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CLIP-170 facilitates the formation of kinetochore–microtubule attachments

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CLIP-170 is a microtubule 'plus end tracking' protein involved in several microtubule-dependent processes in interphase. At the onset of mitosis, CLIP-170 localizes to kinetochores, but at metaphase, it is no longer detectable at kinetochores. Although RNA interference (RNAi) experiments have suggested an essential role for CLIP-170 during mitosis, the molecular function of CLIP-170 in mitosis has not yet been revealed. Here, we used a combination of high-resolution microscopy and RNAi-mediated depletion to study the function of CLIP-170 in mitosis. We found that CLIP-170 dynamically localizes to the outer most part of unattached kinetochores and to the ends of growing microtubules. In addition, we provide evidence that a pool of CLIP-170 is transported along kinetochore–microtubules by the dynein/dynactin complex. Interference with CLIP-170 expression results in defective chromosome congression and diminished kinetochore–microtubule attachments, but does not detectibly affect microtubule dynamics or kinetochore–microtubule stability. Taken together, our results indicate that CLIP-170 facilitates the formation of kinetochore–microtubule attachments, possibly through direct capture of microtubules at the kinetochore.

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Introduction

Microtubules are important for a large variety of cellular functions, including intracellular transport, motility and cell division. During mitosis, microtubules must attach paired sister chromatids to opposite poles of the spindle, which is essential to ensure proper distribution of the sister chromatids over the daughter cells. Fast and accurate attachments are possible because of the highly dynamic nature of microtubules. This is based on the inherent ability of microtubules to undergo changes between periods of growth and shrinkage, termed dynamic instability (Kirschner and Mitchison, 1986). Although dynamic instability is seen throughout the cell cycle, there is a striking increase in microtubule dynamics at the onset of mitosis (Belmont *et al*, 1990).

The dynamics of interphase microtubules are regulated by a group of proteins that binds specifically to the plus ends of growing microtubules, collectively called 'plus end tracking' proteins or +TIPs (Schuyler and Pellman, 2001). CLIP-170 is the founding member of the +TIPs (Perez *et al*, 1999). It has an N-terminal microtubule-binding domain, a long central coiled-coil domain, required for homodimerization, and a C-terminus with two zinc-finger domains. In both budding and fission yeast the CLIP-170 homologs, Bik1 and Tip1 respectively, also localize to the microtubule plus ends (Brunner and Nurse, 2000; Lin *et al*, 2001). However, in contrast to CLIP-170, Bik1 and Tip1 are transported to the plus end along the microtubule fiber by motor proteins, rather than binding to microtubule plus ends directly (Perez *et al*, 1999; Busch *et al*, 2004; Carvalho *et al*, 2004). CLIP-170 was shown to act as a positive regulator of microtubule growth in both yeast and humans (Berlin *et al*, 1990; Brunner and Nurse, 2000; Komarova *et al*, 2002). In addition, CLIP-170 is involved in linking microtubules to the cortex through IQGAP1 and Cdc42 (Fukata *et al*, 2002). Recently, it was also shown that CLIP-170 is involved in the recruitment of dynactin to the microtubule plus ends (Lansbergen *et al*, 2004). Together, these reports show that CLIP-170 is an important component of several different microtubule-dependent processes.

Although significant advances have been made in understanding the role of CLIP-170 in interphase, little is known about the role of CLIP-170 during mitosis. At the onset of mitosis, CLIP-170 localizes to kinetochores, large protein structures that assemble at the centromeres during mitosis (Dujardin *et al*, 1998). During later stages of mitosis, when chromosomes are fully aligned at the metaphase plate, CLIP-170 is no longer detected at these sites. However, the exact timing and mechanism responsible for displacement of CLIP-170 are unknown. In budding yeast, Bik1 was initially reported to bind kinetochores through its C-terminus, independently of microtubules (Lin *et al*, 2001). However, a recent report suggested that Bik1 does not localize to unattached kinetochores (Tanaka *et al*, 2005). Nonetheless, cells with a deletion in the C-terminal domain of Bik1 show defects in chromosome segregation, possibly because of impaired microtubule-binding to kinetochores (Lin *et al*, 2001). This chromosome segregation defect was only observed in polyploid yeast strains, suggesting a minor role for Bik1 in this process. In mammalian cells, inhibition of CLIP-170 function using a dominant-negative approach results in a slight increase of mitotic cells and a relative increase in prometaphase cells (Dujardin *et al*, 1998). A more recent report showed that knockdown of CLIP-170

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proteins levels through RNA interference (RNAi) results in a near complete block of cells in mitosis (Wieland *et al*, 2004), suggesting that CLIP-170 has an important role in mitotic progression. Here, we have addressed the molecular function of CLIP-170 in mitosis and the mechanisms that control its localization.

Results

CLIP-170 is displaced from kinetochores after microtubule attachment

CLIP-170 localizes to kinetochores at the onset of prometaphase (Figure 1A, upper panel), but is absent from kinetochores at metaphase, at which time CLIP-170 staining is observed at the mitotic spindle (Figure 1A, lower panel), consistent with previous findings (Dujardin *et al*, 1998). As kinetochore association correlates with spindle checkpoint status, it is possible that CLIP-170 kinetochore localization is controlled by the spindle checkpoint. To test this hypothesis, the checkpoint was directly inactivated by RNAi-mediated depletion of the checkpoint protein Mad2 (Lens *et al*, 2003). In Mad2-depleted metaphase cells, CLIP-170 was mostly gone from kinetochores (data not shown). However, when Mad2-depleted cells were treated with nocodazole, strong CLIP-170 staining at kinetochores was observed (Figure 1B, lower panel). This shows that spindle checkpoint inactivation *per se* does not result in CLIP-170 displacement from kinetochores. Together, these results suggest that CLIP-170 kinetochore localization is determined by the presence or absence of microtubules rather than the status of the spindle checkpoint.

Possibly, CLIP-170 is displaced from the kinetochore after initial microtubule attachment. Alternatively, CLIP-170 could be displaced when sister kinetochores are under tension as a result of opposing forces of microtubules. To discriminate between the two possibilities, cells were treated with drugs that allow attachment to kinetochores, but inhibit the generation of tension. First, cells were treated with monastrol to investigate CLIP-170 localization in cells with a monopolar spindle. In monastrol-treated cells, CLIP-170 could clearly be seen on some kinetochores, but was not present on others (Figure 1C), similar to CLIP-170 localization in cells overexpressing an N-terminal deletion mutant of CLIP-associated protein 1 (Maiato *et al*, 2003). Some kinetochores could be observed with attached microtubules that lacked CLIP-170 staining, while other, unattached, sister kinetochores showed bright CLIP-170 staining (Figure 1C). In addition, kinetochore pairs could often be seen, of which one kinetochore showed CLIP-170 staining, whereas the adjacent sister kinetochore did not (data not shown). Microtubule attachment can occur at a single kinetochore. In contrast, tension always occurs at both sister kinetochores at the same time. Thus, the fact that CLIP-170 is displaced from one sister kinetochore, but not the other, suggests that CLIP-170 is displaced from the kinetochore by attachment rather than tension.

In a second approach, cells were treated with the microtubule-stabilizing drug taxol. Taxol treatment resulted in displacement of CLIP-170 from most kinetochores (Figure 1D), consistent with the notion that CLIP-170 is displaced by attachment rather than tension. Finally, we investigated kinetochore association of CLIP-170 in cells with normal bipolar spindles using Survivin siRNA. Previously, we have shown that Survivin-depleted cells cannot establish tension over kinetochore pairs (Lens *et al*, 2003). In Survivin-depleted cells, CLIP-170 was displaced from kinetochores (Figure 1E, upper panel), confirming that attachment suffices for CLIP-170 displacement. Survivin-depleted cells, treated with nocodazole, showed strong CLIP-170 staining at kinetochores (Figure 1E, lower panel), indicating that CLIP-170 can be recruited to kinetochores in these cells. Taken together, these results demonstrate that CLIP-170 is displaced from kinetochores after microtubule attachment.

