

# Mal3, the fission yeast EB1 homologue, cooperates with Bub1 spindle checkpoint to prevent monopolar attachment

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**Bipolar microtubule attachment is central to genome stability. Here, we investigate the mitotic role of the fission yeast EB1 homologue Mal3. Mal3 shows dynamic inward movement along the spindle, initial emergence at the spindle pole body (SPB) and translocation towards the equatorial plane, followed by sudden disappearance. Deletion of Mal3 results in early mitotic delay, which is dependent on the Bub1, but not the Mad2, spindle checkpoint. Consistently, Bub1, but not Mad2, shows prolonged kinetochore localization. Double mutants between *mal3* and a subset of checkpoint mutants, including *bub1*, *bub3*, *mad3* and *mph1*, but not *mad1* or *mad2*, show massive chromosome mis-segregation defects. In *mal3bub1* mutants, both sister centromeres tend to remain in close proximity to one of the separating SPBs. Further analysis indicates that mis-segregated centromeres are exclusively associated with the mother SPB. Mal3, therefore, has a role in preventing monopolar attachment in cooperation with the Bub1/Bub3/Mad3/Mph1-dependent checkpoint.**

Keywords: EB1; spindle checkpoint; bipolar attachment; fission yeast; kinetochore

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## INTRODUCTION

For each sister chromatid to be segregated properly, establishment of bipolar attachment of the sister kinetochore to the mitotic spindle is vital, although molecular mechanisms underlying this process are still poorly understood (Tanaka *et al*, 2002). Microtubules have intrinsic polarity (a plus and a minus end), in which dynamic plus ends form different structural conformations, depending on growing, shrinking or pausing states (Desai & Mitchison, 1997). The local control of the microtubule at the plus end is regulated by a distinct class of microtubule accessory factors, called plus-end-tracking proteins (Schuyler & Pellman, 2001). The kinetochore attaches to the plus end of the spindle microtubule, which is required for establishment of chromosome bi-orientation. It is, therefore, postulated that some plus-end-tracking proteins have a crucial role in bipolar microtubule attachment.

EB1, originally identified as a protein interacting with tumour suppressor protein APC (Su *et al*, 1995), belongs to the conserved plus-end-tracking protein family. EB1 is believed to have a role in stabilization of microtubules by promoting growth and/or in regulation of microtubule dynamics by suppressing the pause state (Schuyler & Pellman, 2001). EB1 functions not only in microtubule-dependent cell polarity but also in chromosome stability during mitosis (Lengauer *et al*, 1998; Green & Kaplan, 2003). Despite this, a physiological role for mitotic EB1 remains largely elusive.

The spindle assembly checkpoint (Mad and Bub proteins) monitors spindle–kinetochore interaction, thereby delaying onset of anaphase until bipolar attachment is secured (Millband *et al*, 2002; Cleveland *et al*, 2003). Checkpoint components localize to kinetochores when bi-orientation is not achieved and somehow sense the physical status of these mitotic kinetochores, unattached and/or tensionless. In this study, we characterize a mitotic role of Mal3, fission yeast EB1 homologue, and present evidence that Mal3 has a vital role in the establishment of bipolar attachment in cooperation with a specific subset of spindle checkpoint components.

## RESULTS AND DISCUSSION

### Inward movement of Mal3 on the spindle

To characterize Mal3 function during mitosis, we first examined the dynamics of Mal3 localization in live cells using Mal3–GFP (green fluorescent protein), in which the chromosomal *mal3*<sup>+</sup> gene was tagged with GFP at the carboxyl terminus under the native promoter. As reported previously (Browning *et al*, 2003; Busch & Brunner, 2004), during interphase, Mal3 localizes along cytoplasmic microtubules in punctate patterns, particularly enriched at microtubule tips (Fig 1A, left panel). On entry into mitosis, Mal3 colocalized to spindles and astral microtubules, again as dotted patterns (Fig 1A, middle and right panels).

