Modulated targeting of GFP-AtMAP65-1 to central spindle microtubules during division

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Summary

AtMAP65-1 bundles cortical microtubules and we examined how this property is regulated during division in time-lapse studies of Arabidopsis suspension cells expressing GFP-AtMAP65-1. Spindle fluorescence is diffuse during metaphase, restored to the central spindle at anaphase and then compacted at the midline during late anaphase/early telophase. However, mutagenesis of the microtubule-associated protein (MAP) consensus Cdk site to a non-phosphorylatable form allows premature decoration of microtubules traversing the central region of the metaphase spindle without affecting the timing of the subsequent compaction. This suggests that mutagenesis does not affect compaction but does affect a phosphorylation/dephosphorylation switch that normally targets AtMAP65-1 to the central spindle at the metaphase/anaphase transition. GFP-AtMAP65-1 continues to label the midline of the early phragmoplast, suggesting a structural continuity with the central spindle – both structures being composed of anti-parallel microtubules. However, once the cytokinetic apparatus expands into a ring the MAP becomes depleted at the midline. Despite this, cytokinesis is not arrested and membrane and callose are deposited at the cell plate. It is concluded that AtMAP65-1 plays a role in the central spindle at anaphase to early cytokinesis but is not essential at the midline of the phragmoplast at later stages.

Keywords: AtMAP65-1, microtubule-associated protein, cytokinesis, phragmoplast, cell cycle, mitosis.

Introduction

In higher plants, microtubules form arrays that underpin the processes of cell wall growth during interphase but are reorganized into three quite different assemblies during division (Lloyd and Chan, 2004). Microtubule-associated proteins (MAPs) that unite individual microtubules into complex arrays must be involved in this cyclic remodelling. In this paper we examine the role of a major cross-bridging protein, AtMAP65-1, throughout the division cycle of living suspension cells.

MAP65 was first discovered in tobacco BY-2 suspension cells (Jiang and Sonobe, 1993). Work on carrot cells then showed that a highly purified, biochemically isolated fraction of MAP65 induced brain taxol microtubules to form bundles *in vitro* – the microtubules being held 25–30 nm apart by fine cross-bridges (Chan *et al.*, 1999). The carrot MAP65 fraction was comprised of three bands on 1-D gels but it is now known that there are nine members of this multigene family in the Arabidopsis genome (Hussey and

Hawkins, 2001). These are likely to be regulated according to the cell cycle and to the developmental programme (Lloyd and Chan, 2004). A carrot MAP65 isoform, which was most closely related to AtMAP65-1, was found to be the predominant protein in MAP fractions made from carrot cells undergoing active cell elongation in G1/G0, indicating the potential importance of this isoform for the bundling of cortical microtubules (Chan *et al.*, 2003b). Recently, recombinant AtMAP65-1 was demonstrated to bundle microtubules *in vitro* (Smertenko *et al.*, 2004), consistent with the finding that the AtMAP65-1-like isoform was the most abundant isoform in the carrot MAP65 fraction that bundled microtubules (Chan *et al.*, 2003b). Wicker-Planquart *et al.* (2004) have also demonstrated that recombinant NtMAP65-1b binds and bundles microtubules *in vitro* and Van Damme *et al.* (2004a,b) have shown that AtMAP65-1-GFP decorates bundled cortical microtubules in living tobacco BY-2 cells. The question addressed in this paper is how these interphase

properties of MAP65 are used and modified in division as AtMAP65-1 is variably associated with microtubules in mitosis and cytokinesis (Smertenko *et al.*, 2000, 2004).

It is now apparent that MAP65 belongs to a family of evolutionarily conserved proteins that accumulates in the midline of the 'central spindle' where they are thought to have a role in stabilizing the anti-parallel microtubules of this microtubule array that remains after the chromatids have moved to the poles during anaphase (Kurasawa *et al.*, 2004; Mollinari *et al.*, 2002; Schuyler *et al.*, 2003; Verbrugghe and White, 2004; Verni *et al.*, 2004). These studies have shown that central spindles do not form in the absence of these MAPs. As the central spindle is continuous with the mid-body – the bundle of interdigitating microtubules involved in animal cytokinesis (Euteneuer and McIntosh, 1980) – this family of proteins has also been concluded to function in the cytokinesis of some cells, although this role is not supported for all cell types (e.g. Verbrugghe and White, 2004).

To investigate how the properties of a single MAP65 isoform affect microtubules during mitosis and cytokinesis we transiently expressed GFP-AtMAP65-1; importantly, all cells were monitored by time-lapse to demonstrate that division was not inhibited by transient infection. By muta-genizing a consensus site on GFP-AtMAP65-1 it was possible to show that the timing with which the MAP was targeted to the spindle appears to be regulated by a phosphorylation-sensitive switch. The study also showed that AtMAP65-1 becomes compacted to the midline of the late anaphase spindle and that this concentrated band of labelling continues smoothly into the formation of the early phragmoplast. However, once the phragmoplast begins to open out centrifugally the midline labelling is lost. This shows that if AtMAP65-1 has a role in stabilizing anti-parallel microtubules during cell division then it is likely to be most (but not necessarily exclusively) active at the transition between late mitosis and the setting-up of the phragmoplast.

