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Bub1 and Bub3 promote the conversion from monopolar to bipolar chromosome attachment independently of shugoshin

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The eukaryotic spindle assembly checkpoint (SAC) delays anaphase in the presence of chromosome attachment errors. Bub3 has been reported to be required for SAC activity in all eukaryotes examined so far. We find that Bub3, unlike its binding partner Bub1, is not essential for the SAC in fission yeast. As Bub3 is needed for the efficient kinetochore localization of Bub1, and of Mad1, Mad2 and Mad3, this implies that most SAC proteins do not need to be enriched at the kinetochores for the SAC to function. We find that Bub3 is also dispensable for shugoshin localization to the centromeres, which is the second known function of Bub1. Instead, Bub3, together with Bub1, has a specific function in promoting the conversion from chromosome mono-orientation to bi-orientation.
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INTRODUCTION

The spindle assembly checkpoint (SAC) is a signalling mechanism that delays anaphase until all chromosomes have achieved correct attachment to the mitotic spindle (Musacchio & Salmon, 2007). The core components of the SAC, the proteins Mad1, Mad2, Mad3/BubR1, Bub1, Bub3 and Mps1/Mph1, were first identified in budding yeast and their orthologues have subsequently been found in many other eukaryotes. During mitosis, SAC proteins are concentrated at kinetochores that are not attached to microtubules. A common hypothesis suggests that they become 'activated' at the kinetochores and subsequently, on leaving the kinetochore, generate a diffusible checkpoint signal, which ultimately inhibits the anaphase-promoting complex or cyclosome (Peters, 2006). Recent studies have indicated that Mad2 does indeed need to contact a complex of Mad1 and Mad2 at unattached kinetochores, and that Mad2 and Mad3 are directly involved in inhibiting the anaphase-promoting complex or cyclosome (Millband & Hardwick, 2002; De Antoni *et al*, 2005; Nezi *et al*, 2006; Nilsson *et al*, 2008; Kulukian *et al*, 2009). Although Bub1, Bub3 and Mps1 are also found to be present at unattached kinetochores (Musacchio & Salmon, 2007), their function there and in SAC signalling in general, is less defined.

Several proteins with a crucial role in the SAC have additional functions during mitosis. Human BUBR1, BUB1 and BUB3 are required for stable kinetochore–microtubule attachments (Ditchfield *et al*, 2003; Lampson & Kapoor, 2005; Meraldi & Sorger, 2005; Logarinho *et al*, 2008); however, their molecular role in promoting this attachment is not well understood. A function of Bub1 in ensuring correct chromosome segregation has also been described in yeast (Bernard *et al*, 1998; Warren *et al*, 2002). Bub1 is needed for the localization of shugoshin proteins to the centromere (Kitajima *et al*, 2004), which are also involved in promoting correct chromosome attachment (Indjeian *et al*, 2005; Kawashima *et al*, 2007; Vanoosthuyse *et al*, 2007; Kiburz *et al*, 2008). Consequently, it has been assumed that Bub1 promotes correct chromosome segregation through its action on shugoshin (Fernius & Hardwick, 2007; Hauf *et al*, 2007). Bub3 interacts with Bub1 in all eukaryotes examined so far (Musacchio & Hardwick, 2002), but whether Bub3 is also required for shugoshin localization has not yet been assessed.

Here, we show that, unlike its binding partner Bub1, Bub3 in fission yeast is neither essential for shugoshin localization nor for SAC activity. However, fission yeast Bub3 and its association with Bub1 are required for correct chromosome segregation, and our data indicate that Bub3 and Bub1 together promote the transition from monopolar to bipolar chromosome attachment.