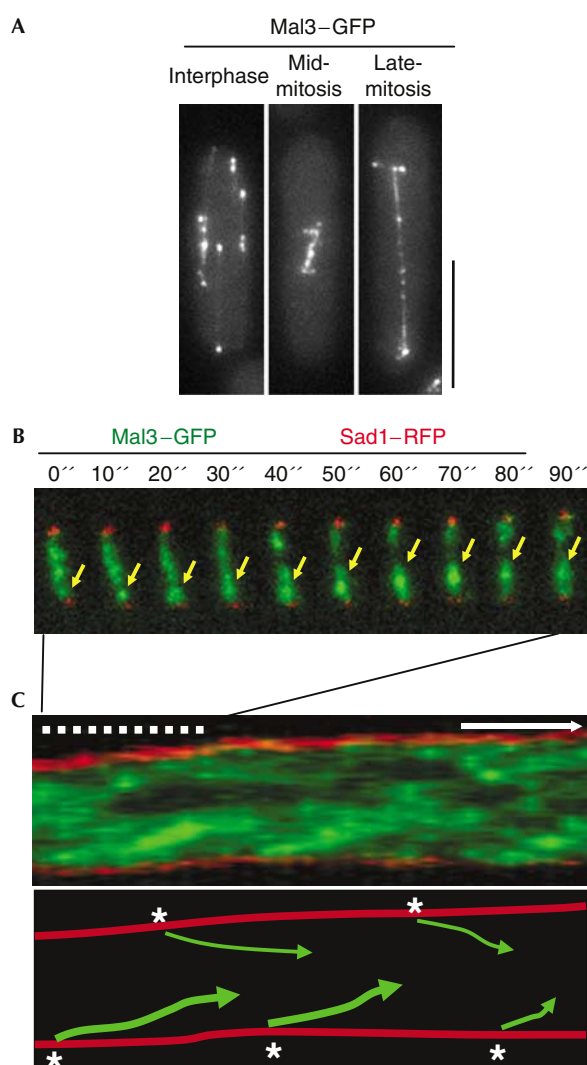
To clarify Mal3 movement during mid-mitosis in more detail, time-lapse live analysis was carried out using a strain that contains Mal3–GFP and Sad1–RFP (red fluorescent protein), in which Sad1 is a marker for the spindle pole body (SPB). During mid-mitosis (the spindle length is ~2 μm), Mal3–GFP appeared as blobs, which showed rapid movement from the vicinity of one SPB towards the middle of the spindle (Fig 1B; supplementary Movie S1 online), similar to mitotic EB1 movement (Tirnauer *et al*, 2002). The kymograph picture assembled from serial time-lapse images clearly indicated the following three points. (i) Mal3–GFP was loaded onto the vicinity of the SPB (Fig 1C, asterisks). (ii) Mal3 was then translocated towards the middle region of the spindle. (iii) Following this inward movement, Mal3 signals suddenly disappeared (Fig 1C, arrow tips; see supplementary Fig S1 and Movie S2 online for more mitotic images of Mal3).

### Genetic interaction with spindle checkpoint genes

*mal3*<sup>+</sup> is not essential, but its deletion results in defects in chromosome stability (Beinhauer *et al*, 1997). We examined a genetic interaction between *mal3* and mutations in the spindle checkpoint. It was found that double mutants between *mal3* and a subset of checkpoint mutants including *bub1*, *bub3*, *mad3* and *mph1* show compromised growth defects, which were viable but temperature sensitive (Fig 2A). In contrast, growth properties of *mal3mad1*, *mal3mad2* or each single mutant were similar to those of wild-type cells (Fig 2A; data not shown). Bub1 is shown to have dual roles in genome stability in a spindle checkpoint-dependent and spindle checkpoint-independent manner, in which checkpoint-independent roles are not shared by Bub3 or Mad3 (Vanoosthuysse *et al*, 2004; Kadura *et al*, 2005). The fact that synthetic phenotypes are observed not only in *bub1* but also in other checkpoint mutants suggests that Mal3-related roles of Bub1 lie in its checkpoint function (see below). This genetic result also shows that the Mad2 spindle checkpoint is not required for maintenance of viability in the absence of Mal3.

