

# Distinct mechanisms govern the localisation of *Drosophila* CLIP-190 to unattached kinetochores and microtubule plus-ends

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## Summary

CLIP-170 was the first microtubule plus-end-tracking protein to be described, and is implicated in the regulation of microtubule plus-ends and their interaction with other cellular structures. Here, we have studied the cell-cycle-dependent mechanisms which localise the sole *Drosophila melanogaster* homologue CLIP-190. During mitosis, CLIP-190 localises to unattached kinetochores independently of spindle-checkpoint activation. This localisation depends on the dynein-dynactin complex and Lis1 which also localise to unattached kinetochores. Further analysis revealed a hierarchical dependency between the proteins with respect to their kinetochore localisation. An inhibitor study also suggested that the motor activity of dynein is required for the removal of CLIP-190 from attached kinetochores. In addition, we found that CLIP-190 association to

microtubule plus-ends is regulated during the cell cycle. Microtubule plus-end association is strong in interphase and greatly attenuated during mitosis. Another microtubule plus-end tracking protein, EB1, directly interacts with the CAP-Gly domain of CLIP-190 and is required to localise CLIP-190 at microtubule plus-ends. These results indicate distinct molecular requirements for CLIP-190 localisation to unattached kinetochores in mitosis and microtubule ends in interphase.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/118/16/3781/DC1>

Key words: CLIP-170, Microtubule, Kinetochore, Dynein, EB1, *Drosophila*

## Introduction

The microtubule cytoskeleton in eukaryotes is essential for different cellular processes, such as cell migration, cell architecture, organelle transport and chromosome segregation. This diversity of functions is made possible by the ability of microtubules to alter their organisation in different cell types. Microtubules are dynamic polymers of tubulin dimers with intrinsic polarity. The minus-end is less dynamic and is generally anchored to microtubule organising centres (MTOCs). By contrast, the plus-end is very dynamic and interacts selectively with other cellular structures, such as kinetochores and regions of the cell cortex. The interactions and dynamics of microtubule plus-ends must be precisely regulated to achieve the correct configuration of microtubule arrays. Microtubule associated proteins (MAPs), both motor and non-motor, are considered to be the primary regulators of microtubule dynamics and organisation.

In the last decade or so, a new special group of MAPs has emerged as crucial regulators of microtubule plus-ends. These MAPs were collectively called microtubule plus-end-tracking proteins, based on their preferential localisation to growing microtubule plus-ends (Schuyler and Pellman, 2001). CLIP-170 was the first MAP to be described with this property (Perez et

al., 1999) and is well conserved among yeast and animals (Pierre et al., 1992; Lantz and Miller, 1998; Brunner and Nurse, 2000). Consistent with its localisation, CLIP-170 is implicated in binding endocytic vesicles to microtubules (Pierre et al., 1992), regulating microtubule dynamics (Brunner and Nurse, 2000; Komarova et al., 2002), kinetochore function (Dujardin et al., 1998; Lin et al., 2001; Coquelle et al., 2002; Tai et al., 2002), and the linkage between microtubules and the cell cortex (Brunner and Nurse, 2000; Akhmanova et al., 2001; Fukata et al., 2002). More families of microtubule-tracking proteins have now been reported, most notably EB1 that has no sequence similarity to CLIP-170 (Mimori-Kiyosue et al., 2000).

Plus-end-tracking by specific MAPs is thought to derive from a higher affinity for growing plus-ends relative to the microtubule lattice (Carvalho et al., 2003; Galjart and Perez, 2003). Recent studies in yeast homologues have suggested an alternative – but not exclusive – mechanism, which utilises a plus-end directed microtubule motor (Browning et al., 2003; Busch et al., 2004; Carvalho et al., 2004). In fission yeast, the EB1 homologue is involved in plus-end localisation of the CLIP-170 homologue (Busch and Brunner, 2004). In higher eukaryotes the involvement of similar mechanisms has not been demonstrated.

Kinetochore localisation of CLIP-170 in mitosis has been shown in higher eukaryotes (Dujardin et al., 1998; Maiato et al., 2002) and in budding yeast (Lin et al., 2001). Involvement of dynein-dynactin and Lis1 in this localisation was demonstrated using overexpression of dominant negative proteins (Dujardin et al., 1998; Coquelle et al., 2002; Tai et al., 2002). These studies revealed a direct interaction between Lis1 and CLIP-170, and a dependency of CLIP-170 kinetochore localisation on Lis1 and the dynein-dynactin complex, but not vice versa. Interestingly, all these molecules have also been shown to be involved in microtubule–cell-cortex interactions (Dujardin and Vallee, 2002) and even to cooperate in this process (Sheeman et al., 2003).

