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Mad1 promotes chromosome congression by anchoring a kinesin motor to the kinetochore

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SUMMARY (150 words):

For proper partitioning of genomes in mitosis, all chromosomes must be aligned at the spindle equator before the onset of anaphase. The spindle assembly checkpoint (SAC) monitors this process, generating a “wait anaphase” signal at unattached kinetochores of misaligned chromosomes. However, the link between SAC activation and chromosome alignment is poorly understood. Here we show that Mad1, a core SAC component, plays a hitherto concealed role in chromosome alignment. Protein-protein interaction screening revealed that fission yeast Mad1 binds the plus-end-directed kinesin-5 motor protein Cut7/Eg5, which is generally thought to promote spindle bipolarity. We demonstrate that Mad1 recruits Cut7 to kinetochores of misaligned chromosomes and promotes chromosome gliding toward the spindle equator. Similarly, human Mad1 recruits another kinetochore motor CENP-E, revealing that Mad1 is the conserved dual-function protein acting in SAC activation and chromosome gliding. Our results suggest that the mitotic checkpoint has co-evolved with a mechanism to drive chromosome congression.

During cell division accurate partitioning of sister chromatids requires the formation of a bipolar spindle, which is assembled depending on molecular motor proteins such as Eg5, a member of the plus-end-directed kinesin-5 family. Eg5 usually forms a homotetramer with pairs of motor domains lying at both ends of a central rod and thus generate outward force by crosslinking antiparallel microtubules ¹⁻⁸. Along with bipolar spindle formation, chromosomes are captured and gradually incorporated into the spindle. Some chromosomes move poleward, while others move inward or glide to the equator along the microtubules. After such complex movements, all chromosomes are finally captured by microtubules emanating from opposite spindle poles (bi-orientation) and aligned on the spindle equator (congression) ⁹. The key interaction between chromosomes and spindle microtubules occurs at the kinetochore, an apparatus assembled on each centromere of paired sister chromatids. One well-characterized congression mechanism is chromosome gliding from the spindle pole to the equator, which is mediated by CENP-E/kinesin-7, a kinetochore-associated plus-end-directed motor ¹⁰⁻¹³. CENP-E forms homodimers and enriches to kinetochores on misaligned chromosomes. Although BubR1 associates with CENP-E ¹⁴, it might not be the kinetochore platform for CENP-E ¹⁵. Therefore, it remains elusive how CENP-E is localized only at misaligned chromosomes.

The spindle assembly checkpoint (SAC) is activated specifically on erroneously attached kinetochores, which correlate with misaligned chromosomes, to initiate a signaling cascade that inhibits Cdc20, an essential cofactor for the anaphase-promoting complex/cyclosome (APC/C) ¹⁶⁻²⁰. Thus, cell enters anaphase in principle only after the last chromosome is aligned on the spindle equator. Mad1, Mad2, Mad3/BubR1, Mps1, Bub1 and Bub3 are considered the core components of the SAC. Mad1 is recruited to unattached kinetochores depending on Aurora B, Mps1, Bub1 and Bub3. Mad1 then recruits Mad2 to assemble the mitotic checkpoint complex (MCC) to inhibit APC/C activation. Once Mad1 is localized to the kinetochore, the SAC is fully activated ²¹. It was recently reported that Mad1-dependent MCC assembly also occurs on the nuclear envelope during interphase, supplying a sufficient pool of anaphase inhibitors upon mitotic entry ²² (ref: Schweizer et al., JCB 2013). Thus, Mad1 plays a central role in SAC activation.

Some SAC components may have roles beyond the SAC. Bub1 kinase in general phosphorylates histone H2A to target shugoshin, an adaptor of the Aurora B kinase complex, to the inner centromere ²³, while also serving as a platform for several SAC proteins ^{24,25}. Indeed, the fission yeast Bub1 mutant causes severer mitotic defects than those observed for mere SAC mutants ^{26,27}. Mammalian BubR1 also plays an important role in chromosome bi-orientation by recruiting PP2A and thereby antagonizing Aurora B and Mps1 kinases at kinetochores ^{15,17,28-31}. Moreover, *Drosophila mad1*-null mutants display higher incidences of lagging chromosomes at anaphase as compared with *mad2*-null mutants, suggesting that Mad1 has some role in chromosome bi-orientation beyond the SAC ³². Despite the accumulation of evidence supporting SAC factor-dependent chromosome bi-orientation, the underlying molecular mechanisms remain largely elusive.

Mad1 associates directly with Cut7/Eg5

Deletion of the SAC components in fission yeast revealed that *mph1Δ*, *bub1Δ*, *bub3Δ* and *mad1Δ* cells show significantly higher TBZ sensitivity as compared to *mad2Δ* or *mad3Δ* cells, although the SAC is completely defective in *mad2Δ* and *mad3Δ* cells ^{27,33,34} (Fig. 1a). A similar tendency of defects was observed in chromosome mis-segregation assays monitoring centromere-targeted GFP (*cen2*-GFP) in normal growth medium (Fig. 1b). Thus, although Mad2 and Mad3 are required solely for the SAC, Mph1, Bub1, Bub3 and Mad1 might have additional mitotic roles in chromosome bi-orientation beyond the SAC. A common feature of these mutant cells is the absence of Mad1 at the kinetochores ^{24,34}, suggesting the possibility that Mad1 has a unique function in chromosome bi-orientation. To explore this, we sought to identify proteins that associate with Mad1. Yeast two-hybrid screening using full-length Mad1 as bait identified its known binding partners, Mad2 and Mad1 itself, and, surprisingly, identified a C-terminal fragment of Cut7/Eg5, a kinesin-5 motor protein that acts to assemble bipolar spindles in fission yeast ³ (Fig. 1c, black arrow). We found that a Cut7 fragment that lacks the motor domain interacts with the N-terminal unstructured extension of Mad1 (Fig. 1c,d). Furthermore, analyses of truncation and alanine substitution mutants revealed that Lys 24 and Lys 25 in the N-terminal domain of Mad1 are important for its association with Cut7 (Fig. 1e and Supplementary Fig. 1a,b). The

direct association between Mad1 and Cut7 was confirmed by an *in vitro* pull-down assay (Fig. 1f).

Mad1 targets Cut7/kinesin-5 to unattached kinetochores

To examine the significance of the interaction between Mad1 and Cut7 *in vivo*, we constructed a *mad1-KAKA* strain in which Lys 24 and Lys 25 of Mad1 are replaced with alanines. Upon entry into mitosis, Mad1 shows predominant signals at kinetochores before the kinetochores are captured by microtubules, which then relocate to the spindle pole body (SPB; fission yeast centrosome) during and after chromosome alignment (Fig. 2a). Similarly, Mad1-KAKA localizes to kinetochores in early prometaphase, while its relocation to the SPBs is somewhat diminished (Fig. 2a). Further, we arrested cells in mitosis by the *nda3-KM311* mutation (cold-sensitive mutation of β -tubulin ³⁵) and measured Mad1 signal intensity at unattached kinetochores. As with wild-type Mad1, Mad1-KAKA localizes on unattached kinetochores (Fig. 2b), implying that not only kinetochore localization but also SAC are intact in *mad1-KAKA* cells (see below). If Cut7 indeed associates with Mad1 *in vivo*, Cut7 might be recruited to the unattached kinetochores together with Mad1. To test this hypothesis, we first examined the Cut7 localization in unperturbed mitosis. However, the Cut7 signals along the spindle and on the SPBs prevented the inspection of centromeric signals (Fig. 2c). When we used the *nda3-KM311* mutant under conditions in which the spindle is abolished, Cut7 indeed appeared at unattached kinetochores, and, strikingly, these centromeric Cut7 signals were nearly completely abolished in *mad1-KAKA* cells (Fig. 2d). These results indicate that Cut7 localizes to unattached kinetochores in Mad1-dependent manner.

