A mitotic role for Mad1 beyond the spindle checkpoint

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Summary
Unattached kinetochores generate an anaphase inhibitor, through the spindle assembly checkpoint (SAC), that allows cells more time to establish proper kinetochore–microtubule (K–MT) linkages and thus avoid aneuploidy. Mad1 is the receptor for Mad2 at kinetochores, where it catalyzes the formation of Mad2–Cdc20 complexes, an essential part of the anaphase inhibitor, but whether it has any other mitotic function is unknown. We have generated a mad1-null mutation in Drosophila. This mutant is SAC defective and Mad2 is no longer localized to either nuclear envelope or kinetochores, but it displays normal basal mitotic timing. Unlike mad2 mutants, which have relatively normal mitoses, mad1 anaphases show high frequencies of lagging chromatids, at least some of which are caused by persistent merotelic linkages. A transgene expressing GFP–Mad1 rescues both the SAC and the anaphase defects. In an attempt to separate the SAC function from the mitotic function, we made a mad1 transgene with a mutated Mad2-binding domain. Surprisingly, this transgene failed to complement the anaphase phenotype. Thus, Mad1 has activity promoting proper K–MT attachments in addition to its checkpoint function. This activity does not require the presence of Mad2, but it does depend on some unknown way on key residues in the Mad2-binding domain of Mad1.

Key words: Mitosis, Spindle assembly checkpoint, Mad2, Dynein, RZZ

Introduction
The kinetochore plays two key roles in mitosis. It is both the platform for attachment of spindle microtubules to chromosomes and the site of action of the spindle assembly checkpoint (SAC) (Santaguida and Musacchio, 2009). Unattached kinetochores generate an inhibitor of anaphase onset, through the SAC, that allows cells more time to establish proper kinetochore–microtubule (K–MT) linkages and thus avoid the generation of aneuploid daughters. Upon mitotic entry, the proteins required for the SAC are recruited to unattached kinetochores. In response to proper K–MT attachment, the SAC apparatus is dismantled and the source of the anaphase inhibitor is shut off.

Mad1 is one of the six original checkpoint proteins first identified in yeast (along with Mad2, Bub1, Bub3, Mad3/BubR1, Mps1). It is usually described as the ‘receptor for Mad2’ at the kinetochore (Chen et al., 1998; Musacchio and Salmon, 2007; Shah et al., 2004) and indeed its role in generating the anaphase inhibitor is crucial. In current models, a kinetochore-bound Mad1–Mad2 ‘core complex’ catalyzes the formation of Mad2–Cdc20 complexes, which are an essential part of the anaphase inhibitor (De Antoni et al., 2005; Mapelli et al., 2007; Sironi et al., 2002; Yang et al., 2008). Moreover, the dynein-mediated removal of Mad1–Mad2 from attached kinetochores (shedding) is believed to contribute to checkpoint shutoff (Howell et al., 2001; Wojcik et al., 2001). Somewhat surprisingly, relatively little is known about how Mad1 is recruited to and released from kinetochores nor is it known whether Mad1 has any other mitotic role besides as a component of the SAC.

It is now clear that many SAC proteins have additional activities necessary for normal mitosis. In fact, Mad2 is, for the moment, the only SAC component that does not appear to have a second function promoting efficient mitosis (Buffin et al., 2007; Rahmani et al., 2009). More typical are those SAC proteins, such as Bub1 and BubR1, that also promote proper K–MT attachment (Lampson and Kapoor, 2005; Meraldi and Sorger, 2005). Specific mutations of these proteins can affect either their SAC or kinetochore activities (Buffin et al., 2007; Elowe et al., 2009; Klebig et al., 2009; Rahmani et al., 2009).

This multifunctionality of SAC proteins can be easily detected genetically in the Drosophila model system, because kinetochore capture and alignment on the spindle is normally so efficient in flies that, even without a functional SAC (such as in a mad2-null mutant), mitosis is nearly normal and chromosomes usually segregate accurately (Buffin et al., 2007; Rahmani et al., 2009). By contrast, any mutation reducing this efficiency leads to mitotic errors and often death in SAC-deficient flies. This explains why null mutations of most SAC components (Bub3, BubR1, Mps1, RZZ) lead to aneuploidy and are generally lethal in flies, whereas mad2-null mutants are euploid and healthy: a null mutation in a multifunctional SAC component simultaneously reduces the efficiency of mitosis and eliminates the SAC.

Because of this property of fly mitosis, Drosophila provides a sensitive assay for determining non-checkpoint functions of ‘checkpoint’ proteins. If the mitotic phenotype of a given SAC mutation is more severe than that of a mad2-null mutant, then it indicates that the protein in question normally provides at least one additional activity contributing to mitotic function.

To better understand the contribution of Mad1 to mitosis, we have generated and characterized a mad1-null mutation in Drosophila. As expected, it is checkpoint deficient and Mad2 is no longer recruited to kinetochores. However, in the absence of Mad1, dividing cells frequently display lagging chromatids at anaphase, at least some of which are due to merotelic linkages persisting into anaphase. Thus, Mad1 seems to contribute directly or indirectly to the establishment of proper K–MT linkages.

