syrovatkina and Tran 2015; Yukawa et al. 2017). Continuous cell division of cut7Δ cells recovered from TBZ-containing plates upon...
germination still required the presence of this drug; no colonies were formed on plates in the absence of TBZ (Figure 5B). This indicates that the viability of cut7Δ cells is not ascribable to the emergence of suppressor mutations (e.g., pkl1 or msd1) during the period of initial TBZ treatment. We found that very low concentrations of TBZ (2.5 μg/ml), in which no growth defects were apparent in wild-type cells, are sufficient to confer the viability of cut7Δ cells. At high concentrations of TBZ (>15 μg/ml), rescuing activity became compromised, as the growth of even wild-type cells was substantially inhibited under these conditions (Figure 5B). The rescue of cut7Δ cells was not specific to TBZ, as the addition of a related microtubule-destabilizing compound, methyl 2-benzimidazolecarbamate (MBC), also rendered cut7Δ cells viable in a concentration-dependent manner like TBZ (Supplemental Figure S7). Therefore, a moderate disturbance of microtubule stability is crucial to bypass the requirement of Kinesin-5 function. Collectively, the impairment of microtubule stability and/or dynamics renders fission yeast cells viable in the absence of Kinesin-5.

Gene deletions perturbing spindle microtubule assembly are incapable of bypassing the essentiality of Cut7/Kinesin-5

Next, we addressed whether complete deletion of skf and other genes involved in microtubule assembly can also bypass the lethality caused by the cut7 deletion. Tetrad dissection indicated that none of these deletions except for pkl1 or msd1 could rescue the lethality of cut7Δ cells (Figure 6A and B). As shown earlier (Figure 2), deletions of these genes led to the reduced levels of Klp2 on the spindles. Consistent with this, it is known that unlike pkl1, the klp2 deletion is incapable of rescuing the lethality of cut7Δ cells (Yukawa et al. 2015). Note that unlike pkl1 or msd1, wdr8 deletion did not rescue the lethality, but cut7Δwdr8Δklp2Δ triple mutants were viable (data not shown). We envisage that even in the absence of Wdr8, cells retain residual Pkl1 activity, thereby rendering cut7Δ inviable only in the absence of Klp2. Together, we conclude that the reduction of Klp2 levels on spindle microtubules by TBZ treatment contributes to but is not sufficient for rescuing cut7Δ lethality.

As shown earlier, unlike Klp2, the levels of Pkl1 (also Msdl and Wdr8) at the mitotic SPB were not lessened in either wild-type or cut7-22 cells upon TBZ treatment (Figure 4D and Supplemental Figure S6). This implies that Pkl1 activity might not be reduced under this condition. To further address the involvement of Pkl1 in TBZ-treated cells, we observed the morphology of mitotic spindles in Pkl1-overproduced klp2Δ cells in the presence of TBZ. Interestingly, we found that the emergence of monopolar spindles was substantially suppressed by TBZ treatment (Supplemental Figure S8). This result suggests that inward force generated by Pkl1 is compromised by TBZ treatment in the absence of Klp2 and points toward the possibility that Pkl1 inactivation might in part contribute to the rescue of cut7Δ by this drug.

In summary, our genetic analysis indicates that two conditions can rescue hypomorphic cut7 mutants. The first is the disruption of Pkl1/Kinesin-14 function, which is consistent with previous studies (Pidoux et al. 1996; Troxell et al. 2001; Olmsted et al. 2014; Syrovatkina and Tran 2015; Yukawa et al. 2017). The second is the impairment of spindle microtubules by mutations/deletions of genes encoding tubulins and a cohort of MAPs that are important for spindle assembly. We show that the second condition lessens Klp2 localization on the mitotic spindle, which could account for the suppression of cut7 ts mutants (Troxell et al. 2001; Yukawa et al. 2018). Markedly, continuous treatment by a low dose of the microtubule-destabilizing drug TBZ is capable of not only suppressing the hypomorphic cut7 mutant but also bypassing the essentiality of Cut7. As even complete deletion of klp2 fails to rescue the cut7 deletion (Yukawa et al. 2018), TBZ must interfere with some functions other than Klp2 downregulation and Pkl1 inactivation might be involved. We posit that Klp2-independent pathway(s) regulating spindle microtubule stability/dynamics is important to render Cut7 dispensable (Figure 6C).

**DISCUSSION**

In this study, we have performed an unbiased genetic screening for suppressors of the cut7-22 ts mutant, and uncovered a genetic network responsible for conferring lethality to this mutant. We have also found that treatment with the microtubule-depolymerizing drug TBZ rescues the cut7-22 ts mutant. Remarkably, a low dose of TBZ is capable of even bypassing the essential function of Cut7. Hence, microtubule dynamics and/or assembly kinetics antagonize with Cut7, and the orchestration between these two factors is required for bipolar spindle assembly.