Mad1 has a dual function in SAC activation and chromosome bi-orientation

To examine the significance of Cut7 recruitment to kinetochores, we characterized *mad1-KAKA* cells in comparison with another point mutant, *mad1-4A* cells, in which Mad1 lacks the ability to bind Mad2 and to activate the SAC ³⁶ (Supplementary Fig. 1c). To investigate the ability to activate the SAC, we scored the mitotic index of cells when spindle formation was abolished by the *nda3-KM311* mutation. In contrast to *mad1-4A* or *mad2 Δ* cells, *mad1-KAKA* cells indeed retained the full ability to activate the SAC (Fig. 3a). Strikingly, however, *mad1-KAKA* cells showed hypersensitivity to TBZ, and the incidence of chromosome mis-segregation was much greater than in *mad1-4A* or

mad2Δ cells (Fig. 3b,c). Thus, Mad1-KAKA retains the ability to activate the SAC, but lacks some function required for chromosome bi-orientation, while *mad1Δ* cells lack both (Fig. 3a). Further, these results suggest that the defect in chromosome bi-orientation alone rather than in combination with the SAC defect causes TBZ hypersensitivity.

Mad1-associated Cut7 promotes chromosome bi-orientation

Because Mad1-KAKA fails to recruit Cut7 to kinetochores, chromosome bi-orientation defects (TBZ hypersensitivity and chromosome mis-segregation) in *mad1-KAKA* or *mad1Δ* cells might originate from the loss of Cut7 function at kinetochores. To explore this possibility, we engineered truncated Cut7 proteins and fused them with the C-terminal sequences of Cnp3/CENP-C (Cnp3C), a peptide targeting to kinetochores (Fig. 4a). Although full length Cut7 fused to Cnp3C (Cut7^{-Cnp3C}) indeed localized to kinetochores even in *mad1-KAKA* cells (Fig. 4a), this tethering did not rescue the chromosome bi-orientation defects of *mad1-KAKA* cells (Fig. 4b,c). Similarly, the Cut7 motor domain fused to Cnp3C (Cut7m^{-Cnp3C}) failed to suppress *mad1-KAKA* (Fig. 4a-c). However, a Cut7 fragment lacking the C-terminal domain fused with Cnp3C (Cut7d^{-Cnp3C}) strikingly suppressed the bi-orientation defects of *mad1-KAKA* cells (Fig. 4a-c). Conversely, Cut7^{-Cnp3C} but not Cut7d^{-Cnp3C} suppressed *cut7-446* (temperature-sensitive mutation) cells, which stop growing at the restrictive temperature because of defects in spindle bipolarity³ (Fig. 4d,e).

The Kinesin-5 family members are known to form a homotetrameric complex with motor domains positioned at each end of the tetramer's long axis, allowing the Kinesin-5 motors to crosslink and cause antiparallel microtubules to slide apart^{2, 6-8, 37}. This mechanism is essential for generating outward forces that establish spindle bipolarity, as shown in several organisms including fission yeast³ (Fig. 4e). Indeed, our *in vitro* cross-linking experiment verifies that full length Cut7 forms a homotetrameric complex, whereas Cut7d predominantly forms a homodimeric complex and Cut7m remains in a monomer (Fig. 4f). Therefore, our Cnp3C fusion experiments suggest that Cut7 is functional only as a homodimeric complex at kinetochores, while functional as a homotetrameric complex at the spindle (see Fig. 8). Because Cut7 interacts with Mad1 through the C-terminal domain, which is required for homotetramerization (Figs 1c and

4a), it is reasonable to consider that the interaction of Mad1 with Cut7 may promote its homodimerization at kinetochores. Importantly, defects in chromosome bi-orientation in *mad1-KAKA* cells were not suppressed by Cut7*d^{-Cnp3C}, which carries mutations in the motor domain (G164A-K165A-T166A) (Supplementary Fig. 2), indicating that chromosome bi-orientation requires the motor activity of Cut7 locating at kinetochores.

Cut7 promotes chromosome gliding

To explore how Cut7 at kinetochores contributes to chromosome bi-orientation, we performed live-cell imaging of mitosis in the presence of a low concentration of TBZ, which attenuates spindle dynamics and thus enhances the defects of chromosome bi-orientation. To deplete Cut7 from kinetochores, we used *mad1-KAKA* cells. Although wild-type cells mostly complete chromosome alignment soon after spindle formation, *mad1-KAKA* cells showed elevated levels of chromosome alignment defects in which sister chromatids were often attached to one spindle pole and not released throughout prometaphase until anaphase (Fig. 5a,b). Remarkably, the expression of Cut7d^{-Cnp3C} restored the alignment defects, while that of motor-dead Cut7*d^{-Cnp3C} did not (Fig. 5a,b). These results suggest that kinetochore-targeted Cut7 plays a role in chromosome gliding along the microtubules from the spindle pole toward the equator.

To set up a system to monitor chromosome gliding from the microtubule minus-end (SPB) to the plus-end, we utilized a monopolar spindle formed in the *cut11-7* temperature-sensitive mutant³⁸, in which kinetochores are mostly attached to the spindle pole (microtubule minus-end) with transient gliding toward the microtubule plus-end (Fig. 5c). By time-lapse imaging, we measured the maximum distance of kinetochore (Mis6) gliding over 9 min (Fig. 5d). The results indicate that chromosome gliding is indeed observed in *mad1*⁺ cells but diminishes in *mad1-KAKA* cells (Fig. 5d,e), suggesting that chromosome gliding occurs depending on centromeric Cut7. In support of this notion, the expression of Cut7d^{-Cnp3C}, but not Cut7*d^{-Cnp3C}, restored efficient chromosome gliding in *mad1-KAKA* cells (Fig. 5d,e). To further support that Cut7 drives chromosome gliding toward microtubule plus-end, we sought to examine whether deletion of counteracting motor would enhance Cut7-dependent gliding. Fission yeast has two kinesin-14s (Klp2 and Pkl1), which are implicated in chromosome transport to the microtubule minus-end (SPB)³⁹. In *k1p2Δ pkl1Δ*

background, chromosome gliding was greatly enhanced and this gliding also depended on kinetochore Cut7 (Supplementary Fig. 3), implying that plus-end directed motor Cut7 indeed counteracts kinesin-14s in chromosome gliding. Taken together these results strongly suggest that Cut7 recruited to kinetochores by Mad1 plays a significant role in chromosome alignment by facilitating chromosome gliding toward the spindle equator (see Fig. 8).