Results
A mad1-null mutation and a functional fluorescent mad1 transgene
Because Mad1 is a relatively understudied component of the metazoan SAC, we generated a mad1-null mutation in Drosophila (Flybase CG2072/Tpx181-like) by imprecise excision of a P transposon in a neighboring gene. This allele (called here mad1")
lacks the entire promoter region and the first 272 amino acids of the Mad1 coding sequence. No detectable protein is expressed. (Fig. 1; supplementary material Fig. S1, see also Materials and Methods). About 20% of homozygote mad1null animals reached adulthood, the rest dying as late pupae. Surviving flies had slightly rough eyes; females were fertile, males were sterile. To eliminate possible effects from homozygous loci unrelated to mad1, we also examined flies transheterozygous for mad1null and Df(2R)w45-30n, an independently generated large deletion spanning several genes around mad1. The mitotic phenotypes described below were identical in mad1null homozygotes and in mad1null/Df(2R)w45-30n flies, confirming the amorphic nature of mad1null. However, 100% of mad1null/Df(2R)w45-30n survived to adulthood in uncrowded conditions and males showed improved fertility. We conclude that the mad1null chromosome contains other mildly deleterious mutations at other loci, affecting viability and male fertility (see also Materials and Methods, and supplementary material Table S1).

We made several different transgenic constructs linking GFP or Cherry to either the N-terminal or C-terminal ends of wild-type Mad1 controlled by its natural promoter (see Materials and Methods; supplementary material Fig. S1). All were fully functional, rescuing in a single copy the lethality, the rough eyes and the mitotic defects of mad1null homozygotes described below (supplementary material Table S1 and data not shown). The fluorescently tagged mad1null transgene recapitulated the distribution of Mad1 previously described using antibodies in Drosophila (Katsani et al., 2008; Lince-Faria et al., 2009) and has a similar distribution to that seen in mammalian cells. Mad1 associates with nuclear envelope and nucleoplasm during interphase, but localizes to kinetochores at mitotic entry. As kinetochores attach, Mad1 begins to shed, or stream, along the K-fibers (Fig. 2A). To our knowledge, this is the first visualization of Mad1 streaming, as has previously been described for Mad2 and RZZ (Buffin et al., 2005; Howell et al., 2000). By anaphase onset, kinetochore-bound Mad1 levels, like those of Mad2, are nearly undetectable. The distribution of Mad1 in mad2null mutant cells (supplementary material Fig. S1). By contrast, in mad1null cells, Mad2 is uniformly distributed in the nucleus and cytoplasm (Fig. 2B), both in interphase and in mitosis, as has been described following RNAi knockdown of Mad1 in vertebrate cells (Luo et al., 2002). This result argues that Mad2 depends principally or entirely on Mad1 for its subcellular localization.

No obvious change in the kinetochore recruitment of RZZ component Rough Deal, Ndc80 complex component Spc25, Mps1, Aurora-B or BubR1 was observed in mad1null (Fig. 3; supplementary material Fig. S3; data not shown).

**mad1 mitotic phenotype: abnormal anaphases and merotelic attachments**

As expected, mad1null mutants lacked a functional SAC (supplementary material Table S1). The mitotic index of mad1null mutant larval brains did not increase after incubation with the microtubule-depolymerizing drug colchicine, in a manner similar to mutants of other SAC components (Rahmani et al., 2009). As is the case for mad2 Drosophila mutants (Buffin et al., 2007), aneuploid cells were very rare (less than 1%, data not shown). Expression of GFP- or Cherry-tagged mad1null transgenes restored a functional SAC (supplementary material Table S1).

Observation of both fixed and live mad1null mutant cells revealed a high frequency of abnormal anaphases. By live cell imaging, about 70% of the monitored mad1null anaphases had one or more severely lagging kinetochores (Table 1) (Fig. 3; supplementary material Fig. S4 and Movie 7). This profound perturbation of anaphase is not seen in the majority of mad2 mutant cells (Buffin et al., 2007; Lince-Faria et al., 2009) (Table 1) (Fig. 3A; supplementary material Movie 1).

In wild-type cells, nearly all Mad1 is removed from attached kinetochores by the time of anaphase onset. It thus seemed likely that the anaphase problems in the mad1null mutant were the consequence of defects occurring during prometaphase or metaphase, when levels of kinetochore-bound Mad1 are more significant. One possible source of lagging chromatids is from chromosomes that have failed to align at the metaphase plate when anaphase starts. However, only about 13% of mad1null cells had unaligned kinetochores at anaphase onset. Thus, a failure to congress cannot explain the high frequency of laggards in anaphase nor can it explain the slow migration of the severely lagging chromatids.

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**Fig. 1. Schematic of the mad1 genomic region, the mad1null mutation and the transgenes used in this study.** The position of the N- or C-terminal GFP and Cherry tags is indicated on the transgenes. The mad1null mutation is a 2.2 kb deletion generated by imprecise excision of the P transposon EY12060. The rep2 mad1 null transgene was used to generate the mad1null–KAP4 point mutation in the Mad2-binding domain of Mad1 (marked by a star).