### Sustained kinetochore localization of Bub1 in *mal3* mutant

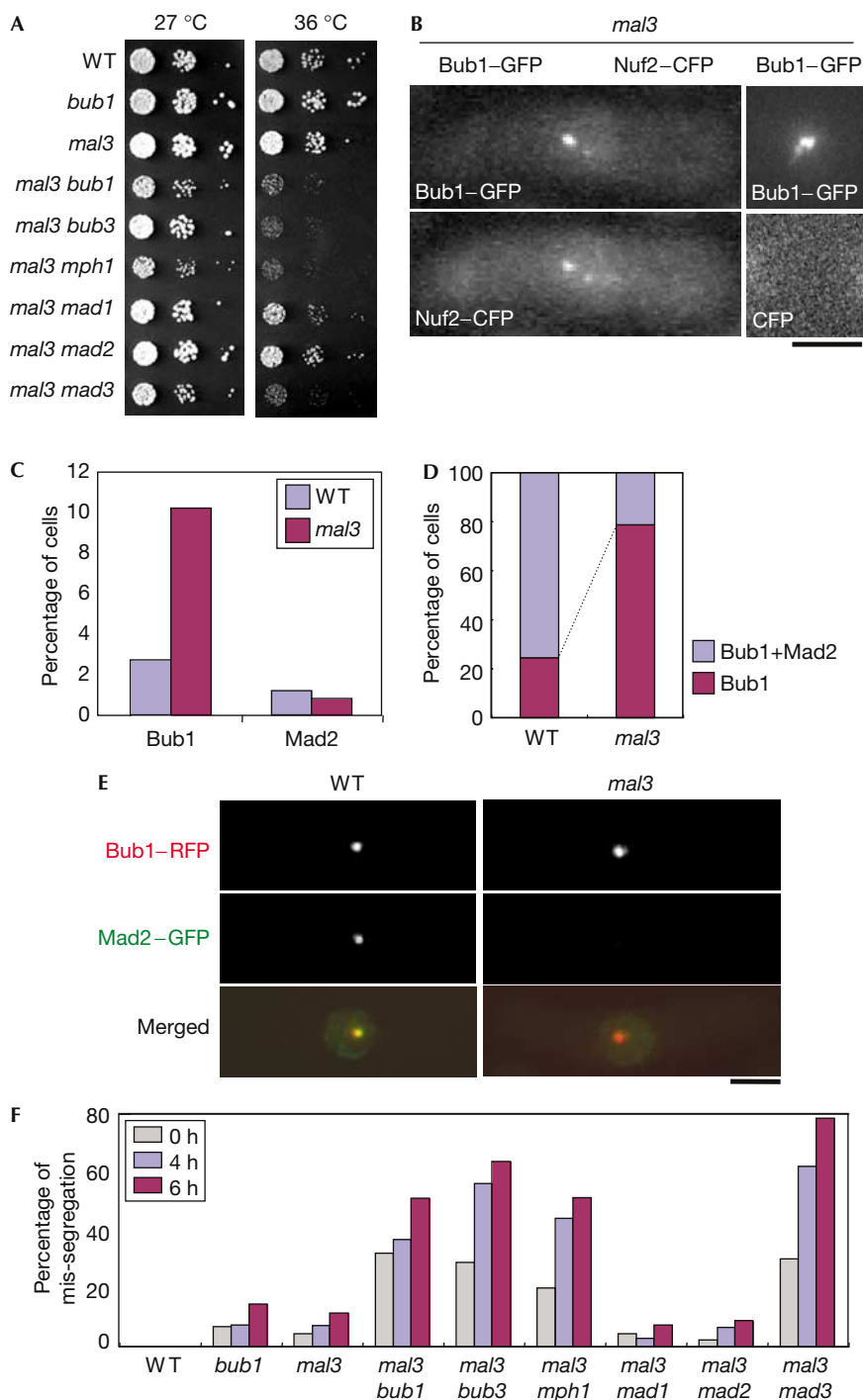
We addressed Bub1 localization in exponentially growing *mal3* mutants. As shown in Fig 2B,C, approximately 10% of *mal3*-deleted cells showed Bub1–GFP at the kinetochore, whereas in wild-type cells, the value was 2.5%. In contrast, cells showing Mad2–GFP localization to the kinetochore were not increased in *mal3* mutants (Fig 2C). To confirm this preferential pattern, strains were constructed in wild type and *mal3* mutants that contained Bub1–RFP and Mad2–GFP simultaneously, and localization of these two proteins was examined in a single cell. In line with the previous analysis, whereas in wild type, 80% of cells