The *Drosophila* genome contains only one CLIP-170 homologue, *Drosophila* CLIP-190 (D-CLIP-190) (Lantz and Miller, 1998). This is in contrast to mammalian genomes, which contain multiple paralogues of CLIP-170. Therefore, *Drosophila* is a good system to study the role and regulation of the CLIP family of proteins. CLIP-190 was originally identified as a protein that binds to myosin VI (Lantz and Miller, 1998), and was later shown to localise to kinetochores (Maiato et al., 2002). Here, we focus on the cell-cycle regulation of CLIP-190 in *Drosophila* cell culture and show that it localises to microtubule plus-ends in interphase and unattached kinetochores in mitosis. Systematic RNAi depletion of potential regulators uncovered distinct mechanisms governing cell-cycle-regulated localisation of CLIP-190.

## Materials and Methods

### Molecular and protein techniques

Standard DNA manipulation and protein techniques were used (Harlow and Lane, 1988; Sambrook et al., 1989). Subregions of CLIP-190 cDNA were cloned into bacterial expression vector pGEX4T-3 (Pharmacia) to express GST-CLIP-190-N, GST-CLIP-190-cc and GST-CLIP-190-C which contain amino acids 1-405, 851-1468 and 1280-1690, respectively. A plasmid expressing full-length EB1 fused with MBP was generously provided by Anne Davidson in our laboratory (Edinburgh, UK). Physical interaction between CLIP-190 and EB1 was examined as follows. Sub-domains of CLIP-190 fused with GST and GST alone were expressed in bacteria, lysed in PBS containing protease inhibitors [Complete EDTA-free (Roche) + 1 mM phenyl methyl sulfonyl fluoride (PMSF)] and purified with glutathione agarose beads (Pharmacia) after the addition of TritonX-100 to 1% final concentration. The beads were divided into two portions and each portion was incubated overnight at 4°C with bacterial lysates (in PBS, inhibitors, 0.1% Triton), containing either MBP or MBP-EB1-expressed proteins. After extensive washing in PBS containing 0.1% Triton, both portions of beads were boiled in sample buffer for analysis by SDS PAGE and immunoblotting.

An antibody against GST-CLIP-190-cc was raised in sheep, and one against GST-CLIP-190-C in rabbit by Diagnostics Scotland (Midlothian, UK). Affinity purification was performed by incubation with the respective antigen bound to nitrocellulose membrane (Smith and Fisher, 1984).

### Cell culture and RNA interference

*Drosophila* Schneider S2 cells were cultured and RNA interference (RNAi) was performed according to published methods (Clemens et al., 2000; Rogers et al., 2002). Double-stranded RNAs (dsRNAs) for RNAi were produced by *in vitro* transcription using the Megascript-T7 kit (Ambion). Templates were made in two rounds of PCR as follows: about 600 bp within an exon of the target gene was first

amplified from genomic DNA or cDNA using gene specific primers with 5'-overhangs of the last 18 nucleotides from the minimum T7 promoter sequence (27 bp), and then further amplified using a primer comprising the full T7 promoter sequence (see supplementary material Fig. S1). dsRNA corresponding to 780 bp of the  $\beta$ -lactamase gene was used as a control RNAi. Primer sequences are shown in supplementary material Fig. S1. Colchicine (6  $\mu$ g/ml final concentration; Sigma) or sodium ortho-vanadate (100  $\mu$ M; final concentration; Sigma) was added to the culture medium and cells were incubated overnight or for 4 hours, respectively, prior to fixation.

### Immunofluorescence microscopy

For immunostaining, S2 cells were cultured on a concanavalin A (conA)-coated coverslip for a minimum of 2 hours with the appropriate drug (see above) and fixed with 90% methanol, 3% formaldehyde, 5 mM NaHCO<sub>3</sub> pH 9 at -80°C as described (Rogers et al., 2002). Antibodies against  $\alpha$ -tubulin (DM1A, 1:200, Sigma), *Drosophila* EB1 (Elliott et al., 2005), Dic (MAB1618, Chemicon; 1:50), Rod (BE40, a kind gift from R. Karess, CNRS, Paris (Scaerou et al., 1999) and BubR1 (a kind gift from C. Sunkel, University of Porto, Porto) (Logarinho et al., 2004) were used as primary antibodies. Rabbit anti-histone-H3-phosphate (06-570, Upstate; 1:500) and affinity purified sheep-anti-CLIP-190cc (this study; 1:50) were also used as primary antibodies. Secondary antibodies, conjugated with Alexa Fluor 488, Cy3 or Cy5 (Molecular Probes or Jackson Lab) and DAPI (Sigma) were used in 1:200, 1:1000 and 1:200 dilution, respectively. DAPI (Sigma) was used at 0.4  $\mu$ g/ $\mu$ l. Images were taken with an Axioplan 2 microscope (Zeiss) attached to a CCD camera (Hamamatsu) controlled by OpenLab 2.2.1 software (Improvision) and were processed with Photoshop (Adobe). The relative plus-end signal for EB1 and CLIP-190 was calculated using the formula  $(S-B) \div B$ , where S is the average pixel intensity for a particular plus-end signal and B is the average pixel intensity of the local background. Since the shape of EB1 comets was variable, we hand-drew the area, tightly surrounding each comet. The local background was also measured by hand-drawn areas on both sides of each comet. The formula  $(S-B) \div B$  was used to compensate for differences in exposure and variations in brightness. A minimum of ten plus-end signals were measured in at least three separate cells for each cell-cycle stage. Measurements were made using OpenLab 2.2.1 (Improvision).