RESULTS AND DISCUSSION

Fission yeast Bub3 is not essential for the SAC

To study SAC activity in fission yeast, we established a live-cell imaging assay using Plo1–GFP (green fluorescent protein). Plo1 localizes to spindle pole bodies (SPBs) specifically from the point of entry into mitosis to the onset of anaphase (Bahler *et al*, 1998;

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Mulvihill *et al*, 1999). To create a situation in which the SAC is active, we used the β-tubulin *nda3-KM311* allele (Hiraoka *et al*, 1984). At restrictive temperature, *nda3-KM311* mutant cells are unable to form microtubules and cells show a delay in mitosis that persists for several hours (Fig 1A; supplementary Fig S1A online). When the SAC is inactivated by the deletion of *mad1, mad2* or *bub1*, the mitotic delay cannot be sustained and cells exit mitosis within 1 h. We observed that deleting *bub3* did not have the same effect and such cells were able to remain in mitosis for considerably longer. We obtained similar results when eliciting SAC activity through conditional mutants of kinesin-5 (*cut7-446*) and cohesin (*psc3-1T*; supplementary information online, note 1; supplementary Fig S2 online). *Drosophila* Bub3 has been shown to have a function in cell-cycle progression (Lopes *et al*, 2005). Therefore, we considered the possibility that *bub3*Δ *nda3-KM311* cells remained in mitosis because Bub3 was required for the efficient progression into anaphase. However, the additional deletion of *mad2* in *nda3-KM311 bub3*Δ cells considerably shortened mitosis (Fig 1A), indicating that the SAC had been active in *nda3-KM311 bub3*Δ cells and that Bub3 was not essential for progression into anaphase. Together, this indicates that fission yeast Bub3 is not essential for SAC activity, which is consistent with studies by two other groups (Tange & Niwa, 2008; V. Vanoosthuyse & K. Hardwick, personal communication). By contrast, two previous studies had reported that Bub3 is necessary for SAC function in fission yeast (Millband & Hardwick, 2002; Vanoosthuyse *et al*, 2004), as it is in many other eukaryotes (Hoyt *et al*, 1991; Kalitsis *et al*, 2000; Campbell & Hardwick, 2003; Meraldi *et al*, 2004; Lopes *et al*, 2005). We think that the use of indirect assays to judge SAC function in these earlier studies led to misinterpretation (supplementary information online, note 2).

Fission yeast Bub3 has been reported to bind to Mad3 and Bub1, and to be required for their localization to kinetochores (Millband & Hardwick, 2002; Vanoosthuyse *et al*, 2004; Kadura *et al*, 2005). In addition, we find that Bub3 is required for the kinetochore localization of Mad1 and Mad2 (Fig 1B; supplementary Fig S3 online). This implies that the SAC can function with undetectable levels of these SAC proteins at the kinetochores, which is not entirely consistent with the standard model (Musacchio & Salmon, 2007; Simonetta *et al*, 2009). It also indicates that fission yeast Bub3 remains intimately linked to the SAC and we hypothesize that, even in fission yeast, Bub3 contributes to SAC signalling but not in an essential manner. Interestingly, *Caenorhabditis elegans* Bub3 becomes dispensable for SAC activity when Mad2 levels are subtly elevated (Essex *et al*, 2009). It is therefore possible that Bub3 has a similar role in the SAC of all eukaryotes, but whether it is essential for SAC activity or not depends on other parameters in the SAC signalling network.

Bub3 is required for correct chromosome segregation

Cells lacking Bub3 are sensitive to the microtubule-destabilizing drug benomyl (Millband & Hardwick, 2002; Vanoosthuyse *et al*, 2004), which is a common phenotype of mutants that cause chromosome segregation defects. Indeed, cells lacking Bub3 showed a delay in mitosis, which was dependent on a functional SAC (Fig 2A). When directly monitoring the segregation of chromosome 2 in *bub3*Δ cells by using live-cell imaging, we observed only a slight increase in mis-segregation compared