**Compromised microtubule stability/dynamics ameliorate Kinesin-5 deficiency**

Previous work in HeLa cells has shown that the perturbation of microtubule stability/dynamics by either knockdown of the chTOG MAP (an ortholog of Alp14 and Dis1) or treatment with low concentrations of microtubule-disruptive drugs (Nocodazole and Vinblastin)
can effectively rescue monopolar spindle phenotypes induced by Eg5/Kif11 inactivation (Kollu et al. 2009; Florian and Mayer 2011), and a similar observation is also reported in U2OS cells (Kollu et al. 2009). These results are wholly consistent with what we observe in fission yeast, indicating evolutionary conservation of Kinesin-5 function. It would, therefore, be worth investigating whether HSET/Kinesin-14 levels on the spindle microtubule are reduced under these conditions, which is observed for fission yeast Klp2/Kinesin-14 under analogous conditions.

Figure 6  Inability of gene deletions to rescue cut7-deleted cells and a schematic model. (A) Tetrad analysis. Tetrad dissections upon crosses between cut7Δpkl1Δ and mutations or deletions of genes encoding tubulin or various MAPs (collectively shown as skf in an outlined frame at the bottom) were performed. Individual spores (a–d) in each ascus (1–4) were dissected on YE5S plates and incubated at 27°C for 3 d. Representative tetrad patterns are shown. Assuming 2:2 segregation of individual markers allows the identification of each segregant. Circles in green: wild type; Dotted circles in green: cut7Δ; Triangles in green: pkl1Δ; Reversed triangles in green: cut7Δpkl1Δ; Squares in green: skfΔ; Dotted circles in red: skfΔcut7Δ; Pentagons in green: skfΔpkl1Δ; Hexagons in green: skfΔcut7Δpkl1Δ. (B) A summary table of suppression profiles toward cut7Δ. 1: data from (Yukawa et al. 2017); 2: data from (Yukawa et al. 2018). (C) A model. Bipolar spindle formation requires collaborative balance between mitotic kinesins and microtubule stability/dynamics. Kinesin-5 (Cut7) generates outward force, while Kinesin-14s (Pkl1 and Klp2) generate opposing inward forces. Spindle microtubule stability/dynamics regulated by a cohort of MAPs play a positive role in Klp2 activity, while Cut7 plays an inhibitory role in microtubule nucleation, polymerization and/or stabilization, thereby suppressing Klp2 activity (left). In the cut7-22 ts mutant, the levels of Klp2 on the mitotic spindles are upregulated. When cut7-22 or cut7Δ cells are treated with a microtubule-depolymerizing drug, compromised microtubule dynamics, the reduced level of Klp2 on the spindle and possibly Pkl1 inactivation collaborate to reduce inward forces, by which these cells no longer require Cut7 for survival.
Cut7/Kinesin-5 regulates microtubule stability and/or dynamics

We show that in the cut7-22 mutant, intensities of spindle microtubules are significantly increased. Furthermore, consistent with these data, cut7-22 cells display hyper-resistance to microtubule-destabilizing TBZ (Yukawa et al. 2015). These results raise the intriguing question as to whether Kinesin-5 molecules per se play any role in microtubule stability and/or dynamics. Previous reports indeed show that Kinesin-5 regulates microtubule stability/dynamics as a microtubule depolymerizing factor (budding yeast Cin8 and human Eg5/Kif11) (Gardner et al. 2008; Wang et al. 2010), conversely as a microtubule stabilizer (budding yeast Kip1) (Fridman et al. 2013) or a microtubule polymerase (Xenopus Eg5) (Chen and Hancock 2015). In fission yeast, mutations in genes encoding the components of γ-TuC other than Alp16 are also capable of rescuing cut7-22 (Rodriguez et al. 2008), and interestingly, Cut7/Kinesin-5 reportedly interacts with γ-TuC (Olmsted et al. 2014). It is, therefore, possible that by physically binding γ-TuC, Cut7 downregulates microtubule nucleating activity, which would explain the increased spindle intensities in the cut7-22 mutant. As the γ-TuC levels at the mitotic SPBs are unaltered in this mutant, Cut7 might act as a nucleation-inhibitory factor of the γ-TuC, instead of as a spatial regulator. It is noteworthy that it was previously claimed that Cut7 potentiates γ-TuC activity (Olmsted et al. 2014); however this notion is somewhat contradictory with genetic results obtained by the same authors (and this study), in which Cut7 and γ-TuC play opposing, rather than collaborative, roles.