**Fig. 2. Behavior of fluorescently tagged Mad1 and Mad2.** (A) Mad1–GFP labels nuclear envelope, nucleoplasm and kinetochores, and sheds along K–MTs during mitosis. Scale bar: 2 μm. (B) Top: typical GFP–Mad2 labeling in wild-type (mad1+/+) interphase and mitotic cells. Middle: GFP–Mad2 no longer localizes to discrete subcellular structures in a mad1null homozygous mutant cell. Bottom: same cell as in middle, Spc25–RFP marks kinetochores (right). Scale bars: 2 μm.
Inadequate dynein motor activity might also contribute to aberrant anaphase migration (Varma et al., 2008; Yang et al., 2007). We therefore asked whether dynein activity was compromised in mad1 mutant cells. Using a transgene encoding GFP–DLIC2 (dynein light intermediate chain 2), we found that dynein was present on lagging chromatids, at approximately the same levels as found in wild type (supplementary material Fig. S3B). Moreover, the poleward transport (‘shedding’) of GFP–Rod, a dynein-dependent process (Karess, 2005; Wojcik et al., 2001), seemed normal (supplementary material Fig. S3C and Movie 6). Taken together, these results suggest that overall kinetochore-bound dynein function seems to be unaffected by the absence of Mad1 and therefore dynein perturbation probably does not contribute to the observed phenotypes.

Merotely, the attachment of a single kinetochore to both poles of the spindle, is another common source of aberrant chromatid migration (Cimini et al., 2001). In mammalian cells, lagging chromatids are believed to be generated almost entirely by merotelic attachments. To determine whether lagging chromatids in mad1 mutants were merotically attached, we examined neuroblasts expressing RFP–Spc25-labeled kinetochores and GFP-tagged spindles (using a GFP insertion allele of the microtubule-associated protein Jupiter) (Fig. 3B–D and Fig. 4A). In favorable cell preparations, severely lagging chromatids were seen associated with K-fibers emanating towards both spindle poles (Fig. 3B; supplementary material Movie 2). In some cases, K-fibers of lagging chromatids were observed to maintain a constant length with K-fibers emanating towards both spindle poles (Fig. 3B; supplementary material Movie 2). Another commonly observed behavior is that one lagging K-fiber fails to shorten at all during the first part of anaphase and then suddenly shortens, which might reflect the severing of an opposing K-fiber. An example of this behavior is shown in the anaphase cell in Fig. 3C (see supplementary material Movie 3). A mad1 cell starting in prometaphase, in which a quartet of associated kinetochores (probably a pair of homologs) generates a pair of lagging non-sister kinetochores that are apparently merotically linked. (Insets: based on their subsequent disjunction, the sister kinetochores can be identified, and are coded blue and green; the non-sisters who will lag are circled in red.) The four kinetochores move as a group and align correctly on the metaphase plate. At anaphase onset, one sister of each pair seems to stick together. As anaphase progresses, one lagging kinetochore (white arrow) is associated with a K-fiber (yellow arrow), which appears to be a merotelic link. A second MT bundle (yellow arrowhead) appears to link the two lagging sisters. See also supplementary material Movie 4.

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Fig. 3. Merotically attached lagging kinetochores are common in mad1 mutant cells. Images were acquired at the indicated time points (minutes:seconds), which are relative to anaphase onset (T=0). Kinetochores are marked with RFP–Spc25 (red), spindles with GFP–Jupiter (green). Scale bars: 2 μm. (A) Near-normal anaphase migration in mad2-null mutant neuroblasts. See also supplementary material Movie 1. (B) A mad1 cell with lagging kinetochores. One (marked by the arrow) is clearly associated with K-fibers emanating towards both spindle poles. See also supplementary material Movie 2. (C) Another mad1 cell. In this example, a lagging kinetochore (boxed in frame 1), associated with K-fibers extending to both poles, remains stationary during the first part of anaphase and become noticeably stretched (insets). At T=03:30, it begins to move, possibly caused by the rupture of the merotelic K-fiber (arrow). Concomitantly, the kinetochore recovers its round shape. This behavior is typical of merotelic kinetochores in mammalian cells (Cimini et al., 2001). See also supplementary material Movie 3. (D) A mad1 cell starting in prometaphase, in which a quartet of associated kinetochores (probably a pair of homologs) generates a pair of lagging non-sister kinetochores that are apparently merotically linked. (Insets: based on their subsequent disjunction, the sister kinetochores can be identified, and are coded blue and green; the non-sisters who will lag are circled in red.) The four kinetochores move as a group and align correctly on the metaphase plate. At anaphase onset, one sister of each pair seems to stick together. As anaphase progresses, one lagging kinetochore (white arrow) is associated with a K-fiber (yellow arrow), which appears to be a merotelic link. A second MT bundle (yellow arrowhead) appears to link the two lagging sisters. See also supplementary material Movie 4.
Table 1. Abnormal anaphases in mad1 and mad2 mutant cells

| Genotype     | n  | Percentage of cells with at least one laggard | Average number of laggards per cell with at least one laggard | Percentage of cells with a bent spindle | Percentage of cells with a clear merotelic chromosome |
|--------------|----|-----------------------------------------------|---------------------------------------------------------------|-----------------------------------------|-------------------------------------------------------|
| Wild type    | 16 | 12.5                                          | 1.0                                                           | 6.3                                     | 0.0                                                   |
| mad1         | 38 | 68.4                                          | 2.2                                                           | 44.7                                    | 28.9                                                  |
| mad1; mad2   | 13 | 61.5                                          | 2.3                                                           | 61.5                                    | ND                                                    |
| mad1-KAPA    | 18 | 66.7                                          | 3.4                                                           | 55.6                                    | 33.3                                                  |
| mad2         | 32 | 28.1                                          | 1.8                                                           | 0.0                                     | 3.1                                                   |