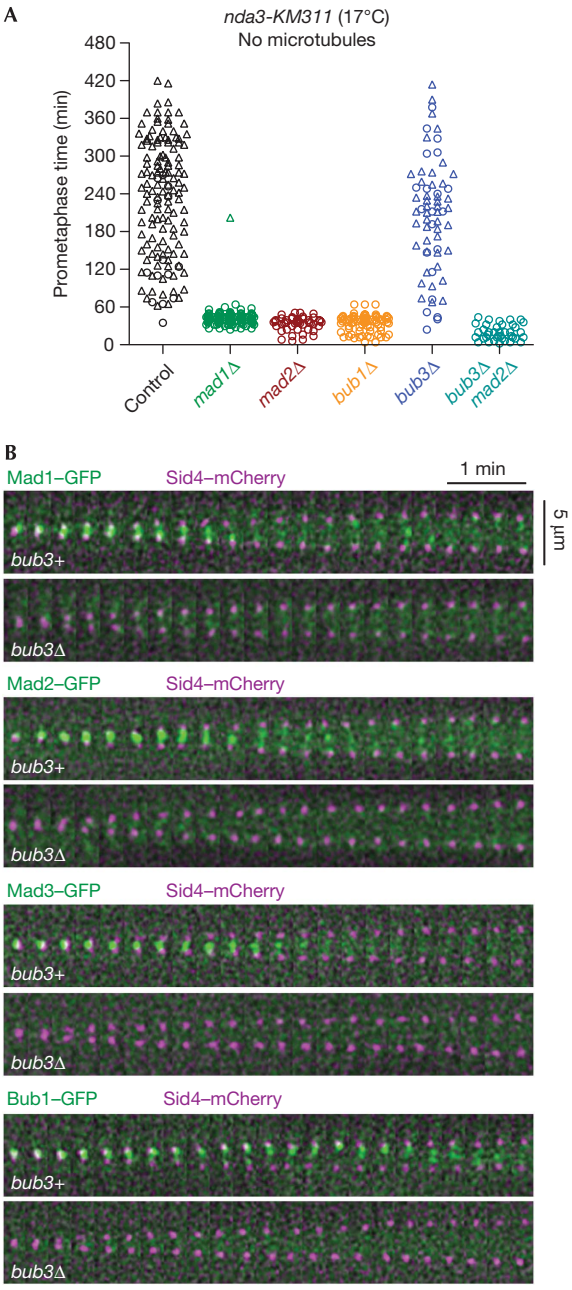

Fig 1 | Bub3 is not essential for the SAC, but for the efficient localization of SAC proteins. (**A**) Cells expressing *plo1–GFP* and the β-tubulin *nda3-KM311* allele were followed by live-cell microscopy at 17 °C. The duration of prometaphase was determined by the presence of Plo1–GFP on the SPBs. Circles indicate cells in which the entire prometaphase took place within the recording time. Triangles indicate cells that had not exited prometaphase when recording was stopped; thus the actual time of prometaphase must be longer than this value. Kymographs of exemplary cells are shown in supplementary Fig S1A online. (**B**) Kymographs of exemplary mitotic cells that were followed by live-cell microscopy at 30 °C using Sid4–mCherry to visualize SPBs. A quantitative analysis is shown in supplementary Fig S3 online. GFP, green fluorescent protein; SAC, spindle assembly checkpoint; SPB, spindle pole body.