Human Mad1 promotes chromosome alignment by recruiting CENP-E to the kinetochore

To explore the conservation of Mad1-dependent chromosome bi-orientation, we then examined the chromosome alignment defect in HeLa cells depleted for human Mad1 (hMad1). As a control, we included cells depleted for CENP-E/kinesin-7, a plus end-directed kinetochore motor protein that plays a role in chromosome gliding to the plus ends of spindle microtubules^{10,40}. Strikingly, hMad1-depleted cells, which were arrested in metaphase by the addition of a proteasome inhibitor, MG132, showed multiple misaligned chromosomes, albeit fewer as compared to CENP-E-depleted cells (Fig. 6a). Similar results were obtained using different siRNAs against hMad1 (Supplementary Fig. 4a-c). Alignment defects in hMad1-depleted cells were confirmed by live-cell imaging (Fig. 6b,c and Supplementary movies 1-4). Further experiments using synchronous mitotic cultures demonstrated that hMad1 is required for initial alignment but not for its maintenance (Supplementary Fig. 4d). Thus, considering the analogy with fission yeast, we speculated that human Eg5 (Cut7) Considering the analogy with fission yeast, we speculated that human Eg5/kinesin-5 (Cut7 homolog) may also localize to unattached kinetochores in a hMad1-dependent manner. However, immunostaining of mitotic HeLa cells revealed that Eg5 localizes mostly at the spindle but never at the kinetochores (Supplementary Fig. 5). Another candidate might be CENP-E/kinesin-7, which localizes to unattached kinetochores and is required for chromosome alignment^{10,41}. Although BubR1 might be functionally related to CENP-E at kinetochores, several recent studies suggest that the BubR1-associated factor required for chromosome alignment is PP2A rather than CENP-E^{15,17,28,29}. Indeed, our RNAi experiments indicate that BubR1 is required for chromosome alignment but dispensable for CENP-E recruitment to kinetochores (Figs 6a and 7a), as reported¹⁵. We then considered the possibility that hMad1 might play a role in recruiting CENP-E to kinetochores. Indeed,

hMad1-depletion decreased CENP-E localization at kinetochores in mitotic cells (Fig. 7a), whereas over-expression of hMad1 increased CENP-E localization at kinetochores (Supplementary Fig. 4e). Further, immunoprecipitation assays indicate that hMad1 and CENP-E form a complex *in vivo* (Fig. 7b).

Noting the similarity between fission yeast and humans in Mad1-kinesin binding, we sought to delineate the kinesin-binding domain in Mad1. By constructing several truncation mutations in the fission yeast Mad1 protein, we narrowed down the amino acid sequences required for Cut7/kinesin-5 binding to a.a. 10-27 of Mad1 (Supplementary Fig. 1a). By carefully inspecting this domain, we found a short amino acid sequence (F/L)xxF(I/L/F) that is conserved from yeasts to mammals (Fig. 7c). Accordingly, the recombinant hMad1 protein associates directly with the kinetochore binding region of CENP-E (CENP-E-KT) *in vitro*, and this association is abolished by introducing a mutation in the conserved sequence of hMad1 (hMad1-5A) (Fig. 7d). Pull-down assays using HeLa cell extracts also support the notion that this conserved sequence of hMad1 is required for its association with CENP-E (Fig. 7e). Consistently, alignment defects in hMad1-depleted cells were rescued by expressing hMad1-WT but not by expressing hMad1-5A (Fig. 7f), indicating the importance of this motif for CENP-E recruitment to kinetochores and for chromosome alignment. Although hMad1 uses its N-terminus to localize to the nuclear envelope, a process required for producing pre-mitotic anaphase inhibitors²², hMad1-5A mutant proteins retain this function (Supplementary Fig. 4f and 6d). We confirmed that the corresponding sequence in fission yeast Mad1 is essential for the Cut7 binding and chromosome bi-orientation (Supplementary Fig. 6). Collectively, these results indicate that hMad1 plays a crucial role in enriching the kinetochore motor protein CENP-E to the unattached kinetochores as fission yeast Mad1 recruits Cut7 (Fig. 8).

DISCUSSION

The original concept of “checkpoints” relied on the principle of an external surveillance mechanism that does not take an active role in the process being monitored⁴². Thus, SAC proteins that monitor kinetochore-microtubule attachments should not play an active role in chromosome bi-orientation, which depends on kinetochore-microtubule attachments. However, in fact, many SAC proteins appear to play a role that extends

from the simple external monitoring of the process, although underlying molecular mechanisms are still elusive.

In the early stage of mitosis, there are many misaligned chromosomes that have not yet achieved bipolar attachment and accumulate the SAC components at the kinetochore. Thus, the necessity for chromosome gliding and activation of the SAC arises at the same time, so that the existence of a molecular link between these two events can be reasonably predicted. We show that fission yeast Mad1 recruits Cut7/Eg5 to kinetochores, thereby promoting chromosome gliding to the spindle equator. Notably, it has been suggested that Bub1 and Bub3 have important roles in chromosome bi-orientation partly through promoting the function of shugoshin-Aurora B ²³, while additional unknown function in bi-orientation are also implicated ²⁷. Because Mad1 fails to localize to kinetochores in *bub1Δ* and *bub3Δ* cells, we assume that the Mad1-Cut7 pathway is the underlying mechanism of Bub1/Bub3-mediated bi-orientation. Indeed, Cut7 tethering significantly suppresses the bi-orientation defects of *bub1Δ* and *bub3Δ* cells as well as cells deleted for Mph1/MPS1, the most upstream component in the hierarchical process of SAC complex assembly ⁴³ (Supplementary Fig. 2f). Thus, although Cut7 usually generates an outward force by crosslinking antiparallel microtubules and promotes spindle bipolarity, Cut7 also plays a crucial role in SAC-associated chromosome bi-orientation. A mutation in Mad1 that abolishes Cut7 binding preserves the ability of SAC activation, whereas a mutation in the Mad2 binding motif preserves intact chromosome bi-orientation, implying that Mad2 and Cut7 are recruited concomitantly to the kinetochore through Mad1. This mechanism ensures the coincidence of SAC activation and chromosome gliding in a single kinetochore.

In interphase, Cut7 disperses throughout the nucleus, whereas Mad1 localizes on the nuclear envelope. Thus, Cut7 might associate with Mad1 in an M-phase specific manner. Kinesin-5 family members possess highly conserved sequences in the tail region called BimC box, which is a target of phosphorylation by Cdk1 and responsible for its spindle localization at least in humans ^{44 45}. Despite the conservation of the phosphorylation site through fungi (Supplementary Fig. 7a), a mutation in this site in fission yeast Cut7 (Cut7-T1011A) does not affect the bipolar spindle formation ⁴⁴. We show that Cut7-T1011A affects neither its kinetochore localization nor binding to Mad1

(Supplementary Fig. 7b-d), leaving open the question of M-phase specific regulation of Mad1-Cut7 association.