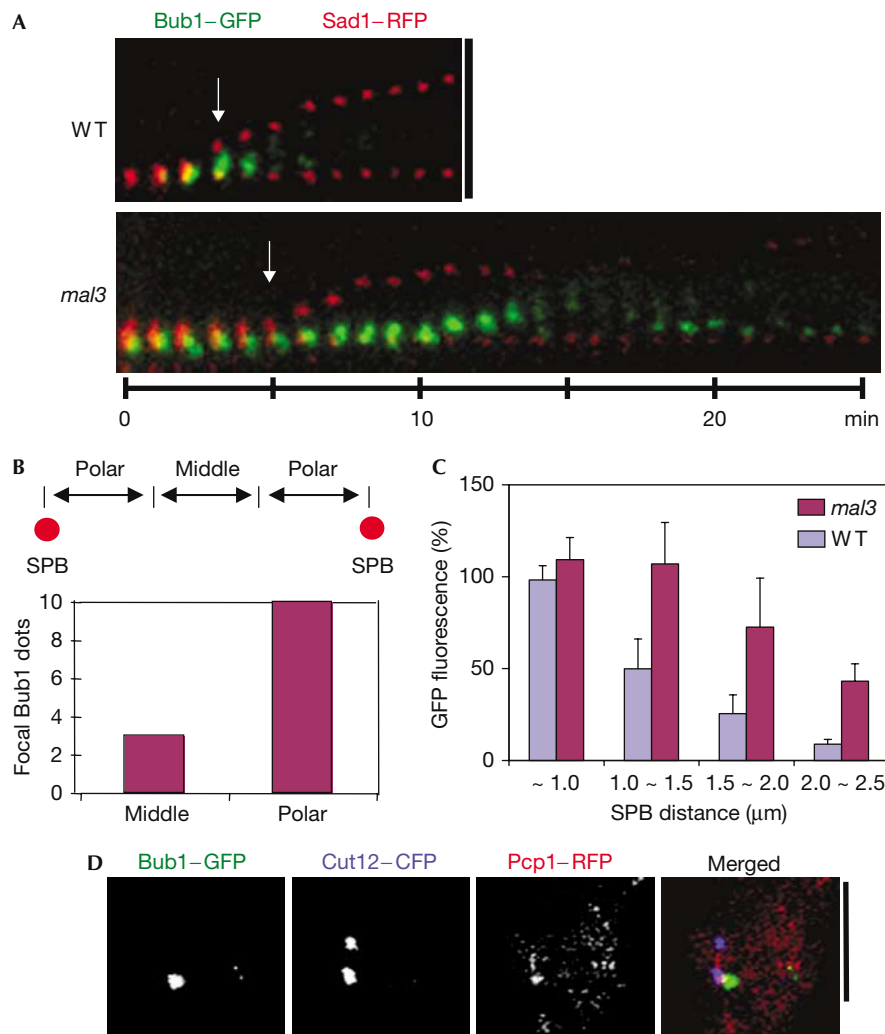
**Figure 1** | Mitotic localization of Mal3. (A) Mal3–GFP (green fluorescent protein) localization during the cell cycle. (B,C) Time-lapse analysis. Mitotic Mal3–GFP was recorded (B, 10 s intervals) and converted to kymograph (C, upper panel). Inward Mal3 blobs are marked by yellow arrows. White dotted lines in (C) correspond to the first 90 s images shown in (B). In the schematic (C, lower panel, arrows), initial Mal3 loading spots are shown with asterisks. The length of the white arrow represents 1 min. Scale bars, 5 μm (A) or 2 μm (B,C).

that contained Bub1–RFP at the kinetochore also contained Mad2–GFP at this site, in the absence of Mal3, this colocalization pattern was reduced to 20% and the remaining 80% of *mal3* cells showed Bub1 dots, but not Mad2 (Fig 2D,E). These data substantiate the idea that in *mal3* mutants, the Bub1-dependent checkpoint is more important than that of Mad2.

Next, phenotypic consequences resulting from the elimination of spindle checkpoint components from *mal3*-deleted cells were examined. A series of mutant strains were grown in liquid cultures at 26 °C, shifted to 36 °C and stained with 4,6-diamidino-2-phenylindole. Consistent with earlier results (Fig 2A), double mutants between *mal3* and *bub1*, *bub3*, *mad3* or *mph1* showed a



**Figure 2** | Dependence of *mal3* deletion mutants on the Bub1, but not the Mad2, checkpoint. (A) Growth properties of double mutants between *mal3* and various spindle checkpoint mutants. WT, wild type. (B) Bub1 localization at the kinetochores. The kinetochore marker (Nuf2-CFP (cyan fluorescent protein)) was used (left panels). As a control, Bub1-GFP (green fluorescent protein) signals, in which Nuf2 was not tagged, were taken using the CFP channel (right panels). (C–E) Kinetochore localization of Bub1 and Mad2. Wild type or *mal3* mutants that contained Bub1-GFP or Mad2-GFP (C) or Bub1-RFP and Mad2-GFP simultaneously (D) were used for quantification, and representative examples are shown (E, Bub1 in red and Mad2 in green). Scale bar, 3  $\mu$ m. (F) Chromosome mis-segregation. The percentage of anaphase cells showing chromosome mis-segregation was quantified ( $n = 50$ ).