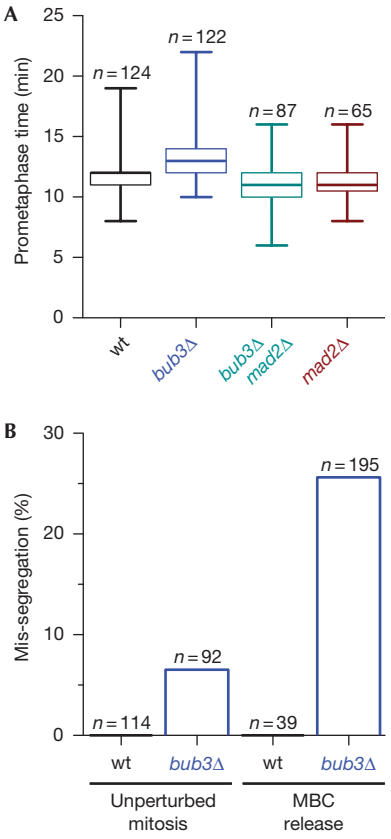

Fig 2 | Bub3 is required for correct chromosome segregation. (**A**) The duration of prometaphase at 30 °C in *bub3*Δ, *bub3*Δ *mad2*Δ, *mad2*Δ and wild-type (wt) cells was determined by live-cell microscopy using Plo1–GFP as a marker. In all box-whisker graphs, the lines from top to bottom are: maximum value, 75th percentile, median, 25th percentile and minimum value. (**B**) Cells carrying *cen2*–GFP and *mCherry–atb2(tubulin)* were synchronized with HU, released from HU arrest and arrested in mitosis by treatment with the microtubule-destabilizing drug MBC for 3.5 h. After washout of MBC, segregation of chromosome 2 (*cen2*–GFP) was followed by live-cell microscopy at 20 °C. Only those cells that were already in mitosis when recording was started were considered. Mis-segregation in unperturbed mitosis was similarly determined by live-cell microscopy at 20 °C after HU release. GFP, green fluorescent protein; HU, hydroxyurea.

to wild-type cells (Fig 2B; Vanoosthuyse *et al*, 2004). In an unperturbed mitosis, kinetochores are in close proximity to SPBs and chromosomes often achieve bi-orientation without any visible intermediate state. By contrast, when microtubules are depolymerized, kinetochores tend to 'uncluster' from SPBs. On microtubule repolymerization, chromosomes need to be retrieved towards the spindle and often undergo a transient state of mono-orientation before reaching bi-orientation (Grishchuk & McIntosh, 2006; Franco *et al*, 2007; Gachet *et al*, 2008). We monitored chromosome segregation after depolymerizing microtubules by using the microtubule-destabilizing drug MBC (methyl-benzimidazole-2-yl carbamate), and then washing out MBC. This led to a strong increase in chromosome mis-segregation in cells lacking Bub3 (Fig 2B), which indicates that Bub3 is more important for correct kinetochore–microtubule attachment in a situation in which kinetochores become unclustered from the SPBs.

Bub3 and Bub1 are required to promote bi-orientation

As chromosome segregation in *bub3*Δ cells was particularly perturbed after microtubule depolymerization and repolymerization, we followed chromosome segregation in these cells more closely. We used the kinetochore protein Mis6 fused to mCherry and GFP–tubulin as markers, which allowed us to follow all three chromosomes simultaneously (Fig 3A). By the time imaging started after washout of the drug, the chromosomes of wild-type cells had often achieved bi-orientation. Only 7% of all chromosomes showed clear mono-orientation for longer than 10 min (Fig 3C) and chromosome attachment was usually corrected to bi-orientation within 30 min (Fig 3B,D; supplementary information online, note 3; supplementary Fig S4A online). By contrast, *bub3*Δ cells more frequently showed mono-oriented chromosomes (20% of all chromosomes; Fig 3C) and, crucially, mono-oriented chromosomes in *bub3*Δ cells only became bi-oriented after a considerable delay, if at all (Fig 3B,D; supplementary Fig S4B–D online). Our results therefore suggest that Bub3 is involved in converting the mono-orientation of chromosomes to bi-orientation. Cells lacking Bub3 delayed anaphase when mono-oriented chromosomes were present (supplementary Fig S5 online), further supporting the idea that the SAC is functional in *bub3*Δ cells (supplementary information online, note 4). To test whether the role of Bub3 in promoting the conversion from mono-orientation to bi-orientation is shared with its interaction partner Bub1, we assayed chromosome segregation under similar conditions using a specific *bub1* mutant (*bub1-ΔGLEBS*), which lacks the Bub3-interacting region (Larsen *et al*, 2007). Bub1-ΔGLEBS did not localize to kinetochores, abolished Bub3 localization to kinetochores (supplementary Fig S6 online), but preserved SAC activity (V. Vanoosthuyse & K. Hardwick, personal communication). Cells expressing the *bub1-ΔGLEBS* mutant showed a defect in converting chromosome mono-orientation to bi-orientation that was similar to *bub3*Δ cells (Fig 3B–D; supplementary Fig S4E online). Thus, Bub1 and Bub3 together promote bipolar chromosome attachment in fission yeast.

