JCB Article

PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone

Cristiana Mollinari,[1] Jean-Philippe Kleman,[1] Wei Jiang,[2] Guy Schoehn,[3] Tony Hunter,[4] and Robert L. Margolis[1]

[1]Institut de Biologie Structurale J-P Ebel (CEA-CNRS), 38027 Grenoble cedex 1, France
[2]Department of Biochemistry, New York University Medical Center, New York, NY 10016
[3]EMBL Grenoble Outstation, 38042 Grenoble cedex, France
[4]The Salk Institute, La Jolla, CA 92037

Midzone microtubules of mammalian cells play an essential role in the induction of cell cleavage, serving as a platform for a number of proteins that play a part in cytokinesis. We demonstrate that PRC1, a mitotic spindle-associated Cdk substrate that is essential to cell cleavage, is a microtubule binding and bundling protein both in vivo and in vitro. Overexpression of PRC1 extensively bundles interphase microtubules, but does not affect early mitotic spindle organization. PRC1 contains two Cdk phosphorylation motifs, and phosphorylation is possibly important to mitotic suppression of bundling, as a Cdk phosphorylation-null mutant causes extensive bundling of the prometaphase spindle. Complete suppression of PRC1 by siRNA causes failure of microtubule interdigitation between half spindles and the absence of a spindle midzone. Truncation mutants demonstrate that the NH_2-terminal region of PRC1, rich in α-helical sequence, is important for localization to the cleavage furrow and to the center of the midbody, whereas the central region, with the highest sequence homology between species, is required for microtubule binding and bundling activity. We conclude that PRC1 is a microtubule-associated protein required to maintain the spindle midzone, and that distinct functions are associated with modular elements of the primary sequence.

Introduction

Mitosis is a highly regulated process characterized by dramatic and coordinated morphological changes to ensure the fidelity of chromosome segregation. Cytokinesis occurs at the final stage of mitosis and is accomplished by the contraction of an acto-myosin ring that leads to daughter cell separation at the midbody (Cao and Wang, 1990). A number of proteins accumulate at the midzone of the mammalian mitotic spindle during late mitosis and have been shown to play a role in cell cleavage by antibody suppression, overexpression, or mutagenesis. Among these proteins are passenger proteins such as INCENP (Mackay et al., 1998) and survivin (Skoufias et al., 2000; Uren et al., 2000); protein kinases such as polo (Lee et al., 1995) and aurora B (Terada et al., 1998); small G-proteins such as Rho (Takada et al., 1996; Drechsel et al., 1997; O'Connell et al., 1999); and microtubule motor proteins such as CENP-E (Yen et al., 1992; Martineau-Thuillier et al., 1998), Rab6-KIFL (Hill et al., 2000), and MKLP1 (Sellitto and Kuriyama, 1988; Nislow et al., 1992). Some interactions among these proteins have been established, but specifically defined functional roles in the cleavage process are still largely unknown. Another protein that accumulates in the spindle midzone and that has a demonstrated role in sustaining cell cleavage, PRC1, has been recently described (Jiang et al., 1998). As is true of most of the other midzone proteins, the precise role of PRC1 in the cleavage process is unknown.

In late anaphase, a central mitotic spindle forms between the two separating sets of chromatids. It consists of a dense network of overlapping antiparallel microtubules (MTs)*. (Mastronarde et al., 1993). The central spindle, the site of accumulation of numerous proteins required for cell cleavage (Glotzer, 1997; Robinson and Spudich, 2000), has been demonstrated to be critical to the completion of cytokinesis (Cao and Wang, 1996; Wheatley and Wang, 1996). This is the only time in the cell cycle that a typical mammalian culture cell generates stable and bundled MTs. The molecular basis for maintenance of the midzone MT bundle is unresolved.

Address correspondence to Robert L. Margolis, Institut de Biologie Structurale J-P Ebel (CEA-CNRS), 41 rue Jules Horowitz, 38027 Grenoble cedex 1, France. Tel.: 33-43-878-9616. Fax: 33-43-878-5494. E-mail: margolis@ibs.fr

Key words: mitosis; Cdk; cytokinesis; microtubule-associated protein; cytoskeleton

*Abbreviations used in this paper: EGFP, enhanced green fluorescent protein; MT, microtubule; NLS, nuclear localization signal; siRNA, short interfering RNA; UTR, untranslated region.

Here we show that PRC1 is required to maintain a stable midzone MT bundle. It is an MT binding and bundling protein in vitro, and it forms extensive MT interphase bundles when overexpressed in mammalian cells. In keeping with these results, introduction of short interfering RNAs (siRNAs) targeting PRC1 profoundly disrupts the formation of the midzone bundle and blocks cytokinesis. The effect of siRNA occurs uniquely during cell cleavage, showing PRC1 is required to maintain interdigitation between the two half spindles during anaphase.

PRC1 has an evident domain structure. The NH_2-terminal region of the protein is largely α-helical with multiple predicted coiled-coil motifs, whereas the COOH-terminal one quarter is predicted to be largely composed of β sheets and turns. Truncation mutants show that PRC1 appears to be modular. The NH_2-terminal half of the protein is required for its association with the cleavage furrow and midbody, whereas sequence within residues 273–486 is required for MT binding. Microinjection of antibody that recognizes the COOH terminus of PRC1 disrupts the function of midzone MT bundles and blocks cleavage, showing the conformation of the COOH terminus may be important to the protein's function. On the basis of our results, we conclude that the function of PRC1 is necessary for spindle integrity during late mitosis, particularly to maintain the midzone MT bundles that are essential for the completion of cell cleavage.

Results

Overexpression of PRC1 modifies the MT array in HeLa cells

PRC1 is a mitotic spindle-associated protein that is required for cytokinesis in mammalian cells (Jiang et al., 1998). Sequence analysis shows that the NH_2-terminal three quarters of the protein is largely α-helical (see Fig. 5 A) with predicted coiled-coil motifs (Fig. 1 B), whereas the COOH-terminal region is predicted to be largely composed of β-sheets and turns. At the junction between these two distinct regions there is a cluster of two Cdk phosphorylation sites, two nuclear localization motifs, and two D boxes and a Ken box (see Fig. 5 A).

In accord with previous findings (Jiang et al., 1998), native PRC1 is intranuclear in interphase (Fig. 1 A, a), and then associates with the spindle in early mitosis, being more enriched on the interdigitating MTs, and finally to the spindle midzone in late mitosis (Fig. 1 A, b and c). We have compared the distribution of PRC1 with that of TD-60, a passenger protein that localizes to the spindle midzone in late mitosis (Andreassen et al., 1991). Double immunofluorescence staining with autoimmune antiserum recognizing TD-60 and with polyclonal anti-PRC1 showed that TD-60 and PRC1 distributed to the kinetochores, and to the entire spindle, respectively, at the beginning of mitosis (Fig. 1 A, d), and then colocalized in the spindle midzone in early telophase, and to the midbody at the end of mitosis (Fig. 1 A, e and f). It is interesting to note that although both proteins are found in the midbody, PRC1 is always at the center of the intercellular bridge, in the so-called Flemming body region (Zeitlin and Sullivan, 2001), compared with the broader TD-60 distribution (Fig. 1 f). The polyclonal anti-PRC1 serum recognizes only one protein in whole-cell extracts, with the predicted mass of PRC1 (Fig. 1 C, right).

For functional analysis, we began by constructing several plasmids to express wild-type and mutant PRC1 (Fig. 1 B), including a full-length cDNA chimera with NH_2-terminal enhanced green fluorescent protein (EGFP), and a chimera with COOH-terminal EGFP. Both the $PRC1^{AA}$ mutant and $PRC1^{EE}$ mutant in which the two threonine Cdk phosphorylation sites were mutated, respectively, to alanine (T470A; T481A) or to glutamic acid (T470E; T481E), were expressed as either NH_2- or COOH-terminal EGFP chimeras. The COOH-terminal EGFP constructs have been truncated by 35 amino acids at the COOH terminus, without apparent effect on PRC1 function or localization.

The polyclonal antiserum recognizes the extreme COOH terminus of PRC1 (Jiang et al., 1998), and thus reveals overexpression of the NH_2-terminal chimera after transfection into HeLa, but does not recognize the COOH-terminal chimera (Fig. 1 C, right). The levels of overexpression of the COOH-terminal chimeras compared with the endogenous protein can be estimated by the relative intensities of the PRC1–EGFP and EGFP–PRC1 bands in cell extracts detected with anti-GFP antibody (Fig. 1 C, left).

Overexpression of the PRC1 chimeras yielded a striking phenotype. Although the endogenous protein was normally confined to the nucleus in interphase, a substantial fraction of the overexpressed protein was cytosolic and localized to brightly stained fiber arrays that ring the nucleus (Fig. 1 D, a). On entry into mitosis the filaments disappeared, and PRC1 associated with a normal mitotic spindle, with a higher concentration in the zone of overlap between antiparallel MT sets during metaphase (Fig. 1 D, b). The in vivo localization of both native and tagged-PRC1, shows an enrichment in the MTs between the two half spindles at metaphase (Fig. 1, A, d and D, b). During anaphase PRC1 remained enriched in the spindle midzone, and finally localized exclusively in the midbody during cell cleavage (Fig. 1 D, c and d). After normal cleavage, perinuclear rings reformed in the two daughter cells (Fig. 1 D, d). The rings are MT arrays rearranged by the presence of PRC1, as shown by the colocalization of PRC1 with tubulin in the rings (Fig. 2 A). After a 4-h exposure to nocodazole, an MT depolymerizing drug, both tubulin and PRC1 were dispersed (Fig. 2 A), and PRC1 was largely intranuclear. At lower doses of nocodazole, PRC1 filaments were resistant to depolymerization compared with control MTs, suggesting that PRC1 overexpression also stabilizes MTs (unpublished data).