CLIP-170 localizes to microtubule plus ends during mitosis

To further investigate the localization of CLIP-170 in mitosis, we used fluorescence time-lapse confocal microscopy to visualize a GFP-CLIP-170 fusion protein in living cells. In interphase, GFP-CLIP-170 moved through the cell in comet-like structures at plus ends of growing microtubules (data not shown), consistent with previous observations (Perez *et al*, 1999). However, we were unable to detect these comet-like structures in mitotic cells transfected with GFP-CLIP-170. To enhance the possible microtubule-associated signal of GFP-CLIP-170, cells expressing an siRNA-resistant form of GFP-CLIP-170 were depleted of endogenous CLIP-170 by siRNA. Under these conditions, dashes of GFP-CLIP-170, emanating from both poles, could be observed moving through the mitotic cell in all directions (Figure 1F and Supplementary movie 1). To confirm that these moving dashes indeed represented the ends of growing microtubules, rather than kinetochores, the speed of the moving dots was calculated. Dashes of GFP moved at a speed of 12.5 ± 4.3 μm/min ($n = 5$) (Figure 1E). This corresponds well with published data of microtubule growth in mammalian cells during mitosis (Piehl and Cassimeris, 2003). As a control, we visualized growing microtubules using EB1-GFP, which moved at a comparable, albeit slightly higher speed (16.3 ± 4.8 μm/min ($n = 5$)), possibly due to the antipausing effect of EB1. Thus, we conclude that CLIP-170 localizes to growing microtubule plus ends during mitosis. To our knowledge, this is the first time dynamic plus end localization of CLIP-170 is shown in mitosis in mammalian cells.

CLIP-170 dynamically localizes to the outer part of the kinetochore

CLIP-170 localizes to kinetochores during mitosis (Dujardin *et al*, 1998), but the localization of CLIP-170, relative to other kinetochore proteins, has not yet been determined. To study this, nocodazole-treated cells were stained with CREST and Mad1 as markers for the centromere and the outer kinetochore, respectively (Maiato *et al*, 2004). CLIP-170 staining was observed substantially further outward from the inner centromere than Mad1 (Figure 2A), suggesting that CLIP-170 localizes to the most outer part of the kinetochore. In addition, CLIP-170 kinetochore localization was compared with that of the dynactin subunit p150glued. CLIP-170 and p150glued staining mostly overlapped at the kinetochore (Figure 2B), suggesting that CLIP-170 localizes at the same position at the kinetochore as p150glued.

CLIP-170 can interact with the dynein-associated protein Lis1 and p150glued both *in vitro* and *in vivo* (Lansbergen *et al*, 2004) and disruption of the dynein/dynactin complex

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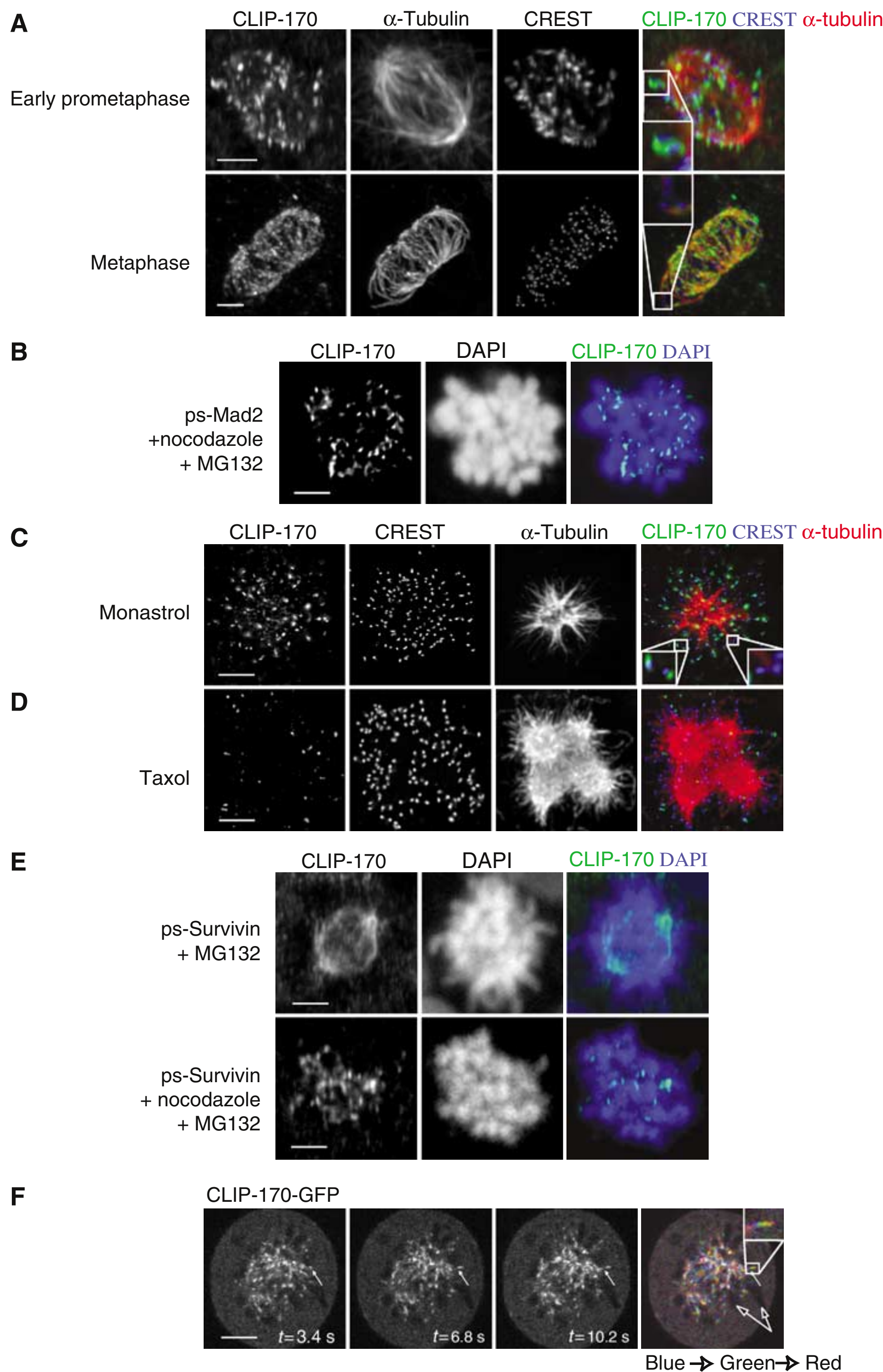

Figure 1 CLIP-170 localizes to unattached kinetochores and growing microtubule plus ends. U2OS cells were fixed with Triton/FA fixation and stained with the indicated antibodies. (**A**) In early prometaphase, a punctuate CLIP-170 staining is observed that localizes adjacent to centromeres, as visualized with CREST (upper panel). In late prometaphase/metaphase, CLIP-170 staining could no longer be detected on most kinetochores (lower panel and inlay). (**B**) U2OS cells were transfected with ps-Mad2 to inactivate the spindle checkpoint. Cells were subsequently treated with nocodazole to prevent microtubule–kinetochore attachments, in combination with the proteasome inhibitor MG132 to prevent mitotic exit. CLIP-170 is present at kinetochores of cells with an inactivated spindle checkpoint. (**C**) In monastrol-treated cells, CLIP-170 is no longer present at a subset of kinetochores. Single kinetochores can be seen that have a microtubule attachment and do not show CLIP-170 staining (right inlay), while other kinetochores that lack attachment show bright CLIP-170 staining (left inlay). (**D**) In taxol-treated cells, most kinetochores lack CLIP-170, but some kinetochores that appear unattached still show CLIP-170 staining. (**E**) U2OS cells were transfected with ps-Survivin and treated as in (B). CLIP-170 was absent from most kinetochores in Survivin-depleted cells (upper panel), but CLIP-170 was present at kinetochores when Survivin-depleted cells were treated with nocodazole (lower panel). (**F**) U2OS cells were transfected with CLIP-170-GFP and ps-CLIP-170. Confocal images were acquired every 3.4 s. Far right image shows an overlay of three time points; 3.4 s is blue, 6.8 s is green and 10.2 s is red. Directed movement can be observed (inlay). Arrows indicate chromosomes. Bars indicate 5 μm.