**Figure 3** | Mitotic localization of Bub1. (A) Time-lapse images. Bub1-GFP (green fluorescent protein) and Sad1-RFP (red fluorescent protein) localization during mitosis in wild type (WT; upper panel) or the *mal3* mutant (lower panel) was recorded ( $n=20$ ) and converted to a kymograph. Arrows indicate the time points when spindle pole body (SPB) separation initiated. (B) Quantification of focal Bub1 signals. The region between the two SPBs was divided into three regions ('polar' and 'middle') and localization patterns of Bub1-GFP foci were examined ( $n=40$ ). (C) Bub1-GFP signals between the two SPBs. The intensity of Bub1-GFP foci just before SPB separation was regarded as 100% ( $n=8$ ). (D) Close association between Bub1 and Pcp1. Wild-type cells containing Bub1-GFP, Pcp1-RFP and Cut12-CFP (cyan fluorescent protein) were grown at 26 °C, starved for nitrogen and resuspended in fresh media to allow re-growth. In the merged image, Bub1-GFP (green), Pcp1-RFP (red) and Cut12-CFP (blue) are shown ( $n=30$ ). Scale bars, 5 μm (A) or 2 μm (D).

high rate of chromosome mis-segregation at 36 °C (40–80% of anaphase cells; Fig 2F; see supplementary Fig S2 online) and, even at 26 °C, mis-segregation phenotypes were evident (20–30%). In sharp contrast, in *mal3mad1* or *mal3mad2* mutants, segregation defects were marginal compared with those in *mal3* or *bub1* single mutants (Fig 2F). Taken together, these results indicate that Mal3 regulates mitotic progression, and in its absence, only a subset of spindle checkpoint components including Bub1, Bub3, Mad3 and Mph1 are activated.

### Bub1 localizes to the vicinity of one SPB

Bub1 is known to localize to the kinetochore during early mitosis (Bernard *et al*, 1998; Toyoda *et al*, 2002). To address mitotic Bub1

localization in more detail, we carried out time-lapse analysis of Bub1-GFP in wild type and *mal3* mutants that contained Bub1-GFP and Sad1-RFP. In wild-type cells, Bub1-GFP signals appeared as a blob in the vicinity of the unseparated SPB, disappearing shortly afterwards (Fig 3A, upper panel; supplementary Movie S3 online). The average duration of Bub1 at the kinetochore is thus transient ( $6 \pm 1.2$  min,  $n=20$ ). In *mal3* cells, conversely, the duration of the focal Bub1-GFP signal was extended threefold (Fig 3A, lower panel,  $18 \pm 1.5$  min,  $n=20$ ; supplementary Movie S4 online) and was retained in the vicinity of one SPB (Fig 3B,C).

Recently, it has been reported that fission yeast SPB is duplicated in a conservative manner (Grallert *et al*, 2004). The

**Table 1** Dynamics of spindle elongation and mitotic phases

Strain	Anaphase A	Anaphase B		Sister chromatid mis-segregation
	From SPB separation to anaphase A onset (min)	Duration (min)	Rate of spindle elongation ( $\mu\text{m}/\text{min}$ )	
Wild type	$11.6 \pm 1.3$ ( $n = 8$ )	$15.6 \pm 1.3$ ( $n = 5$ )	$0.65 \pm 0.1$ ( $n = 5$ )	0/20
<i>mal3</i>	$18.4 \pm 3.7$ ( $n = 8$ )	$21.3 \pm 5.7$ ( $n = 5$ )	$0.46 \pm 0.1$ ( $n = 9$ )	0/15
<i>mal3 bub1</i>	$13.9 \pm 2.6$ ( $n = 9$ )	$20.8 \pm 4.0$ ( $n = 6$ )	$0.51 \pm 0.1$ ( $n = 6$ )	3/12
<i>mal3 mad2</i>	$15.3 \pm 3.5$ ( $n = 4$ )	$19.5 \pm 1.3$ ( $n = 4$ )	$0.41 \pm 0.1$ ( $n = 6$ )	0/13