*All cells expressed the kinetochore marker RFP–Spc25 and microtubule marker GFP–Jupiter. ND, not determined.

Mitotic timing was no faster in mad1-null neuroblasts (average 7.6 minutes) than in the wild type (7.7 minutes for the peak time) (Fig. 5) (see Materials and Methods for details). By contrast, timing for mad2 mutants averaged 6.2 minutes (P<0.005). Thus, Mad1 does not seem to participate in basal timing and the mitotic abnormalities we observed are unlikely to be caused by inappropriately short prometaphase.

**Mutations in the Mad2-binding domain of Mad1 also affect anaphase**

The mitotic phenotypes described above indicate that, in addition to its role in the SAC, Mad1 normally confers an activity required for proper anaphase chromosome behavior, possibly affecting kinetochore function in the generation of correct K–MT attachments or in the repair of improper attachments. It was formally possible, however, that the observed phenotypes were not caused by the lack of Mad1 per se, but rather by the consequent abnormally high pool of free Mad2, which, in a wild-type cell, would normally be tightly bound to Mad1. This explanation predicts, however, that a mad1; mad2 double mutant would resemble the mad2 mutant, which is viable, fertile and has near-normal mitoses despite the complete absence of SAC activity. In fact, the double mutants are only poorly viable and have at least as many anaphase abnormalities as in mad2 mutants alone (supplementary material Table S1). This result excludes the alternative explanation and also reinforces our conclusion that Mad1 has SAC-independent activity.

The Mad1–Mad2 core complex catalyzes the formation of Cdc20–Mad2, a component of the anaphase inhibitor (De Antoni et al., 2005; Simonetta et al., 2009; Sironi et al., 2002; Yang et al., 2008). Sironi et al. (Sironi et al., 2002) delimited the Mad2-binding region of mammalian Mad1 to residues 486–565 and further identified two crucial residues in this region, K541 and P549.
Mad11 (called here Mad1-KAPA) was expressed from a transgene in a function, we mutated both K552 and P560 to alanine. This protein would remove the SAC activity of Mad1 but retain its kinetochore of lagging chromatids is greatly elevated over that seen in mad2-null neuroblasts. At least some of these lagging chromatids are caused by persistent merotelic linkages.

Null mutations in most SAC components in Drosophila are lethal, because they lead to greatly increased rates of aneuploidy, precisely because they reduce the efficiency of the mitotic apparatus to capture kinetochores and simultaneously eliminate the surveillance system that might catch mitotic errors. The sole exception is Mad2, which has no detectable role in mitosis except as a component of the SAC and the timer. Mad1 presents an intermediate case, as mutant mad1 is still viable and aneuploidy is still infrequent, but nevertheless the mitotic apparatus is compromised. One likely explanation for the relatively benign phenotype of mad1 lies in the nature of the mitotic defects. Lagging chromatids still usually migrate to the correct poles. In fact, in mammalian cells, merotelically attached chromatids often segregate correctly, after a delay (Cimini et al., 2004). By contrast, chromosome attachment failure is far more likely to lead to aneuploidy.

Our conclusion that Mad1 does have a SAC-independent mitotic function is reinforced by the fact that, unlike mad2 mutants, mad1 cells do not have a faster basal mitotic timer. That is, there are more merotelic and anaphase abnormalities in mad1 mutants than in mad2 mutants, even though mad2 cells have a shorter prometaphase (and thus would have less time to correct attachment errors). The increased lethality of mad1; mad2 double homozygotes is most likely explained by simultaneous increase in aberrant K–MT linkages and shortening of basal timing.

Lagging chromatids at anaphase have been reported after siRNA depletion of Mad1 in mammalian cells (Luo et al., 2002; Meraldi et al., 2004). However, these studies could not distinguish whether this phenotype was specifically due to Mad1 depletion or just a consequence of a compromised SAC, because the phenotype of inactivated Mad2 also generates highly abnormal anaphases in cultured mammalian cells. On the other hand, Mad1 inactivation has no effect at all in sensitive mitotic assays of the early Caenorhabditis elegans embryo (Essex et al., 2009; Kitagawa, 2009). This might be a consequence of the holocentric nature of worm chromosomes.

The mutant phenotype we describe here demonstrates that Mad1 is required in Drosophila not only for a functional SAC, but also for a fully functional mitotic apparatus. In its absence, the frequency of lagging chromatids is greatly elevated over that seen in mad2-null neuroblasts. At least some of these lagging chromatids are caused by persistent merotelic linkages.