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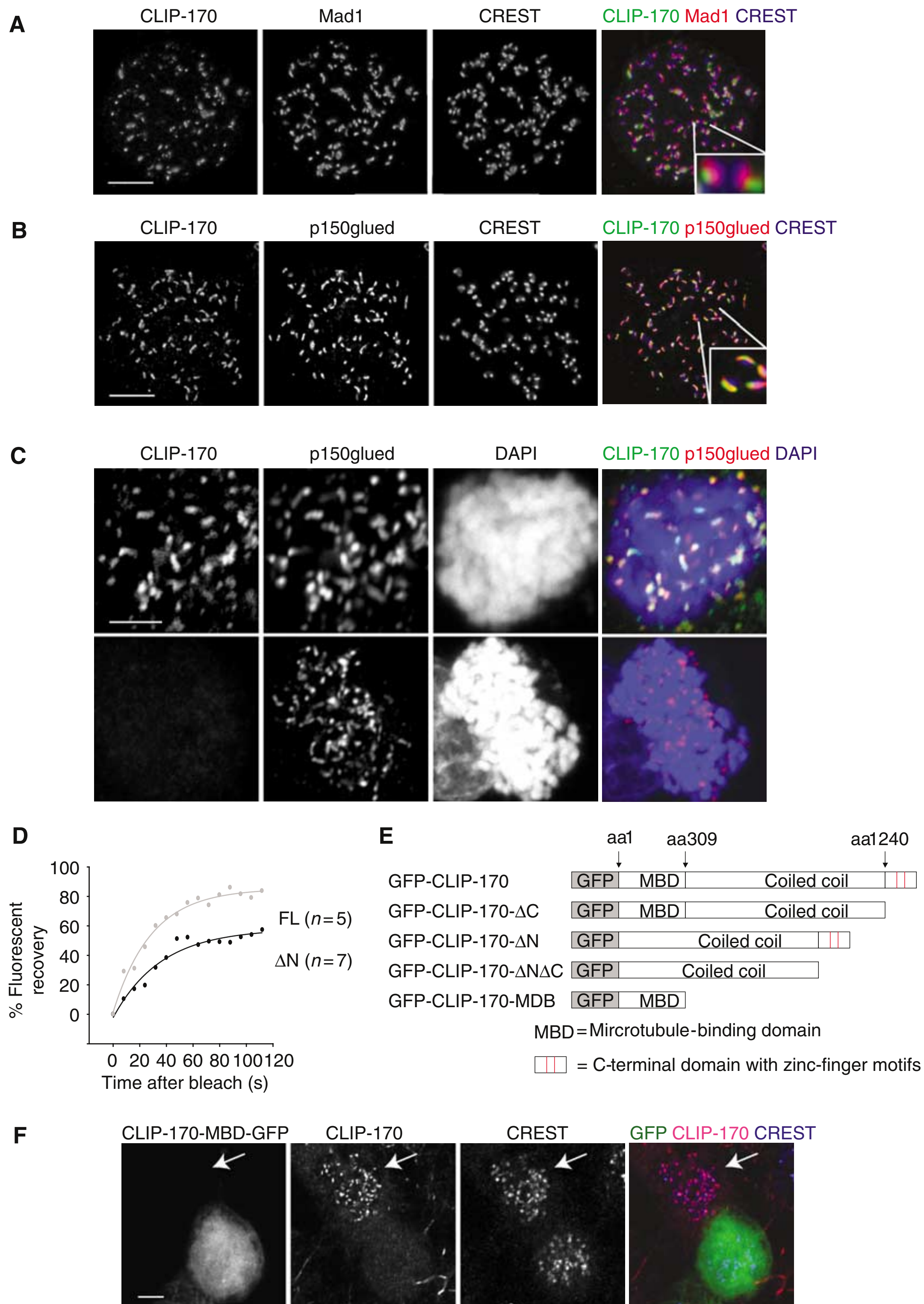

Figure 2 CLIP-170 dynamically localizes to the outer part of the kinetochore. U2OS cells were treated with nocodazole for 3 h (**A**, **B**, **E**). Cells were fixed with Triton/FA (A–C) or with methanol/acetone (**F**). (A, B) Cells were stained for CLIP-170 and Mad1 (A) or p150glued (B) combined with CREST. (**C**) Mock-transfected (upper panel) or CLIP-170 siRNA-transfected cells (lower panel) were stained for CLIP-170 and p150glued and DNA was stained with DAPI. (**D**) U2OS cells were transfected with either GFP-CLIP-170 or with GFP-CLIP-170-ΔN (see E). After bleaching, images were acquired every 8 s. Graph represents averages of either 5 (GFP-CLIP-170) or 7 (GFP-CLIP-170-ΔN) experiments. (E) Schematic representation of the different CLIP-170 deletion mutants used in this study. (F) Cells were transfected with GFP-CLIP-170-MBD and stained for endogenous CLIP-170 and CREST antibodies. Bars indicate 5 μm.

displaces CLIP-170 from the kinetochore (Coquelle *et al*, 2002). It is therefore likely that CLIP-170 is targeted to the kinetochore by the dynein/dynactin complex. However, it is unknown if dynein/dynactin also requires CLIP-170 for kinetochore localization. To test this, CLIP-170 was depleted with siRNA. In mock-transfected cells, CLIP-170 and p150glued

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colocalize at kinetochores (Figure 2C). After CLIP-170 depletion, CLIP-170 was no longer detectable at kinetochores (Figure 2C, lower panel). However, p150glued was still recruited (Figure 2C, lower panel). Similarly, dynein intermediate chain localized to kinetochores after CLIP-170 depletion (data not shown). We therefore conclude that targeting of the dynein/dynactin complex to the kinetochore is not dependent on CLIP-170.

Next, the dynamic behavior of CLIP-170 at the kinetochore was assayed by measuring the fluorescent recovery after photobleaching (FRAP) of GFP-CLIP-170. Nocodazole was added to cells 1 h prior to the experiment to ensure that all kinetochores were unattached. After photobleaching of the GFP signal at kinetochores, CLIP-170 recovered to half of the fluorescence ($t_{1/2}$) in 20.1 ± 3.3 s ($n = 5$; Figure 2D). Maximum fluorescence recovery was 83 ± 14% at 2 min after bleaching. We were unable to image cells longer, because it proved difficult to follow kinetochores for more than 2 min, due to kinetochore drift in the *Z*-axis of the cell. These results show that CLIP-170 association with the kinetochore is highly dynamic, suggesting that CLIP-170 is not a structural component of the kinetochore, but rather a dynamic protein that transiently associates with the outer part of the kinetochore.

Both full-length GFP-CLIP-170 and GFP-CLIP-170 lacking the N-terminal MBD (ΔN) (Figure 2D) localize to the kinetochore, but GFP-CLIP-170 lacking the C-terminal domain (ΔC) does not (Dujardin *et al*, 1998). This suggests that the C-terminal domain targets CLIP-170 to the kinetochore, while the N-terminal domain does not bind to the kinetochore. To examine this in more detail, we determined the dynamics of GFP-CLIP-170-ΔN at the kinetochore by FRAP analysis. Surprisingly, we found that GFP-CLIP-170-ΔN showed a significantly slower ($P = 0.02$) recovery than full-length GFP-CLIP-170 ($t_{1/2} = 25.0 \pm 4.4$ s, $n = 7$). In addition, the total fluorescence recovery of GFP-CLIP-170-ΔN was only 60 ± 15% of the original fluorescence at 2 min after photobleaching. The increase in half-time of fluorescent recovery and the lower maximum recovery indicate that GFP-CLIP-170-ΔN binds with a higher affinity to kinetochores than full-length GFP-CLIP-170. These results suggest that the N-terminal domain of CLIP-170 influences the dynamic association of the protein with kinetochores.