Results

Overexpression of GFP-AtMAP65-1 in interphase cells

First, to establish that the expressed protein was capable of bundling microtubules in this cell system *in vivo*, we examined transiently infected Arabidopsis suspension cells during interphase. Figure 1 shows the fusion protein has a dramatic effect on the bundling of interphase microtubules. It induces excessive bundling of both the cortical (Figure 1a) and the nucleus-associated radial microtubule arrays (Figure 1b), drawing the normally evenly distributed microtubules into only a few groups. Figure 1(c) shows that this enhanced bundling occurs in cells containing longitudinal as well as transverse arrays. By contrast, untransformed control cells stained with anti-tubulin antibodies did not contain bundled microtubules (not shown) nor did cells transformed
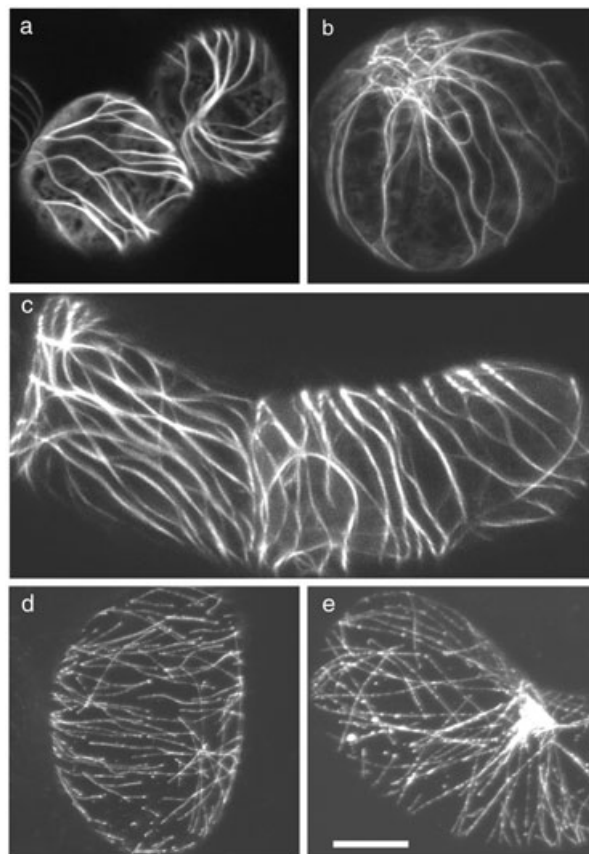

Figure 1. Overexpressed GFP-AtMAP65-1 induces heavy bundling of inter-phase microtubules.
Overexpression induces bundling of cortical (a) and nucleus-associated radial microtubules (b) and bundles microtubules of various orientations (c). Contrast this with the single, unbundled microtubules whose free ends seen are labelled with AtEB1-GFP (d, e). (e) z-section projection showing microtubules labelled with AtEB1-GFP emanating from the nucleus. Scale bar = 6.5 μm.

with another fusion protein, AtEB1-GFP (Figure 1d,e); Figure 1(d) illustrates a cortical array and Figure 1(e) illustrates a radial array in which microtubules emerge from the nuclear surface.

AtMAP65-1 is spatially and temporally regulated during cell division

By tracking cells undergoing cell division at 3-min time intervals it was possible to demonstrate that GFP-AtMAP65-1 was both spatially and temporally regulated during mitosis and cytokinesis (see Movie 1). Regulation was first observed at prometaphase/metaphase in that spindle labelling was diminished as shown in the immunofluorescence study of Smertenko *et al.* (2004) and Figure S2. In contrast to the clearly labelled cortical and PPB microtubules (Figure 2a), it became difficult at prometaphase/metaphase to discern specific microtubule fluorescence against the dim outline of
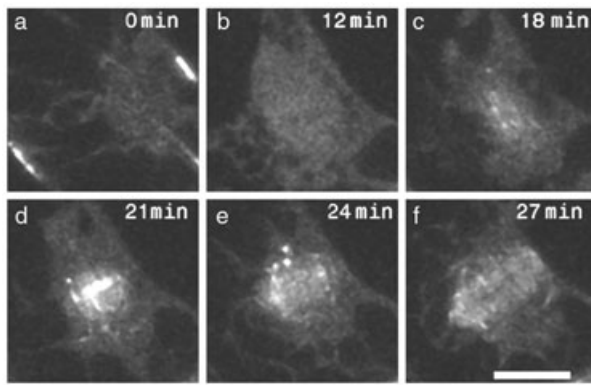
Figure 2. GFP-AtMAP65-1 is spatially and temporally regulated during cell division.
Successive stills from a time-lapse movie. During cell division GFP-AtMAP65-1 accumulates on the microtubules of the preprophase band (a). During metaphase (b) it can be seen that GFP-AtMAP65-1 only diffusely labels the spindle area. However, at early anaphase (c) GFP-AtMAP65-1 starts to re-associate with the central spindle where it concentrates in the midzone from which the phragmoplast develops (d). But as the phragmoplast matures the midline staining is replaced with a more general microtubular staining pattern with a zone of depleted labelling in the midline of the phragmoplast (e, f). Scale bar = 14 μm (a) and 10 μm (b–f).

Figure 3. Compaction of GFP-AtMAP65-1 at the anaphase to telophase transition. GFP-AtMAP65-1 is targeted to the central spindle at anaphase (a) and then becomes further concentrated to the midline a few minutes later (b). Scale bar = 7.3 μm.

the spindle (Figure 2b). The diminution was most clearly seen by comparing fluorescence levels within a time-lapse series, not by single images taken from different cells at different microscope settings. Fluorescence intensity measurements of three such cells that could be tracked from preprophase through to cytokinesis confirmed that while the PPB was 5.18 times brighter than the general cytoplasm, the fluorescence in the region of the prophase/metaphase spindle was only 2.23 times brighter than the cytoplasmic level.

Clear fibrillar fluorescence patterns then reappeared in early anaphase in the central spindle region (Figure 2c). Then, during late anaphase, the GFP-AtMAP65-1 became concentrated in a zone in the midline amongst the phragmoplast initials (Figure 2d). The midline labelling was, however, transient as it then redistributed amongst the microtubules of the two phragmoplast halves, leaving a zone depleted of labelling in the midline as the phragmoplast matured (Figure 2e,f). This compaction of label at the late anaphase/telophase transition is better illustrated in Figure 3.

We tested whether these cell-cycle-specific staining patterns represented changes in protein level or cellular distribution of GFP-AtMAP65-1 by measuring the fluorescence intensities of the nuclear region (encompassing both the spindle and its surrounding cytoplasm) of cells as they underwent spindle morphogenesis. However, despite the fact that the spindle-associated fluorescence was 5.19 times higher than the cytoplasmic level at early anaphase and 8.45 times higher at late anaphase, the total level of fluoresce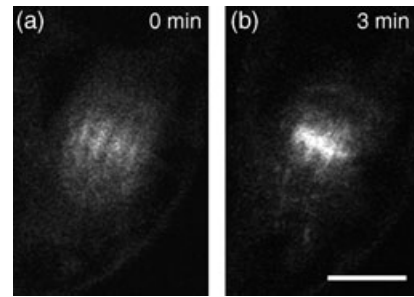nce hardly varied. From metaphase to early anaphase the GFP signal only varied by 1:1.13 and from early anaphase to late anaphase/telophase by 1:0.94. This suggests that the changed labelling pattern is due to a redistribution rather than a general increase in the amount of fluorescent protein.