In madi neuroblasts expressing madi-KAPA, Mad2 did not localize to any subcellular structure. In fact, Mad2 was uniformly distributed throughout the cell during both interphase and mitosis, as it is in the madi mutant (Fig. 6B). This confirms that the mutated Mad1 residues are required for Mad2 localization in vivo. Consistent with the crucial role for the Mad1–Mad2 core complex in activating the checkpoint, madi-KAPA was checkpoint defective (supplementary material Table S1).

Unexpectedly, the mitotic phenotype of madi-KAPA was similar to that of the madi-null mutant itself (Fig. 6A) (Table 1). The frequency of abnormal anaphases remained very high and lagging chromatids were found with merotelic linkages. This failure to restore normal anaphase (data not shown). The simplest, although somewhat surprising, explanation is that the Mad2-binding region of Mad1 is involved not only in binding Mad2, but also in some other activity important for proper kinetochore function.

Discussion

The mutant phenotype we describe here demonstrates that Mad1 is involved in activating the checkpoint, mad1-KAPA cannot rescue the aberrant anaphases of madi. (A) Asynchronous anaphases in madi cells expressing GFP–Mad1-KAPA. Top: kinetochores marked by RFP–Spc25. Bottom: the mutant Mad1-KAPA protein behaves like wild-type Mad1. It is correctly recruited to kinetochores in prometaphase and disappears prior to anaphase onset. Images were acquired at the indicated time points (minutes:seconds), which are relative to anaphase onset (T=0). Scale bar: 2 μm. See also supplementary material Movie 5. (B) Mad2 cannot bind to Mad1-KAPA. In mad1-KAPA cells, Mad2 does not localize to any subcellular structure. The dotted circle at bottom left outlines an interphase cell and the dotted circle top right outlines a mitotic cell. Scale bar: 2 μm.
(Bakhoum et al., 2009). Instability seems to be important for efficient error correction and the Aurora-B-dependent error-correction mechanism is at least partly based on increasing K–MT turnover rates (Cheeseman et al., 2006; Cimini et al., 2006). Experimental perturbation of K–MT attachment stability has demonstrated a link between inappropriate hyperstability and increased rates of merotely (Bakhoum et al., 2009).

One way to understand the increased frequency of merotely in mad1 mutants is to imagine that Mad1 normally helps maintain proper levels of K–MT instability. The relatively high incidence of bent spindles we observe in mad1 mutants might also be a consequence of inappropriate MT stability. Indeed, bent spindles were also observed after perturbation of microtubule regulators such as the microtubule plus-end tracking proteins (+TIPs) Orbit/CLASP and APC, Aurora kinases, the dynein-associated protein nudE, and kinesins Klp67A and Ncd (Endow et al., 1994; Gandhi et al., 2004; Inoue et al., 2000; Rusan et al., 2008; Savoian et al., 2004; Wang et al., 2006). The natural decline in kinetochore-bound Mad1 by anaphase onset would then help to stabilize K–MT attachments, but in the mad1 mutant, prometaphase kinetochores might have inappropriate ‘anaphase levels’ of K–MT stability, thus leading to increased merotely. Mad1 might do this by directly affecting MT binding, because mammalian Mad1 reportedly interacts with the Hecl subunit of the Ndc80 complex according to the two-hybrid assay (Martin-Lluesma et al., 2002). Alternatively, Mad1 might in some way promote the error-correction activity of Aurora-B.

Clearly identifiable merotelic attachments could not be found on all severely lagging kinetochores. This might be partly because Drosophila kinetochores have fewer MTs than typical mammalian kinetochores (Maiato et al., 2006), and the ability to detect a merotelic K-fiber is partly dependent on the number of MTs involved in the linkage. In any case, a merotelic attachment consisting of a single MT would not be detectable. However, there might well be other defects in mad1 mutant cells that also contribute to the observed abnormalities. The fact that we see anaphase bridges (Fig. 4B) suggests that the absence of Mad1 contributes in some indirect way to inappropriate associations of chromosomes.

Our data predict intriguingly that crucial residues required for this non-SAC mitotic junction reside in the Mad2-binding domain of Mad1. In normal cells, this domain might never be ‘empty’, as Mad1 is believed to be saturated by bound Mad2 during both interphase and mitosis (Shah et al., 2004). Only in the abnormal situation of a mad2-null mutant would the domain be unoccupied. However, mad2 cells do not display the profound disruption of chromatid movement seen in mad1-KAPA, and thus this phenotype cannot be a consequence of simply having an unoccupied binding site. Although a portion of the Mad1–Mad2 core complex structure has been solved (Sironi et al., 2002), its geometry with respect to other kinetochore components is unknown. An interaction between residues forming the Mad2-binding domain and, for example, the Ndc80 complex, which binds microtubules, is thus not excluded. Disrupting such an interaction might explain the phenotype of mad1-KAPA.