It was recently shown that the C-terminal domain of CLIP-170 can associate with its own MBD in an intramolecular interaction. This self-association inhibits binding of the MBD to microtubules (Lansbergen *et al*, 2004). Thus, the C-terminus can bind either kinetochores or its N-terminus. We therefore hypothesized that when CLIP-170 is bound to the kinetochore with its C-terminus, the free MBD of CLIP-170 could self-associate with the kinetochore-bound C-terminal domain, thereby disrupting the interaction between the C-terminal domain of CLIP-170 with the kinetochore and thus displacing the molecule from the kinetochore. In this model, the MBD of CLIP-170 stimulates the release of CLIP-170 from the kinetochore. To test if the MBD can interfere with the interaction between CLIP-170 and the kinetochore, we overexpressed the MBD of CLIP-170 and investigated the localization of endogenous CLIP-170. In untransfected cells, endogenous CLIP-170 can be readily detected at kinetochores of nocodazole-treated cells (Figure 2F, arrow). However, in cells transfected with GFP-CLIP-170-MBD, the amount of endogenous CLIP-170 at kinetochores was strongly reduced, although GFP-CLIP-170-MBD itself did not localize to kinetochores (Figure 2F). These results show that the MBD of CLIP-170 can interfere with the interaction of CLIP-170 with the kinetochore.

CLIP-170 localizes to microtubule plus ends and along spindle microtubules

CLIP-170 localizes to the mitotic spindle at late prometaphase and metaphase (Dujardin *et al*, 1998), and here we showed that CLIP-170 dynamically localizes to microtubule plus ends during mitosis (Figure 1A, lower panel). It is therefore possible that spindle staining consists of a large amount of microtubule plus ends that are labeled with CLIP-170. However, when single *Z*-planes of deconvolved confocal images of late prometaphase or metaphase cells were analyzed, CLIP-170 could be detected along the lattice of kinetochore–microtubules (Figure 3A, arrows). To further investigate if a fraction of CLIP-170 localizes along the microtubule lattice, metaphase cells were given a cold shock for 10 min at 4°C to depolymerize all unattached microtubules. Although CLIP-170 staining in the spindle area was diminished after cold-shock, likely due to loss of CLIP-170 plus end staining, some CLIP-170 staining remained. This pool of CLIP-170 colocalized with microtubules of the spindle and could be seen along kinetochore–microtubules (Figure 3B, arrows). Although we cannot completely exclude the possibility that the dot-like staining pattern along kinetochore–microtubule bundles, in fact, represents the plus ends of different microtubules in a single kinetochore–microtubule bundle, these results suggest that CLIP-170 can localize along kinetochore–microtubules.

CLIP-170 can localize to spindle microtubules independently of its MBD

Direct binding of CLIP-170 to microtubules plus ends is dependent on the MBD of CLIP-170 (Pierre *et al*, 1992). We were therefore interested to see if CLIP-170 localization along the microtubule lattice was dependent on the MBD. To test this, deletion mutants of GFP-CLIP-170 were used, lacking either the N-terminal MBD (ΔN), the C-terminal domain (ΔC) or both the N- and the C-terminal domain (ΔN-ΔC) (see Figure 2E). When interphase cells were examined that express full-length GFP-CLIP-170 or GFP-CLIP-170-ΔC, clear comet-like staining was observed at the ends of microtubules (Figure 3C). In contrast, neither GFP-CLIP-170-ΔN nor GFP-CLIP-170-ΔN-ΔC localized to microtubule plus ends in fixed interphase cells (Figure 3C), confirming that the N-terminal domain is essential for microtubule plus end binding in interphase. When mitotic cells were examined, GFP-CLIP-170-ΔC was detected in a punctate pattern throughout the spindle (Figure 3D, upper panel). While no significant microtubule association of GFP-CLIP-170-ΔN was detected in interphase cells, GFP-CLIP-170-ΔN was detected along microtubules in mitotic cells in both U2OS and HeLa cells (Figure 3D, middle panel and data not shown). The spindle localization of the CLIP-170-ΔN-GFP mutant was dependent on the C-terminal domain, since the GFP-CLIP-170-ΔN-ΔC mutant failed to localize to the spindle (Figure 3D, lower panel). This also suggests that GFP-CLIP-170-ΔN does not localize to the spindle through heterodimerization with

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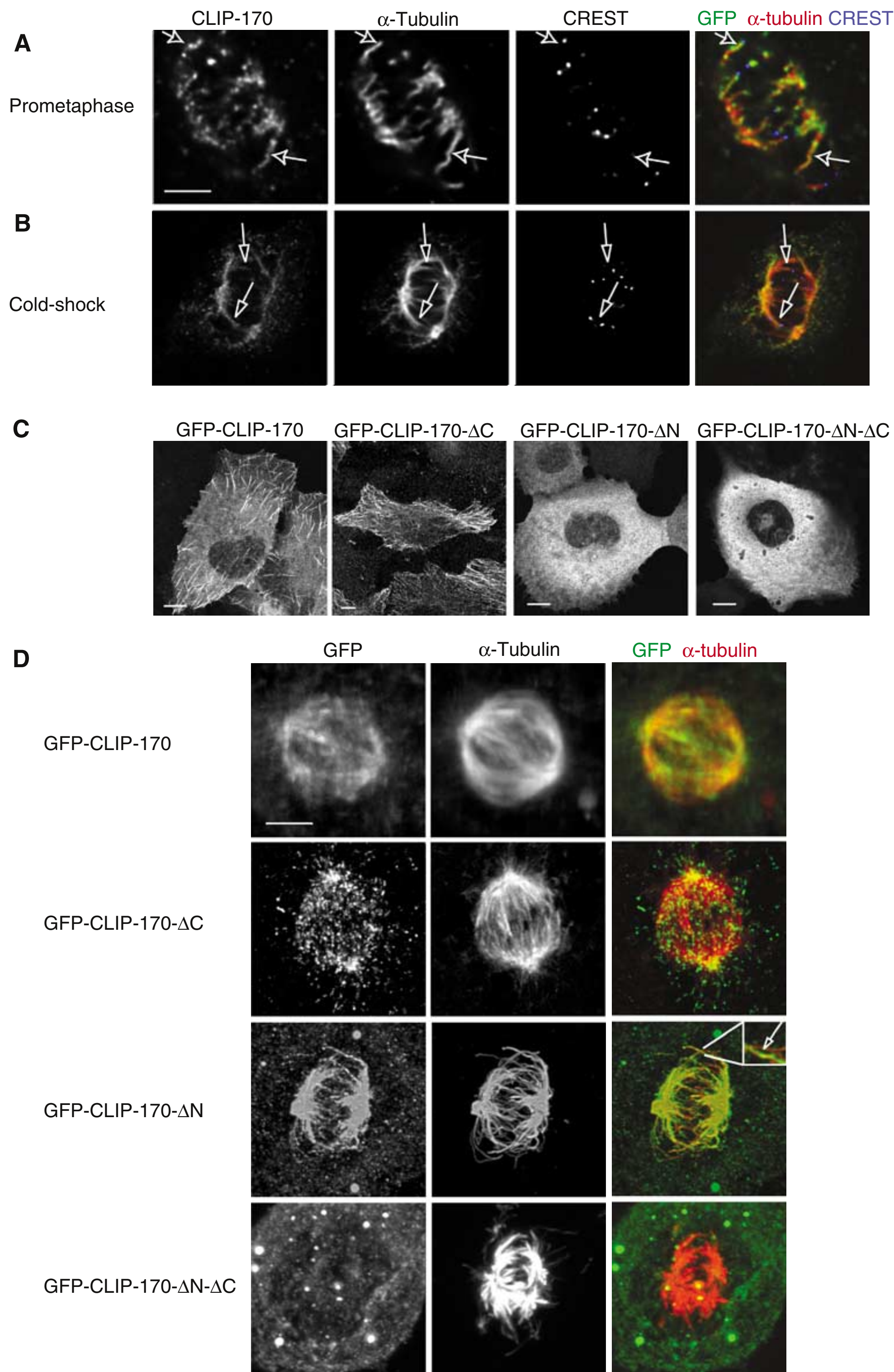

Figure 3 CLIP-170 localizes along spindle microtubules through its C-terminal domain. Cells were fixed with Triton/FA (A, B and D, lower two panels) or with methanol/acetone, which completely preserves microtubule plus ends (D, upper two panels). (**A**) Single *Z*-plane of a prometaphase cell stained for CLIP-170, α-tubulin and CREST. Arrows indicate kinetochore–microtubules decorated with CLIP-170. (**B**) Cells were incubated at 4°C for 10 min before fixation to depolymerize all nonkinetochore microtubules. CLIP-170 staining was still observed on the spindle and along kinetochore–microtubules in cold-treated cells (arrows). (**C**, **D**) Cells were transfected with indicated constructs and stained with α-GFP antibody. Representative examples of interphase (C) and mitotic (D) cells are shown. Inlay in (D) shows GFP-CLIP-170-ΔN along a single microtubule. Bars indicate 5 μm.

endogenous CLIP-170, since the dimerization domain is located in the coiled-coil, which is present in both GFP-CLIP-170-ΔN and the GFP-CLIP-170-ΔN-ΔC. The spindle localization was also not due to aspecific binding of GFP to the spindle, since an HA-tagged CLIP-170-ΔN mutant also localized along spindle microtubules (data not shown).