Mutagenesis of the Cdk site

In view of the time-lapse data showing that the labelling of the metaphase spindle by GFP-AtMAP65-1 was diminished compared to the rest of mitosis we examined the possibility that the MAP was modified at this stage. Jiang *et al.* (1998) have examined the role of Cdk in regulating the MAP65 homologue, PRC1, during division and Mollinari *et al.* (2002) examined this further by mutagenizing the consensus Cdk sites. We therefore mutagenized the corresponding consensus Cdk phosphorylation site of AtMAP65-1 between amino acid residues 503 and 506: [S/T]-P-X-[K/R] (Kennelly and Krebs, 1991). We mutated serine 503 to non-phosphorylatable alanine and transfected Arabidopsis cells with the GFP-AtMAP65-1[A] mutant. The mutated AtMAP65-1 was still able to induce heavy bundling of cortical microtubules and these cells could be shown to complete division (Movie 2). Overall, the mutated GFP-AtMAP65-1[A] showed a similar pattern of decoration to the control in that the spindle labelling was less intense than on the preprophase band but then concentrated again on the midzone of the nascent phragmoplast at the late anaphase to telophase transition (Figure 4). The timings were also similar in both cases. By tracking three cells at 3-min time intervals we found that it took an average of 18.7 min for control cells to progress from prometaphase (defined as the first frame after the disappearance of the PPB) to a stage in late anaphase (the first frame with intense central spindle staining) and it took 22 min for the mutant. The intense, discrete labelling in the central spindle region then redistributed within 6.3 min in the control versus 5 min in the mutant background.
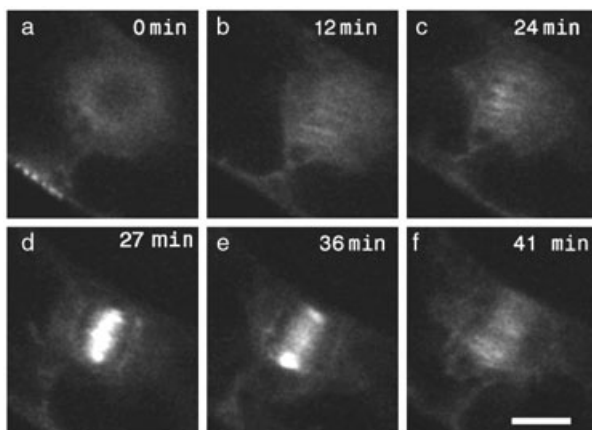
Figure 4. Time-lapse analysis of cell expressing mutagenized GFP-AtMAP65-1[A].

The general labelling pattern resembles that of wild-type MAP65-1 cells in that fluorescence diminishes during prometaphase (b) and metaphase (c) compared to the PPB (a), and then transiently increases during anaphase/telophase (d–f) at the midzone of late anaphase spindle. However, a significant difference is that central spindle microtubules are labelled earlier in the mutant cells. Scale bar = 10 μm.

However, there was a qualitative difference in the staining pattern of spindles. Spindles labelled by mutant GFP-AtMAP65-1[A] produced a fibrous staining pattern in the central region of the spindle throughout spindle morphogenesis in contrast to control cells in which unmutagenized fusion protein only appeared to accumulate at the central spindle from anaphase onwards (compare Figures 2b and 4b). This difference is particularly evident in the detailed sequence of spindle morphogenesis illustrated in Figure 5 (also see Movie 2). Mutant GFP-AtMAP65-1[A] labelled fibres of the central spindle region within 3 min (one frame) of PPB breakdown whereas it took an average of 16 min in the sampled wild-type cells.

This early targeting of mutant GFP-AtMAP65-1[A] to the central spindle was also investigated by taking fluorescence profiles along the spindle axis. Figure 6 compares the profiles of GFP-AtMAP65-1 versus mutant GFP-AtMAP65-1[A] spindles 6 min after the disappearance of the PPB. As can be seen, GFP-AtMAP65-1 yielded relatively flat traces across the spindle axis consistent with the staining pattern being diffuse and non-polarized at this stage. By contrast, the fluorescence in GFP-MAP65-1[A] mutant cells was more focused to the centre of the spindle – consistent with the presence of fibrous staining patterns in this area.

Overexpression induces bundling of phragmoplast microtubules

Antibodies to AtMAP65-1 have shown that the protein is concentrated in the midzone of the phragmoplast (Smertenko et al., 2004) but in the present study overexpression

changed this phenotype. In transformed cells, GFP-AtMAP65-1 was not concentrated in the midline but appeared to label the entire phragmoplast (Figure 7). Notably, the fusion protein induced extensive microtubule bundling during cytokinesis (Figure 7a–d): normally the phragmoplast microtubules are difficult to resolve but overexpression caused them to be drawn into countable bundles (Figure 7c). This bundling was apparent at all stages of cytokinesis, from late anaphase (when the phragmoplast initials first appear out of the spindle midzone: Figure 7a) to the later stage of phragmoplast expansion depicted in Figure 7(c,d) [see also Figure S3(d)–(g)]. Overexpression of GFP-AtMAP65-1 also promoted the bundling of the aster-like microtubules that interact with the cell cortex (Figure 7d).

Phragmoplasts with bundled microtubules make cell plates

Movie 3 and Figure S3 show that despite the bundling of the phragmoplast microtubules and the poor/non-labelling of the midline, the cytokinetic apparatus was still capable of centrifugal outgrowth and transition to interphase. These data suggest that it may not be essential for AtMAP65-1 to be compacted at the midline of the phragmoplast for the cytokinetic apparatus to expand. To check whether cells plates were actually deposited under such conditions, transfected cells were stained with the fluorescent membrane dye, FM4-64, which has been shown to label membrane of forming cell plates in plant suspension cells (Bolte et al., 2004).

Figure 8(a) shows the GFP-AtMAP65-1 signal of a cell undergoing a polarized form of cytokinesis where the phragmoplast has already contacted the mother wall at the left. The leading edge of the phragmoplast to the right is marked by abnormal, curled microtubule bundles and in Figure 8(b) the FM4-64 staining of the same cell shows that membranous material is contributing to the formation of the cell plate that has contacted the left-hand wall of the mother cell. To check that the membranous disk contained cell plate polysaccharide, transformed cells were stained with the callose dye, aniline blue. Figure 8 confirms that forming (Figure 8c,d) and completed cell plates (Figure 8e,f) contain disk-shaped deposits of aniline blue-positive material.