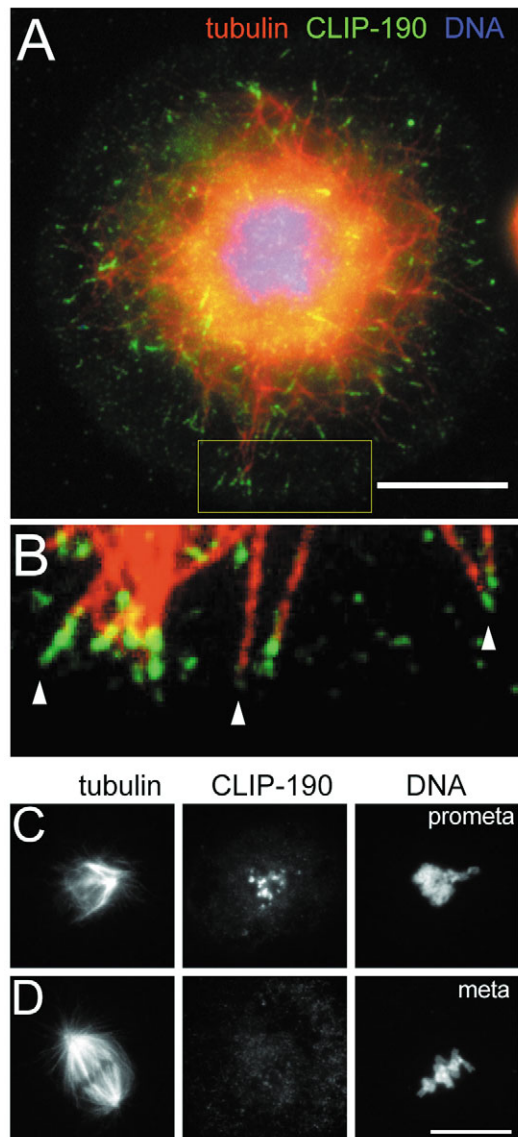
## Results

### Cell-cycle-dependent localisation of CLIP-190

To investigate cellular localisation of CLIP-190 in detail, we used *Drosophila* S2 cells spread on conA-treated coverslips (Rogers et al., 2002), which allowed us to resolve individual microtubule ends. We raised antibodies against two parts of CLIP-190 in rabbits and used these for immunostaining of S2 cells together with a tubulin antibody. Both CLIP-190 antibodies, but not the preimmune sera, gave the same results.

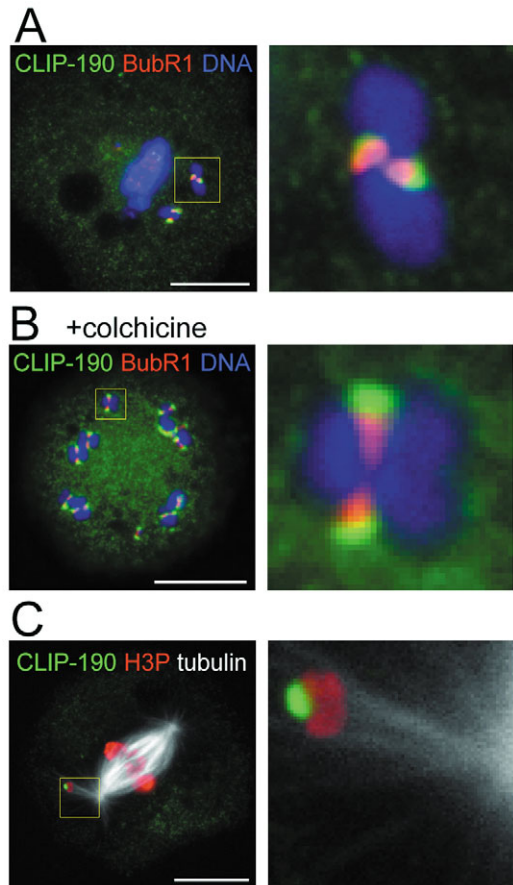
After growing on a conA-coated surface, S2 cells spread and flatten, with interphase or astral microtubules extending towards the cell periphery. During interphase, CLIP-190 mainly accumulated at microtubule plus-ends but also localised weakly along microtubules (Fig. 1A,B). Accumulation of CLIP-190 at the microtubule plus-ends was variable both in intensity and length, which may reflect a difference in microtubule dynamics. The results indicate that CLIP-190, like all CLIP-170 homologues, is a microtubule plus-end-binding protein during interphase.