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CLIP-170-ΔN spindle localization is dependent on the dynein/dynactin complex

Since CLIP-170 targeting to the kinetochore is dependent on the dynein/dynactin complex (Coquelle *et al*, 2002) and dynein localizes to the mitotic spindle at late prometaphase and metaphase (Steuer *et al*, 1990), it is possible that GFP-CLIP-170-ΔN is targeted to the spindle through the dynein/dynactin complex. Indeed, GFP-CLIP-170-ΔN colocalized with dynein at kinetochores and at the spindle (Figure 4A). In prometaphase cells, often only a subset of kinetochores were positive for dynein, but kinetochores positive for dynein always showed GFP-CLIP-170-ΔN staining as well (Figure 4A). In contrast, GFP-CLIP-170-ΔC did not show significant overlap with dynein at kinetochores or the spindle (data not shown). These data support the idea that a fraction of CLIP-170 is bound to the spindle through association with the dynein/dynactin complex.

To further test if CLIP-170 is indeed targeted to the spindle through the dynein/dynactin complex, we overexpressed the dynactin subunit p50/dynamitin, which disrupts the dynein/dynactin complex (Echeverri *et al*, 1996). When HA-CLIP-170-ΔN and H2B-GFP were cotransfected with high levels of p50/dynamitin, we found that HA-CLIP-170-ΔN spindle localization was substantially reduced (Figure 4B, lower panel), when compared to cells transfected with HA-CLIP-170-ΔN and H2B-GFP alone (Figure 4B, upper panel). This strongly suggests that HA-CLIP-170-ΔN binds to the spindle indirectly, through the dynein/dynactin complex.

CLIP-170 is required for efficient kinetochore–microtubule formation

Since we found that CLIP-170 localizes to the outer most part of unattached kinetochores through its C-terminus and can also bind microtubules with its N-terminus, we hypothesized that CLIP-170 can act as an initial linker between incoming microtubules and the kinetochore, and thus plays an important role in microtubule capture at kinetochores. To test this, HeLa cells stably expressing H2B-YFP were treated with CLIP-170 siRNA. At 4 days after transfection, CLIP-170 proteins level had decreased substantially (Figure 5A). In control cells, we found that in >90% of cells the chromosomes were aligned within 30 min after DNA condensation and cells proceeded to anaphase within 60 min (Figure 5B). In contrast, in CLIP-170-depleted cells, in <25% of cells chromosomes were aligned within 60 min. To determine in which percentage of cells we filmed CLIP-170 had been extensively repressed, we scored the knockdown of CLIP-170 in a parallel experiment on fixed samples. In all, 68% of cells had an extensive knockdown, 26% a partial knockdown and 6% had no obvious knockdown. A representative CLIP-170-depleted cell is shown in Figure 5C. At 62 min after DNA condensation, many chromosomes still remained misaligned (Figure 5C; 1:02). Some misaligned chromosomes eventually aligned at the metaphase plate, but up to 7 h after mitotic entry, multiple misaligned chromosomes were observed (Figure 5C; 7:40). The cell shown in Figure 5C remained in mitosis for the full duration of the movie. However, we found that some CLIP-170-depleted cells (<50%) eventually aligned all chromosomes and proceeded to anaphase. These results were reproduced with a second independent siRNA, confirming the specificity of the RNAi (data not shown). Together, these results demonstrate that CLIP-170 is required for normal chromosome alignment in human cell lines.

Next, we focused on the dynamics of the misaligned chromosomes. To investigate the defect in chromosome alignment of CLIP-170-depleted cells in more detail, we

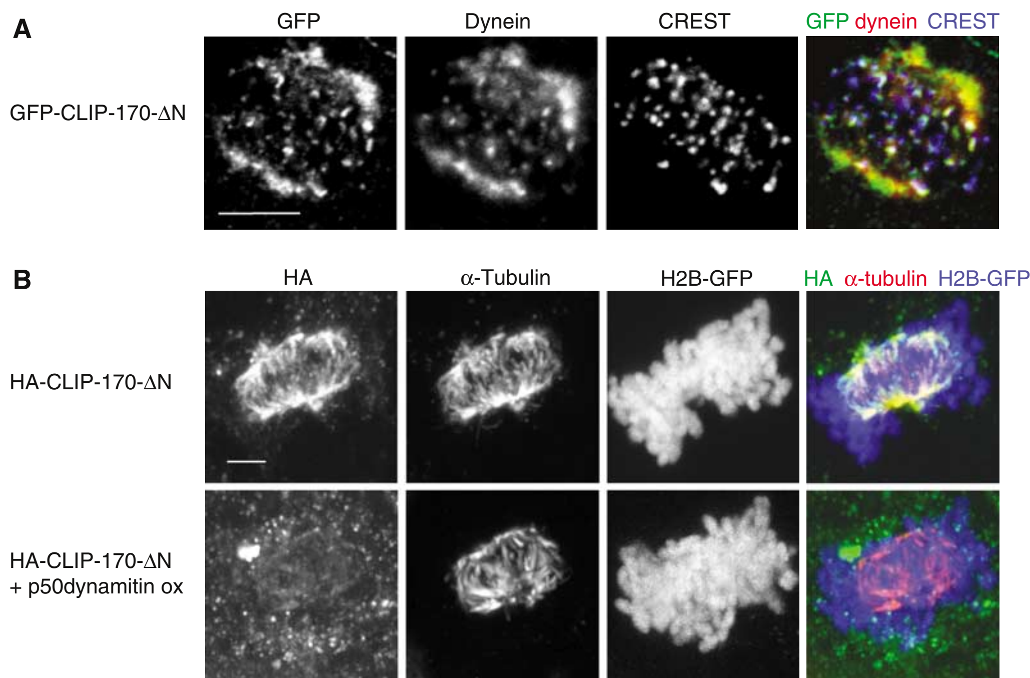

Figure 4 GFP-CLIP-170-ΔN is targeted to the spindle by the dynein/dynactin complex. (**A**) Cells were transfected with GFP-CLIP-170-ΔN and stained with the indicated antibodies. A single *Z*-plane is shown. GFP-CLIP-170-ΔN and dynein colocalize at the spindle and at kinetochores. (**B**) Cells were transfected with HA-CLIP-170-ΔN and H2B-GFP alone (upper panel) or in combination with high levels of p50/dynamitin (lower panel). Cells transfected with p50/dynamitin show strongly decreased HA-CLIP-170-ΔN staining on the spindle (ox is overexpression). Bars indicate 5 μm.