Discussion

MAP65 is a microtubule bundling protein shown to cross-bridge microtubules into groups (Chan et al., 1996, 1999; Jiang and Sonobe, 1993). That the group 1 sub-class MAP65s are active in interphase has been demonstrated with biochemically isolated (Chan et al., 2003b) and recombinant (Smertenko et al., 2004; Wicker-Planquart et al., 2004) protein *in vitro* and with GFP fusion protein *in vivo* (Van Damme et al., 2004a,b).

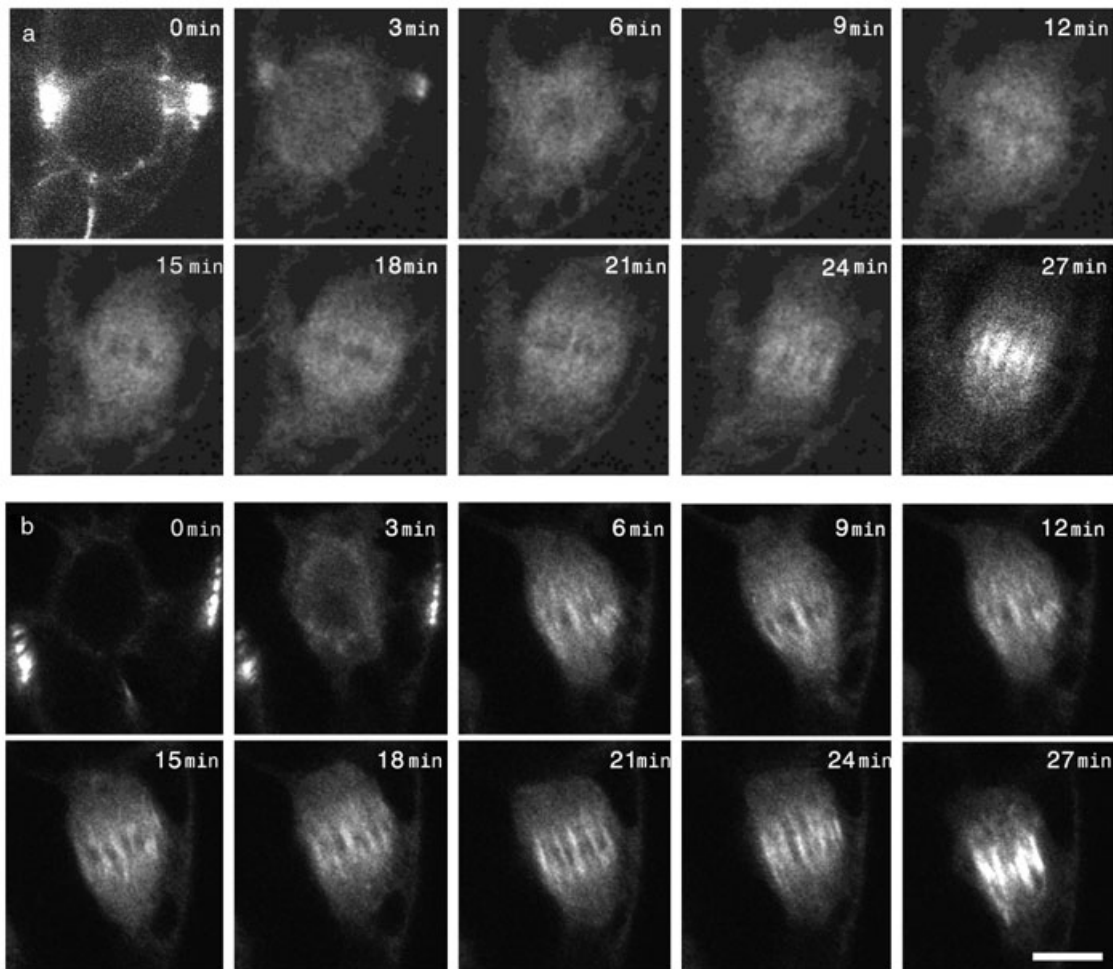The phenotype shown here for Arabidopsis suspension cells overexpressing GFP-AtMAP65-1 is particularly pro-

Figure 5. Time-lapse images showing the early targeting of mutant GFP-AtMAP65-1[A] to the central region of the spindle.
(a) In wild-type cells, central spindle labelling becomes apparent 15-min post-PPB breakdown.
(b) Fibres that traverse the two halves of the spindle are evident after only one frame (3 min) following PPB breakdown in the mutant background. Scale bar = 7.3 µm.

nounced, drawing the cortical microtubules into just a few bundles. MAP65 forms cross-bridges of 25–30 nm between microtubules (Chan *et al*., 1999) and Smertenko *et al.* (2004) recently showed *in vitro* that recombinant AtMAP65-1 can span this gap, probably as a homodimer. The MAP65 homologue in *Saccharomyces cerevisiae*, Ase1p, has also been reported to form homodimers (Schuyler *et al.*, 2003). The extensive cross-linking induced in the present study by overexpression of a single MAP65 isoform is consistent with homodimerization as a mechanism for cross-linking although heterodimerization cannot be ruled out in the milieu of the cell.

The polarity of plant microtubules is often difficult to judge but several studies have shown that endoplasmic microtubules radiate out from the surface of the plant cell's nucleus (see Schmit, 2002 for a review). For example, Stoppin *et al.* (1994) have shown that neurotubulin is

polymerized from the nuclear surface *in vitro* with the growing plus ends of the microtubules distal to the nuclear surface. This conclusion is also supported by our own studies using AtEB1-GFP (Chan *et al.*, 2003a) showing microtubule plus ends grow towards the cortex so that the minus ends are therefore proximal to the nuclear surface. The fact that GFP-AtMAP65-1 bundles such microtubules suggests that the MAP is capable of cross-linking parallel microtubules – a conclusion supported by the bundling of microtubules in the separate halves of the phragmoplast, which are presumed to have uniform polarity (e.g. Figure 7c,d).