In prometaphase, CLIP-190 was observed as multiple foci



**Fig. 1.** Localisation of CLIP-190 in the cell cycle. S2 cells were immunostained with CLIP-190 and  $\alpha$ -tubulin antibodies, DNA was counterstained with DAPI. (A) In interphase cells, CLIP-190 is mainly localised to microtubule plus-ends. (B) Magnified view of the region marked by the rectangle in A. Three arrowheads indicate the variation in CLIP-190 localisation at microtubule plus-ends, which may reflect differences in microtubule dynamics. (C) In prometaphase, CLIP-190 is localised to kinetochores. (D) In metaphase, CLIP-190 disappears from kinetochores although it still associates weakly with spindle microtubules. Bars, 10  $\mu$ m.

on chromosomes (Fig. 1C). These foci were never seen before nuclear-envelope breakdown. To test whether these foci corresponded to kinetochores, we double-stained S2 cells with an antibody against a known kinetochore protein, BubR1 (Fig. 2A). CLIP-190 colocalised with BubR1 on the primary constriction of some chromosomes, indicating that CLIP-190 is localised to kinetochores. Consistent with previous reports in mammalian and *Drosophila* cells (Dujardin et al., 1998; Maiato et al., 2002), kinetochore localisation of CLIP-190 was



**Fig. 2.** Localisation of CLIP-190 to unattached kinetochores. S2 cells were immunostained without (A,C) or after (B) incubation with colchicine. The right panels show magnified images of the regions marked with squares in the left panels. (A) Kinetochore localisation of CLIP-190. Staining of CLIP-190 overlaps with the one of BubR1, a known kinetochore protein. (B) Microtubule-independent localisation of CLIP-190 to kinetochores. CLIP-190 and BubR1 both localise to primary constrictions of all chromosomes in the absence of microtubules. Note that CLIP-190 localises to the outer regions of kinetochores relative to BubR1. (C) Localisation of CLIP-190 to unattached kinetochores. Chromosomes were visualised with an antibody against phosphorylated histone H3. Only one of the sister kinetochores, the one facing away from the pole, has CLIP-190. Bars, 10  $\mu$ m.

not observed in metaphase or later stages of mitosis (Fig. 1D). Interestingly, during mitosis, CLIP-190 only weakly localised to plus-ends of astral microtubules and to spindle microtubules (see below).

In summary, CLIP-190 shows cell-cycle-dependent localisation to microtubule plus-ends in interphase and kinetochores in early mitosis. We describe the molecular mechanisms involved in localising CLIP-190 in mitosis and interphase below.

#### CLIP-190 is localised to unattached kinetochores

To further study kinetochore localisation of CLIP-190, we examined mitotic cells that only had some chromosomes aligned at the metaphase plate. CLIP-190 localised to