Thus, when overexpressed, PRC1 has the capacity to rearrange the normally radial MT arrays in HeLa cells, as well as in several other cell types (unpublished data), bundling interphase MTs into perinuclear rings. This bundled ring rearrangement is commonly observed during overexpression of many MT-associated proteins in mammalian culture cells (Weisshaar et al., 1992; Barlow et al., 1994; Waterman-Storer et al., 1995; Mandelkow et al., 1996; Togel et al., 1998; Koonce et al., 1999; Smith et al., 2000).

PRC1 binds and clusters Taxol-stabilized MTs in vitro

The rearrangement of the interphase MT array and the coassociation of PRC1 with MTs suggest a specific interaction

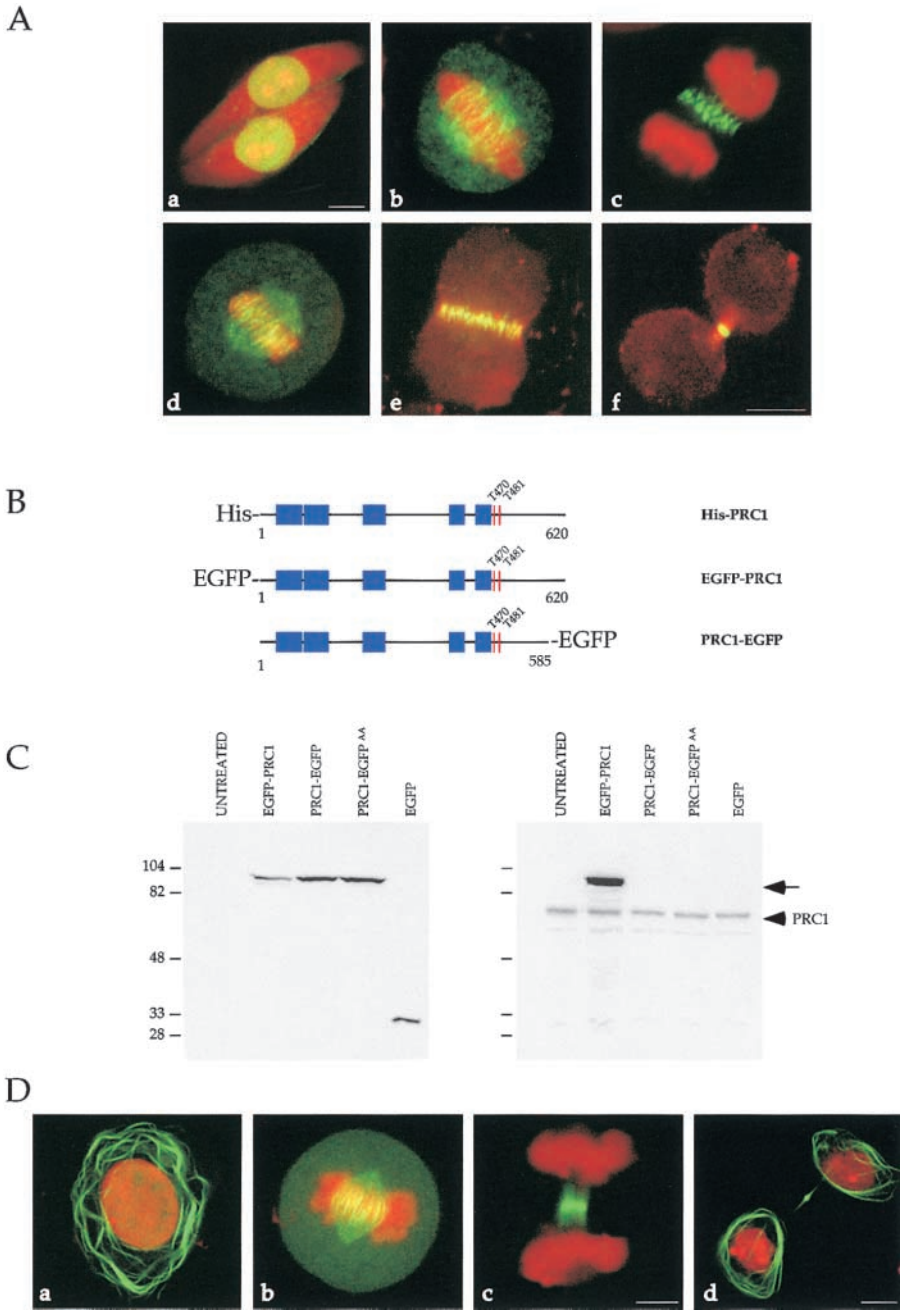

Figure 1. **The distribution of the endogenous PRC1 and its chimeras in HeLa cells.** (A) The localization of the endogenous PRC1 and TD-60. (a–c) HeLa cells were labeled with the affinity-purified antibody against PRC1 (green) and with propidium iodide for DNA (red). PRC1 is present in nuclei in interphase cells (a), and then becomes localized to the spindle upon entry into mitosis (b), and concentrates in the spindle midzone during late anaphase (c). (d–f) Double labeling of PRC1 (green) and TD-60 (red) during mitosis. (d) In metaphase, TD-60 is bound to the kinetochores at the metaphase plate, whereas PRC1 is associated with the spindle, enriched on interdigitating MTs. In late mitosis, the two antigens largely colocalize at the cleavage furrow (e) and midbody (f). PRC1 appears restricted to the Flemming body. Bars, 10 μm. (B) Schematic representation of the PRC1 chimeras. PRC1 was expressed in bacteria or HeLa cells as a tagged protein. For the first two constructs, the complete coding region of PRC1 (1–620 aa) was fused downstream to a 6×-His tag (His-PRC1) or an EGFP tag (EGFP–PRC1). The last construct allowed the expression of a COOH terminus truncation of PRC1 fused upstream to the EGFP (PRC1–EGFP). Both types of EGFP chimeras were used to overexpress the PRC1 mutant proteins. The PRC1^{AA} protein is a null phosphorylation mutant, with both Thr 470 and Thr 481 (red vertical bars) mutated into Ala. The PRC1^{EE} protein, with both the Thr 470 and Thr 481 mutated into Glu, mimics phosphorylation. The blue boxes indicate the regions of PRC1 with a high probability of multicoil formation (residues 35–85, 89–135, 210–251, 384–411, and 437–464). (C) Expression of the endogenous PRC1 and chimeras in HeLa cells. Extracts from HeLa, either untreated or transfected with one of the PRC1 chimeras (EGFP–PRC1, PRC1–EGFP, and PRC1–EGFP^{AA}), or with a control plasmid expressing only EGFP, were subjected to electrophoresis on 8% polyacrylamide gels and immunoblotted with the anti-EGFP antibody (left) or anti-PRC1 antibody (right). (Right) The anti-PRC1 antibody recognizes the endogenous PRC1 (arrowhead) in all extracts along with a band at the expected molecular mass of the EGFP–PRC1 fusion protein (arrow). PRC1–EGFP and the mutant PRC1–EGFP^{AA} fusions were not recognized because they lack the COOH- terminal residues recognized by this antibody. In contrast, the anti-EGFP antibody recognizes the unfused EGFP as well as all the three PRC1 fusion proteins. (D) The distribution of the PRC1-EGFP fusion protein in HeLa cells. During interphase, the overexpressed PRC1–EGFP protein (green) induces the formation of circular filaments around nuclei (DNA, red). In common with endogenous PRC1, overexpressed PRC1 is also in nuclei. During mitosis, the PRC1–EGFP protein is associated to a normal spindle (b), and then becomes associated to the midzone (c) and midbody at late telophase (d), similar to endogenous PRC1. The perinuclear ring filaments are evident in interphase (a) and at the end of telophase (d) but are dispersed during mitosis. Bar, 10 μm.

between PRC1 and MTs. Thus, we probed for PRC1 binding to MTs in an in vitro assay, and found that His-tagged, bacterially expressed PRC1 was entirely pelleted by Taxol-stabilized pure tubulin MTs (Fig. 2 B). In contrast, in the absence of MTs, PRC1 remained in the supernatant (Fig. 2 B). Direct microscopic observation of MTs after PRC1 binding shows complete colocalization of the two antigens, and reveals that the MTs have become extensively bundled in the presence of PRC1 (Fig. 2 C).

We have also analyzed the Taxol-stabilized MTs in the presence of His-PRC1 by electron microscopy. The bundles were extremely dense, but in favorable regions could be seen to contain clusters of aligned MTs (Fig. 2 D, left, arrow). Examination of these regions at higher magnification re-