Labelling of the spindle midzone

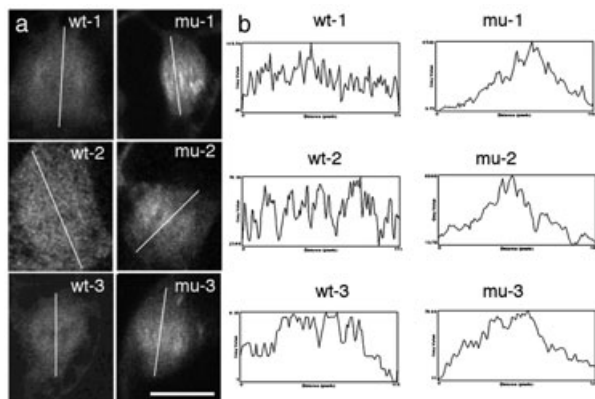By tracking individual cells, it could be seen that GFP-AtMAP65-1 decorates the interphase cortical array and

Figure 6. Fluorescence profiles of cells 6 min after PPB breakdown
(a) Six-min-old spindles labelled by GFP-AtMAP65-1 (wt) and GFP-AtMAP65-1[A] (mu) and the longitudinal axes used for analysis.
(b) Fluorescence profiles of the spindle axes depicted in (a). Fluorescence intensities are more focused to the centre of the spindle in mutant cells indicating precocious labelling of microtubules in this region. Scale bar in (a) = 10.4 μm.

the preprophase band but that the labelling at metaphase becomes diffuse before reappearing strongly in the midzones of the late anaphase spindle and the early stage phragmoplast. This 'metaphase effect' is consistent with the poor immunolabelling of fixed Arabidopsis suspension cells at this stage (Smertenko *et al.*, 2004 and Figure S2). This changed labelling pattern suggested that the binding or bundling of AtMAP65-1 is downregulated specifically in prometaphase/metaphase as has been shown for other members of this extended family of central spindle proteins: SPD-1 in *C. elegans* (Verbrugghe and White, 2004)
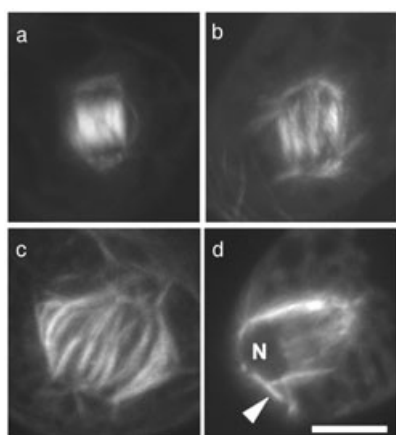

Figure 7. Effects of overexpressed GFP-AtMAP65-1 on division.
Formation of the phragmoplast, from the anaphase midzone (a) to the outgrowth of the phragmoplast (b). Note the thick, elongated microtubule bundles of the phragmoplast in (c). The aster-like microtubules that radiate from the spindle pole and contact the cortex (arrowed in d) are also bundled. N denotes the position of the nuclei. Scale bar = 9.2 μm.
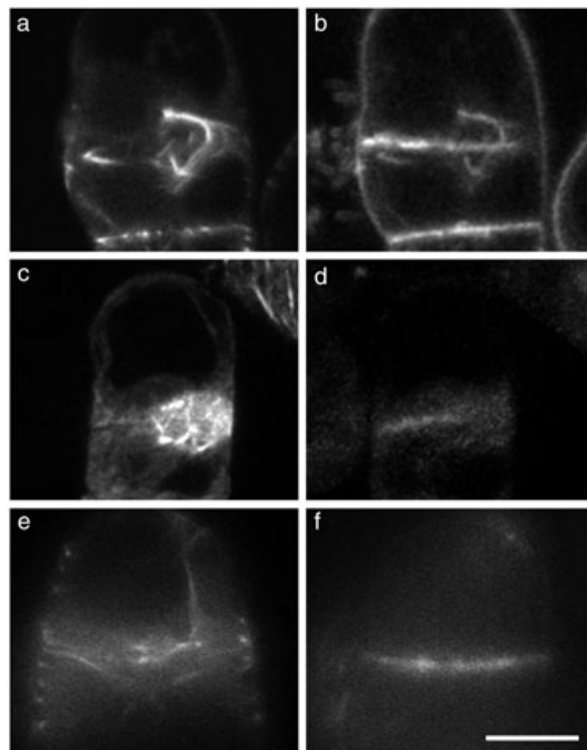

Figure 8. Cell plate formation in cells overexpressing GFP-AtMAP65-1.
(a) Phragmoplast with irregular and bundled GFP-labelled microtubules. Double-labelling with the fluorescent membrane dye FM4-64. (b) A Membranous plate has formed in this polarized phragmoplast that has already fused with the mother cell wall on the left. In (c), the GFP-AtMAP65-1 labels another phragmoplast that has already fused with the mother wall to the left; the aniline blue staining of the same cell in (d) indicates the formation of a callose-containing cell plate. (e) A later stage (GFP-AtMAP65-1) and its aniline blue-dyed plate (f). Scale bar = 10 μm (a–d, g, h); 6 μm (e, f).

and Feo in *Drosophila* (Verni *et al.*, 2004). However, this differs from human PRC1, which is constitutively on the metaphase spindle (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). In these Arabidopsis cells, following the phase of diffuse labelling, GFP-AtMAP65-1 then labels the central spindle at anaphase – the subset of overlapping non-kinetochore microtubules that gives rise to the midbody in animal cells and appears to give rise to the phragmoplast in somatic plant cells. A key feature of this late anaphase/early telophase transition is the compaction of the fluorescence to the midzone of the central spindle/nascent phragmoplast. As quantification revealed there was no more fluorescence at either early or late anaphase than at metaphase, it seems that the reappearance of microtubular labelling at the central spindle is more likely to be due to modification of its associative properties than to changes in the amount of protein.

By mutagenizing serine 503 in the consensus Cdk site to a non-phosphorylatable alanine we were able to change the labelling pattern as the GFP-AtMAP65-1[A]

prematurely labelled the central spindle within 3 min of PPB breakdown. Intriguingly, the labelling appeared to be concentrated towards the midline rather than the poles, decorating the microtubule bundles that traversed the metaphase plate. Serine 503 would therefore seem to be involved in the targeting of AtMAP65-1 to the spindle. As this occurs prematurely in the mutant, in which the Cdk site cannot be phosphorylated, the restoration of labelling at anaphase in the wild type could be controlled by a phosphatase switch at the metaphase to anaphase transition, as discussed for animal cells (Mishima *et al.*, 2004).

In plants, the possibility has similarly been raised that Cdk plays some part in modifying the properties of microtubules during the transition between mitosis and cytokinesis (Weingartner *et al.*, 2004). They demonstrated in tobacco BY-2 cells that ectopic expression of a non-degradable cyclin B1 caused the midzone microtubules to disappear during anaphase with the subsequent failure of phragmoplast formation. Weingartner *et al.* (2004) suggested that this could have been caused by changes in the phosphorylation status of MAP65-1. Our results are consistent in that mutagenesis of the consensus Cdk site induced premature labelling by GFP-AtMAP65-1 of the central spindle; however, as mutagenesis did not induce the premature compaction of labelling (which occurred at late anaphase/telophase in wild type and mutant alike), microtubule binding and compaction seem to be under separate control.