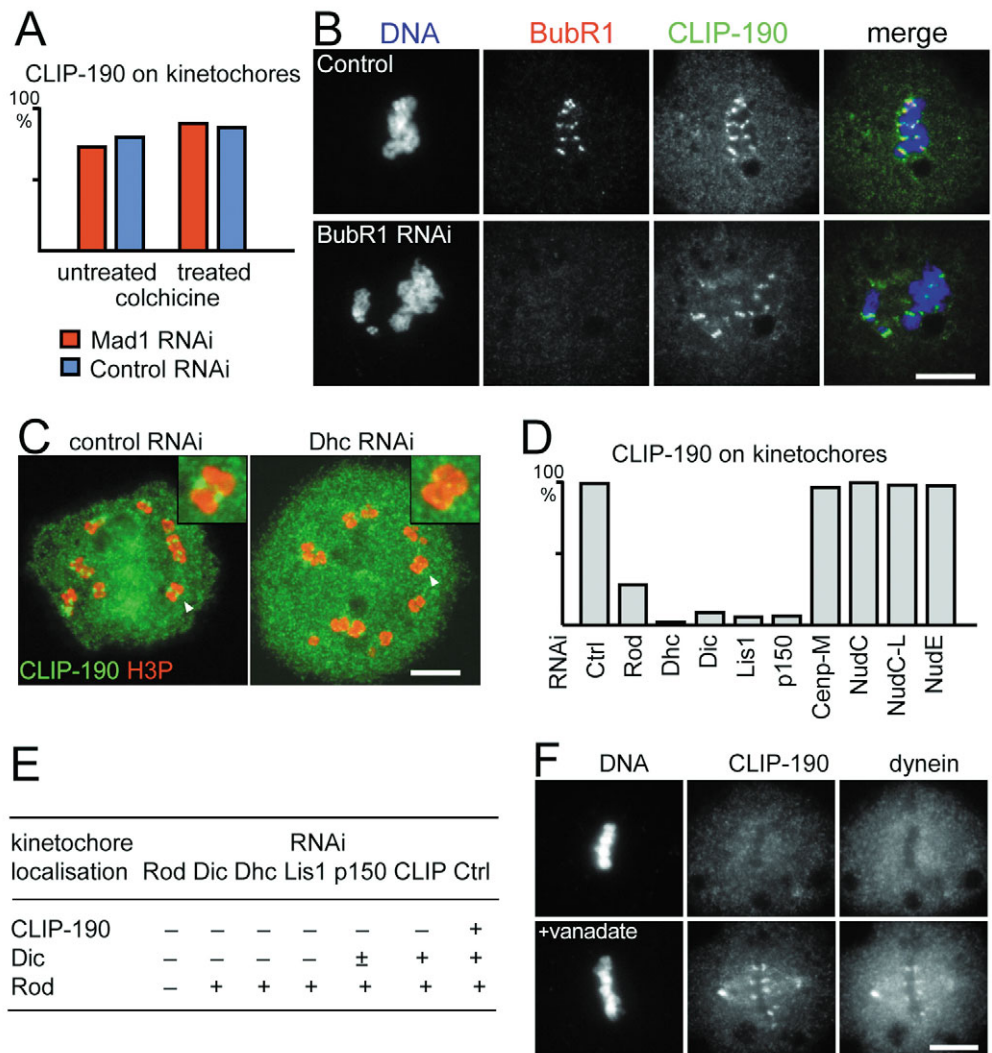
kinetochores of unaligned chromosomes, but was generally absent from aligned chromosomes (Fig. 2A,C). In many cases, CLIP-190 localised only to one sister kinetochore facing away from the poles, suggesting that microtubule attachment, not tension, is crucial for the loss of CLIP-190 (Fig. 2C). To examine the microtubule requirement for kinetochore localisation, S2 cells were incubated with the microtubule-depolymerising drug colchicine. Complete depolymerisation of microtubules resulted in the accumulation of S2 cells in mitosis. In these mitotically arrested cells, all kinetochores accumulated CLIP-190 signals (Fig. 2B), indicating that CLIP-190 localisation to unattached kinetochores is microtubule independent. Close inspection of the images indicated that CLIP-190 is localised outside BubR1 on unattached

kinetochores, both in colchicine-treated and non-treated cells (Fig. 2A,B).

**CLIP-190 localisation to unattached kinetochores depends on the dynein-dynactin complex, Lis1 and Rod, but is independent of an active spindle checkpoint**

CLIP-190 localisation to unattached kinetochores is similar to that of proteins involved in the spindle checkpoint. To test whether activation or inactivation of the checkpoint pathway regulates localisation of CLIP-190, S2 cells were depleted for the checkpoint protein Mad1 by RNAi. We chose Mad1 because its depletion was recently shown to effectively inactivate the spindle checkpoint in mammalian cells – without

**Fig. 3.** CLIP-190 localisation to kinetochores requires the dynein complex. (A) The proportion of cells having CLIP-190 on at least one kinetochore after depletion of *Drosophila* Mad1 ( $n=100$  cells). Mad1 was depleted by RNAi and cells were immunostained with CLIP-190 before and after colchicine treatment. A  $\chi^2$ -test indicated no significant difference between Mad1-depleted cells and mock-depleted cells. Mad1-mediated spindle checkpoint is not required for the localisation of CLIP-190 to unattached kinetochores. (B) Kinetochore localisation of CLIP-190 after control or BubR1 RNAi. Cells were immunostained for BubR1 and CLIP-190 after colchicine treatment. Both BubR1 and CLIP-190 localise to kinetochores in control RNAi. After BubR1 RNAi, BubR1 signals on kinetochores were undetectable, whereas CLIP-190 localises robustly to kinetochores. (C) S2 cells depleted of Dhc were immunostained with CLIP-190 after colchicine treatment. Chromosomes marked by arrowheads are magnified in the top right corners. CLIP-190 was not localised to kinetochores in Dhc depleted cells. (D) The proportion of mitotic cells that have CLIP-190 on at least one kinetochore ( $n=100$  cells scored in each category) after depletion of the following proteins; Ctrl (control,  $\beta$ -lactamase), Dhc (dynein heavy-chain), Dic (dynein intermediate-chain), Lis1, p150 (p150<sup>Glued</sup>), Cenp-M (Cenp-meta), NudC, NudC-L (NudC-like: CG31251), and NudE (CG8104). Cells were immunostained with CLIP-190 after colchicine treatment at day 5 of RNAi. All residual CLIP-190 kinetochore signals were weak in Rod-depleted cells. (E) Localisation dependency among CLIP-190, dynein-dynactin complex and Rod. Kinetochore localisation of CLIP-190, Dic and Rod were examined after depletion of each protein by RNAi. + indicates that kinetochore localisation is retained, while – indicates kinetochore localisation is abolished or greatly reduced. Dic after p150 depletion gave reduced but consistent signals on kinetochores and is therefore marked with  $\pm$ . (F) Metaphase cells without (upper panel) or with (lower panel) vanadate treatment, an inhibitor of dynein-motor-activity. S2 cells were treated with vanadate before immunostaining for Dic, CLIP-190 and DNA. Dynein and CLIP-190 is lost from kinetochores of metaphase chromosomes without vanadate treatment, whereas both proteins accumulated on the kinetochores as well as spindle microtubules and poles after vanadate treatment. Bars, 10  $\mu$ m.