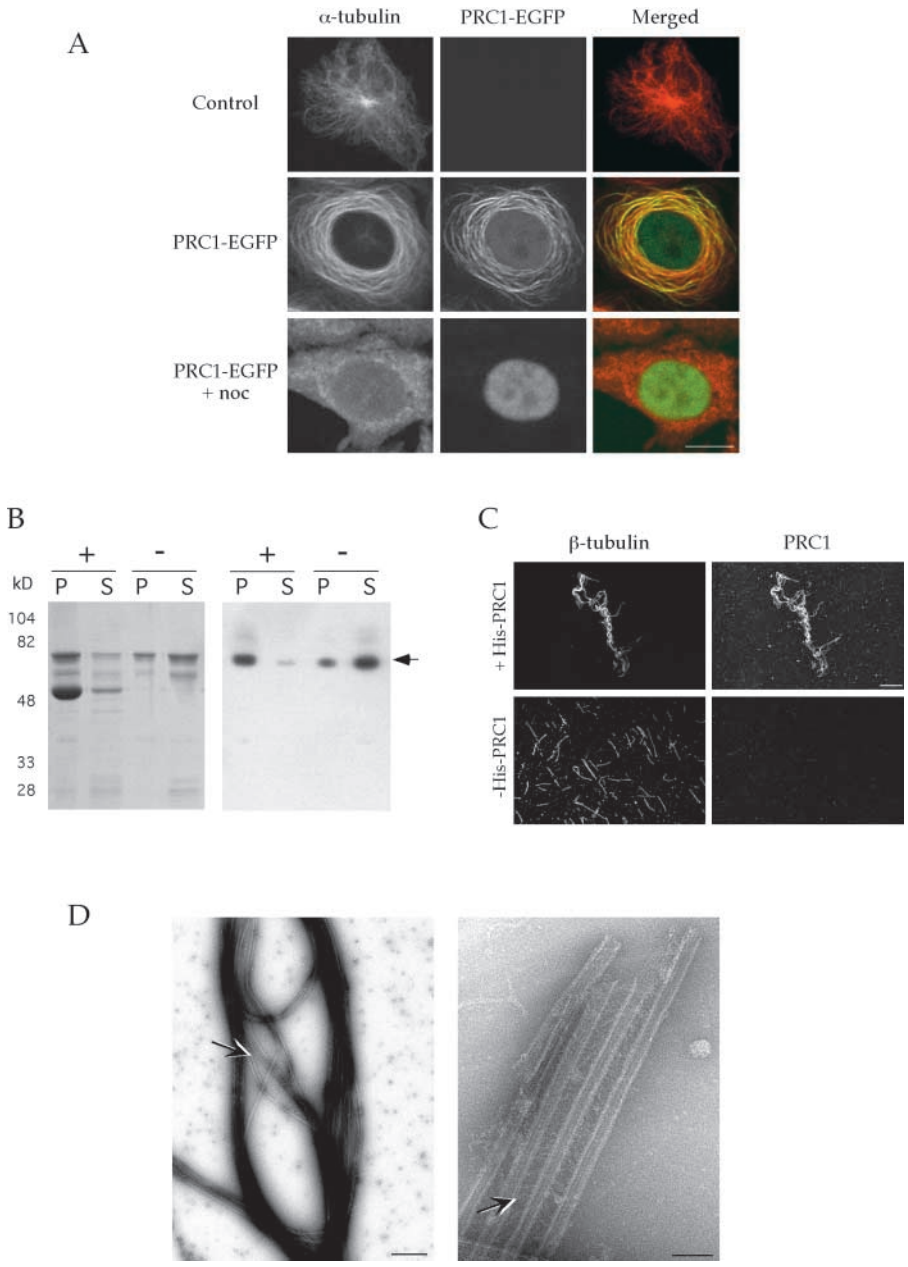

Figure 2. **PRC1 is a MT bundling protein.** (A) The overexpression of PRC1–EGFP chimera induces a circular rearrangement of the MT array. Starting from the top, the panel shows the MTs (red) focused on the centrosome in an untransfected HeLa cell. In an interphase cell overexpressing the PRC1-EGFP fusion protein (green), the circular filaments correspond to MTs (red) rearranged into a circular pattern around the nucleus. After a 4-h incubation in 1 μg/ml of nocodazole, the circular filaments are dispersed. Bar, 10 μm. (B) His-PRC1 binds in vitro MTs. His-PRC1 was incubated 10 min at 37°C in the absence (−) or presence (+) of MTs then subjected to a brief centrifugation. Pellets (P) and supernatants (S) were recovered in equal volumes of SDS-PAGE loading buffer, and samples were subjected to electrophoresis (left, Coomassie gel) and immunoblotted with the anti-PRC1 antibody (right). His-PRC1 protein is completely pelleted in the presence of MTs, whereas in the absence of MTs PRC1 remains soluble. (C) His-PRC1 induces MT bundling in vitro. Taxol-stabilized MTs were incubated in presence or absence of His-PRC1. The mixture was then deposited on top of a poly-lysine–treated coverslip which was then methanol fixed and incubated with anti–β-tubulin and anti-PRC1 antibodies. MTs appear short and unbundled in the absence of His-PRC1. After addition of His-PRC1, MTs form long highly bundled filaments. Bar, 20 μm. (D) Negatively stained electron microscopy samples. Low magnification view of bundled MTs. Arrow indicates MTs with clearly visible alignment (left). Bar, 400 nm. High magnification of aligned MTs shows PRC1 forming extensive intermicrotubule bridges (right, arrow). Bar, 40 nm.

vealed MTs in regularly spaced arrays interconnected by filamentous projections (Fig. 2 D, right). The average distance between the MTs was ~19 nm. The PRC1 filaments linking the MTs appeared to form a constant angle (~38°) with respect to the longitudinal MT axis (Fig. 2 D, right, arrow).

PRC1 is an MT-associated protein whose bundling activity is cell cycle regulated

It is remarkable that, despite a gross rearrangement of the interphase MT array, overexpressed PRC1 is associated with mitotic spindles of normal appearance (Fig. 1 D, b). The spindles of cells overexpressing PRC1 also exhibited normal function, as transfected cells routinely proceeded through normal anaphase and telophase (Fig. 1 D, c and d). In keeping with this dichotomy in interphase/mitotic behavior, we found that recently divided daughter cells, connected by a midbody following normal mitosis, contained perinuclear MT ring arrays (Fig. 1 D, d).

The capacity of PRC1 to form interphase rings but participate in normal spindle function suggests that its capacity to bundle MTs is specifically suppressed, either directly or indirectly, during early mitosis. In contrast, PRC1 strongly associates with the MT bundle that normally forms at the spindle equator during anaphase. These observations indicate that the bundling capacity of mitotic spindle-associated PRC1 might be downregulated during early mitosis, and then activated during late mitotic stages when the midzone MT bundle, necessary for cell cleavage (Cao and Wang, 1996; Wheatley and Wang, 1996), is formed. Thus, we have conducted tests to determine if there is specific downregulation of PRC1 in early mitosis, followed by a requirement for PRC1 to bundle the spindle midzone in late mitosis.

Human PRC1 has two Cdk phosphorylation sites, at T470 and T481 (Jiang et al., 1998). An attractive explanation for our observations is that Cdc2/cyclin B phosphorylation specif-

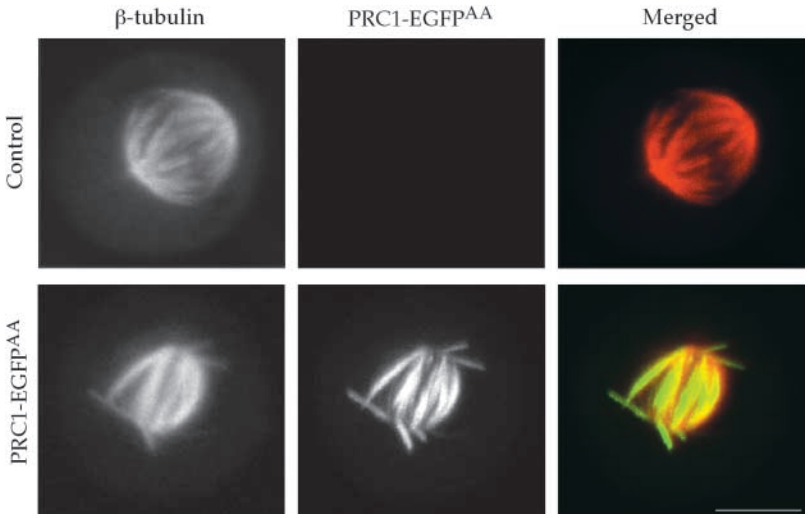

Figure 3. **Overexpression of a double PRC1 mutant lacking the two Cdk phospho-threonines creates multiple MT bundles in mitotic spindles.** A control untreated cell (top) and a cell (bottom) overexpressing PRC1–EGFP^{AA} (green), double labeled with anti–β-tubulin antibody (red), are shown. Although PRC1 distribution shows the MTs are highly bundled, the mitotic spindle preserves a bipolar shape. Bar, 10 μm.

ically downregulates PRC1 bundling capacity in early mitosis. We mutated the two Cdk-phosphorylated threonine residues to alanine, and transfected HeLa with plasmids expressing the nonphosphorylatable PRC1–EGFP^{AA} mutant. The result was striking. In early mitosis, mutant PRC1 caused extensive bundling of the MTs of the mitotic spindle (Fig. 3, bottom) compared with the morphology of a control spindle (Fig. 3, top). Despite extensive bundling, the MT arrays largely preserve a bipolar orientation. Approximately 37% of the prometaphase cells overexpressing the mutant showed such bundled spindles associated with highly condensed DNA, and no mitotic cells with such bundles were observed in anaphase, indicating the mutant blocks mitotic progression.

We also constructed a phosphorylation mimic PRC1–EGFP^{EE} mutant to determine if it had a distinct effect. Expression of this construct gave results identical to those obtained with the PRC1–EGFP^{AA} mutant (unpublished data). The EE mutant also had no effect on MT binding (see Fig. 5 C) or bundling (unpublished data) of PRC1 in vitro. We interpret these results as indicating that glutamate substitution in this case is not a good phosphorylation mimic, but instead interferes with a critical function at the phosphorylation site.

The absence of PRC1 affects midzone formation during anaphase

PRC1 is clearly required for cell cleavage, as microinjection of anti-PRC1 antibody causes cleavage failure (Jiang et al., 1998). To determine the specific role that PRC1 plays in cell cleavage, we transfected cells with siRNA to block PRC1 translation, and followed cells in which PRC1 was suppressed. Immunofluorescence analysis showed that PRC1 was substantially diminished in 30–35% of transfected cells after 24 h. Many cells could be found in which PRC1 was apparently completely absent as shown by paired PRC1-negative and -positive metaphase and early (Fig. 4 A, a) and late anaphase (Fig. 4 A, b) cells. In both cases, one cell of the pair has no detectable PRC1 (Fig. 4 A a, arrow and b, arrowhead). In accord with these observations, Western blots showed that PRC1 was strongly suppressed in the entire cell population after transfection (Fig. 4 B).

In the absence of PRC1, cells were able to progress normally in mitosis to metaphase (Fig. 4 A, a, arrow), and underwent normal chromatid segregation in anaphase (Fig. 4 A, b, arrowhead). However, cells lacking PRC1 always showed aberrant anaphase spindle morphology (Fig. 4 C). Interpolar MTs were radially dispersed, and interdigitating MTs were generally absent between the two half spindles (Fig. 4 C, a). Interestingly, even in the absence of a midzone MT bundles, cells were able to separate their sets of sister chromatids and showed partial furrowing (Fig. 4 C, b and c).