The mad1 allele phenotype calls into question certain assumptions about the mitotic phenotypes associated with depleting the RZZ complex and dynein. RZZ is needed for kinetochore recruitment of both dynein and Mad1–Mad2 (Buffin et al., 2005; Karess, 2005; Kops et al., 2005; Starr et al., 1998). It had been assumed that the aberrant anaphases characteristic of RZZ mutant phenotypes reflected the lack of kinetochore-bound dynein. However, the fact that Mad1 itself affects chromosome segregation suggests that part of the RZZ mutant phenotype previously ascribed to the failure to recruit dynein to kinetochores might in fact be due to kinetochore changes caused by failure to recruit Mad1. Furthermore, dynein has been implicated in the maintenance or establishment of stable end-on K–MT attachments (Varma et al., 2008). As removal of Mad1 from attached kinetochores depends on dynein activity, interfering with this removal might be a contributing explanation for the effect of dynein on stable attachment formation.

Finally, our finding that basalt mitotic timing is not accelerated in mad1-mutant cells has implications for the nature of the timer. Assuming that the timer reflects the presence of Mad2–Cdc20 complexes formed independently of the SAC, this result suggests that Mad1 does not participate in the generation of such complexes outside of the unattached kinetochore, even though Mad1 is part of the catalyst for the generation of Mad2–Cdc20 complexes as part of the SAC. Perhaps only a low threshold level of Mad2–Cdc20 suffices for normal timing, a level obtained in the absence of the Mad1 catalyst.

This work has revealed that Mad1 contributes to the proper functioning of mitosis, in addition to its role as the kinetochore-bound receptor for Mad2. It presumably does so by interacting with other kinetochore components, in part through residues in the Mad2-binding domain. However, although kinetochore recruitment of Mad1 depends on the activity of the RZZ complex (Buffin et al., 2005; Kops et al., 2005), Mps1 (Liu et al., 2003), Ndc80 complex (Martin-Lluesma et al., 2002) and Bub1 (Brady and Hardwick, 2000; Sharp-Baker and Chen, 2001), no clear kinetochore-binding molecular partners of Mad1 are known. Obtaining such information will be important to determining how it carries out its dual roles.

Materials and Methods

Generation of a mutant mad1 allele

The mad1 allele was generated by imprecise excision of the P-element P(E-Pyg-2)EY12060 inserted in the adjacent Rep2 gene (CG1975). This original P-element insertion was homozygous and viable. Imprecise excision was identified by a white eye color and was sequenced by PCR using primers corresponding to genomic sequences flanking the insertion region. The deletion is 2225 base pairs long, starting from the P-element insertion site, and encompasses the entire intergenic region (including the promotor) and the first 814 base pairs of the mad1 coding sequence, corresponding to the first 272 of the 730 codons of mad1.

Rep2 encodes a predicted caspase-activated nuclease. No phenotype is associated with any of the P-transposon insertions in the Rep2 gene, as described in Flybase. There are two annotated transcription start sites, but only the first transcript should be affected by the mad1 deletion.

The following experiments exclude a contribution of Rep2 to the mitotic phenotype we describe for the mad1 allele. We made a transgenic construct encoding the entire Rep2 gene. This transgene does not complement the checkpoint defect or the anaphase defect of homozygous mad1 flies. By contrast, a transgene encoding a GFP–Mad1 fusion protein rescued both the checkpoint and anaphase defects of mad1 flies.

Homozygous mad1 male flies are entirely sterile. The following evidence argues that this sterility is unrelated to the lesion in mad1. Neither the Rep2 transgene nor the GFP–mad1 transgene, nor both together complemented the male sterility. Flies transheterozygous for mad1/Df(2R)45-30b displayed abnormal anaphases and checkpoint failure indistinguishable from that seen in mad1 homozygotes, but male survivors consistently showed some (low) fertility. Normal fertility in these animals was restored by the GFP–mad1 transgene, but not by the Rep2 transgene. We conclude that the total male sterility of mad1 homozygotes is unrelated to mad1 or Rep2, but is caused by a linked mutation outside the genomic region delimited by Df(2R)45-30b.

mad1 transgenes

Several transgenes carrying the mad1 gene and its endogenous promoter fused or not with a fluorescent tag have been constructed. An mCherry tag was fused to the N or C terminus of Mad1; a GFP tag was fused only to the C terminus. These three transgenes were cloned into pCasper4, which inserts randomly in the fly genome. Transgenes rep2, rep2 mad1, rep2 gfp::mad1, rep2 mcherry::mad1, rep2 mad1-KAPA, rep2 and rep2 gfp::mad1-KAPA were cloned into P-element constructs obtained from Johannes Bischof (Institute for Molecular Biology, Zurich, Switzerland). This vector...
allows site-directed integration using the PhiC31 system (Bischoff et al., 2007). The four wild-type constructs were inserted in yw flies at site 2A, whereas the four mutant constructs were inserted at site 86F.