affecting other mitotic parameters such as transition time (Meraldi et al., 2004). As Mad1 homologues had not previously been identified in *Drosophila*, we searched the *Drosophila* genome and found one putative Mad1 homologue (CG2072/TXBP181-like) as judged by homology and its coiled-coil structure prediction. RNAi of this gene resulted in a checkpoint-defective phenotype, a low mitotic index in an asynchronously growing cell population, lagging chromosomes and chromosome bridges in late anaphase or telophase, and a failure to accumulate mitotic cells after overnight colchicine treatment (supplementary material Fig. S3). In these checkpoint-defective cells, CLIP-190 showed kinetochore signals in prometaphase cells at levels comparable to those in control cells (Fig. 3A), both with and without colchicine treatment. To further confirm checkpoint independent localisation of CLIP-190, BubR1 was depleted by RNAi and then cells were simultaneously stained with both CLIP-190 and BubR1 antibodies. In control cells, the spindle checkpoint was functional and both BubR1 and CLIP-190 localised to unattached kinetochores (Fig. 3B). RNAi of BubR1 resulted in a checkpoint-deficient phenotype (supplementary material Fig. S4) but CLIP-190 still localised to unattached kinetochores, despite BubR1 being undetectable at the same kinetochores (Fig. 3B). These results indicate that a functional spindle checkpoint is not required for the localisation of CLIP-190 to the kinetochores.

To investigate the molecular requirements for the kinetochore localisation of CLIP-190, various kinetochore proteins were depleted by RNAi and the CLIP-190 localisation was examined in colchicine-arrested mitotic cells. We found that the localisation to kinetochores was abolished or greatly reduced, without altering the level of CLIP-190 protein, by depletion of Rough deal (Rod) (a subunit of the Rod-ZW10-Zwilch checkpoint complex), dynein heavy chain (Dhc), dynein intermediate chain (Dic), p150<sup>Glued</sup> (a dynactin subunit) or Lis1 (Fig. 3C,D). Depletion of CENP-meta (the CENP-E homologue) had no effect on CLIP-190 localisation (Fig. 3D). Since the kinetochore proteins required for localisation of CLIP-190 are components of the dynein-dynactin complex or associated proteins, we examined the requirement of other putative dynein interactors: the NudE homologue (CG8104), NudC (CG9710) and a protein sharing sequence similarity to NudC that we here call NudC-like (CG31251). Depletions of these three proteins by RNAi did not affect kinetochore localisation of CLIP-190 (Fig. 3D).

In all the above depletion experiments, CLIP-190 localisation to microtubule plus-ends of cells not treated with colchicine was not affected (data not shown), indicating that distinct mechanisms are involved in localising CLIP-190 either to kinetochores or to microtubule plus-ends.

#### Hierarchical relationships among proteins localising to unattached kinetochores

Our results show that CLIP-190 localisation to kinetochores requires dynein-dynactin, Lis1 and Rod. Among these proteins, some physical and dependency relationships were reported by several studies using overexpression of dominant negative proteins or mutational analysis in *Drosophila* (Dujardin et al., 1998; Starr et al., 1998; Coquelle et al., 2002). To clarify the relationship among these proteins, we depleted Rod, p150<sup>Glued</sup>,

Dic, Dhc, Lis1 or CLIP-190 by RNAi, and immunostained with Rod, Dic and CLIP-190 antibodies (Fig. 3E). In each case, depletion did not affect the amount of the other proteins (data not shown).

Rod depletion abolished localisation of all other proteins, but Rod localisation itself was independent of the presence of all other proteins. Localisation of CLIP-190 to kinetochores depended on all proteins tested, whereas localisation of any of the other proteins was not affected by CLIP-190 depletion. On the other hand, depletion of Dhc, Dic, p150<sup>Glued</sup> and Lis1 abolished kinetochore localisation of Dic and CLIP-190, but not that of Rod protein, indicating that the dynein-dynactin complex and Lis1 link Rod and CLIP-190. Interestingly, we found that Lis1 depletion abolished localisation of dynein (Dic) to kinetochores. A requirement for Lis1 in dynein kinetochore localisation has so far not been shown in any system. Since Lis1 kinetochore localisation depends on dynein in mammalian cells (Coquelle et al., 2002), Lis1 and dynein may be mutually dependent on each other for their localisation.