Four strains (wild type, *mal3*, *mal3bub1* and *mal3mad2*) containing *cen2*-GFP (green fluorescent protein) and Sad1-RFP (red fluorescent protein) were grown and time-lapse live analysis was performed. SPB, spindle pole body.

mother (old) SPB could be marked specifically by an SPB component Pcp1 tagged with RFP. Given the asymmetrical association of Bub1-GFP with one of the two segregating SPBs, we were interested in whether Bub1-GFP showed a preference in its association with the old or new SPB. To this end, wild-type cells that contained Bub1-GFP, Pcp1-RFP and Cut12-CFP (cyan fluorescent protein; Cut12 is an SPB marker) were constructed. We found that during early mitosis when Bub1-GFP is recruited to the kinetochore, it located in close proximity to Pcp1-RFP that colocalized to one of the two separating SPBs ( $n = 30$ ; Fig 3D). These results showed that even in wild-type cells, Bub1 locates in close association with the mother SPB during early mitosis.

### Mitotic delay is dependent on Bub1

We sought to examine the kinetics of mitotic spindle elongation in wild type and *mal3* mutants. To visualize a pair of specific sister centromeres, we used a centromere-marking GFP-LacI system (Nabeshima *et al*, 1998). Time-lapse imaging showed that the duration between initiation of SPB separation and onset of anaphase A was extended by 60% in *mal3* cells compared with that in wild-type cells (Table 1; supplementary Fig S3 online). We found that the delay was mostly, although not completely, abolished in *mal3bub1* (20% longer than wild type). During this analysis, we noticed that 3 out of the 12 *mal3bub1* cells observed live failed to segregate *cen2*-GFP equally (Table 1; see below). In contrast, *mal3mad2* cells showed substantial mitotic delay, although with some reduction (17%). These results indicate that mitotic phases are deferred in the absence of Mal3 function and, consistent with genetic data, this delay is mainly dependent on the Bub1 checkpoint.

### Live imaging of chromosome mis-segregation

To address the mitotic defects of *mal3bub1* mutants in more detail, we examined the patterns of sister chromatid segregation using the *cen2*-GFP Sad1-RFP systems. Consistent with earlier results (Fig 2F; Table 1), even at 26 °C, unequal segregation of *cen2*-GFP signals was observed in approximately 25% of mitotic cells ( $n = 1,000$ ; Fig 4A). Next, we examined the mitotic behaviour of *cen2*-GFP with time-lapse live imaging. In wild-type cells, *cen2*-GFP oscillated between the two poles during prophase and prometaphase (Fig 4B, left panel; supplementary Movie S5 online), then, following anaphase onset, started to move towards opposite poles, and reached two SPBs, followed by further spindle elongation in anaphase B (the kymograph image is shown in Fig 4B, right panel). In clear contrast, *mal3bub1* double mutants

showed *cen2*-GFP signals being retained in close proximity to one of the SPBs (3 out of 12 samples; Fig 4C; supplementary Movie S6 online). These data unambiguously show that the persistent association with one of the SPBs is the reason why chromosome mis-segregation is imposed in *mal3bub1* cells.