Because budding yeast Eg5 homologs are also required for chromosome alignment⁴⁶, the Mad1-Eg5 pathway might be conserved in fungi. Our analyses in human cells, however, reveal that CENP-E, but not Eg5, localizes to kinetochores depending on its binding to hMad1. Consistent with the fact that CENP-E forms a parallel homodimer, our results suggest that Cut7 binds Mad1 as a parallel homodimer at kinetochores, otherwise it forms tetramers (anti-parallel two dimers) on the spindle microtubules to promote spindle bipolarity (Fig. 8). Given that both Eg5 and CENP-E are plus-end directed kinesins that exhibit processive movement⁴⁷, and yeasts have no CENP-E homologue, we assume that CENP-E is a specialized kinesin that evolved to replace the centromeric Eg5 function in animals. Notably, a recent study has reported that the unusually long coiled-coil stalk of CENP-E is essential for its alignment function because CENP-E with a shortened stalk is incapable of achieving a stable lateral attachment, a prerequisite for chromosome gliding⁴⁸. Thus, it is reasonable to speculate that the stalk of the kinetochore kinesin must be longer in higher eukaryotes because their chromosomes assemble much larger kinetochores than those of yeasts. This spatial restriction might have promoted the adaptive evolution of CENP-E, which possesses an extraordinarily long stalk.

Our data in fission yeast suggest that while Mad1 recruits Cut7 to kinetochores, Cut7 localized at the SPBs acts to recruit Mad1 (Fig. 2a,c). Also in mammalian cells, the SAC components and CENP-E localize to the spindle pole depending on dynein^{14, 49, 50}. Although the biological significance of hMad1 or CENP-E localization to the spindle pole remains unknown, we speculate that the Mad1-kinesin complex appearing near the spindle pole might facilitate loading of the ‘glider complex’ onto the misaligned chromosomes, which often locate near the pole (Figs 5a and 6a)¹¹.

In summary, our study reveals that the conserved SAC component Mad1 functionally links chromosome gliding and SAC activation, a mechanism that facilitates and ensures chromosome congression in mitosis. Our results further suggest that the mitotic

checkpoint have co-evolved with a kinesin anchorage mechanism, which drives rather than checks chromosome congression.

Figure legends

Figure 1. Identification of a Mad1 interacting protein.

(a) Serial dilution assay of SAC component mutant cells (TBZ 10 $\mu\text{g/ml}$). (b) The indicated strains were cultured in YE medium for 24 h at low temperature (18°C). Chromosomes marked with *cen2*-GFP were monitored for segregation in bi-nucleate cells (> 350 cells per strain were analyzed). Error bars, s.e.m. for $n = 3$ independent experiments. Statistical significances (t -test, two-tailed) relative to wild-type were assessed (* $P < 0.05$, ** $P < 0.001$). (c) Domain organization of Mad1 and Cut7. The Mad1 N-terminal region (blue) represents the unstructured domain, which is followed by the coiled-coil. The conserved Mad2 and Bub1 binding sequences (dark grey) locate in the C-terminal region of Mad1^{24,36}. Cut7 carries the N-terminal motor domain (light blue) and the C-terminal tail domain (red), which are connected by a stalk. The bidirectional arrow indicates the range of Cut7 ΔN obtained by the yeast two-hybrid screening. (d) A yeast two-hybrid assay shows that the N-terminal unstructured domain of Mad1 is important for Cut7 interaction. The pair of p53 and T-antigen serves as a positive control. (e) A yeast two-hybrid assay shows Lys 24 and Lys 25 in the N-terminal domain of Mad1 are important for Cut7 interaction. (f) His-Cut7 was pulled down by GST-Mad1. Cut7 was efficiently pulled down by wild-type Mad1, whereas to a lesser extent by Mad1-KAKA or Mad1 ($\Delta 18-27$). Statics source data for Fig. 1b can be found in Supplementary Table 2. Uncropped images of blots are shown in Supplementary Fig. 8.

Figure 2. Mad1 targets Cut7/kinesin-5 to unattached kinetochores.

(a) Kinetochores and SPB localization of Mad1 was observed in the indicated cells expressing Mad1-GFP or Mad1-KAKA-GFP, Mis6-2mCherry (kinetochore) and Sfi1-CFP (SPB). Representative kymographs are shown. Note that Mad1-KAKA localizes to the kinetochores but much less to the SPBs. (b) The GFP signals were measured in the mitotic (*nda3-KM311*) cells expressing Mad1-GFP or Mad1-KAKA-GFP, Mis6-2mCherry (kinetochore) and Sfi1-CFP (SPB). 30 kinetochores detached from SPB from

10 cells were analyzed. Error bars, s.e.m. for $n = 3$ independent experiments. **(c)** Cut7 localization was observed in wild-type and *mad1-KAKA* cells expressing Cut7-3GFP, Mis6-2mCherry (kinetochore) and Sfi1-CFP (SPB). Representative kymographs are shown. **(d)** The GFP signals were measured in the indicated mitotic (*nda3-KM311*) cells expressing Cut7-3GFP, Mis6-2mCherry (kinetochore) and Sfi1-CFP (SPB). 30 kinetochores detached from SPB from 10 cells were analyzed. Error bars, s.e.m. for $n = 3$ independent experiments. Statics source data for Fig. 2b,d can be found in Supplementary Table 2. Scale bars, 3 μm .

Figure 3. The Mad1-KAKA mutant unable to recruit Cut7 is defective in chromosome bi-orientation but intact in SAC activation.

(a) The indicated cells carrying the *nda3-KM311* mutation were cultured at the restrictive temperature and scored for Plo1-GFP-positive cells (over 100 cells per strain were analyzed). Note that Plo1 signals at the SPB appear only in M-phase. Error bars, s.e.m. for $n = 3$ independent experiments. **(b)** Serial dilution assay (TBZ 15 $\mu\text{g/ml}$). Note that *mad1-KAKA* mutant cells show similar TBZ sensitivity to that of *mad1 Δ* cells. **(c)** The indicated strains were analyzed for chromosome segregation defect as in Fig. 1b. Over 500 cells per strain were analyzed. Error bars, s.e.m. for $n = 3$ independent experiments. Statics source data for Fig. 3a,c can be found in Supplementary Table 2. Scale bars, 4 μm .

Figure 4. Dimer-Cut7 at kinetochores promotes chromosome bi-orientation.

(a) Schematic depiction of Cut7 fragments used for Cnp3C fusion constructs (upper panel). The dimer and tetramer domains are assigned according to the studies of budding yeast Eg5 homolog Cin8³⁷ and *Drosophila* KLP61F⁵¹. Photos showing the representative localization of Cut7-CFP^{Cnp3C} constructs (lower panel). Note that all constructs constitutively localize at the centromere. **(b)** Serial dilution assay (TBZ 15 $\mu\text{g/ml}$). Note that only when *cut7d-cnp3C* is expressed is the TBZ hypersensitivity of *mad1-KAKA* mutant cells suppressed. **(c)** The indicated strains were analyzed for chromosome segregation defect as in Fig. 1b. Over 500 cells per strain were analyzed. Error bars, s.e.m. for $n = 3$ independent experiments. **(d)** Serial dilution assay. Note that only when *cut7-cnp3C* is expressed, the temperature sensitivity of *cut7-446* mutant is suppressed. **(e)** The indicated strains were cultured in YE medium at a permissive

temperature (26.5°C) and then shifted to a restrictive temperature (30°C). 2 h after the shift, mitotic spindles (mCherry-Atb2) were monitored to count the frequency of monopolar spindle formation. (> 100 cells per strain were analyzed). Error bars, s.e.m. for n = 3 independent experiments. **(f)** Purified His-tagged Cut7, Cut7d and Cut7m were incubated with or without EGS cross-linker, loaded on the gel and examined by immunoblot using an anti-His antibody. Monomer (green circle), dimer (blue circle) and tetramer (red circle) are marked. Statics source data for Fig. 4c,e can be found in Supplementary Table 2. Uncropped images of blots are shown in Supplementary Fig. 8. Scale bars, 4 μ m.