Differing degrees of severity of the phenotype were always associated with either complete or reduced levels of PRC1 expression. In some cases, midzone MTs were present, but in disarray. More often, MTs displayed no interdigitation at the spindle equator (Fig. 4 C, b), and as a result the half spindles and their chromosomes frequently rotated away from their normal alignment orthogonal to the spindle equator. However, even in the absence of midzone MTs, cells seemed to initiate furrowing then regress before completing cleavage (Fig. 4 C, d). As a result of furrow regression, cells lacking PRC1 became increasingly binucleate with time (Fig. 4 D), reaching values consistent with the PRC1 suppressed population. We conclude that the absence of PRC1 disturbs neither MT assembly nor chromosome segregation, but severely alters interzonal bundling in anaphase.

Domain structure of PRC1

The primary sequence of PRC1 has several striking features (Fig. 5 A, wt). The NH_2-terminal three quarters of the protein is predicted to be largely composed of α-helical sequence (within which are predicted coiled-coil motifs, Fig. 1), whereas the COOH-terminal region mostly contains β-sheets and turns. At the junction between these two regions there are two Cdk consensus phosphorylation sites clustered with two nuclear localization signals (NLSs). A central region of the protein, spanning residues 240–440, is highly conserved among eukaryotic species, and can be assumed to be important to the function of PRC1. PRC1 also contains putative consensus sequences for ubiquitination-dependent proteolysis, including two D boxes and a KEN box (Fig. 5 A) (Glotzer et al., 1991; Pfleger and Kirschner, 2000).

To determine the relative roles of these distinct regions of the primary structure in PRC1 function, we constructed sev-

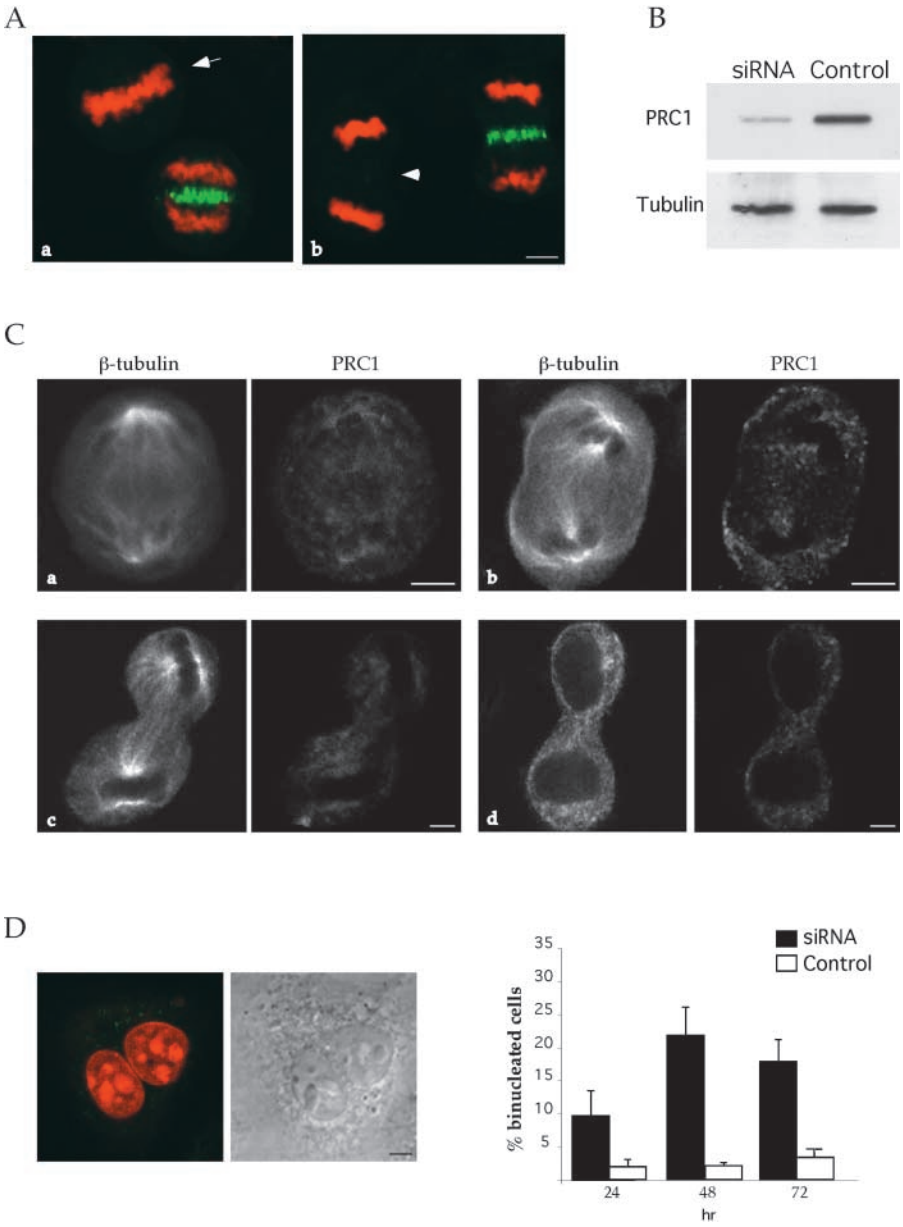

Figure 4. **Suppression of PRC1 function by siRNA disrupts midzone MTs in HeLa cells.** (A) Panels a (arrow) and b (arrowhead) show two examples of mitotic cells negative for PRC1 (green) side by side with cells expressing endogenous PRC1. (B) Western blot showing a specific decrease of PRC1 protein after siRNA transfection compared with untreated cells. The same blot was then reprobed with an anti-tubulin antibody to show equal loading of the samples. (C) HeLa cells negative for PRC1 have an altered spindle midzone. Interdigitating MTs are absent (a) or drastically reduced and disorganized (b and c) and as a consequence the two half spindles are rotated relative to the spindle axis. Cells with altered spindle midzone initiate furrowing (b and c) but do not complete cleavage (d). (D) A binucleated cell negative for PRC1. Bar, 5 μm. A histogram showing the increase in binucleated cells after siRNA transfection. A peak is reached 48 h after transfection.

eral truncation mutants (Fig. 5 A) and expressed them as GFP-tagged chimeras to assay their properties in mammalian cells. Western blots of PRC1 chimeras, expressed after transfection, showed that truncated proteins of the correct size were expressed, and that all constructs were being expressed at approximately the same level (Fig. 5 B).

Results with the different constructs are summarized in Table I. In brief, we found that MT binding during mitosis correlated with the presence of the conserved central region. Further, NH_2-terminal sequence upstream of the conserved region was required for association with the midzone of the cleavage furrow and for localization to the center of the midbody. Nuclear localization during interphase correlated with the presence of the NLS consensus sequence, as expected.

Immunofluorescence images of the different chimeras demonstrate their distinct capacities for localization (Fig. 6). The shortest COOH-terminal construct, C439, neither localizes to the mitotic spindle nor to the cleavage midzone. The construct M273, containing the central conserved region, localizes to the whole mitotic spindle but lacks sequence required to address it to the center of the midbody at the end of mitosis.

Like M273, the longer COOH-terminal construct C273 also localizes to the mitotic spindle but not to the center of the midbody in telophase. It is noteworthy that inclusion of downstream sequence causes C273 to induce stronger MT association in the mitotic spindle than M273 (Fig. 6 A). In contrast to the COOH-terminal constructs, the NH_2-terminal constructs N305 and N486 both specifically localize to the center of the midbody at the end of mitosis (Fig. 6 B, insets), whereas the longer construct, N486, localizes to the entire mitotic spindle at metaphase, the shorter N305 does not (unpublished data), reinforcing the importance of the central region in MT binding. The result with N305 demonstrates that MT binding capacity is not required for localization to the center of the midbody. These in vivo results were reinforced by in vitro MT binding assays (Fig. 5 C),