Sgo2-independent bi-orientation by Bub3 and Bub1

In fission yeast, Sgo2 is the only mitotic shugoshin protein and its localization depends on Bub1 (Kitajima *et al*, 2004). Deletion of *sgo2* also increases mis-segregation specifically after microtubule depolymerization and repolymerization (Kawashima *et al*, 2007; supplementary Fig S7 online). As Bub1 interacts with Bub3 (Vanoosthuyse *et al*, 2004; Kadura *et al*, 2005), it is possible that the defect in bi-orienting chromosomes in *bub3*Δ and *bub1-ΔGLEBS* cells resulted from a loss of Sgo2 function. However, unlike in cells lacking Bub1, Sgo2 still localized to the centromeres in the *bub3*Δ or *bub1-ΔGLEBS* mutants (Fig 3E; supplementary information online, note 5). To determine whether the bi-orientation defect seen in *bub3*Δ and *bub1-ΔGLEBS* cells could nevertheless be due to the loss of Sgo2 function, we followed chromosome segregation in *sgo2*Δ cells after release from MBC arrest. We found that cells lacking Sgo2 had pronounced defects in attaching and segregating chromosomes correctly (supplementary Movie online). However, *sgo2*Δ cells

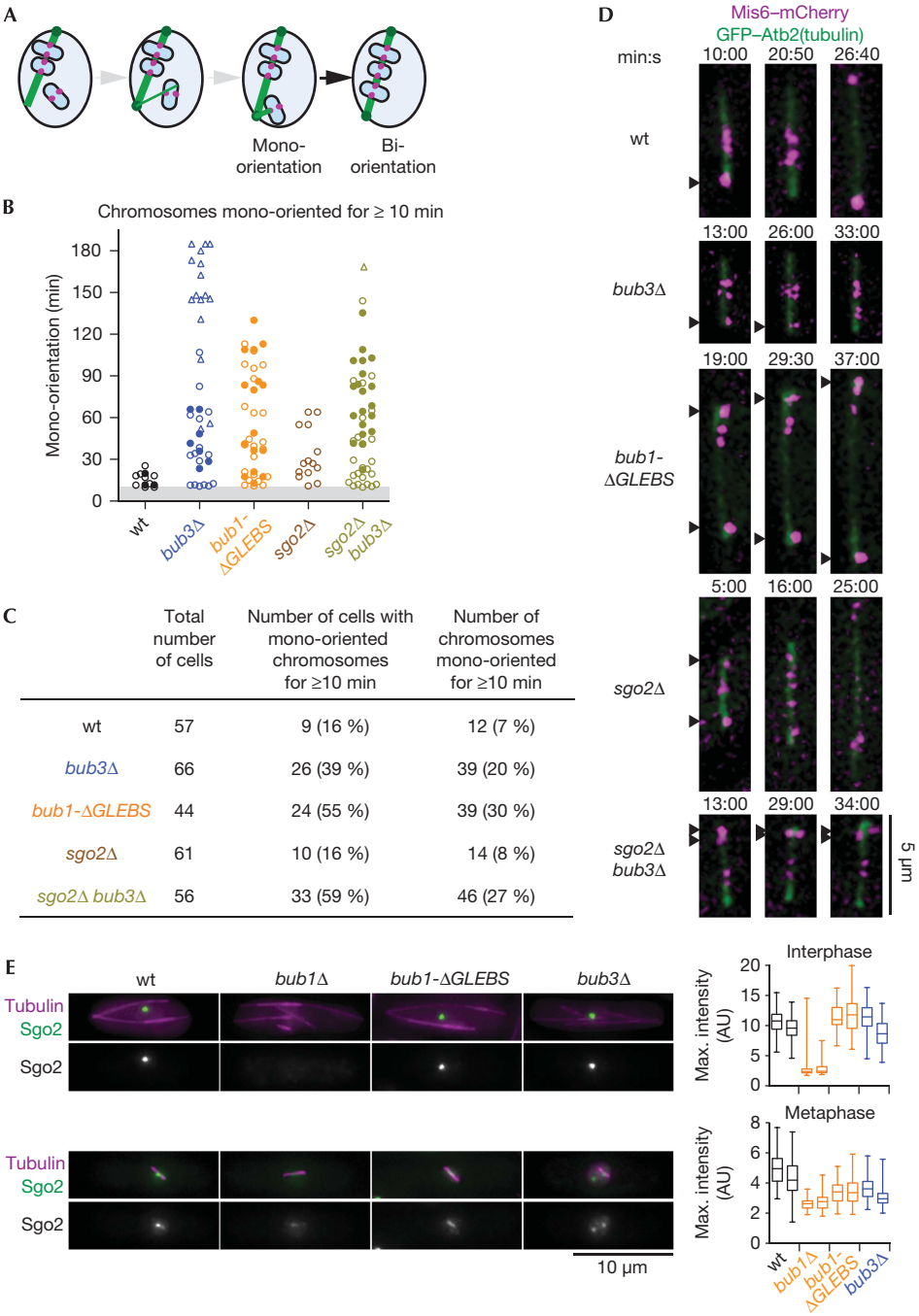

Fig 3 | Bub3 and its interaction with Bub1 are required for chromosome bi-orientation in an Sgo2-independent manner. (**A–C**) Cells expressing *GFP–atb2(tubulin)* and the kinetochore marker *mis6–mCherry* were pre-synchronized and treated with MBC as described in Fig 2B. After washout of MBC, segregation of the chromosomes was monitored by using live-cell microscopy at 20 °C. (**B**) Chromosomes that persisted close to an SPB for at least 10 min were followed and the time until they became bi-oriented was determined (open circles). Filled circles indicate chromosomes that apparently never achieved bi-orientation; triangles indicate chromosomes that had failed to achieve bi-orientation by the end of the recording. The total number of cells observed, the number of cells showing mono-oriented chromosomes for at least 10 min and the number of mono-oriented chromosomes are given