In conclusion, our RNAi analysis revealed co-dependency of recruitment to kinetochores, with Rod protein first, followed by dynein-dynactin and Lis1, and then CLIP-190. Our results also indicate for the first time that Lis1 is essential for dynein localisation to kinetochores.

#### Involvement of dynein ATPase activity in the removal of CLIP-190 from attached kinetochores

Next, we examined how CLIP-190 is removed from kinetochores upon microtubule attachment. It has been proposed that dynein motor activity is responsible for removing some kinetochore proteins, such as Rod and CENP-E, from attached kinetochores by transporting them along microtubules (Howell et al., 2001). Although a physical interaction between dynein-dynactin and CLIP-190 has been reported (Komarova et al., 2002; Lansbergen et al., 2004), the removal of CLIP-190 or other homologues from attached kinetochores by dynein has not been investigated.

To test this possibility, we used sodium ortho-vanadate to inhibit the motor activity of dynein, as depletion of dynein abolishes CLIP-190 localisation in the first place (Fig. 3F). S2 cells were treated with 100  $\mu$ M vanadate for 4 hours and immunostained with dynein and CLIP-190 antibodies. As we expected, vanadate treatment did not prevent dynein localisation to kinetochores, but resulted in an abnormal accumulation of dynein on kinetochores of aligned chromosomes. In addition, dynein was found to accumulate along spindle microtubules and especially at the spindle poles. In vanadate treated cells, CLIP-190 exactly colocalised with dynein at the kinetochores of aligned chromosomes as well as along spindle microtubules and the poles. Although the effect of vanadate on dynein localisation was not completely penetrant and vanadate probably has multiple targets, CLIP-190 mislocalisation was tightly correlated to that of dynein. This suggests that inhibition of dynein motor activity is the primary cause for the failure of CLIP-190 removal from attached kinetochores.

#### EB1 and CLIP-190 show overlapping localisation to microtubule plus-ends

Depletion of proteins required for kinetochore localisation of

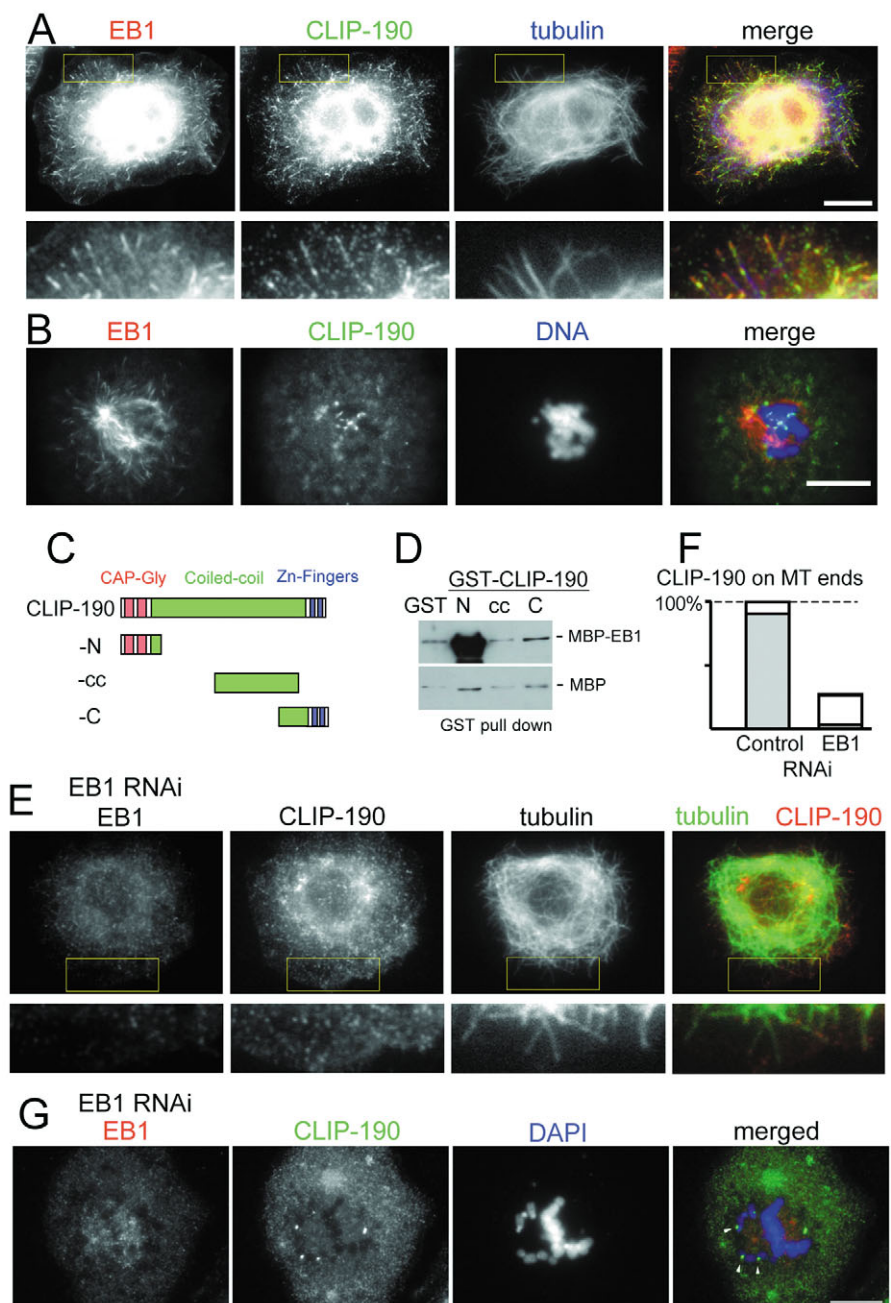
CLIP-190 did not affect the localisation of CLIP-190 to microtubule plus-ends in interphase, indicating the involvement of distinct mechanisms. To understand how CLIP-190 localises to microtubule plus-ends, we first examined the spatial relationship of CLIP-190 with EB1, another microtubule plus-end-tracking protein. Although these proteins represent two major families of microtubule plus-end-tracking proteins, their exact localisation relative to each other has not been examined. For this purpose, we raised an anti-CLIP-190 antibody in sheep to allow simultaneous staining with a rabbit anti-EB1 antibody. We found that the localisation of the two proteins to microtubule plus-ends substantially overlapped, although not completely (Fig. 4A). Compared to EB1, which stained as a comet, CLIP-190 gave more punctate staining and appeared to have a longer tail. By contrast, there was distinct