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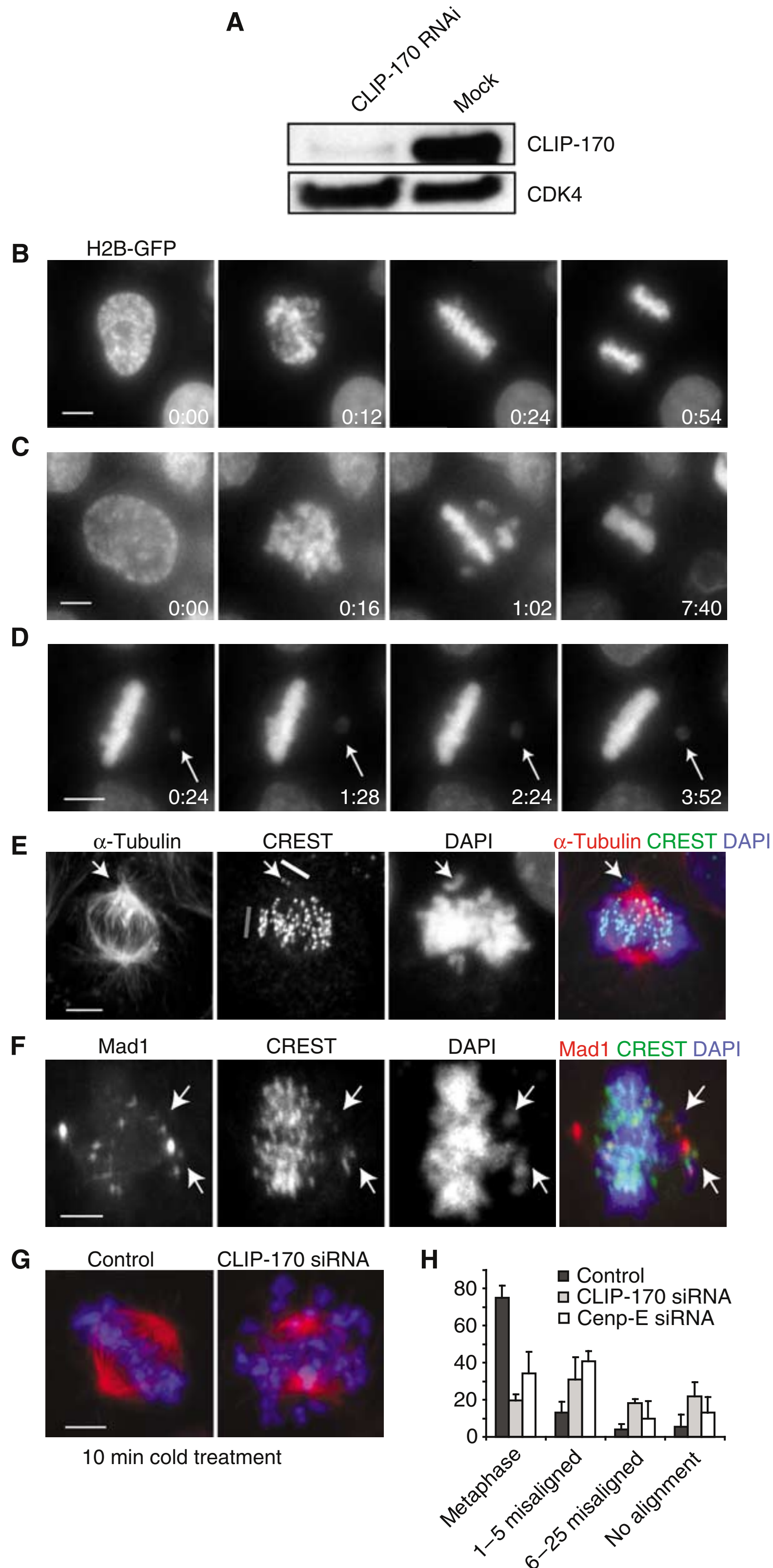

Figure 5 CLIP-170 is required for proper kinetochore–microtubule attachments and chromosome alignment in HeLa and U2OS cells. (**A**) CLIP-170 protein levels of mock-transfected cells and CLIP-170 siRNA-transfected cells. (**B–D**) Selected images of fluorescence time-lapse movie of HeLa cells stably expressing H2B-YFP. (B) Mock-transfected cell. (C–D) CLIP-170 siRNA-transfected cells. Arrows indicate misaligned chromosomes. (**E, F**) Confocal image of a fixed U2OS cell treated with CLIP-170 siRNA. (E) Arrow points at a kinetochore that is unattached. (F) Misaligned chromosomes are unattached as indicated by high Mad1 levels at kinetochores of unaligned chromosomes (arrows). (**G**) Control and CLIP-170 siRNA transfected cells were given a cold treatment at 4°C for 10 min and stained for α-tubulin. (**H**) The number of misaligned chromosomes in CLIP-170 siRNA treated cells was compared to that in Cenp-E siRNA treated cells.

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analyzed cells in which most chromosomes had aligned at the metaphase plate and single misaligned chromosomes could be followed. Figure 5D and Supplementary movie 2 show a HeLa cell in which most but not all chromosomes were aligned. The misaligned chromosome (indicated by an arrow in Figure 5D) did not move for over 4 h. Since the chromosome is completely immobile for a prolonged period of time, it is likely that it is unattached to microtubules. In addition, we often found that polar chromosomes would suddenly move rapidly towards the metaphase plate, indicating that they obtained an attachment (data not shown) Together, these data suggest that CLIP-170-depleted cells are impaired in the establishment of microtubule–kinetochore interactions.

To examine the misaligned chromosomes in more detail, U2OS cells were analyzed by confocal microscopy. In CLIP-170 siRNA-treated cells, chromosomes could often be observed near spindle poles (see Figure 5F). Although it was difficult to determine if the poleward-facing kinetochore was attached to the nearest spindle pole, the kinetochore facing away from the pole was clearly unattached (Figure 5E, arrow). Consistent with this, sister kinetochores of bi-oriented chromosomes were aligned along the pole-to-pole axis (gray bar), while the sister kinetochores of the unaligned chromosome were not (white bar). In addition, CLIP-170-depleted cells were stained for the kinetochore protein Mad1, which localizes to unattached kinetochores. High levels of Mad1 were seen on misaligned chromosomes (Figure 5F), further confirming that misaligned chromosomes did not have proper attachments. Finally, to directly show that CLIP-170 is required for efficient formation of kinetochore–microtubules, HeLa cells were given a cold-shock for 10 min at 4°C (Yao *et al*, 2000). In most control cells (>80%), a robust spindle in which most chromosomes appeared to be attached to microtubules was observed. In contrast, CLIP-170-depleted cells showed spindles with a substantially reduced number of kinetochore–microtubules (Figure 5G). Thus, CLIP-170-depleted cells are unable to form proper kinetochore–microtubules, even though they arrested in mitosis for a prolonged period of time.

To determine the relative importance of CLIP-170 in the formation of kinetochore–microtubules, we directly compared chromosome alignment in CLIP-170-depleted cells with Cenp-E-depleted cells, which have been shown to have a decreased number of kinetochore–microtubules (Putkey *et al*, 2002). We used a previously described Cenp-E siRNA that efficiently reduces Cenp-E levels (Harborth *et al*, 2001; Weaver *et al*, 2003) (Supplementary Figure S1). Our results confirm that depletion of Cenp-E causes alignment defects. Interestingly, we found that depletion of CLIP-170 caused a more severe defect in chromosome alignment (Figure 5H). Taken together, these results further demonstrate that CLIP-170 has an important role in the formation of kinetochore–microtubules.

To further investigate the role of CLIP-170 in chromosome congression and microtubule attachments at kinetochores, U2OS cells were treated with nocodazole to depolymerize all microtubules. Subsequently, cells were released from the nocodazole block and stained for Mad1 and CREST at regular intervals. 67±10% of control cells had completely aligned their chromosomes at 30 min postrelease (Figure 6A). Indeed, Mad1 was mostly absent from kinetochores in these cells (Figure 6B). However, only 13±9% of CLIP-170 siRNA-treated cells had aligned all chromosomes 30 min after release. Strikingly, 50±19% of CLIP-170-depleted cells showed no alignment at all at that time (Figure 6A). In addition, these cells had high Mad1 levels on kinetochores (Figure 6B). This was not due to a previously failed mitosis, since we only scored cells with bipolar spindles and two centrosomes. This shows that loss of CLIP-170 not only results in the inability to align polar chromosomes but also leads to a global chromosome congression defect. In addition, these results suggest that the defect in alignment is due to a problem in efficiently forming attachments. At later time points ($t=60$, 120 and 240 min), most CLIP-170-depleted cells were able to form a metaphase plate, although often one or more chromosomes remained misaligned (74±15, 60±3 and 40±10%, respectively) (Figure 6A). Taken together, these results demonstrate that CLIP-170 is required for efficient formation of kinetochore–microtubule attachments and chromosome congression.