**Western blot analysis**

Western blots of mad11 mutant and transgenic mad11 variants were performed as previously described (Buffin et al., 2007) using mouse anti-Mad1 (Katsanis et al., 2008). In brief, protein extracts from 50 to 60 brains of wild type, mad1-KAP4A and GFP-mad1 (each expressed in the GFP–mad1 background), and mad1 homozygous or mad1/Df(2R)A45-30n third instar larvae were loaded onto 10% SDS-acrylamide gels and electroblotted to nitrocellulose membrane. Membranes were blocked for 1 hour in TBST (TBS and 0.1% Tween) with 3% dry milk and incubated overnight at 4°C with anti-Mad1 diluted at 1:200 in TBST plus 1% milk. After washing in TBST, the blot was incubated for 1 hour at room temperature with secondary antibody goat anti-mouse IgG conjugated with horseradish peroxidase. After washing in TBST, the blot was incubated for 1 hour at room temperature with secondary antibody goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000, Bio-Rad Laboratories). Immunodetection was performed with the SuperSignal kit (Thermo Fisher Scientific). Membranes were stripped and incubated with mouse anti-ubiquitin (1:4000, Sigma) as a loading control.

**Cytology**

For simple cytology of mitotic cells, third instar larval brains were fixed and stained in aceto-orcein as described previously (Buffin et al., 2007).

**In vivo observation of larval neuroblasts**

The kinetics of mitosis in living larval neuroblasts was determined as previously described (Buffin et al., 2005). Fluorescent time-lapse movies were acquired with an Olympus IX-70 inverted microscope, a focused Xenon lamp and a Hamamatsu OrcaER camera, piloted by the Olympus Cell-R hardware and software system. Acquisition times per frame were between 50 ms and 100 ms. Stacks of 3 to 7 planes at 0.5 µm intervals were collected every 15 seconds with a 60x NA 1.4 objective. Images depicted are maximum intensity projections and have been adjusted for brightness and contrast. Microtubules were visualized using the GFP-Jupiter fusion allele (Karpova et al., 2006). Kinetochores were labeled by expression of an Spc25–mRFP1 transgene (Schnittlenn et al., 2007).

For mitotic timing, quantification was performed as described (Buffin et al., 2007); Rahmani et al. (2009). NEB was defined as when the GFP–Rd signal began to be visible on kinetochores. Anaphase onset was defined as the moment sister kinetochores (marked with the GFP–Rod signal) began to separate. The basal mitotic time, or ‘peak time’, is the average of wild-type cells excluding the slowest 20%, which are delayed by the SAC. The retained 80% (the ‘peak’) can be fit to a normal curve assuming a homogenous population lacking the SAC [see discussion in Meraldi et al. (Meraldi et al., 2004)].

**References**

Bakoum, S. F., Thompson, S. L., Manning, A. L. and Compton, D. A. (2009). Genistein stability is ensured by temporal control of kinetochores-microtubule dynamics. *Nat. Cell Biol.* 11, 27-33.

Bischoff, J., Maeda, R. K., Hediger, M., Karch, F. and Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proc. Natl. Acad. Sci. USA* 104, 3312-3317.

Brady, D. M. and Hardwick, K. G. (2000). Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. *Curr. Biol.* 10, 675-687.

Buffin, E., Lifeline, C., Huang, J., Gagou, M. E. and Karess, R. E. (2004). Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. *Curr. Biol.* 15, 1233-1250.

Buffin, E., Emre, D. and Karess, R. E. (2007). Flies without a spindle checkpoint. *Nat. Rev. Mol. Cell Biol.* 8, 59-71.

Macleijowski, J., George, K. A., Terret, M. E., Zhang, C., Shkatov, K. M. and Jallepalli, P. V. (2010). Mps1 directs the assembly of Cdc20 inhibitory complexes during interphase and mitosis to control M phase timing and spindle checkpoint signaling. *J. Cell Biol.* 190, 89-100.

Maistro, H., Hergert, P. J., Mouhtinho-Pereira, S., Dong, Y., Vandenbeldt, K. J., Rieder, C. L. and McEwen, B. F. (2006). The ultrastructure of the kinetochore and kinetochore fiber in Drosophila somatic cells. *Chromosoma* 115, 469-480.

Meraldi, P., Massimiliano, L., Santaguida, S. and Musacchio, A. (2007). The Mad2 spindle checkpoint protein facilitates chromosome segregation and chromosome congression. *Curr. Biol.* 17, 2149-2155.

McKee, B. D. (2004). Homologous pairing and chromosome dynamics in meiosis and mitosis. *Biochim. Biophys. Acta* 1677, 165-180.

Meraldi, P. and Seger, P. K. (2005). A dual role for Bub1 in the spindle checkpoint and chromosome congression. *Curr. Biol.* 15, 1621-1633.

Meraldi, P., Draviam, V. M. and Seger, P. K. (2004). Timing and checkpoints in the spindle-assembly checkpoint. *Curr. Biol.* 14, 2149-2155.

Karpova, N., Bobinnec, Y., Fouix, S., Huitorel, P. and Debec, A. (2008). Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. *Curr. Biol.* 18, 301-312.

Klebig, C., Corinith, K. and Meraldi, P. (2009). Bub1 regulates chromosome segregation in a kinetochore-independent manner. *J. Cell Biol.* 185, 841-858.