Figure 5. Cut7 facilitates chromosome alignment by promoting chromosome gliding toward the spindle equator.

(a) The indicated cells marked for *cen2*-GFP and expressing mCherry-Atb2 (tubulin) were filmed during mitosis. Representative kymographs are shown. **(b)** The indicated strains were analyzed for chromosome alignment defect judged by persistent *cen2*-GFP dots at the SPBs throughout mitosis (over 40 cells for each strain). Error bars, s.e.m. for n = 3 independent experiments. **(c)** Schematic depiction of the chromosome gliding assay using the *cut11-7* temperature-sensitive mutant that produces a monopolar spindle. Chromosome gliding from the SPB toward the spindle microtubule plus-end was monitored by visualizing Mis6-2mCherry (kinetochore) and GFP-Atb2 (tubulin). **(d)** Representative time-lapse images and kymographs of the chromosome gliding assay. Yellow arrowheads indicate gliding. **(e)** Quantification of chromosome gliding during time-lapse recording (9 min). We categorized the gliding in three groups according to the gliding length (over 30 cells per strain were analyzed). Error bars, s.e.m. for n = 3 independent experiments. Statistical significances (*t*-test, two-tailed) were assessed (**P* < 0.05, ***P* < 0.005). Statics source data for Fig. 5b,e can be found in Supplementary Table 2. Scale bars, 2 μ m.

Figure 6. Human Mad1 is required for chromosome alignment.

(a) HeLa cells treated with the indicated siRNAs were arrested in metaphase by MG132 and examined for chromosome alignment by immunostaining. Representative images are shown (left). Frequencies of cells exhibiting fewer than five or more than five mis-aligned chromosomes were measured (right; 50 cells for each RNAi). Error bars, s.e.m.

for $n = 3$ independent experiments. Statistical significances (t -test, two-tailed) were assessed ($**P < 0.005$). **(b)** Selected frames of a representative imaging are presented with bars denoting prometaphase (dark blue), metaphase (light blue) and metaphase plate with unaligned chromosomes (metaphase-like) (orange). Proper anaphase (green) and abnormal anaphase (red) are also shown. The time (hr: min) after nuclear envelope breakdown is shown at the right bottom corner of each frame. Yellow arrowheads indicate unaligned chromosomes. Note that the CENP-E-depleted cell showed mitotic arrest due to the multiple mis-aligned chromosomes, and did not enter anaphase during imaging. **(c)** Mitotic progression in observed cells. Note that hMad1 RNAi causes mitotic acceleration, making it difficult to observe alignment defects. Only in cells staying in mitosis for a longer period (hMad1 RNAi effect may be partial) allowed us to detect unaligned chromosomes. About half of metaphase cells showed alignment defects, which is consistent with the result obtained in fixed-samples (Fig. 6a). Statics source data for Fig. 6a can be found in Supplementary Table 2. Uncropped images of blots are shown in Supplementary Fig. 8. Scale bars, 5 μm .

Figure 7. Human Mad1 targets CENP-E/kinesin-7 to unattached kinetochores.

(a) HeLa cells treated with the indicated siRNAs arrested in mitosis by nocodazole and MG132 were examined by immunostaining (left). Localization was quantified as the ratio of the fluorescence intensity of CENP-E to that of CENP-C (100 kinetochores from 10 cells). Error bars, s.e.m. for $n = 3$ independent experiments (right). Statistical significances (t -test, two-tailed) were assessed ($**P < 0.001$). **(b)** Cell extracts were prepared from HeLa cells synchronized in mitosis by double-thymidine block and release followed by nocodazole treatment. hMad1 was immunoprecipitated (IP) by anti-hMad1 antibodies and analyzed by immunoblot using the indicated antibodies. **(c)** Sequence alignment of Mad1 N-terminus. Highlighted residues are conserved from yeast to human. Asterisks indicate the residues replaced by alanine in the 5A mutant. Red letters show the position of residues replaced in the *mad1-KAKA* mutant. *H. sapiens* (NP_001013859.1), *G. gallus* (XP_004945193.1), *O. latipes* (XP_004066129.1), *X. tropicalis* (AAH76947.1), *S. pombe* (NP_595516.1), *S. japonicus* (XP_002175955.1), *S. cerevisiae* (CAA96791.1) data are from the NCBI protein data base. **(d)** Domain organization of hMad1 and CENP-E (top). The blue region in hMad1 represents the unstructured domain including the conserved motif. KT,

kinetochore binding region¹⁴. CENP-E-KT was used to examine the interaction with hMad1. CENP-E-KT was pulled down by GST-hMad1 and GST-hMad1-5A (bottom). (e) Mitotic HeLa cell extracts expressing either hMad1 or hMad1-5A were used for the extract pull-down assay. The graph indicates the quantification of the pull-down efficiency. Error bars, s.e.m. for n = 3 independent experiments. (f) hMad1-depleted HeLa cells were reconstituted by RNAi-resistant hMad1-3Flag-HA or hMad1-5A-3Flag-HA, and examined for chromosome alignment by immunostaining as in Fig. 6a. (30 cells for each sample). Error bars, s.e.m. for n = 3 independent experiments. Statics source data for Fig. 7a,e,f can be found in Supplementary Table 2. Uncropped images of blots are shown in Supplementary Fig. 8. Scale bars, 5 μ m.

Figure 8. Schematic depiction of Mad1-kinesin interplay in *S. pombe* and human cells.

In fission yeast, Cut7 tetramers crosslink and walk along antiparallel microtubules, promoting spindle bipolarity. On the other hand, a Cut7 dimer at kinetochores promotes chromosome bi-orientation. In human cells, hMad1 utilizes CENP-E to align polar chromosomes. Eg5, which may function specifically in bipolar spindle formation, does not localize at kinetochores.

METHODS

Schizosaccharomyces pombe strains.

All strains used are listed in Supplementary Table 1. Deletion of *mad1*⁺, *mad2*⁺, *mad3*⁺, *bub3*⁺, *bub1*⁺, *mph1*⁺, *pkll*⁺ and *klp2*⁺ and tagging of *mad1*⁺ and *cut7*⁺ by GFP and 3GFP respectively were performed using a PCR-based targeting method for *S. pombe*⁵². To generate *cut7-T1011A*-GFP strain, T1011 was changed to alanine using Prime STAR Mutagenesis Basal Kit (TaKaRa). Then, the genomic *cut7* fragment fused to GFP was transformed using a PCR-based targeting method for *S. pombe*. To generate the *mad1-KAKA*, -4A or -5A strains, K24, K25 or R510, V511, L512 and Q513 or L15, P16, R17, F18 and L19 were changed to alanines using PrimeSTAR Mutagenesis Basal Kit (TaKaRa). The genomic *mad1* fragments carrying the mutations were then transformed into *mad1::ura4*⁺ cells and the integration at *mad1::ura4*⁺ was selected by 5-fluoroorotic acid (5-FOA) resistance and confirmed by PCR. To express Cut7-CFP-Cnp3C constructs, a sequence encoding CFP and Cnp3C (amino acids 384–642) was

fused to the carboxy terminus of *cut7* fragments and cloned under the promoter *Padh41* (a weak version of the *adh1*⁺ promoter). The resulting plasmid (pHBCA41-*cut7*⁺-CFP-*cnp3C*) was linearized and integrated into the locus adjacent to the SPAC26F1.12c gene of chromosome 1 (we refer to this as the *C* locus) using the *hyg*^r marker. To express mCherry-tubulin, a sequence encoding mCherry was fused to the amino terminus of *atb2*⁺, cloned under the promoter *Padh15* (a weak version of the *adh1*⁺ promoter), and integrated into the locus adjacent to the *zfs1*⁺ gene of chromosome 2 (we refer to this as the *Z* locus) using the *nat*^r marker.