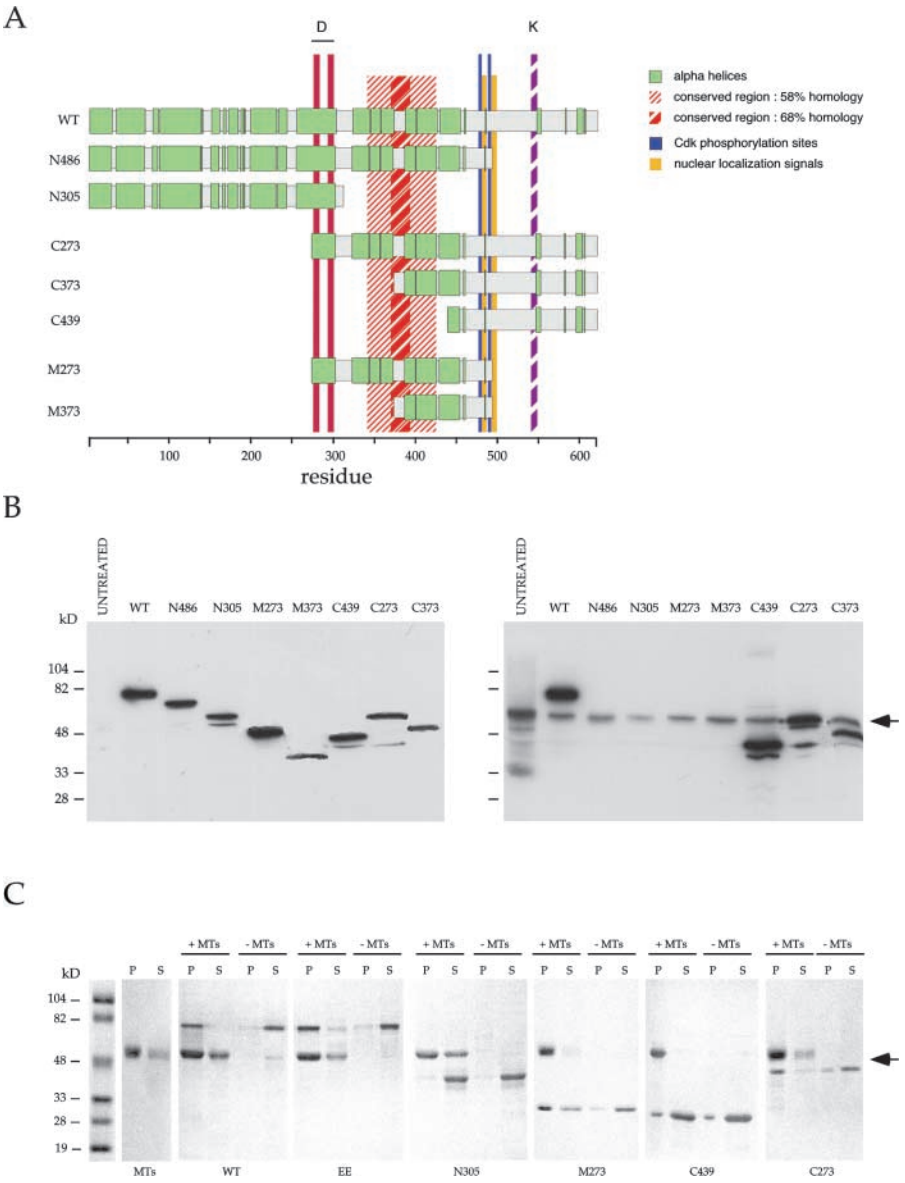

Figure 5. **Expression and properties of the PRC1 domains.** (A) Scheme representing the WT PRC1 protein and its NH_2- and COOH-terminal truncations. The different helical regions of PRC1 are indicated in the scheme along with two Cdk substrate threonines adjacent to two distinct nuclear localization signals (TPSKRRGLAPNTPGKARKL from aa 470 to 488). (D and K) Degradation boxes. Names given to the different mutants are C for COOH-terminal (number indicating the residue where the mutant commences), N for NH_2-terminal (number indicating the residue where the mutant terminates), or M for middle region (number indicating the residue where the mutant commences). The indicated percent homology is by comparison, using ClustalW, with the following sequences: AAC02688 and AAH05140 *Homo sapiens*; AAH05475 *Mus musculus*; AAF47965 and *AAF47966 Drosophila melanogaster*; CAC17794/17795/17796 *Nicotiana tabacum*; CAB82688 and BAB08676 *Arabidopsis thaliana*. Table I summarizes the distinct properties of the WT PRC1 and its truncation mutants. (B) Western blots of the different GFP-PRC1 proteins, both wild-type and mutants, expressed in HeLa cells, recognized with anti-GFP antibody (left) or anti-PRC1 antibody (right). The anti-PRC1 antibody recognizes the WT and COOH-terminal truncations along with the endogenous PRC1 (arrow). Expression levels of the different constructs are all approximately the same. (C) Coomassie-stained gels of different His-PRC1 constructs after MT binding assay in vitro. WT PRC1 and $PRC1^{EE}$ both pellet in presence of MTs. M273 and C273 also pellet (P) in presence of MTs, whereas C439 and N305 remain mostly in the supernatant (S). The arrow indicates the position of tubulin.

which showed that C273 was almost entirely bound to MTs, whereas M273 exhibited intermediate binding. In contrast, N305 and C439 did not bind to MTs in vitro.

Although the COOH-terminal domain of PRC1 does not display any particular localization or activity, it apparently influences the rest of the protein, as suggested by the difference between C273 and M273 in MT association. Further, remarkably, an antibody directed against a COOH-terminal peptide is able to disrupt cell cleavage (Jiang et al., 1998).

Table I. Properties and localization of PRC1 domains

	Interphase		Mitosis		
	MT bundling	**Nuclear[a]**	**MT localization**	**Cleavage midzone**	**Central midbody**
WT	Yes	Yes	Yes	Yes	Yes
N486	Yes	No	Yes	Yes	Yes
N305	No	No	No	Yes	Yes
C273	Yes	Yes	Yes	No	No
C373	No	Yes	No	No	No
C439	No	Yes	No	No	No
M273	Yes	No	Yes	No	No
M373	No	Yes	No	No	No

Summary of the properties and localization of wild-type PRC1 and its truncation mutants in HeLa cells. The different constructs are described in Fig. 5 A.
[a]Specific concentration.

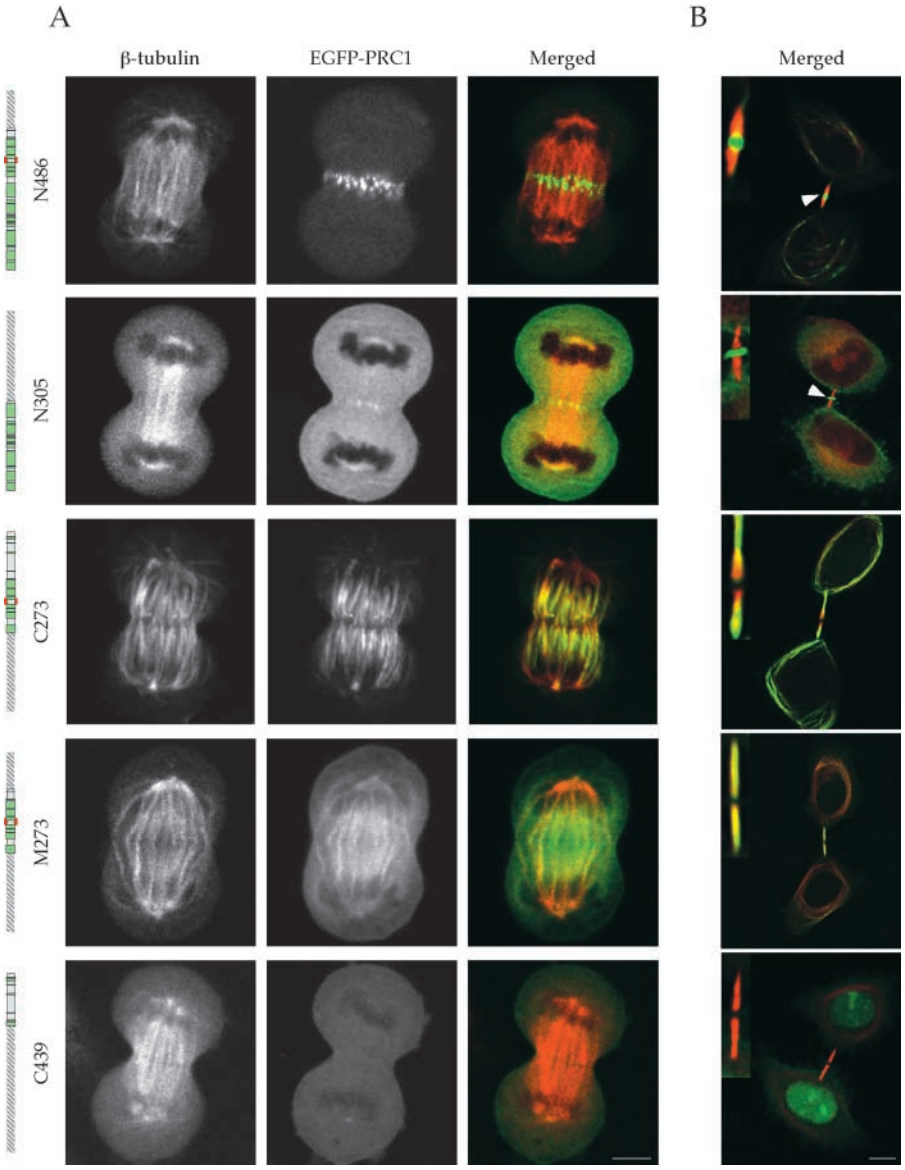

Figure 6. **PRC1 contains distinct domains with different functional capacities as determined by immunofluorescence.** To the left of the panels is shown a scheme of the different constructs studied. For each construct, anaphase (A) and telophase (B) cells are shown, stained for β-tubulin (red) and PRC1 (green). Removal of the COOH-terminus of PRC1 does not affect its ability to bind MTs (M273) nor its enrichment at the Flemming body during telophase (N486-N305) (B, arrowheads). In fact, the COOH terminus alone does not seem to be directly involved in any PRC1 activity assayed. Constructs C273 and M273 contain the region responsible for binding and bundling MTs, but they lack the signal to focus on the center of the midzone in anaphase (A), or the Flemming body during telophase (B). In (B) a closeup of the midbody (inset) is shown for each telophase cell. Bars, 5 μm.

Upon microinjection of affinity-purified anti-PRC1 antibodies, we found that spindle morphology and function were normal in early mitosis (Fig. 7, a and b), and all stages up to telophase were unchanged. However, at telophase, the usual broad MT bundle was greatly diminished by comparison to normal mitotic controls, and did not appear to extend to the cell cortex (Fig. 7 c). Thus, the effect of a COOH-terminal directed antibody is quite similar to that of PRC1 suppression by siRNA, except that the phenotype is more severe with siRNA, which affects the earlier step of midzone formation during anaphase with consequent suppression of cytokinesis. We find that ~50% of the microinjected cells fail in cleavage, as previously noted (Jiang et al., 1998).

Discussion

We have shown that human PRC1 is a MT-associated bundling protein. Both in vivo and in vitro evidence support this conclusion. The requirement for PRC1 in cell cleavage, and the bundling that is associated with PRC1 presence in the midzone of the late mitotic spindle, lead us to conclude that PRC1 functions to stabilize the midzone MT bundle, permitting completion of cell cleavage.

Many proteins concentrate at the equatorial region of the late mitotic central spindle for the purpose of participating in the cleavage event. Although many of these proteins, like PRC1, are known to be required for the cleavage process, their specific roles in cell cleavage have not as yet been defined. Some of these proteins have defined functions, acting as MT motors (Ohkura et al., 1997; Adams et al., 1998; Williams et al., 1995; Raich et al., 1998), small GTP-binding proteins and their regulators (Kishi et al., 1993; Drechsel et al., 1997; Prokopenko et al., 1999; Tatsumoto et al., 1999; Hill et al., 2000; Jantsch-Plunger et al., 2000), or protein kinases (Lee et al., 1995; Carmena et al., 1998; Madaule et al., 1998; Terada et al., 1998; Yasui et al., 1998). Their overall functions must be either to maintain the central spindle, recruit required proteins to this site, create the physical means by which the central spindle communicates to the cell cortex for the controlled deposition of myosin II and actin, or to generate the signal for the cleavage event.