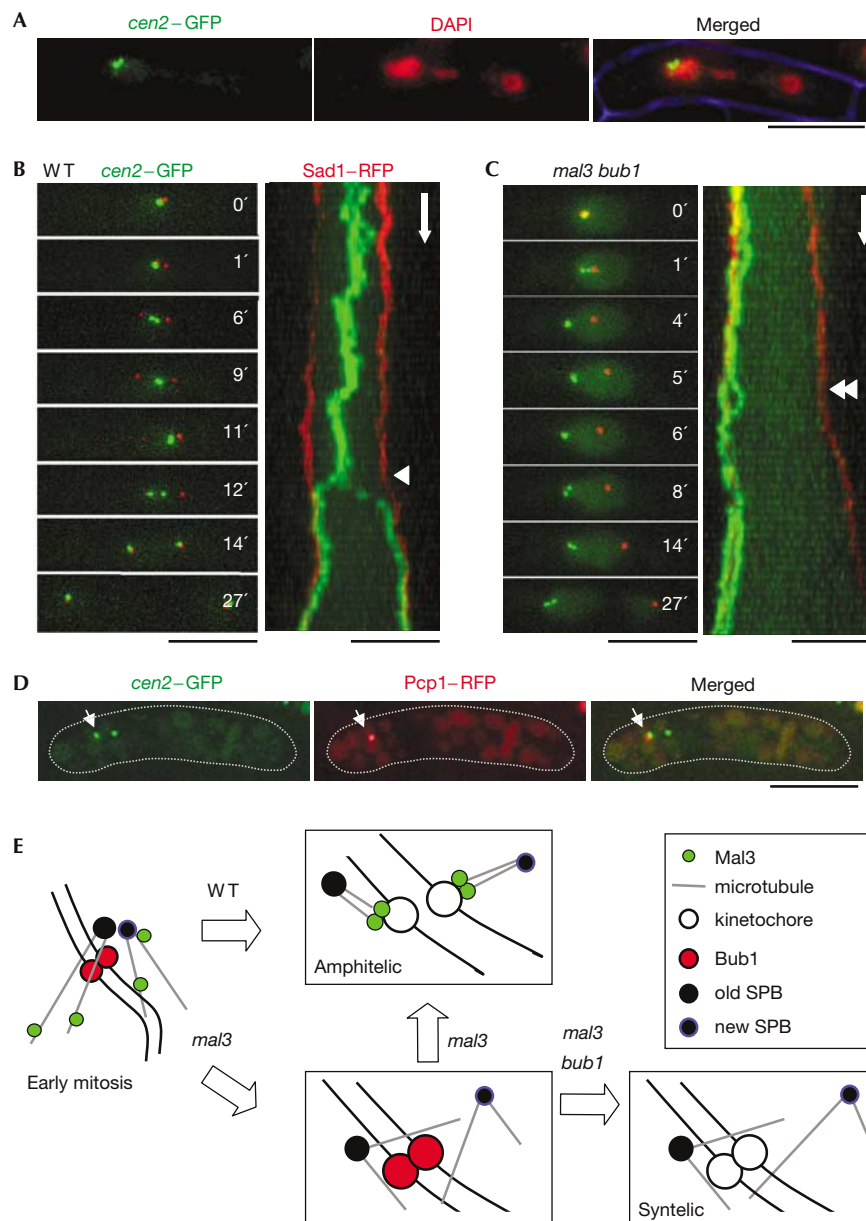
### Chromosome mis-segregation to the mother SPB

We next investigated whether mis-segregated *cen2*-GFP had a preference in its association with the old SPB similar to Bub1-GFP during early mitosis in wild-type cells (Fig 3D). For this purpose, a *cen2*-GFP strain was constructed, which contained Pcp1-RFP in *mal3bub1* mutants. Out of 30 cells that showed sister chromatid mis-segregation, all showed mis-segregated *cen2*-GFP that was associated with the old SPB, in which Pcp1-RFP signals were detected (Fig 4D, arrowheads). This analysis suggests that, together with Bub1, Mal3 is involved in the kinetochore attachment to the spindle emanating from the new SPB, and in the absence of both proteins, two sister kinetochores tend to be associated with only the old SPB.

### Possible roles of Mal3 and Bub1 in bipolar attachment

In this study, we explore the function of the fission yeast EB1 homologue Mal3 in bipolar microtubule attachment. A single *mal3* mutant shows prolonged mitotic delay during the early mitotic phase, in which Bub1, but not Mad2, localizes to the kinetochore. Although these *mal3* mutants show a somewhat enhanced chromosome instability (Kerres *et al*, 2004), in most (>90%) of these single mutants, sister chromatids segregate equally. Conversely, cells deleted for Mal3 and a subset of checkpoint components (Bub1/Bub3/Mad3/Mph1) simultaneously show severe chromosome mis-segregation defects. In the case of *mal3bub1* double mutants, both sister chromatids tend to remain associated in the vicinity of the mother SPB. This indicates that Mal3 has a role, in cooperation with the Bub1-dependent checkpoint, in equal sister chromatid segregation, in particular, attachment of the kinetochore to the spindle emanating from the new SPB.