Synchronous culture of fission yeast.

For prometaphase arrest, we used the *nda3-KM311* mutation and cells cultured at 17°C for 15 h³⁵. To measure the frequency of *cen2*-GFP mis-segregation at anaphase, cells were cultured at low temperature (18°C). 24 h later, the cells were fixed and stained with DAPI. To measure the frequency of monopolar spindle formation, *cut7-446* temperature sensitive cells were first cultured at permissive temperature (26.5°C) and then shifted to restrictive temperature (30°C). 2 h later, the cells were fixed and stained with DAPI. Images in Fig. 1, 3, 4 and Supplementary Fig. 6 are representative ones, repeated at least three times.

Two-hybrid assay.

Full-length *mad1*⁺ cDNA was subcloned into the pGBKT7 vector and used as bait for screening. An *Schizosaccharomyces pombe* cDNA library constructed on pVP16 vector was cotransformed into *Saccharomyces cerevisiae* AH109 strain with bait, and positive transformants were selected on the SC-trp-leu-his-ade plate containing 1 mM 3-amino-1,2,4-triazole. Prey plasmids were extracted from candidate clones and sequenced. The constructs of *mad1* were amplified by PCR and cloned into pGBKT7 vector and used as bait. *cut7*⁽⁴⁷⁵⁻¹⁰⁸⁵⁾ and *mad2* cDNA was amplified by PCR and cloned into pGADT7 vector and used as prey. These plasmids were transformed into AH109 strain. Plates lacking histidine or both histidine and adenine with the addition of appropriate amounts of 3-amino-1,2,4-triazole were used as the selective media.

Time-lapse imaging.

To measure the frequency of chromosome mis-alignment, cells were cultured in minimal medium at 30°C, mounted onto a glass-bottomed dish (Matsunami) and the medium was exchanged to minimal medium containing 7.5 µg/ml TBZ (Sigma-Aldrich). 1.5 h later, live cell recordings were performed on a Delta Vision P system (Applied Precision) using a microscope (IX71, Olympus) equipped with a Plan Apo N ×60 NA 1.42 objective and CoolSNAP HQ2 camera (Photometrics) in a chamber maintained at 26.5°C by a temperature controller (Precision Control). For live cell imaging of Mad1-GFP and Cut7-3GFP, cells were cultured in minimal medium at 30°C, then the cells were mounted onto a glass-bottomed dish, and live cell recordings were performed on a Delta Vision P system in a chamber maintained at 26.5°C. Images in Fig. 2 are representative ones, repeated at least three times.

Chromosome gliding assay.

Cells were cultured in minimal medium at 25°C, and then mounted onto a glass-bottomed dish and incubated in an air-conditioned chamber maintained at 37°C to inactivate Cut11. Thirty minutes later, live cell recordings were obtained on a Delta Vision P system. Images were acquired every 1 min for 9 min. Chromosome gliding distance was measured by softWoRx software (Delta Vision). Images in Fig. 5 are representative ones, repeated at least three times.

Quantification of fluorescent signals.

To quantify the fluorescent signals at kinetochores, in-focus images of Mad1-GFP and Cut7-3GFP cells were taken with Axio Vision imaging software (Carl Zeiss). We measured the average intensity of the nuclear dots associated with Mis6-2mCherry dots (kinetochore) and subtracted the average background intensity. The intensities of protein signals were quantified in 30 kinetochores detached from SPB from 10 cells and averaged over 3 independent experiments. Images in Fig. 2 are representative ones, repeated at least three times.

***In vitro* pull-down assay.**

DNA fragments encoding 6His-Cut7, GST-Mad1, GST-Mad1-KAKA, GST-Mad1 (Δ 18-27), GST-Mad1-5A, GST-hMad1, GST-hMad1-5A and 6His-CENP-E (1958-2663) were amplified by PCR and inserted into a pET19b or pGEX-6P-3 vector. The resulting

plasmids were cotransformed into *E. coli*. Cultured *E. coli* were harvested, re-suspended in lysing buffer (20 mM Tris-HCl (pH 7.5), 600 mM NaCl, 1 % Triton X-100, 1 mM β -mercaptoethanol, 1 mM PMSF, complete protease inhibitors). *E. coli* was lysed by sonication and the supernatants were collected after centrifugation. GST-fused proteins were purified on Glutathione-Sepharose beads. After washing 3 times with lysing buffer, Cut7 or CENP-E binding was analyzed by western blotting with anti-His H-3 (1:1000; Santa Cruz Biotech, sc-8036) or anti-CENP-E (1:2000, Sigma, C-7488) antibodies.

Chemical crosslinking.

Chemical crosslinking assays were performed as previously described^{53, 54}; any modifications are detailed below. Purified proteins at around 1 mM were incubated in HGNE buffer (1 mM β -mercaptoethanol, 100 mM NaCl, 0.2 mM EDTA, 0.05 % NP-40, 10 % glycerol and 25 mM HEPES (pH 8.0)) containing 500 mM EGS (Ethylene glycol-bis Succinimidylsuccinate) for 30 min at 37°C. The reaction was quenched by adding Tris-Glycine buffer (100 mM Tris and 100 mM glycine pH 7.5) and incubating for 30 min at 37°C. The products were analyzed by western blotting.

Immunoprecipitation using HeLa cell extracts.

HeLa cells were synchronized by double thymidine block and release. At the second thymidine release, 1 μ M nocodazole was added to the medium and the cells were cultured for 16 h. Cells collected from culture dishes were washed once with PBS, and resuspended in ice-cold cell resuspension buffer (CRB, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.3 % Triton X-100, 10 % glycerol, supplemented with complete protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail, 1 mM PMSF, 4 μ M Okadaic acid, 4 μ M Microcystin-LR and 1 mM β -mercaptoethanol). Cells were incubated for 30 min at 4°C, followed by the addition of 1 mM CaCl_2 and micrococcal nuclease (Sigma). Incubated at 30°C for 15 min, and centrifuged for 10 min at 4°C. The supernatants were collected and incubated with 5 μ g anti-hMad1 antibodies (GeneTex, GTX109519) or control rabbit IgG for 2 h, and additionally incubated with Protein G beads for 1 h. The beads were washed 6 times with CRB and resuspended in SDS-PAGE sample buffer.

Pull down assay using HeLa cell extracts.