CLIP-170 regulates microtubule stability in interphase cells by promoting microtubule rescue (Komarova *et al*, 2002). Thus, the observed defect in the formation of kinetochore–microtubules could be due to a global defect in microtubule stability, leading to a reduced capture at kinetochores. To exclude this possibility, U2OS cells were released from a nocodazole block and fixed 5 min after release, at which time robust microtubule asters were visible (data not shown). Microtubules were stained after pre-permeabilization, which removes cytoplasmic tubulin subunits. The total amount of microtubule polymer was calculated by quantifying the tubulin fluorescence in the spindle. We found that control cells had an identical amount of microtubule polymer as CLIP-170-depleted cells (Figure 6C). This suggests that the defect in chromosome congression in CLIP-170-depleted cells, observed after nocodazole release, was not due to an impaired microtubule nucleation or stability.

To determine if more subtle defects in microtubule dynamics occur after depletion of CLIP-170, we quantified the number of growing microtubules in the spindle. For this, cells were stained for EB1, which binds selectively to growing microtubules (Morrison *et al*, 1998; Tirnauer and Bierer, 2000). When the total fluorescence of spindle-associated EB1 of control cells was compared to that of CLIP-170-depleted cells, we found no differences (Figure 6D). As a control, cells were treated with an ultralow dose of nocodazole, either 25 or 100 nM. Although this did not visibly effect the spindle (data not shown), a clear reduction in EB1 staining could be observed, confirming the sensitivity of this assay.

An alternative explanation for our data is that kinetochore–microtubules are unstable in CLIP-170-depleted cells, resulting in loss of formed kinetochore–microtubules. To test this, we measured the stability of kinetochore–microtubules using photoactivatable α-tubulin (Salic *et al*, 2004). A bar-shaped region was activated across the spindle of metaphase cells and loss of spindle fluorescence was measured over time (Figure 6E, and for an example, see Supplementary movie 3). Only CLIP-170 siRNA-treated cells that showed misaligned chromosomes were imaged. The observed loss of fluorescence was biphasic. The initial loss corresponds to diffusion of activated cytoplasmic α-tubulin and turnover of the highly dynamic non-kinetochore microtubules, while the second

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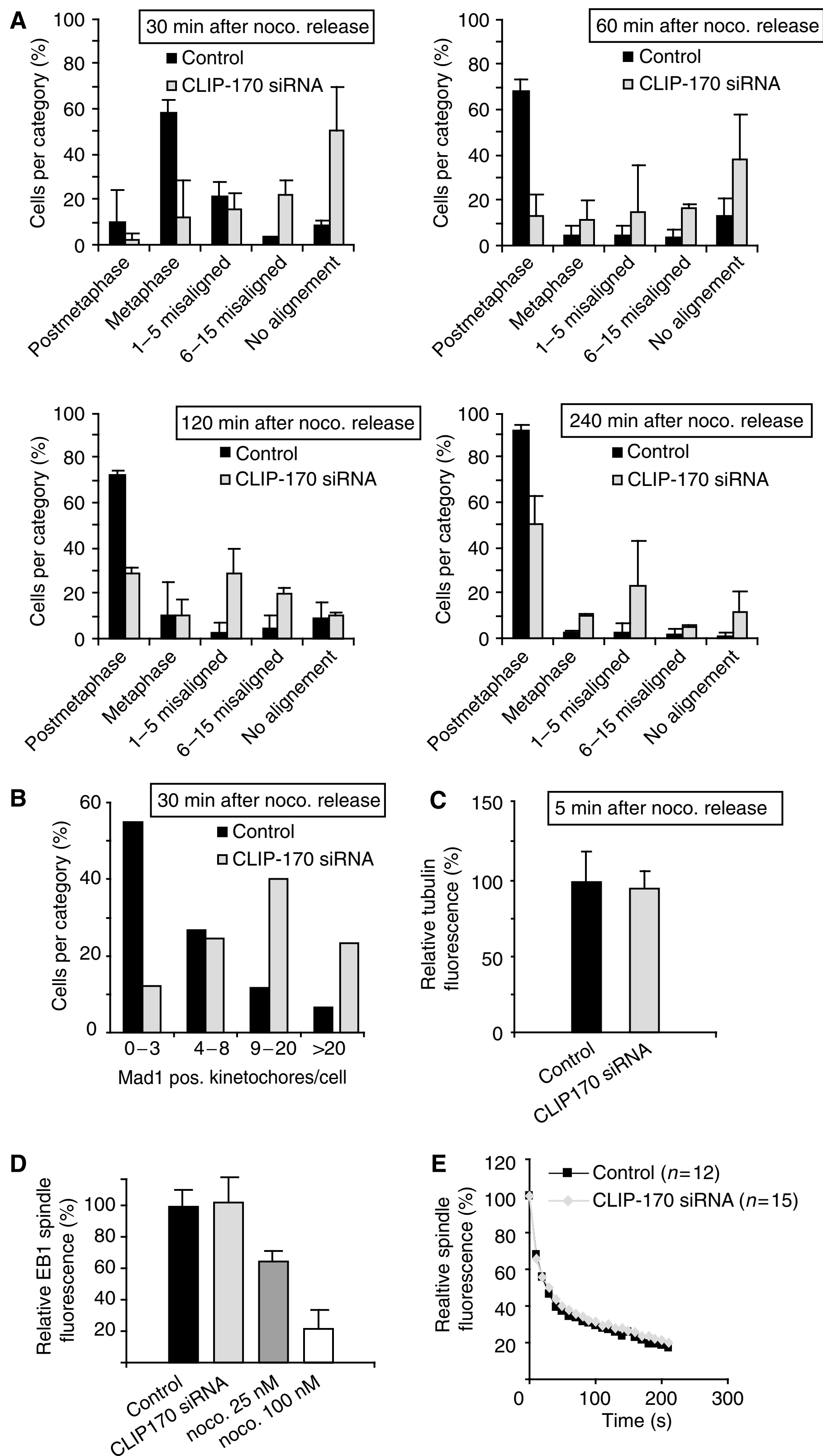

Figure 6 Defects in the formation of kinetochore–microtubule attachments are not due to defects in either global or kinetochore-microtubule stability. (**A–E**) U2OS cells. (A) Cells were released from a nocodazole block and harvested 30, 60, 120 or 240 min later. The number of misaligned chromosomes per cell was scored (average of three independent experiments). (B) Cells were treated as in (A) and were stained for Mad1. The number of Mad1-positive kinetochores per cells was scored (four independent experiments). (C) Cells were treated as in (A) and were fixed 5 min after release and stained for α-tubulin. The total amount of tubulin polymer was quantified. (D) Cells were stained for EB1 and the total spindle fluorescence of EB1 was quantified. (E) Cells were transfected with photoactivatable α-tubulin and H2B-RFP. A bar was activated across the spindle and the fluorescence decay was measured over time. Only CLIP-170-depleted cells were measured that showed misaligned chromosomes.

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phase corresponds to the turnover of kinetochore microtubules (Salic *et al*, 2004). Thus, the slope of the second phase fluorescence decay represents the kinetochore–microtubule turnover. We found that the turnover of kinetochore–microtubules is identical in control cells versus CLIP-170-depleted cells (Figure 6E). These results suggest that the chromosome congression defect was not due to loss of existing kinetochore–microtubules.

Taken together, these data suggest that CLIP-170 is involved in the formation of kinetochore–microtubule attachments, independently of the regulation of microtubule stability or the maintenance of kinetochore–microtubules and are consistent with a role for CLIP-170 in direct microtubule capture at kinetochores.

Discussion

CLIP-170 localization and dynamics during mitosis

Here, we show that CLIP-170 localizes to microtubule plus ends in mitosis in an MBD-dependent manner, while it can also interact with the mitotic spindle independently of the MBD. This latter localization is dependent on the dynein/dynactin complex, as disruption of this complex displaces a CLIP-170 mutant lacking the MBD from the spindle. Interestingly, CLIP-170 associates with kinetochores in a dynein/dynactin-dependent manner (Coquelle *et al*, 2002) and dynein was shown to be displaced from the kinetochore, like CLIP-170 (Figure 2B and C), after microtubule attachment (King *et al*, 2000). It is therefore possible that a pool of kinetochore-bound CLIP-170 is transported by the dynein/dynactin complex onto kinetochore–microtubule bundles and towards the spindle pole after attachment. Dynein/dynactin-dependent transport of CLIP-170 appears to occur only in mitosis, since in interphase cells the dynein/dynactin-binding domain of CLIP-170 does not localize to microtubules. Possibly, cell cycle-regulated, post-translational modifications influence the interaction of CLIP-170 with the dynein/dynactin complex along microtubules. This could occur by negatively regulating the interaction of CLIP-170 with microtubules during mitosis, thereby increasing its association with dynein/dynactin. It is important to note that we found that CLIP-170 localizes to microtubule plus ends in mitosis, but it is possible that its affinity for microtubules is decreased compared to interphase cells.