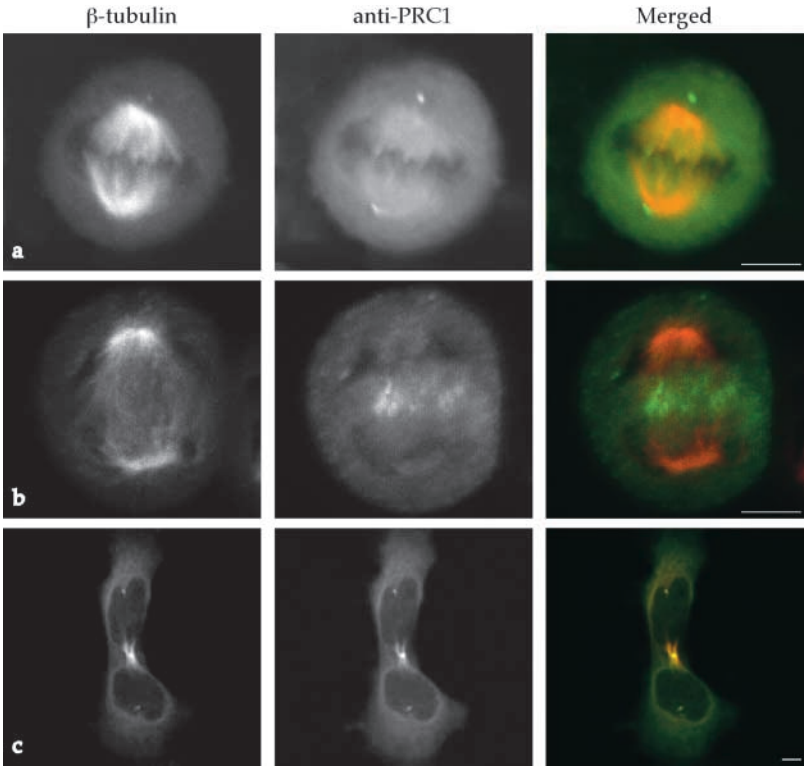

Figure 7. **The microinjection of the anti-PRC1 antibody specifically affects the formation of the midbody during telophase.** HeLa cells microinjected with the anti-PRC1 antibody, labeled with the anti–β-tubulin antibody (red) along with the anti–rabbit IgG antibody (green), are shown in the panel. Antibody microinjection does not affect the early steps of spindle formation. PRC1 is associated with the entire spindle at metaphase (a), and moves to the spindle midzone during anaphase (b), as in controls. However, during telophase (c), the midzone spindle appears as a narrow and unstructured bundle of MTs that has detached from the cell cortex. Note anti-PRC1 antiserum crossreacts with centrosomes, as previously noted (Jiang et al., 1998). Bars, 5 μm.

Considering that interdigitated midzone MTs are required for successful cytokinesis, and that passenger proteins with roles in cell cleavage collect at the center of the central spindle in late anaphase, PRC1 may be essential for these proteins to correctly localize to the cleavage furrow. Indeed, our preliminary data indicate that PRC1 has a key role in targeting the passenger proteins involved in cleavage (unpublished data). The capacity of PRC1 to specifically localize to the Flemming body in telophase, independent of its association with MTs, suggests there may be a second and important function for PRC1 during cleavage.

PRC1 phosphorylation sites and their potential function

A notable aspect of PRC1 behavior is that, despite bundling MTs when overexpressed in interphase cells, it permits normal spindle function. This result suggests that PRC1 MT bundling function is strongly downregulated in early mitosis, and then reactivated in late mitosis for the purpose of stabilizing the midzone MTs. Inactivation in early mitosis is in accord with siRNA experiments that show suppression of PRC1 does not interfere with any aspect of mitosis until cell cleavage. Among the proteins that bundle interphase MTs when overexpressed, PRC1 is, to our knowledge, unique with respect to its suppression during early mitosis.

PRC1 has two Cdk phosphorylation sites, and a null phosphorylation mutant yields an early mitotic phenotype consistent with the interpretation that phosphorylation suppresses PRC1 bundling activity, as the mutant generates MT bundles throughout the mitotic spindle. PRC1 has been shown to be a good substrate for several Cdks in vivo and in vitro (Jiang et al., 1998). It is reasonable to speculate that the mitosis-specific regulation is mediated by Cdc2 activity. Although several observations indicate a physiological role for phosphorylation of PRC1, we were unable to clearly demonstrate this because overexpression of a phosphorylation mimic EE mutant of PRC1 generates the same bundled mitotic spindle phenotype seen with the phosphorylation-null mutant, suggesting that the EE mutant is not an adequate mimic of phosphorylation status.

Domain structure of PRC1

PRC1 contains multiple α-helical regions with the potential for formation of multicoils that may figure in interprotein linkages (Fig. 1 B). It shares 57% homology with the budding yeast protein Ase1 that localizes to the anaphase spindle midzone and is required for many aspects of mitosis (Juang et al., 1997). Together with another nonmotor MT-associated protein, Ase1 is required for anaphase B, the elongation of the spindle and separation of spindle poles (Pellman et al., 1995). Loss of Ase1 protein function destabilizes the spindle during telophase (Juang et al., 1997). Significant sequence homology (56%) is also shared with a *Nicotiana tabacum* MT-associated protein, Map65 (GenBank/EMBL/DDBJ accession no. CAC17794, CAC17795, and CAC17796). The homologue of Map65 in carrots has been demonstrated to form regular inter-MT linkages, thus generating MT bundles (Chan et al., 1999). Similarly, our data indicate that PRC1 forms bundles of aligned MTs where inter-MT linkage is made through filamentous projections at a constant angle with respect to the longitudinal MT axis. MT bundling may require dimers or higher oligomers of PRC1, a possibility we are currently exploring. In preliminary experiments, we have found that PRC1 runs as a single included peak on sizing columns, with a mass of ~300 kD (unpublished data), suggesting that it forms small oligomers in vitro. The primary se-

quence of PRC1 gives a clear indication that the protein has distinct domains. Truncation mutants confirm this impression, and demonstrate that distinct regions of PRC1 have distinct roles. The central region is clearly implicated in MT binding. In contrast, the NH_2-terminal region does not bind MTs, but is required for association of PRC1 with the Flemming body at the center of the midbody in late cleavage. These results show that association of PRC1 with the Flemming body does not require MT association. In fact, one of the truncation mutants of PRC1, N305, neither associates with MTs in vitro nor with the spindle MTs in vivo, but clearly associates with the Flemming body during cleavage.

Thus, it is possible that PRC1 has two distinct functions in cleavage, one for MT bundling and another relating to association with the Flemming body at the last stages of cleavage. Cleavage has two distinct stages, the first involving the initial cortical contraction, and the second, resolution and final cell separation (Zeitlin and Sullivan, 2001). PRC1 may play a role in each of these distinct events. siRNA experiments show that cleavage can fail at an early stage in the absence of PRC1, but this result does not exclude a further role for PRC1 in the final stage of cleavage.

Potential association with other cleavage proteins

The spindle-associated motor protein MKLP1 has been speculated to play a role in bundling the late mitotic central spindle based on its in vitro capacity to bundle MTs (Nislow et al., 1992). MKLP1 homologues in lower eukaryotes play a role in cytokinesis, as mutants of these homologues, pavarotti (Adams et al., 1998) and ZEN-4 (Raich et al., 1998; Severson et al., 2000), exhibit derangements in cleavage. Our results suggest that MKLP1 alone is not sufficient to maintain the midzone MT bundle. In fact, the role of MKLP1 is complex, as an alternatively spliced form, CHO1, must bind actin to complete the terminal step in cleavage (Kuriyama et al., 2002).

Cyk-4, a Rho GAP, interacts specifically with ZEN-4 in *Caenorhabditis elegans*, and both proteins appear to be required for formation of the midzone spindle in late mitosis (Mishima et al., 2002). A human Cyk-4 orthologue, HsCYK-4, has recently been shown to form a heterotetramer with MKLP-1 and to bundle microtubules in vitro (Mishima et al., 2002).

Additionally, the PRC1 truncation mutant N305 does not bind directly to MTs, but does associate with the spindle midzone. Thus, it is possible that PRC1, in addition to binding directly to MTs, also binds as part of a protein complex at the late mitotic spindle midzone.

Our evidence supports a role for in the formation of midzone MT bundles during anaphase. Further work will establish what proteins PRC1 associates with and, specifically, if there is interaction and cooperation between PRC1, Cyk-4, and MKLP1 in maintaining the spindle midzone, as well as in the terminal stage of cleavage.

Materials and methods

Cloning and mutagenesis

Wild-type PRC1 cDNA in pCL (Jiang et al., 1998) was used to generate different constructs. Using a BamHI internal site, PRC1 cDNA, including the 5′ untranslated region (UTR) up to nucleotide 1836, was subcloned into the EcoRI-BamHI sites of the pEGFP-N1 vector (CLONTECH Laboratories, Inc.). This construct encoded the PRC1 protein, lacking the last 35 amino acids at the COOH terminus, fused upstream to the EGFP protein. To generate a wild-type Histidine-PRC1 (His-PRC1), the entire coding sequence of PRC1 was amplified by PCR and subcloned into the EcoRI-NotI of pHAT2 (Peränen et al., 1996). To generate an EGFP–PRC1 fusion protein, the fragment EcoRI-NotI in pHAT2 was cut with NotI, filled with Klenow (Biolabs) and then ligated into the EcoRI-SmaI sites of pEGFP-C2. The $PRC1^{AA}$ and $PRC1^{EE}$ mutants in which Thr 470 and Thr 481 were respectively substituted by Ala or Glu, were generated by PCR. For $PRC1^{AA}$, two independent PCR reactions were performed using oligonucleotides 5′-CCGGAATTCATGAGGAGAAGTGAGGTGCTG-3′, 5′-TGCTCGAGGAGCGCTGCCATACAG-3′ and 5′-CCTAGCAAGCGGCGAGGACTGGCTCCCAATGCCCC-3′, 5′-TTTATAGCGGCCGCTCAGGACTGGATGTTGGTTG-3′.