Compaction to the midline has been shown in animal cells to depend upon the involvement of the plus end kinesin Kif4 whose interaction with PRC1 seems to be regulated by phosphorylation/dephosphorylation (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005). The observed compaction of AtMAP65-1 in the midline supports the intervention of a Kif4-like kinesin in plants and although a plant homologue exists (At5g60930) its function remains to be proved.

The role of AtMAP65-1 in early cytokinesis

In some cells, this key role of stabilizing the anti-parallel microtubules of the central spindle (the anti-parallel spindle microtubules remaining at anaphase) continues into early cytokinesis. For example, the cross-bridging activity of PRC1 is restored in late anaphase and becomes localized to the middle of the cytokinetic midbody (Mollinari *et al.*, 2002) much like AtMAP65-1 is concentrated in the midzone of the late anaphase spindle and the early column-stage phragmoplast (Smertenko *et al.*, 2004 and present results). Hook decoration studies have established that both of the latter microtubule arrays of plants, which overlap in space and time in somatic cells (Zhang *et al.*, 1990), are composed of anti-parallel microtubules with plus ends overlapping in the middle (Euteneuer and McIntosh, 1980). Our observations therefore support the idea that this extended family of cross-bridging MAPs is active where overlapping sets of anti-parallel microtubules are stabilized, not only during late mitosis but also during early cytokinesis (Mollinari *et al.*, 2002; Schuyler *et al.*, 2003). This reinforces the view that the phragmoplast and midbody, which both evolve out of the central spindle, are homologous organelles.

However, once the phagmoplast grows into a ring the GFP signal becomes depleted at the line of microtubule overlap, and so our results only provide positive support for a role for AtMAP65-1 in stabilizing anti-parallel microtubules during the restricted transitional period between late mitosis and the early stages of cytokinesis. In untransformed Arabidopsis suspension cells, antibodies specific for AtMAP65-1 label a restricted mid-zone region across the two halves of the ring-stage phragmoplast (Smertenko *et al.*, 2004), which is broader than the narrow line of overlap labelled by antibodies to AtMAP65-3 (Muller *et al.*, 2004) and by AtMAP65-3-GFP (Van Damme *et al.*, 2004a). This differs from our inability to decorate the midline with GFP-AtMAP65-1 during later stages of cytokinesis. This apparent exclusion of GFP-AtMAP65-1 from the midline is accompanied by the induced bundling and more general labelling of microtubules in the phragmoplast. Although this phenotype may have been induced by overexpression, the fact that these cells form cell plates and complete cytokinesis demonstrates that it is not essential for AtMAP65-1 to be concentrated in a narrow or even broad midline during the latter stages of phragmoplast formation in order to undergo successful cytoplasmic division.

We can envisage two general explanations for the apparent depletion of GFP-AtMAP65-1 at the midline once the phragmoplast has developed to the ring-stage, depending on the presence or absence of microtubules at this locus. First, if microtubules do overlap at the midline then, under the present conditions, either GFP-AtMAP65-1 is excluded from the extreme plus ends of all overlapping microtubules or it does not appear to decorate a subpopulation of microtubules that do overlap at the midline but are difficult to visualize by fluorescence microscopy. Alternatively, the failure of GFP-AtMAP65-1 to produce a restricted labelling of the midline could reflect the absence of microtubules from the midline once the phragmoplast develops and the cell plate becomes deposited between the two halves. Recent high-resolution EM studies with high-pressure frozen/freeze substituted material indicate that in later-stage phragmoplasts the plus ends of microtubules are embedded in the cell plate matrix material with little evidence for microtubule overlap (Segui-Simarro *et al.*, 2004; L. A. Staehelin, personal communication). Absence (or diminution) of opposing microtubules that cross over within the plate is consistent with the presence of a dark, poorly labelled midline in phragmoplasts of living Arabidopsis cells transformed with GFP-tubulin (Ueda *et al.*, 2003). Unlike the dark line often observed in phragmoplasts labelled with fluorescent

antibodies (e.g. Clayton and Lloyd, 1984), which may be explained as poor penetration of antibody into a congested area, the diminished fluorescence from the site where overlapping microtubules should give the strongest signal cannot be easily explained by apparent exclusion of the GFP-tubulin. But if microtubules do not meet in the midline, how to explain the fact the cytokinetically important isoform AtMAP65-3 (Muller *et al.*, 2004; Van Damme *et al.*, 2004a) and the kinesin, AtPAKRP1 (Pan *et al.*, 2004), do form a thin line of positive staining along the midline of the phragmoplast? Further work is required to decide whether such proteins are still marking the plus ends of a minority of hard-to-see phragmoplast microtubules (which would be beyond the ends of the thick bundles seen in the present study) or whether these proteins have become detached from the ends of microtubules to become associated with cell plate matrix material.

Whichever explanation proves to be correct, the expression phenotype of GFP-AtMAP65-1 is consistent with the hypothesis that the binding/bundling properties of AtMAP65-1 are downregulated during metaphase, prior to its targeting to the central spindle during anaphase and then focussing to the central spindle in late anaphase/early telophase. Serine 503 seems to be involved in the targeting of AtMAP65-1 to the central spindle at anaphase, as this occurs prematurely in the mutant, in which the Cdk site cannot be phosphorylated – consistent with the operation of a phosphatase switch at the metaphase-to-anaphase transition. These results support the hypothesis that, like its homologues in animal cells, MAP65 is a key organizer of the central spindle, necessary for proper cytokinesis.

Experimental procedures

Plant cells

The *Arabidopsis thaliana* 'Col-0' cell suspension (Mathur *et al.*, 1998) was grown on a shaker, in ATN medium, at 21 °C in the dark and sub-cultured weekly (see Chan *et al.*, 2003a).

Cloning and transformation of GFP-AtMAP65-1 in Arabidopsis cells

The Arabidopsis *AtMAP65-1* gene (Smertenko *et al.*, 2004) (*AT5g55230*) was cloned by RT-PCR into the plant expression vector, pBinGWC, using the Gateway system (Invitrogen, Carlsbad, CA, USA) as described (Chan *et al.*, 2003a). The primers used were: forward – AAAAAGCAGGCTTCATGGCAGTTACAGATACTG; reverse – AGAAAGCTGGGTCTCATGGTGAAGCTGGAAC.