Recently, it has become clear that the XMAP215/Dis1/TOG microtubule polymerase family is also a part of a microtubule nucleator; XMAP215 and Alp14 bind γ-TuC directly and indirectly, respectively, thereby promoting the very first step of microtubule assembly (Flor-Parra et al. 2018; Thawani et al. 2018). It is tempting to speculate that the microtubule nucleator complex includes both positive (the XMAP215/Dis1/TOG family) and negative regulators (Kinesin-5) in addition to its canonical γ-TuC components (Kollman et al. 2011; Paz and Luders 2018). Overall, whether and how Cut7 impacts γ-TuC-mediated microtubule nucleation and elongation is an outstanding issue that should be addressed in the future.

A low dose of the microtubule-destabilizing reagent bypasses the requirement of Kinesin-5

One surprising observation made in this study is the rescue of cut7Δ cells by treatment with a low concentration of TBZ or MBC. As cut7Δ/klp2Δ double mutant cells are inviable, the inhibition of Klp2 activity with this drug treatment is not sufficient to fully account for the suppression, though it would make an important contribution. One possibility is the down-regulation of Pkl1-mediated inward force. However, the measurement of Pkl1 intensities in TBZ-treated cells does not support this notion (Figure 4D). Nonetheless, it is possible that Pkl1 activity is suppressed by TBZ treatment, which is observed in Pkl1-overproduced klp2Δ cells (Supplemental Figure S8). In both animal and fission yeast cells, treatment with low concentrations of microtubule-disruptive drugs interferes with microtubule dynamics and/or increases the concentration of free (unpolymerized) tubulins (Vasquez et al. 1997; Mary et al. 2015). Thus, compromised microtubule dynamics per se likely suppress the generation of lethal inward force in the absence of Kinesin-5, thereby rendering Kinesin-5 dispensable.

Alp14 and Dis1 microtubule polymers play both positive and negative roles in kinesin-dependent bipolar spindle assembly

We show here that the deletion of either alp14 or dis1 rescues the hypomorphic cut7 mutant phenotype, indicating that Alp14/Dis1-dependent microtubule polymerization counteracts Cut7-mediated outward force. Curiously and apparently paradoxically, our previous work showed that either of these two MAPs becomes essential for cell viability in the absence of both Cut7 and Pkl1 (cut7Δ/pkl1Δ) (Yukawa et al. 2017), showing that Alp14 and Dis1 play a cooperative role with Cut7 in the absence of Kinesin-14. In this latter situation where Cut7 and Alp14/Dis1 (and other MAPs) functionally collaborate (Rincon et al. 2017; Yukawa et al. 2017), we previously argued that the plus ends of polymerizing microtubules push the respective opposite SPB, thereby generating outward force. This force is sufficient to separate two SPBs in cut7Δ/pkl1Δ cells.

In the hypomorphic cut7 mutant cells, on the other hand, microtubule polymerization/stabilization mediated by Alp14 or Dis1 is detrimental to these cells. We hypothesize that this adverse impact elicited by Alp14 or Dis1 is mediated by Klp2, which generates antagonistic inward force against Cut7-dependent outward force (Yukawa et al. 2018). Taken together, bipolar spindle formation is under the control of a complex network consisting of multiple kinesins and MAPs, and individual molecules display different characters in a context-dependent manner.

Concluding remarks

Much attention has been focused on mitotic kinesins as druggable targets in cancer therapeutics (Cosenza and Kramer 2016). In particular, inhibitors against Kinesins-5 and -14 are thought to be promising pharmacological reagents, and several small-molecule inhibitors have been clinically evaluated (Huszar et al. 2009). One of downsides, however, is the emergence of a cell population that is resistant to drug treatment. Results obtained in this work provide novel insight into the usage of these inhibitors; the combined treatment of Kinesin-5 inhibitors and microtubule stabilizing reagents such as Paclitaxel (Taxol), which by itself is widely used in cancer chemotherapy (Shi et al. 2008; Mitchison 2012; Weaver 2014), would be beneficial for the treatment of drug resistant cancers.

ACKNOWLEDGMENTS

We thank Paul Nurse, Iain Hagan and J. Richard McIntosh for strains, and Tetsushi Iida and Masaru Ueno for the instruction for the isolation of genomic DNAs from revertants and data analysis of whole genome sequences with the Mudi method. We are grateful to Kyle Pan and Vismaya Kharkar for critical reading of the manuscript. 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.)]. M.Y. and T.T. designed research. M.Y. and Y.Y. performed experiments and analyzed the data. M.Y. and T.T. wrote the manuscript, and Y.Y. made suggestions.

LITERATURE CITED


Asakawa, K., M. Toya, M. Sato, M. Kanai, M. Kume et al., 2005 Mal3, the fission yeast EB1 homolog, cooperates with Bub1 spindle checkpoint to prevent monopolar attachment. EMBO Rep. 6: 1194–1200. https://doi.org/10.1038/sj.embor.7400540

Bähler, J., J. Wu, M. S. Longtine, N. G. Shah, A. McKenzie, III et al., 1998 Heterologous modules for efficient and versatile PCR-based gene

278 I M. Yukawa, Y. Yamada, and T. Toda