These oligonucleotides were designed to substitute both Thr 470 and Thr 481 by Ala along with two silent mutations generating SmaI and XhoI restriction sites. An identical PCR strategy was used to generate the $PRC1^{EE}$. To generate the EGFP–$PRC1^{AA}$ and EGFP–$PRC1^{EE}$ fusions, the fragments EcoRI-NotI in pHAT2 were cut with NotI, filled with Klenow and then ligated into the EcoRI-SmaI sites of pEGFP-C2. To create the truncations PRC1–$EGFP^{AA}$ and PRC1–$EGFP^{EE}$, the fragments EcoRI-BamHI from the pEGFP-C2 constructs were subcloned into the EcoRI-BamHI sites of pEGFP-N1. The different truncations were generated either by PCR, introducing premature stop codons, or by restriction enzymes to cut within the PRC1 coding region. C273 was generated by cutting the wt EGFP-PRC1 construct with BspEI and XhoI followed by mung bean nuclease treatment and ligation. M273 was similarly obtained, starting from the N486 construct. C373 was obtained by cutting the wt EGFP–PRC1 construct with HindIII followed by mung bean nuclease and ligation. M373 was obtained as described for C373 starting from the N486 construct. N305, M273, C373, and C439 were cloned into pHAT2 for protein expression. All the constructs were verified by DNA sequencing (Eurogentec).

Cell culture and transfection

HeLa cells were grown as a monolayer in DME (GIBCO BRL) supplemented with 10% fetal bovine serum (Hyclone), and maintained in a humid incubator at 37°C in a 5% CO_2 environment. HeLa cells (3×10^6), attached to coverslips in 10-mm dishes, were transfected by Exgen (Euromedex) or Lipofectamine 2000 (GIBCO BRL), with 4 μg of one of the PRC1 fusion constructs, or with a control (pEGFP-N1) plasmid, according to manufacturer's instructions. For the preparation of the cell extracts, in order to obtain a better efficiency of transfection (~70%), cells were transfected with a mixture of 20 μg of each of the plasmids and the Lipofectamine 2000. HeLa cells transfected with the PRC1–EGFP plasmid were selected in 1.5 mg/ml geneticin G418 (GIBCO BRL). Nocodazole was dissolved in DMSO and used at 0.04 μg/ml or 1 μg/ml. For nocodazole treatment, HeLa cells transiently transfected with the PRC1–EGFP plasmid were exposed to 0.04 or 1 μg/ml nocodazole for 4 h before fixation.

siRNA oligonucleotides and transfection

To generate single-stranded, gene-specific annealed RNA oligomers (Dharmacon Research), we used 5′-AAATATGGGAGCTAATTGGGA-3′ as the human PRC1 cDNA sequence to be targeted by the oligonucleotides. Transfection conditions were as described by Elbashir and colleagues (2001). HeLa cells were plated on coverslips in a 24-well plate and transfected using oligofectamine (GIBCO BRL). Cells were fixed at different time points after transfection and processed for immunofluorescence. For Western blots, cells were harvested with trypsin and washed in PBS before adding SDS-PAGE sample buffer.

Immunofluorescence microscopy

Cells grown on poly-D-lysine–coated glass coverslips for immunofluorescence microscopy were fixed with 2% paraformaldehyde-PBS, or alternatively, with cold methanol, followed by a step of rehydration in PBS. Cells were then processed with primary and secondary antibodies and counterstained with propidium iodide. For protocol detail see Martineau-Thuillier et al. (1998). Affinity-purified PRC1 COOH-terminal antibody (Jiang et al., 1998), anti–β-tubulin monoclonal antibody (T4026; Sigma-Aldrich), anti-tyrosinated α-tubulin rat monoclonal antibody (Lafanechere et al., 1998) (YL 1/2), and JH human autoimmune serum, used to detect TD-60 (Andreassen et al., 1991), were diluted 500, 400, 500 and 300×, respectively. Secondary antibodies, including FITC-conjugated affinity-purified goat anti–rabbit IgG, rhodamine-conjugated

anti–human IgG (Jackson Laboratories), Texas red–conjugated sheep anti–mouse IgG, and rhodamine-conjugated goat anti–rat IgG (Cappel), were used at 2.5 μg/ml. Images were collected with a MRC-600 Laser Scanning Confocal Apparatus (Bio-Rad Laboratories) coupled to Nikon Optiphot microscope.

Cell extracts and immunoblotting

24 h after transfection, cells were trypsinized, collected by centrifugation, and washed in PBS before the addition of SDS-PAGE loading buffer. After a short sonication, 10 μg/lane of cell extract was resolved on 8 or 10% polyacrylamide gels using a minigel apparatus (Bio-Rad Laboratories) and transferred to nitrocellulose. Affinity-purified rabbit antibodies against PRC1 (Jiang et al., 1998) were diluted 1,000-fold to detect the endogenous and the overexpressed fusion proteins. Anti-EGFP polyclonal antibody (CLONTECH Laboratories, Inc.), diluted 500-fold, was used to detect expression of the fusion proteins. The anti–β-tubulin monoclonal antibody was diluted 1,000-fold. Blots were then exposed to HRP-conjugated goat anti–rabbit IgG (TAGO), diluted 2,500-fold, for 1 h, and then developed by ECL (Pierce Chemical Co.).

Antibody microinjection

For microinjection, HeLa cells were grown on glass coverslips as previously described (Jiang et al., 1998). Interphase cells were injected in the cytoplasm with affinity-purified anti-PRC1 antibodies (3.8 mg/ml), using a semiautomatic microinjector (Eppendorf). After a 21-h incubation, coverslips were fixed and stained with FITC-conjugated goat anti–rabbit IgG, along with anti–β-tubulin monoclonal antibody.

Nickel affinity chromatography

All the constructs in pHAT2 were expressed in BL21 DE3. Bacteria were induced at 37°C for 4 h in the presence of 0.5 mM isopropylthio-β-D-galactoside (IPTG). Lysis and binding to nickel-Sepharose beads (Hitrap chelating; Amersham Pharmacia Biotech) was performed in a phosphate buffer (50 mM NaH_2PO_4, pH 7.6, 300 mM NaCl, 5 mM imidazole, 0.1 mM PMSF, and 10 mg/ml aprotinin). The proteins were eluted with 250 mM imidazole, analyzed by SDS-PAGE, and used for further MT binding assays.

In vitro MT binding assays and electron microscopy

The His-PRC1 constructs from nickel-Sepharose purification were diluted to <0.2 μg/μl in 20 μM Taxol-PEM (80 mM Pipes, pH.6.8, 1 mM EGTA, 1 mM $MgCl_2$). Pure tubulin MTs (6.4 mg/ml; provided by Dr. L. Wilson [University of California, Santa Barbara, CA]), isolated from bovine brain (Farrell et al., 1987), were assembled at 37°C, stabilized with 5 μM Taxol, and then mixed with His-PRC1 to a final concentration of 0.6 μg/μl in a total volume of 20 μl. The mixture was then incubated 10 min at 37°C. For SDS-PAGE and Western blot analysis of MT bundles, PRC1 constructs were cosedimented with MTs in a short centrifugation step at 16,000 *g* (cold, 5 min). The pellets were rinsed twice in PEM, both pellets and supernatants were recovered in equal volumes of SDS-PAGE loading buffer, and samples subjected to electrophoresis.

For immunofluorescence studies, 1 vol of the incubated MTs/PRC1 (wild-type His-PRC1 or mutant His-PRC1$^{EE)}$ mix was diluted in 60 vol of prewarmed PEM-Taxol containing 0.05% glutaraldehyde. The solution was deposited on a polylysine-coated glass coverslip, and then fixed in 100% methanol and prepared as for immunofluorescence microscopy. A control MT preparation without PRC1 was treated identically.

For electron microscopy, protein samples at 0.05 μg/μl, prepared as described above, were applied to the clean side of carbon on mica (carbon/mica interface) and negatively stained with 2% uranyl acetate. Micrographs were taken under low-dose conditions with a JEOL 1200 EX II microscope at 100 kV at a nominal magnification of 40,000×.

We thank Dr. R. Wade and Dr. R.W.H. Ruigrok for assistance with electron microscopy. We also thank Dr. L. Wilson for providing bovine brain PC-tubulin.

This work was supported by funding from La Ligue Nationale Contre le Cancer (Laboratoire Labelliseé), and Association pour la Recherche contre le Cancer (5338; R.L. Margolis). T. Hunter is a Frank and Else Schilling American Cancer Society Research Professor. C. Mollinari is supported by a Telethon fellowship (Telethon Fondazione Onlus, Rome, Italy). J.P Kleman is a researcher of the Commissariat à l'Energie Atomique. W. Jiang is a scholar of Mallinckrodt Foundation.

Submitted: 13 November 2001
Revised: 3 May 2002
Accepted: 8 May 2002

References

Adams, R.R., A.A. Tavares, A. Salzberg, H.J. Bellen, and D.M. Glover. 1998. Pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev.* 12:1483–1494.

Andreassen, P.R., D.K. Palmer, M.H. Wener, and R.L. Margolis. 1991. Telophase disc: a new mammalian mitotic organelle that bisects telophase cells with a possible function in cytokinesis. *J. Cell Sci.* 99:523–534.

Barlow, S., M.L. Gonzalez-Garay, R.R. West, J.B. Olmsted, and F. Cabral. 1994. Stable expression of heterologous microtubule-associated proteins (MAPs) in Chinese hamster ovary cells: evidence for differing roles of MAPs in microtubule organization. *J. Cell Biol.* 126:1017–1029.