The construct of GFP-AtMAP65-1 was confirmed by sequencing. Arabidopsis cells were transformed by *Agrobacterium* (GV3101) harbouring the GFP-AtMAP65-1 expression construct. Log phase bacteria were incubated 1 h in the solution containing 10 μM acetosyringone (Sigma, St Louis, MO, USA) and 100 mM magnesium chloride. These were then added to a 1-day-old Arabidopsis cell suspension and expression of GFP-AtMAP65-1 was detected after 2 days.

Site-directed mutagenesis

The cdc2 phosphorylation null mutant, AtMAP65-1[A], was obtained using the GeneTailor site-directed mutagenesis system (Invitrogen) according to the manufacturer's instructions. Serine 503 was substituted by alanine by PCR using the AtMAP65-1 entry clone as a template. The primers were: cdcF1 – GCC TTT AGC ACC AGG CCA GCT CCT GCA AGA C; cdcR1 – TGG CCT GGT GCT AAA GGC AGA TTC TTG CTC.

After fusing with GFP, the GFP-AtMAP65-1[A] Cdk mutant was used to transform Arabidopsis cells as above.

Fluorescence microscopy

Cells were fixed for 1 h in PME (50 mM PIPES, 5 mM magnesium sulphate and 5 mM EGTA) containing 4% (w/v) formaldehyde as described (Chan *et al.*, 2003a).

Rabbit antibodies against the conserved peptide CEEESWLE-DYNR were made by Biogenes GmbH (Berlin, Germany), secondary antibody used was Alexa 568 conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). Anti-tubulin YOL 1/34 was purchased from Abcam (Cambridge, UK), secondary antibody used was CY3 conjugated anti-rat IgG (Amersham, Little Chalfont, UK).

Protein analysis

SDS-PAGE and western blotting were carried out according to Chan *et al.* (1996). Antibodies [anti-GFP (Clontech, Basingstoke, UK) and the anti-CEEE peptide antibody] were used at 1:1000 for immunoblotting. The primary antibody was detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) using horseradish peroxidase-conjugated secondary antibodies diluted 1:10 000 (DAKO, Ely, Denmark).

Confocal microscopy and image analysis

Transformed Arabidopsis cells were imaged using either a Leica SP2 (Milton Keynes, UK), or Bio-Rad MRC 1024 (Hemel Hempstead, UK) confocal microscopes. GFP was excited using 488 nm light from either an argon ion or argon krypton laser and imaged using emission filters 500–550 nm (Leica SP2) or 515LP (Bio-Rad MRC 1024). Aniline blue staining was excited using 405 nm light from a violet laser diode and imaged using 420–490 nm filter (Leica SP2). FM4-64 was excited using 568 nm light from an argon krypton laser and imaged using a 585LP filter (Bio-Rad MRC1024). CY3 and Alexa 568 was excited using 543 nm light from a green helium neon laser and imaged using 560–620 nm filter (Leica SP2). Images from z sections were 'max' projected using either the Leica software or ImageJ programme (http://rsb.info.nih.gov/ij/). Time-lapse imaging of living cells were carried out in a modified chamber, constructed using a gas-permeable membrane (bioFOLIE, VivaScience, Hannover, Germany). Time-lapse images were acquired at 3–5-min intervals and the images processed using ImageJ. The intensity of fluorescence within and outside microtubule arrays (in five different locations, of fixed box size) was quantified using the 'measure' tool of ImageJ. Mean fluorescence intensities were expressed relative to the general cytoplasmic level. The total amount of fluorescence within the nuclear region of metaphase and anaphase cells was measured using the 'freehand selection' and measure tools of ImageJ.

Cell plate staining

Aniline blue (Acros organics, c/o Fisher Scientific UK, Manchester, UK) was used to stain callose by adding it to living cells at 0.1 mM in culture medium for 1 h at room temperature. FM4-64 (Molecular Probes) was used to stain membranes by adding at 1 μm to living cells for 3 h at room temperature.

Acknowledgements

Supplementary Material

The following supplementary material is available for this article online:
Figure S1. Specificity of the anti-AtMAP65-1 peptide antibody.
Figure S2. Immunolabelling of Arabidopsis suspension cells using the anti-At MAP65-1 peptide antibody.
Figure S3. Stills from a time-lapse series (see Movie 2) of a transformed cell undergoing division.
Movie 1. GFP-AtMAP65-1 is spatially and temporally regulated during cell division.
Movie 2. Mutagenized GFP-AtMAP65-1[A] prematurely labels the central spindle region in dividing cells.
Movie 3. Phragmoplast morphogenesis is not inhibited by microtubule bundling.

References

Bolte, S., Talbot, C., Boutte, Y., Catrice, O., Read, N.D. and Satiat-Jeunemaitre, B. (2004) FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. *J. Microsc.* **214**, 159–173.

Chan, J., Rutten, T. and Lloyd, C. (1996) Isolation of microtubule-associated proteins from carrot cytoskeletons: a 120 kDa map decorates all four microtubule arrays and the nucleus. *Plant J.* **10**, 251–259.

Chan, J., Jensen, C.G., Jensen, L.C., Bush, M. and Lloyd, C.W. (1999) The 65-kDa carrot microtubule-associated protein forms regularly arranged filamentous cross-bridges between microtubules. *Proc. Natl Acad. Sci. USA*, **96**, 14931–14936.

Chan, J., Calder, G.M., Doonan, J.H. and Lloyd, C.W. (2003a) EB1 reveals mobile microtubule nucleation sites in Arabidopsis. *Nat. Cell Biol.* **5**, 967–971.

Chan, J., Mao, G., Smertenko, A., Hussey, P.J., Naldrett, M., Bottrill, A. and Lloyd, C.W. (2003b) Identification of a MAP65 isoform involved in directional expansion of plant cells. *FEBS Lett.* **534**, 161–163.

Clayton, L. and Lloyd, C.W. (1984) The relationship between the division plane and spindle geometry in Allium cells treated with CIPC and griseofulvin: an anti-tubulin study. *Eur. J. Cell Biol.* **34**, 248–253.

Euteneuer, U. and McIntosh, J.R. (1980) Polarity of midbody and phragmoplast microtubules. *J. Cell Biol.* **87**, 509–515.