in (**C**). (**D**) Exemplary cells from this experiment. Arrowheads indicate mono-oriented chromosomes. For the *bub3*Δ cell, correction of mono-orientation to bi-orientation can be seen at 33 min. In the *bub1-ΔGLEBS* cell, mono-orientation was never corrected and the cell entered anaphase after 37 min. In the *sgo2*Δ cell, bi-orientation was achieved after about 15 min. The *sgo2*Δ *bub3*Δ cell failed to bi-orient two chromosomes and delayed entry into anaphase for more than 1 h (supplementary Fig S4 online). (**E**) Living cells from the indicated strains expressing *mCherry–atb2(tubulin)* and *sgo2–GFP* were imaged by fluorescence microscopy. For each genotype, two independent strains were used. The maximum signal intensity in the nucleus was determined. Interphase or metaphase cells were identified by the characteristic microtubule signal. *bub3*Δ and *bub1-ΔGLEBS* cells often showed more than one Sgo2–GFP signal in mitosis (supplementary Fig S9 online). GFP, green fluorescent protein; SPB, spindle pole body; wt, wild type.

showed a similar fraction of mono-oriented chromosomes as wild-type cells (Fig 3C), and rarely showed a delay in bi-orienting chromosomes that had been pulled towards one SPB (Fig 3B,D; supplementary Fig S4F online). When *bub3* was deleted in addition to *sgo2*, mono-orientation was prolonged, similar to the *bub3*Δ single mutant (Fig 3B,D; supplementary Fig S4G online), making it unlikely that Sgo2 acts downstream of Bub3 in promoting chromosome bi-orientation. Therefore, we suggest that Bub1 and Bub3 together have a Sgo2-independent role in promoting the conversion from chromosome mono-orientation to bi-orientation. In human cells, knock down of BUB1 or BUB3 by RNA interference also causes a chromosome congression defect (Ditchfield *et al*, 2003; Meraldi & Sorger, 2005; Logarinho *et al*, 2008), which might not be caused exclusively by the loss of shugoshin from the kinetochores (Klebig *et al*, 2009). Thus, this function of Bub1 and Bub3 might be conserved between yeast and humans.