Mitotic HeLa cell extracts expressing 3flag-HA-tagged hMad1 constructs were obtained as in immunoprecipitation assay. Extracts expressing 3flag-HA-tagged hMad1 and hMad1-5A were incubated with MBP-CENP-E-KT bound beads for 2.5 h. The beads were washed 4 times with CRB and resuspended in SDS-PAGE sample buffer. Images in Fig. 7 are representative ones, repeated at least three times.

Plasmids, Transfection, siRNA and Cell Synchronization.

3flag-HA-tagged hMad1 constructs were expressed from the modified pcDNA5/TO vector, which carries a weaker version of the CMV promoter. For transient overexpression of 3flag-HA constructs, cells were transfected using Lipofectamine LTX reagent (Invitrogen). For the RNAi of hMad1, a mixture of two oligonucleotides was used; Silencer Select Pre-designed siRNA s15906 (Invitrogen) and the other siRNA (5'-CUCACCUUGUGAAAUAAAATT-3') obtained from JbioS. The siRNAs of CENP-E, BubR1 and hMad1-1 were also obtained from JbioS. The siRNA sequences are CENP-E: 5'-CCACUAGAGUUGAAAGAUATT-3', BubR1: 5'-CGGGCAUUUGAAUAUGAA ATT-3', hMad1-1: 5'-CCAAAGUGCUGCACAUGAGTT-3'. siRNAs were transfected at 25 nM using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). For hMad1 depletion, siRNA was transfected twice in 6 days. For depletion of CENP-E and BubR1, siRNA was transfected twice in 3 days. For the rescue assay of hMad1 RNAi, siRNA and RNAi-resistant hMad1-3flag-HA cloned into modified pcDNA5/TO vector were co-transfected using Lipofectamine 3000 reagent (Invitrogen). Following transfection, the HeLa cells were synchronized at G1/S by adding 2 mM thymidine for 24 h. Cells were released into fresh medium for 11 h, further incubated with 1 μ M nocodazole and 20 μ M proteasome inhibitor MG132 for 2 h, and examined by immunostaining.

Immunostaining of HeLa cells and quantification of fluorescent signals.

HeLa cells were detached and collected from dishes treated with 0.3 % EDTA, and spun onto glass slides by cytospin for 5 min at 1500 rpm before fixed with PBS containing 2 % PFA and 0.2 % Triton X-100. Subsequently, cells were washed with PBS once, and incubated in 0.2 % Triton X-100/PBS for 10 min. Immunostaining was performed in 3 % BSA/PBS. The cells were stained with primary antibody at room temperature for 1 h.

The following primary antibodies were used: CENP-C (1:1000; MBL, PD030), anti-hMad1 (1:1000; GeneTex, GTX109519), anti-CENP-E (1:250; Abcam, ab5093), anti-Eg5 (1:1000; Abcam, ab51976), anti-HA 3F10 (1:1000; Roche, 11867423001) and anti-BubR1 (1:1000; Abcam, ab70544). Cells were washed twice for 5 min in PBS prior to incubation with secondary antibodies. The secondary antibodies were: Alexa 488-conjugated goat anti-rabbit, Alexa 568-conjugated goat anti-mouse and Alexa 647-conjugated goat anti-guinea pig (Invitrogen). DNA was counterstained with Hoechst 33342 (Dojindo) at a concentration of 50 ng/ml. To quantify CENP-E signals, fluorescent intensities at the centromere were divided by fluorescence intensities of CENP-C and the average background intensity was subtracted. The intensities of protein signals were averaged over 10 centromeres in each cell and the average value for 10 cells is shown. Images were deconvolved and projected. Images in Fig. 6 and 7 are representative ones, repeated at least three times.

Live imaging

HeLa cells transfected with pcDNA5/TO plasmids (invitrogen) harboring H2B-mCherry were imaged in a Lab-Tek Chambered Coverglass System (Nunc) in phenol red-free Leibovitz's L-15 medium (GIBCO) at 37°C under 5 % CO₂. Exposures of 0.05 sec with a 2 x 2 bin were acquired every 3 min using a 40x NA 0.75 objective on an Olympus IL-X71 Applied Precision DeltaVision microscope. Images in Fig. 6 are representative ones, repeated at least three times.

Code availability

H. sapiens (NP_001013859.1), *G. gullus* (XP_004945193.1), *O. latipes* (XP_004066129.1), *X. tropicalis* (AAH76947.1), *S. pombe* (NP_595516.1), *S. japonicus* (XP_002175955.1), *S. cerevisiae* (CAA96791.1), *S.pombe* [x57513], *H. sapiens* [x85137], *X. laevis* [x71864] , *A.nidulans* [M32075] data are from the NCBI protein data base.

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AUTHOR CONTRIBUTIONS

T.A. designed and performed most experiments. Y.G. isolated Cut7 as a Mad1 interactor. M.S., M.Y. and Y.W. supervised the project. T.A. and Y.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Supplementary Figures

Supplementary Figure 1: Mad1 directly associates with Cut7.

- (a) Amino acid sequence of the N-terminal region of fission yeast Mad1. Amino acids sequence highlighted in blue represents the region required for Cut7 binding (Top). Yeast two-hybrid assay for mapping the minimal Cut7 interaction domain in Mad1 N-terminus (Bottom).
- (b) Yeast two-hybrid assay showing KAKA mutation abolished the interaction between Mad1 N-terminal region and Cut7.
- (c) Yeast two-hybrid assay showing *mad1-4A* mutation (R510A, V511A, L512A, Q513A) abolished the interaction between Mad1 and Mad2.

Supplementary Figure 2: Cut7 motor activity is essential for its kinetochore function.

- (a) The signals of CFP-*Cnp3C*, Cut7d-CFP-*Cnp3C* and Cut7*d-CFP-*Cnp3C* were observed in the indicated cells. The centromeres were visualized by *cen2*-GFP.
- (b) Serial dilution assay showing that motor-dead version of Cut7d (Cut7*d) cannot suppress the TBZ hypersensitivity of *mad1-KAKA* cells (TBZ 15 μ g/ml).
- (c) The indicated cells were monitored for *cen2*-GFP segregation in bi-nucleate cells as in Fig. 1b. Over 500 cells per strain were analyzed. Error bars, s.e.m. for $n = 3$ independent experiments.
- (d) Serial dilution assay showing that *cut7d^{cnp3C}* suppresses the TBZ hypersensitivity of *mad1 Δ* cells (10 μ g/ml).
- (e) The indicated cells were monitored for *cen2*-GFP segregation in bi-nucleate cells as in Fig. 1b (over 500 cells per strain were analyzed). Error bars represent s.e.m. for $n = 3$ independent experiments.
- (f) Serial dilution assays showing that centromere targeting of Cut7 can suppress the TBZ hypersensitivity of *bub1 Δ* , *bub3 Δ* , and *mph1 Δ* cells.
- Scale bars, 4 μ m.

Supplementary Figure 3: Deletion of counteracting kinesins enhances Cut7-mediated chromosome gliding.

Chromosome gliding assays were performed as in Fig.5c-e in the absence of minus-end directed kinesin-14 motors (Pkl1 and Klp2 in fission yeast). Kinesin-14 motors were implicated in chromosome movement toward the spindle poles in several organisms including fission yeast³⁶. Note that greater chromosome gliding observed in Kinesin-14

deleted cells, supporting the idea that Cut7 promotes plus-end directed gliding. Around 20 cells per strain were analyzed. Scale bars, 2 μ m.