Hussey, P.J. and Hawkins, T.J. (2001) Plant microtubule-associated proteins: the HEAT is off in temperature-sensitive mor1. *Trends Plant Sci.* **6**, 389–392.

Jiang, C.-J. and Sonobe, S. (1993) Identification and preliminary characterization of a 65 kDa higher plant microtubule-associated protein. *J. Cell Sci.* **105**, 891–901.

Jiang, W., Jimenez, G., Wells, N.J., Hope, T.J., Wahl, G.M., Hunter, T. and Fukunaga, R. (1998) PRC1: a human mitotic spindle-associated CDK substrate protein required for cytokinesis. *Mol. Cell.* **2**, 877–885.

Kennelly, P.J. and Krebs, E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**, 15555–15558.

Kurasawa, Y., Earnshaw, W.C., Mochizuki, Y., Dohmae, N. and Todokoro, K. (2004) Essential roles of KIF4 and its binding partner PRC1 in organized central spindle midzone formation. *EMBO J.* **23**, 3237–3248.

Lloyd, C. and Chan, J. (2004) Microtubules and the shape of plants to come. *Nat. Rev. Mol. Cell Biol.* **5**, 13–22.

Mathur, J., Szabados, L., Schaefer, S., Grunenberg, B., Lossow, A., Jonas-Straube, E., Schell, J., Koncz, C. and Koncz-Kalman, Z. (1998) Gene identification with sequenced T-DNA tags generated by transformation of Arabidopsis cell suspension. *Plant J.* **13**, 707–716.

Mishima, M., Pavicic, V., Gruneberg, U., Nigg, E.A. and Glotzer, M. (2004) Cell cycle regulation of central spindle assembly. *Nature*, **430**, 908–913.

Mollinari, C., Kleman, J.P., Jiang, W., Schoehn, G., Hunter, T. and Margolis, R.L. (2002) PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone. *J. Cell Biol.* **157**, 1175–1186.

Muller, S., Smertenko, A., Wagner, V., Heinrich, M., Hussey, P.J. and Hauser, M.T. (2004) The plant microtubule-associated protein AtMAP65-3/PLE is essential for cytokinetic phragmoplast function. *Curr. Biol.* **14**, 412–417.

Pan, R., Lee, Y.R. and Liu, B. (2004) Localization of two homologous Arabidopsis kinesin-related proteins in the phragmoplast. *Planta*, **220**, 156–164.

Schmit, A.C. (2002) Acentrosomal microtubule nucleation in higher plants. *Int. Rev. Cytol.* **220**, 257–289.

Schuyler, S.C., Liu, J.Y. and Pellman, D. (2003) The molecular function of Ase1p: evidence for a MAP-dependent midzone-specific spindle matrix. Microtubule-associated proteins. *J. Cell Biol.* **160**, 517–528.

Segui-Simarro, J.M., Austin, J.R. II, White, E.A. and Staehelin, L.A. (2004) Electron tomographic analysis of somatic cell plate formation in meristematic cells of Arabidopsis preserved by high-pressure freezing. *Plant Cell* **16**, 836–856.

Smertenko, A., Saleh, N., Igarashi, H., Mori, H., Hauser-Hahn, I., Jiang, C.J., Sonobe, S., Lloyd, C.W. and Hussey, P.J. (2000) A new class of microtubule-associated proteins in plants. *Nat. Cell Biol.* **2**, 750–753.

Smertenko, A.P., Chang, H.-Y., Wagner, V., Kaloriti, D., Fenyk, S., Sonobe, S., Lloyd, C., Hauser, M.-T. and Hussey, P.J. (2004) The Arabidopsis microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling capacity. *Plant Cell*, **16**, 2035–2047.

Stoppin, V., Vantard, M., Schmit, A.C. and Lambert, A.M. (1994) Isolated plant nuclei nucleate microtubule assembly: the nuclear surface in higher plants has centrosome-like activity. *Plant Cell*, **6**, 1099–1106.

Ueda, K., Sakaguchi, S., Kumagai, F., Hasezawa, S., Quader, H. and Kristen, U. (2003) Development and disintegration of phragmoplasts in living cultured cells of a GFP::TUA6 transgenic *Arabidopsis thaliana* plant. *Protoplasma*, **220**, 111–118.

Van Damme, D., Bouget, F.Y., Van Poucke, K., Inze, D. and Geelen, D. (2004a) Molecular dissection of plant cytokinesis and phragmoplast structure: a survey of GFP-tagged proteins. *Plant J.* **40**, 386–398.

Van Damme, D., Van Poucke, K., Boutant, E., Ritzenthaler, C., Inze, D. and Geelen, D. (2004b) In vivo dynamics and differential microtubule-binding activities of MAP65 proteins. *Plant Physiol.* **136**, 3956–3967.

Verbrugghe, K.J. and White, J.G. (2004) SPD-1 is required for the formation of the spindle midzone but is not essential for the completion of cytokinesis in C. elegans embryos. *Curr. Biol.* **14**, 1755–1760.

Verni, F., Somma, M.P., Gunsalus, K.C., Bonaccorsi, S., Belloni, G., Goldberg, M.L. and Gatti, M. (2004) Feo, the *Drosophila* homolog of PRC1, is required for central-spindle formation and cytokinesis. *Curr. Biol.* **14**, 1569–1575.

Weingartner, M., Criqui, M.C., Meszaros, T., Binarova, P., Schmit, A.C., Helfer, A., Derevier, A., Erhardt, M., Bogre, L. and Genschik, **P.** (2004) Expression of a nondegradable cyclin B1 affects plant development and leads to endomitosis by inhibiting the formation of a phragmoplast. *Plant Cell*, **16**, 643–657.

Wicker-Planquart, C., Stoppin-Mellet, V., Blanchoin, L. and Vantard, M. (2004) Interactions of tobacco microtubule-associated protein MAP65-1b with microtubules. *Plant J.* **39**, 126–134.

Zhang, D., Wadsworth, P. and Hepler, P.K. (1990) Microtubule dynamics in living dividing plant cells: confocal imaging of microinjected fluorescent brain tubulin. *Proc. Natl Acad. Sci. USA*, **87**, 8820–8824.

Zhu, C. and Jiang, W. (2005) Cell cycle-dependent translocation of PRC1 on the spindle by Kif4 is essential for midzone formation and cytokinesis. *Proc. Natl Acad. Sci. USA*, **102**, 343–348.