There are several possible explanations for the involvement of Mal3 in bipolar microtubule attachment. As the loading of Mal3 onto spindle microtubule is often uneven in its intensity, Mal3 may regulate the dynamics of a subset of spindle microtubules. It is possible that in the absence of Mal3, spindles are less stable or dynamic, resulting in inefficient attachment of the kinetochore to the spindle, in particular, interaction between the kinetochore and the spindle emanating from the new SPB. Alternatively, although



**Figure 4** | Mono-orientated segregation of sister centromeres in *mal3bub1* double mutants. (A) Mis-segregation of sister chromatids. *mal3bub1* mutants containing *cen2*-GFP (green fluorescent protein) were grown at 26 °C (left panel, *cen2*-GFP in green; middle panel, 4,6-diamidino-2-phenylindole (DAPI) in red; right panel, merged). (B,C) Time-lapse analysis. Representative images of wild type (WT; B) and *mal3bub1* (C) are shown (*cen2*-GFP in green and Sad1-RFP (red fluorescent protein) in red). Corresponding kymograph pictures are shown on the right-hand side. The timing of anaphase A onset in wild type and that of anaphase B onset in *mal3bub1* cells are marked with a white arrowhead (B) and a double arrowhead (C), respectively. The length of the white arrow represents 1 min. (D) Association of mis-segregated *cen2*-GFP with the old SPB. Pictures of *cen2*-GFP (green, left panel), Pcp1-RFP (red, middle panel) and the merged image (right panel) are shown ( $n = 30$ ). Scale bars, 2  $\mu\text{m}$  (B,C, right panels) or 5  $\mu\text{m}$  (B,C left panels), A,D). (E) Role of Mal3 and the Bub1 spindle checkpoint in sister chromatid segregation. See the text for details.

not mutually exclusive, Mal3 might have a more direct role in chromosome bi-orientation through the interaction with kinetochore proteins (Kerres *et al*, 2004). Mal3 may be involved in a specific mode of spindle–kinetochore interaction, for example, switch from lateral attachment to end-on interaction, as observed in animal and budding yeast cells (Merdes & De Mey, 1990; Tanaka *et al*, 2005).

Kinetochore recruitment of Bub1 occurs only during early mitosis, when the kinetochore is closely situated in the vicinity of two separating SPBs in both wild-type and *mal3* cells. We show that at least in wild-type cells, during this stage, Bub1 exclusively localizes in close proximity to the mother SPB. We presume that Bub1 is recruited to the kinetochore that has not achieved amphitelic attachment, but is instead unattached or syntelic, in

which spindles from one pole interact with both of the sister kinetochores (Fig 4E). We speculate that the absence of Mal3 might result in prolonged syntelic attachment, in which Bub1 localizes to these kinetochores. Nonetheless, under these conditions, chromosome segregation defect is effectively prevented in a Bub1/Bub3/Mad3/Mph1 checkpoint-dependent manner, and further elimination of this checkpoint leads to chromosome mis-segregation, with two sister chromatids being closely connected to the old SPB. It is of note that the pattern of preferential requirement of a subset of the spindle checkpoint components is similar to that reported in the spindle orientation checkpoint (Gachet et al, 2001; Rajagopalan et al, 2004; Tournier et al, 2004). It is possible that the molecular event that the spindle orientation checkpoint senses involves syntelic/monopolar attachment. Bub1 is also required to protect cohesion at the centromere/kinetochore by Shugoshin (Kitajima et al, 2004). It is formally possible that chromosome mis-segregation phenotypes induced in *mal3bub1* double mutants are linked to cohesion defects at kinetochores. Clearly, more work will be necessary to clarify these points. The behaviour of sister centromeres in *mal3bub1* mutants indicates a remarkable similarity to that in the budding yeast Aurora B kinase *ip11* mutant (Tanaka, 2002; Tanaka et al, 2002). It would be of great interest to address whether Mal3 or Mal3 binding partners are regulated by Aurora B-dependent phosphorylation. As mutations in EB1-binding APC show a high rate of chromosome instability, one of the hallmarks of colorectal tumour cells (Lengauer et al, 1998; Green & Kaplan, 2003), it is tempting to speculate that the principal, if not sole, defect of these cancer cells is the failure in establishment of chromosome bi-orientation.

## METHODS

**Strains, media and genetic methods.** Strains used in this study are listed in supplementary Table S1 online. Rich YES5 and minimal EMM2 media were used. A temperature of 30 °C was used for most of the experiments, unless otherwise stated. For live imaging analysis, room temperature (22 °C) was used.

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

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