Separate pathways for the correction of mono-orientation

Little is known about the molecular events that occur during conversion from chromosome mono-orientation to bi-orientation in any organism. Mono-oriented chromosomes might be captured directly by microtubules reaching over from the other pole (direct pathway; Fig 4A). In vertebrate cells, mono-oriented chromosomes can move on pre-existing kinetochore microtubules towards the centre of the spindle (indirect pathway; Fig 4A; Kapoor *et al*, 2006). As Bub3 localizes to mono-oriented chromosomes up to the moment of bi-orientation (Fig 4B), and the abolishment of Bub3 and Bub1 localization to the kinetochores in the *bub1-ΔGLEBS* mutant leads to persistent mono-orientation (Fig 3B–D), we favour the idea that Bub3 and Bub1 promote bi-orientation by modulating kinetochore–microtubule interactions at the mono-oriented chromosome (indirect pathway). An additional observation supports the view that the direct pathway is functional in *bub3*Δ cells: when one or more chromosomes remain mono-oriented or unattached, spindles often undergo cycles of elongation and shrinkage (Fig 5; supplementary information online, note 6; supplementary Figs S4D, S8A online). Interestingly, mono-oriented chromosomes in *bub3*Δ cells often achieved bi-orientation when the spindle was short (Fig 5C,D). Our interpretation is that mono-oriented chromosomes in *bub3*Δ cells are able to achieve bi-orientation through capture from the opposite spindle pole (direct pathway), which is more likely to happen when spindles are short (Fig 5A). This also explains why chromosome segregation of *bub3*Δ cells is only slightly affected in an unperturbed mitosis (Fig 2B). In this situation, chromosomes are clustered close to the separating SPBs at the onset of mitosis and, hence, can rapidly achieve bi-orientation through the 'direct' pathway while the spindle is still short. After MBC release, by contrast, chromosomes first need to be retrieved towards the SPB by astral microtubules, and the spindle elongates while this happens. Once mono-oriented chromosomes reach the SPB, direct capture becomes unlikely owing to the paucity of long microtubules coming from the opposite pole (Grishchuk *et al*, 2007). Bi-orientation, therefore, needs a mechanism to move chromosomes closer to the opposing SPB, which depends on Bub3 and Bub1 (Fig 4A; supplementary information online, note 7). How Bub3 affects kinetochore–microtubule interactions of mono-oriented chromosomes remains an open question. A study in budding yeast did not find any evidence for a direct interaction between Bub3 and microtubules (Guenette *et al*, 1995). One candidate for a mediator between Bub3 and microtubules is dynein, which has been found to interact with Bub3 in the mammalian system (Lo *et al*, 2007) and to show a genetic interaction in the fungus *Aspergillus nidulans* (Efimov & Morris, 1998). In fission yeast, deletion of the dynein heavy chain (*dhc1*) causes a defect in the transition from chromosome mono-orientation to bi-orientation that resembles that seen in *bub3*Δ cells (Grishchuk *et al*, 2007). For *C. elegans*, it has been proposed that dynein is involved in the regulation of microtubule attachment during both mono-orientation and bi-orientation (Gassmann *et al*, 2008). Thus, Bub1, Bub3 and dynein might cooperate at the kinetochores to promote chromosome bi-orientation.