Kops, G. J., Kim, Y., Weaver, B. A., Yao, Y., McLeod, L., Yates, J. R., 3rd, Tagaya, M. and Cleveland, D. W. (2005). ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J. Cell Biol.* 169, 49-60.

Lampson, M. A. and Kapoor, T. M. (2005). The human mitotic checkpoint protein Bub3 regulates chromosome-spindle attachments. *Nat. Cell Biol.* 7, 93-98.

Liu, S. T., Chan, G. K., Hittle, J. C., Fujii, G., Lees, E. and Yen, T. J. (2003). Human MPS1 kinase is required for mitotic arrest induced by the loss of CENP-E from kinetochores. *J. Cell Biol.* 165, 647-657.

Liao, X., Tang, Z., Rizo, J. and Yu, H. (2002). The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Mol. Cell* 9, 59-71.

Meraldi, P., Draviam, V. M. and Sorger, P. K. (2004). Timing and checkpoints in the spindle-assembly checkpoint. *Curr. Biol.* 14, 2149-2155.

McKee, B. D. (2004). Homologous pairing and chromosome dynamics in meiosis and mitosis. *Biochim. Biophys. Acta* 1677, 165-180.

Meraldi, P. and Seger, P. K. (2005). A dual role for Bub1 in the spindle checkpoint and chromosome congression. *Curr. Biol.* 15, 1621-1633.

Meraldi, P., Draviam, V. M. and Seger, P. K. (2004). Timing and checkpoints in the mitigation progression. *Dev. Cell* 7, 45-60.

Musacchio, A. and Salmon, E. D. (2007). The spindle-assembly checkpoint in space and time. *Nat. Rev. Mol. Cell Biol.* 8, 338-344.

Kops, G. J., Kim, Y., Weaver, B. A., Yao, Y., McLeod, L., Yates, J. R., 3rd, Tagaya, M. and Cleveland, D. W. (2005). ZW10 links mitotic checkpoint signaling to the structural kinetochore. *J. Cell Biol.* 169, 49-60.

Lampson, M. A. and Kapoor, T. M. (2005). The human mitotic checkpoint protein Bub3 regulates chromosome-spindle attachments. *Nat. Cell Biol.* 7, 93-98.
Rusan, N. M., Akong, K. and Peifer, M. (2008). Putting the model to the test: are APC proteins essential for neuronal polarity, axon outgrowth, and axon targeting? J. Cell Biol. 183, 203-212.

Santaguida, S. and Musacchio, A. (2009). The life and miracles of kinetochores. EMBO J. 28, 2511-2531.

Savoian, M. S., Gatti, M. K., Riparbelli, M. G., Callaini, G. and Glover, D. M. (2004). Drosophila Klp67A is required for proper chromosome congression and segregation during meiosis I. J. Cell Sci. 117, 3669-3677.

Schittenhelm, R. B., Heeger, S., Althoff, F., Walter, A., Heidmann, S., Mechtler, K. and Lehner, C. F. (2007). Spatial organization of a ubiquitous eukaryotic kinetochore protein network in Drosophila chromosomes. Chromosoma 116, 385-402.

Shah, J. V., Botvinick, E., Bonday, Z., Furnari, F., Berns, M. and Cleveland, D. W. (2004). Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. Curr. Biol. 14, 942-952.

Sharp-Baker, H. and Chen, R. H. (2001). Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. J. Cell Biol. 153, 1239-1250.

Simonetta, M., Manzoni, R., Mosca, R., Mapelli, M., Massimiliano, L., Vink, M., Novak, B., Musacchio, A. and Giliberto, A. (2009). The influence of catalysis on mad2 activation dynamics. PLoS Biol. 7, e10.

Sironi, L., Mapelli, M., Knapp, S., De Antoni, A., Jeang, K. T. and Musacchio, A. (2002). Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a ‘safety belt’ binding mechanism for the spindle checkpoint. EMBO J. 21, 2496-2506.

Starr, D. A., Williams, B. C., Hays, T. S. and Goldberg, M. L. (1998). ZW10 helps recruit dynactin and dynein to the kinetochore. J. Cell Biol. 142, 763-774.

Varma, D., Monzo, P., Stehman, S. A. and Vallee, R. B. (2008). Direct role of dynein motor in stable kinetochore-microtubule attachment, orientation, and alignment. J. Cell Biol. 182, 1045-1054.

Wang, Y., Toppari, J., Parvinen, M. and Kallio, M. J. (2006). Inhibition of Aurora kinases perturbs chromosome alignment and spindle checkpoint signaling in rat spermatocytes. Exp. Cell Res. 312, 3459-3470.

Wojcik, E., Basto, R., Serr, M., Scaerou, F., Karess, R. and Hays, T. (2001). Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. Nat. Cell Biol. 3, 1001-1007.

Yang, M., Li, B., Liu, C. J., Tomchick, D. R., Machius, M., Rizo, J., Yu, H. and Luo, X. (2008). Insights into mad2 regulation in the spindle checkpoint revealed by the crystal structure of the symmetric mad2 dimer. PLoS Biol. 6, e50.

Yang, Z., Tulu, U. S., Wadsworth, P. and Rieder, C. L. (2007). Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. Curr. Biol. 17, 973-980.