Cao, L.G., and Y.L. Wang. 1990. Mechanism of the formation of contractile ring in dividing cultured animal cells. I. Recruitment of preexisting actin filaments into the cleavage furrow. *J. Cell Biol.* 110:1089–1095.

Cao, L.G., and Y.L. Wang. 1996. Signals from the spindle midzone are required for the stimulation of cytokinesis in cultured epithelial cells. *Mol. Biol. Cell.* 7:225–232.

Carmena, M., M.G. Riparbelli, G. Minestrini, A.M. Tavares, R. Adams, G. Callaini, and D.M. Glover. 1998. *Drosophila* polo kinase is required for cytokinesis. *J. Cell Biol.* 143:659–671.

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

Drechsel, D.N., A.A. Hyman, A. Hall, and M. Glotzer. 1997. A requirement for Rho and Cdc42 during cytokinesis in *Xenopus* embryos. *Curr. Biol.* 7:12–23.

Elbashir, S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 411:494–498.

Farrell, K.W., M.A. Jordan, H.P. Miller, and L. Wilson. 1987. Phase dynamics at microtubule ends: the coexistence of microtubule length changes and treadmilling. *J. Cell Biol.* 104:1035–1046.

Glotzer, M. 1997. The mechanism and control of cytokinesis. *Curr. Opin. Cell Biol.* 9:815–823.

Glotzer, M., A.W. Murray, and M.W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature.* 349:132–138.

Hill, E., M. Clarke, and F.A. Barr. 2000. The Rab6-binding kinesin, Rab6-KIFL, is required for cytokinesis. *EMBO J.* 19:5711–5719.

Jantsch-Plunger, V., P. Gonczy, A. Romano, H. Schnabel, D. Hamill, R. Schnabel, A.A. Hyman, and M. Glotzer. 2000. CYK-4: a Rho family GTPase activating protein (GAP) required for central spindle formation and cytokinesis. *J. Cell Biol.* 149:1391–1404.

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

Juang, Y.L., J. Huang, J.M. Peters, M.E. McLaughlin, C.Y. Tai, and D. Pellman. 1997. APC-mediated proteolysis of Ase1 and the morphogenesis of the mitotic spindle. *Science.* 275:1311–1314.

Kishi, K., T. Sasaki, S. Kuroda, T. Itoh, and Y. Takai. 1993. Regulation of cytoplasmic division of *Xenopus* embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J. Cell Biol.* 120:1187–1195.

Koonce, M.P., J. Kohler, R. Neujahr, J.M. Schwartz, I. Tikhonenko, and G. Gerisch. 1999. Dynein motor regulation stabilizes interphase microtubule arrays and determines centrosome position. *EMBO J.* 18:6786–6792.

Kuriyama, R., C. Gustus, Y. Terada, Y. Uetake, and J. Matuliene. 2002. CHO1, a mammalian kinesin-like protein, interacts with F-actin and is involved in the terminal phase of cytokinesis. *J. Cell Biol.* 156:783–790.

Lafanechere, L., C. Courtay-Cahen, T. Kawakami, M. Jacrot, M. Rudiger, J. Wehland, D. Job, and R.L. Margolis. 1998. Suppression of tubulin tyrosine ligase during tumor growth. *J. Cell Sci.* 111:171–181.

Lee, K.S., Y.L. Yuan, R. Kuriyama, and R.L. Erikson. 1995. Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. *Mol. Cell. Biol.* 15:7143–7151.

Mackay, A.M., A.M. Ainsztein, D.M. Eckley, and W.C. Earnshaw. 1998. A dominant mutant of inner centromere protein (INCENP), a chromosomal protein, disrupts prometaphase congression and cytokinesis. *J. Cell Biol.* 140:991–1002.

Madaule, P., M. Eda, N. Watanabe, K. Fujisawa, T. Matsuoka, H. Bito, T. Ishizaki, and S. Narumiya. 1998. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature.* 394:491–494.

Mandelkow, E.M., O. Schweers, G. Drewes, J. Biernat, N. Gustke, B. Trinczek, and E. Mandelkow. 1996. Structure, microtubule interactions, and phosphorylation of tau protein. *Ann. NY Acad. Sci.* 777:96–106.

Martineau-Thuillier, S., P.R. Andreassen, and R.L. Margolis. 1998. Colocalization

of TD-60 and INCENP throughout G2 and mitosis: evidence for their possible interaction in signaling cytokinesis. *Chromosoma.* 107:461–470.

Mastronarde, D.N., K.L. McDonald, R. Ding, and J.R. McIntosh. 1993. Interpolar spindle microtubules in PTK cells. *J. Cell Biol.* 123:1475–1489.

Mishima, M., S. Kaitna, and M. Glotzer. 2002. Central spindle assembly and cytokinesis requires a kinesin-like protein/RhoGAP complex with microtubule bundle activity. *Dev. Cell.* 2:41–54.

Nislow, C., V.A. Lombillo, R. Kuriyama, and J.R. McIntosh. 1992. A plus-end-directed motor enzyme that moves antiparallel microtubules in vitro localizes to the interzone of mitotic spindles. *Nature.* 359:543–547.

O'Connell, C.B., S.P. Wheatley, S. Ahmed, and Y.L. Wang. 1999. The small GTP-binding protein rho regulates cortical activities in cultured cells during division. *J. Cell Biol.* 144:305–313.

Ohkura, H., T. Torok, G. Tick, J. Hoheisel, I. Kiss, and D.M. Glover. 1997. Mutation of a gene for a *Drosophila* kinesin-like protein, Klp38B, leads to failure of cytokinesis. *J. Cell Sci.* 110:945–954.

Pellman, D., M. Bagget, Y.H. Tu, G.R. Fink, and H. Tu. 1995. Two microtubule-associated proteins required for anaphase spindle movement in *Saccharomyces cerevisiae. J. Cell Biol.* 130:1373–1385.

Peränen, J., M. Rikkonen, M. Hyvönen, and L. Kääriäinen. 1996. T7 vectors with a modified T7lac promoter for expression of proteins in *Escherichia coli. Anal. Biochem.* 236:371–373.

Pfleger, C.M., and M.W. Kirschner. 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev.* 14:655–665.

Prokopenko, S.N., A. Brumby, L. O'Keefe, L. Prior, Y. He, R. Saint, and H.J. Bellen. 1999. A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in *Drosophila*. *Genes Dev.* 13:2301–2314.

Raich, W.B., A.N. Moran, J.H. Rothman, and J. Hardin. 1998. Cytokinesis and midzone microtubule organization in *Caenorhabditis elegans* require the kinesin-like protein ZEN-4. *Mol. Biol. Cell.* 9:2037–2049.

Robinson, D.N., and J.A. Spudich. 2000. Towards a molecular understanding of cytokinesis. *Trends Cell Biol.* 10:228–237.

Sellitto, C., and R. Kuriyama. 1988. Distribution of a matrix component of the midbody during the cell cycle in Chinese hamster ovary cells. *J. Cell Biol.* 106:431–439.

Severson, A.F., D.R. Hamill, J.C. Carter, J. Schumacher, and B. Bowerman. 2000. The aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. *Curr. Biol.* 10:1162–1171.

Skoufias, D.A., C. Mollinari, F.B. Lacroix, and R.L. Margolis. 2000. Human survivin is a kinetochore-associated passenger protein. *J. Cell Biol.* 151:1575–1582.

Smith, D.S., M. Niethammer, R. Ayala, Y. Zhou, M.J. Gambello, A. Wynshaw-Boris, and L.H. Tsai. 2000. Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. *Nat. Cell Biol.* 2:767–775.

Takada, M., N. Morii, S. Kumagai, and R. Ryo. 1996. The involvement of the rho gene product, a small molecular weight GTP-binding protein, in polyploidization of a human megakaryocytic cell line, CMK. *Exp. Hematol.* 24:524–530.

Tatsumoto, T., X. Xie, R. Blumenthal, I. Okamoto, and T. Miki. 1999. Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis. *J. Cell Biol.* 147:921–928.

Terada, Y., M. Tatsuka, F. Suzuki, Y. Yasuda, S. Fujita, and M. Otsu. 1998. AIM-1: a mammalian midbody-associated protein required for cytokinesis. *EMBO J.* 17:667–676.

Togel, M., G. Wiche, and F. Propst. 1998. Novel features of the light chain of microtubule-associated protein MAP1B: microtubule stabilization, self interaction, actin filament binding, and regulation by the heavy chain. *J. Cell Biol.* 143:695–707.

Uren, A.G., L. Wong, M. Pakusch, K.J. Fowler, F.J. Burrows, D.L. Vaux, and K.H. Choo. 2000. Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype. *Curr. Biol.* 10:1319–1328.

Waterman-Storer, C.M., S. Karki, and E.L. Holzbaur. 1995. The p150Glued component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1). *Proc. Natl. Acad. Sci. USA.* 92:1634–1638.

Weisshaar, B., T. Doll, and A. Matus. 1992. Reorganisation of the microtubular cytoskeleton by embryonic microtubule-associated protein 2 (MAP2c). *Development.* 116:1151–1161.

Wheatley, S.P., and Y. Wang. 1996. Midzone microtubule bundles are continuously required for cytokinesis in cultured epithelial cells. *J. Cell Biol.* 135:981–989.

Williams, B.C., M.F. Riedy, E.V. Williams, M. Gatti, and M.L. Goldberg. 1995. The *Drosophila* kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. *J. Cell Biol.* 129:709–723.

Yasui, Y., M. Amano, K. Nagata, N. Inagaki, H. Nakamura, H. Saya, K. Kaibuchi, and M. Inagaki. 1998. Roles of Rho-associated kinase in cytokinesis; mutations in Rho-associated kinase phosphorylation sites impair cytokinetic segregation of glial filaments. *J. Cell Biol.* 143:1249–1258.

Yen, T.J., G. Li, B.T. Schaar, I. Szilak, and D.W. Cleveland. 1992. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature.* 359:536–539.

Zeitlin, S.G., and K.F. Sullivan. 2001. Animal cytokinesis: breaking up is hard to do. *Curr. Biol.* 11:R514–R516.