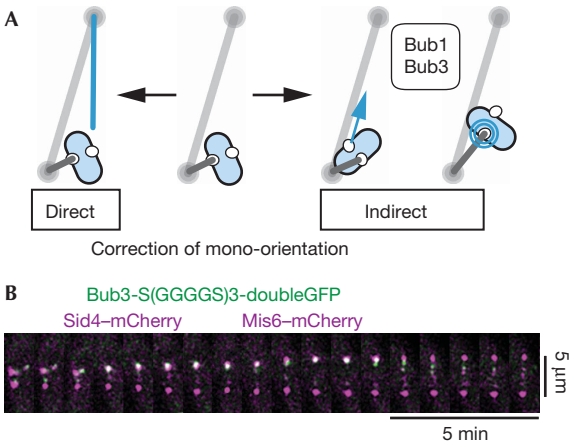

Fig 4 | Possible pathways for the correction of chromosome mono-orientation. (**A**) When chromosomes are clustered close to the SPBs at the beginning of mitosis or when spindles are short, direct capture by a microtubule from the opposite SPB might be the predominant way of correcting mono-orientation (left). When spindles become elongated, further mechanisms might be needed (right). We propose that Bub1 and Bub3 are involved in moving mono-oriented chromosomes closer to the centre of the spindle either by promoting movement along pre-existing kinetochore microtubules or by modulating kinetochore–microtubule attachment of the mono-oriented chromosome to the proximal pole. (**B**) Kymograph of a *bub3–GFP*-expressing cell treated with MBC after release from HU-arrest as described in Fig 2B. An initially unattached chromosome was captured by microtubules, pulled towards an SPB and subsequently bi-oriented. Bub3–GFP localized to one or both kinetochores of this chromosome until the chromosome had achieved bi-orientation. GFP, green fluorescent protein; HU, hydroxyurea; SPB, spindle pole body.

METHODS

Strains, media and imaging conditions. Strains of fission yeast used in this study are listed in supplementary Table S1 online. The *bub1-ΔGLEBS* mutant was constructed by deleting the bases corresponding to amino-acids 264–299 (GKRV...SSIQ). For live-cell imaging, cells were grown in Edinburgh minimal medium (Moreno *et al*, 1991) containing the necessary supplements and mounted in glass-bottom culture dishes (Ibidi (Martinsried, Germany) or MatTek (Ashland, MA, USA)). Live cell imaging

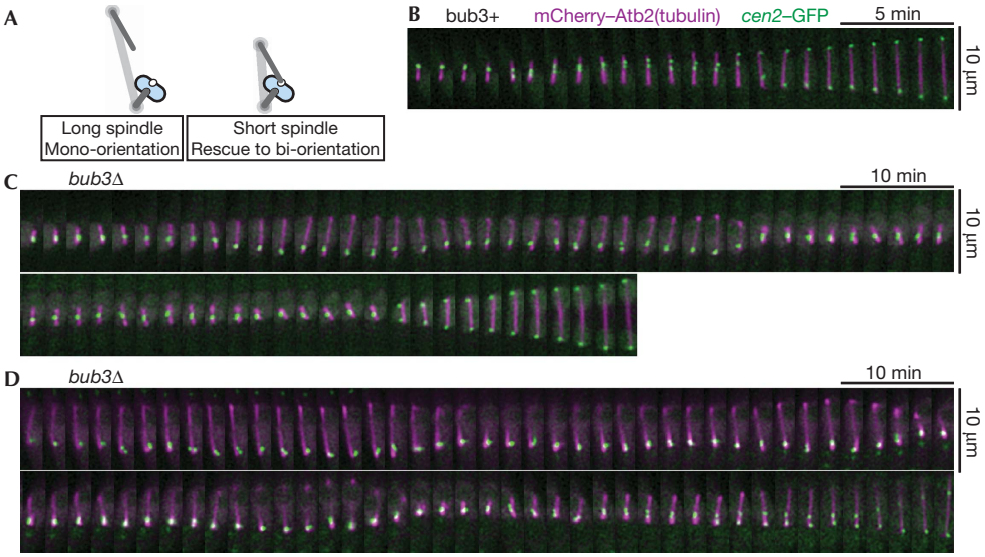

Fig 5 | Mono-orientation of chromosomes in *bub3*Δ cells can be rescued when mitotic spindles are short. (**A**) Model for the possible mechanism of rescue: when the mitotic spindle is long, microtubules from the opposite spindle pole cannot reach the mono-oriented chromosome, whereas they can if the spindle is short. A second, indirect mechanism of rescue (Fig 4A) is presumably not functional in *bub3*Δ cells. (**B–D**) Exemplary kymographs of (**B**) wild-type or (**C,D**) *bub3*Δ cells carrying *cen2*–GFP and expressing *mCherry–atb2(tubulin)* from the experiment described in Fig 2B. GFP, green fluorescent protein.

was carried out on a DeltaVision Core system (Applied Precision, Issaquah, WA, USA) equipped with a climate chamber. MBC (Sigma, St Louis, MO, USA, 45368) was used at a final concentration of 20 μg/ml. Detailed descriptions of culture conditions, live-cell imaging and image analysis are available in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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