In addition, we found that CLIP-170 has a relatively high turnover at the kinetochore. The rapid turnover of CLIP-170 at kinetochores would allow kinetochores that have lost an attachment to reaccumulate rapidly CLIP-170 and recapture a microtubule. Surprisingly, we found that a CLIP-170 mutant lacking the MBD has a higher affinity for the kinetochore. In addition, overexpression of the MBD displaces endogenous CLIP-170 from kinetochores. This suggests that the MBD of CLIP-170 can fold back onto its C-terminus, thereby displacing the molecule from the kinetochore. Moreover, distinct pools of CLIP-170 appear to exist at the kinetochore, as a fraction of CLIP-170 lacking the MBD does not recover after photobleaching. Indeed, CLIP-170 can either interact with Lis1 or p150glued, both of which are present at kinetochores. However, the interaction of CLIP-170 with p150glued is substantially stronger than with Lis1 *in vitro* (Lansbergen *et al*, 2004). Therefore, the intramolecular interaction of CLIP-170 might be strong enough to compete with the interaction between CLIP-170 and Lis1, but not with the interaction of CLIP-170 and p150glued.

CLIP-170 is involved in the formation of kinetochore–microtubules

Detailed live analysis of cells depleted of CLIP-170 showed that CLIP-170 is needed for normal chromosome alignment. In addition, fixed cell imaging revealed that many chromosomes did not obtain correct microtubule attachments. Since CLIP-170 binds to both the very outer part of kinetochores and to microtubules, localizes to unattached kinetochores only and is known to link microtubules to subcellular structures (Pierre *et al*, 1992; Fukata *et al*, 2002), the most likely explanation for our results is that CLIP-170 is involved in the initial interaction between kinetochores and microtubules (Figure 6B).

We did not find any differences in overall microtubule stability after depletion of CLIP-170, in contrast to what has been reported in interphase cells (Komarova *et al*, 2002). Thus, it seems that the microtubule-stabilizing effect of CLIP-170 is specifically inhibited during mitosis or that other microtubule-associated proteins compensate for the loss of CLIP-170. Second, it is known that CLIP-170 can crosslink and organize microtubules both upon overexpression and also *in vivo*, in the microtubule manchette of the mouse testis (Pierre *et al*, 1994; Akhmanova *et al*, 2005). It is therefore possible that CLIP-170 has a second role in the formation of kinetochore–microtubules by organizing or crosslinking microtubules. Interestingly, we found evidence that CLIP-170 is transported onto kinetochore–microtubules by the dynein/dynactin complex, where it would be ideally localized to guide growing microtubules towards the kinetochore. This hypothesis will require further investigation.

It should be noted that we also found an increase in multipolar spindles in CLIP-170-depleted cells (unpublished observation), but these cells were consistently excluded from our quantifications.

Redundancy in CLIP-170 function

Here, we found that CLIP-170 is important for chromosome alignment and for the formation of kinetochore–microtubule attachments in two different human cell lines. However, we found that most chromosomes eventually aligned, suggesting that redundant pathways exist for the formation of kinetochore–microtubules. Interestingly, fibroblasts from CLIP-170 knockout mice have only minor defects in chromosome alignment (Akhmanova *et al*, 2005). Although it is possible that this is due to species-specific differences, these results also suggest that redundant pathways exist for kinetochore–microtubule interaction. One such pathway might involve the motor protein Cenp-E. Loss of Cenp-E results in very similar defects in chromosomes alignment as loss of CLIP-170 (Putkey *et al*, 2002) (Figure 5H). However, chromosome alignment defects after loss of CLIP-170 are not due to loss of Cenp-E at the kinetochore, since Cenp-E localizes normally at kinetochores that lack CLIP-170 (Tai *et al*, 2002). It will therefore be interesting to investigate whether CLIP-170 indeed has partially redundant functions with Cenp-E or other kinetochore proteins.

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Materials and methods

Expression constructs and RNAi

Expression constructs of CLIP-170 are based on rat brain CLIP-170 (Accession no. CAB92974). Constructs GFP-CLIP-170 and GFP-CLIP-170 deletion mutants (Komarova *et al*, 2002), ps-Mad2, ps-Survivin, photoactivatable α-tubulin (Salic *et al*, 2004) and histone H2B-GFP (Lens *et al*, 2003), have all been described previously. p50/dynamitin-GFP was a kind gift of Dr J Neefjes. dsRNA oligonucleotide sequences used for CLIP-170 RNAi were: (1) GCACAGCTCTGAAGACACC and (2) CTGCAATGACGACGAAACC. ps-CLIP-170 was based on sequence 1.

Antibodies

CREST anti-serum was from Cortex Biochem and was used 1:1000. CLIP-170 antibody #2360 has been described previously (Coquelle *et al*, 2002). Anti-GFP antibody (a gift from Dr J Neefjes) was used 1:500. Mad1 antibody (a gift from Dr Musacchio) (Steensgaard *et al*, 2004) was used 1:10. α-tubulin antibody (Sigma) was used 1:750. The p150glued, EB1 antibodies (Transduction Laboratories) and anti-dynein intermediate chain (Sigma) were used 1:200. Secondary antibodies for immunofluorescence were Alexa-488, Alexa-568 and Alexa-647 (Molecular probes). The secondary anti-body for Western blot was peroxidase-conjugated goat-α-rabbit (Dako).

Cell culture and transfection

U2OS and HeLa cells were cultured in DMEM (Gibco) with 6% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. DNA constructs were transfected using standard calcium-phosphate transfection protocol. dsRNA was used at a final concentration of 100 nM and was transfected with Dharmafect1 according to the manufacturer's guidelines (Dharmacon). Where indicated, cells were treated with 1 μM nocodazole, 1 μM of taxol or 200 μM of monastrol (Sigma).

Confocal microscopy

Before staining, cells were pre-permeabilized with 0.1% Triton in PEM buffer (20 mM PIPES, pH 6.8, 0.2% Triton X-100, 1 mM $MgCl_2$, 10 mM EGTA) for 60 s and subsequently fixed in 3.7% formaldehyde in PEM for 5 min (Triton/FA fixation) or cells were fixed in −20°C methanol/acetone 1:1 for 30 s (methanol/acetone fixation). Coverslips were blocked in PBS with 3% BSA for 1 h. All primary antibodies were incubated at room temperature overnight and secondary antibodies were incubated for 1 h at room temperature. Coverslips were mounted using Vectashield mounting fluid with DAPI (H-1200, Vectorlabs). Confocal images were acquired on a Leica TCS SP2 AOBS (Leica Microsystems) with a HCX Plan Apo 63× lambda-blue NA 1.4 objective. If indicated, images were deconvolved with Huygens Essential version 2.6.0p0, Scientific Volume Imaging (SVI). Brightness and contrast were adjusted with Photoshop 6.0 (Adobe). Images are maximum intensity projections of all *Z*-planes, unless stated otherwise. Live confocal microscopy was performed in a heated culture chamber at 37°C incubated with 5% CO_2.

Time-lapse microscopy

Time-lapse microscopy was performed as described previously (Lens *et al*, 2003) and images were processed using MetaMorph software (Universal Imaging).

Fluorescence recovery after photobleaching

Fluorescence intensities were measured with MetaMorph. Fluorescence recovery was corrected for *Z*-axis drift and bleaching due to imaging by simultaneous measurement of two non-bleached kinetochores. Fitting of data was carried out with Sigmaplot. Half recovery times ($t_{1/2}$) were calculated according to $t_{1/2} = \ln(2)/k$ and maximum recovery was calculated by averaging the final two data points.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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