Supplementary Figure 4: CENP-E is targeted to the kinetochores by hMad1.

(a) HeLa cells treated with the indicated siRNAs were examined by immunoblot using the indicated antibodies. Note that hMad1-1 siRNA is less effective than hMad1 siRNA, which is used in Fig. 6 and 7.

(b) HeLa cells treated with control siRNA or hMad1-1 siRNA were arrested in metaphase by MG132 and examined for chromosome alignment as in Fig. 6a. Error bars, s.e.m. for $n = 3$ independent experiments. Statistical significances (t -test, two-tailed) were assessed ($*P < 0.05$).

(c) HeLa cells treated with control siRNA or hMad1-1 siRNA arrested in mitosis by nocodazole and MG132 were examined by immunostaining (left). Localization was quantified as the ratio of fluorescence intensity of CENP-E to the value of CENP-C in 10 kinetochores from each cell (right). Error bars, s.e.m. for $n = 9$ cells for each. Statistical significances (t -test, two-tailed) were assessed ($*P < 0.05$).

(d) HeLa cells treated with indicated siRNAs were arrested by Monastrol and released into MG132 after the washout of Monastrol. To prevent premature mitotic exits in hMad1-depleted cells treated with Monastrol, MG132 was added to the culture 1 hr after the Monastrol addition. In this synchronous mitotic culture, alignment defects in hMad1-depleted cells appeared transiently only after 1 hr after release but not anymore at 2 hr. This contrasts to the results in CENP-E-depleted cells, in which alignment defects were persistent. Because CENP-E is only partly displaced from centromeres in hMad1-depleted cells (Fig 7a), this residual CENP-E might finally complete the alignment. Over 30 cells were analyzed for each.

(e) HeLa cells expressing hMad1-3Flag-HA or hMad1-5A-3Flag-HA arrested in mitosis by nocodazole were examined by immunostaining (left). Localization was quantified as the ratio of fluorescence intensity of CENP-E to the value of CENP-C in 10 kinetochores from each cell (right). Error bars, s.e.m. for $n = 7$ cells for each. Statistical significances (t -test, two-tailed) were assessed ($**P < 0.005$). hMad1 overexpression enhanced the CENP-E accumulation at kinetochores in N-terminal motif-dependent manner.

(f) hMad1-depleted HeLa cells expressing RNAi-resistant hMad1-3Flag-HA or hMad1-5A-3Flag-HA were examined for its nuclear envelope localization by immunostaining. Single sections of the images are shown. Uncropped images of blots are shown in Supplementary Fig. 8. Scale bars, 4 μ m.

Supplementary Figure 5: Eg5 does not localize at kinetochores.

Non-treated or nocodazole-treated HeLa cells were examined for Eg5 localization by immunostaining. Note that Eg5 was not detected at the kinetochores even in the absence of attachment (+ Nocodazole). Scale bars, 2 μ m.

Supplementary Figure 6: Fission yeast Mad1 uses the N-terminal conserved motif for Cut7 binding.

(a) His-Cut7 was pulled down by GST-Mad1. Cut7 was efficiently pulled down by wild-type Mad1, whereas it was pulled down to a lesser extent by Mad1-5A.

(b) The indicated cells carrying the *nda3-KM311* mutation were cultured at the restrictive temperature and scored for Plo1-GFP-positive cells. Note that Mad1-5A can efficiently activate the SAC. Over 100 cells per strain were analyzed. Error bars, s.e.m. for n = 3 independent experiments.

(c) Serial dilution assay showing that *mad1-5A* mutant shows hypersensitivity to TBZ as *mad1* Δ and *mad1-KAKA* cells (TBZ 15 μ g/ml).

(d) The single section of interphase cells expressing Mad1-GFP, Mad1-KAKA-GFP or Mad1-5A-GFP. Note that both Mad1-KAKA-GFP and Mad1-5A-GFP properly localize to the nuclear envelope. Uncropped images of blots are shown in Supplementary Fig. 8. Scale bar, 3 μ m.

Supplementary Figure 7: Potential Cdk1 phosphorylation site T1011 in Cut7 is not involved in Cut7-Mad1 interaction.

(a) Alignment of conserved BimC motif in Cut7 family motor proteins. Cdk1 phosphorylation site in human ⁴⁵ is highlighted in red. Identical or similar residues are highlighted in blue. Alignments of 997-1019 from *S.pombe* Cut7 [gene bank number x57513], 912-934 from *H.sapiense* Eg5 [x85137], aa.923-945 from *X.laevis* Eg5 [x71864], 992-1014 *A.nidulans* BimC [M32075].

- (b) A yeast two-hybrid assay shows that the potential Cdk1 phosphorylation sites (T1011) in Cut7 is not important for Mad1 interaction.
- (c) The GFP signals were measured in the indicated mitotic (*nda3-KM311*) cells expressing Cut7-GFP or Cut7-T1011A-GFP, Mis6-2mCherry (kinetochore) and Sfi1-CFP (SPB). Note that kinetochore localization of Cut7-T1011A mutant was intact. Error bars, s.e.m. for $n = 30$ kinetochores detached from SPB from 10 cells.
- (d) Serial dilution assay (28°C, 34°C, TBZ 15 µg/ml at 28°C). Note that *cut7-T1011A* mutant cells show temperature sensitivity at 34°C, and does not show TBZ sensitivity unlike *mad1-KAKA*. Scale bar, 3 µm.

Supplementary Figure 8: uncropped images of blottings and gels.

Supplementary Video 1: Live imaging of mitotic HeLa cell expressing H2B-mCherry.

Original Live-cell movie of HeLa cell shown in Fig. 6b (Control). Exposures of 0.05 sec with a 2 x 2 bin were acquired every 3 min.

Supplementary Video 2: Live imaging of CENP-E-depleted-mitotic HeLa cell expressing H2B-mCherry.

Original Live-cell movie of CENP-E-depleted HeLa cell shown in Fig. 6b (CENP-E RNAi). Exposures of 0.05 sec with a 2 x 2 bin were acquired every 3 min.

Supplementary Video 3: Live imaging of hMad1-depleted-mitotic HeLa cell expressing H2B-mCherry.

Original Live-cell movie of hMad1-depleted HeLa cell shown in Fig. 6b (hMad1 RNAi example 1). Note that cell exited from mitosis prematurely. Exposures of 0.05 sec with a 2 x 2 bin were acquired every 3 min.

Supplementary Video 4: Live imaging of hMad1-depleted-mitotic HeLa cell expressing H2B-mCherry.

Original Live-cell movie of hMad1-depleted HeLa cell shown in Fig. 6b (hMad1 RNAi example 2). Note that although in the presence of unaligned chromosomes, cell entered

anaphase due to the defective SAC. Exposures of 0.05 sec with a 2 x 2 bin were acquired every 3 min.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Supplementary
Figure 1

Supplementary
Figure 2

Supplementary
Figure 3

Supplementary
Figure 4

Supplementary
Figure 5

Supplementary
Figure 6

Supplementary
Figure 7

Supplementary
Figure 8

Fig. 1f

Fig. 4f

Fig. 7b

Fig. 7d

Fig. 7e

Supplementary Fig. 4a

Supplementary Fig. 6a