localisation of the proteins during mitosis (Fig. 4B). EB1 mainly localised to spindle and astral microtubules with no accumulation at kinetochores, whereas CLIP-190 localised to spindle microtubules only weakly and showed strong kinetochore accumulation.

#### The CAP-Gly domain of CLIP-190 directly interacts with EB1

Since CLIP-190 and EB1 show overlapping localisation to microtubule plus-ends, and we have previously shown that CLIP-190 from S2 cell extracts can bind to EB1 (Rogers et al., 2004), we tested for direct physical interaction by expressing these two proteins in bacteria. CLIP-190 and its mammalian homologues have three separate domains (Fig. 4C): the N-

**Fig. 4.** The plus-end localisation of CLIP-190 depends on EB1. S2 cells were immunostained with antibodies against EB1, CLIP-190 and  $\alpha$ -tubulin. (A) Colocalisation of CLIP-190 and EB1 to the microtubule plus-ends during interphase. Both proteins accumulate near microtubule plus-ends and weakly localise along the microtubules. The region marked with a rectangular box is magnified below each panel. (B) Distinct localisation of CLIP-190 and EB1 in mitosis. CLIP-190 localises to the kinetochores at prometaphase, whereas EB1 localises to spindle and aster microtubules without accumulation at kinetochores. (C) Conserved domain structure of CLIP-190. CLIP-190 has two CAP-Gly domains in its N-terminus, followed by a coiled-coil region and two zinc finger motifs in its C-terminus. Subregions of CLIP-190 shown here were expressed in bacteria as GST-fusion proteins. (D) Physical interaction between CLIP-190 and EB1. GST-tagged subdomains of CLIP-190 and GST alone were expressed in bacteria and bound to glutathione beads. After washing, the beads were incubated in bacterial lysates containing MBP-EB1 or MBP alone (to control for the MBP-part of the fusion protein). The glutathione beads were washed again and analysed by immunoblotting using an MBP antibody. The N-terminal domain (CAP-Gly domain) of CLIP-190 directly interacts with EB1. (E) Delocalisation of CLIP-190 in EB1-depleted cells. S2 cells were immunostained for CLIP-190,  $\alpha$ -tubulin and EB1 after EB1 depletion. The region marked with a box is magnified below each panel. Localisation of CLIP-190 to microtubule plus-ends was abolished (compare with A). (F) The proportion of cells with CLIP-190 at microtubule plus-ends after control and EB1 RNAi ( $n=100$  cells scored each). Grey boxes indicate robust localisation of CLIP-190 to microtubule plus-ends, whereas open boxes indicate a greatly reduced signal. (G) The localisation of CLIP-190 to unattached kinetochores is unaffected in EB1 RNAi. S2 cells were immunostained for DNA, CLIP-190 and EB1 after EB1 depletion. CLIP-190 was still seen on unattached kinetochores in EB1 RNAi (arrowheads; compare with B). Bars, 10  $\mu$ m.



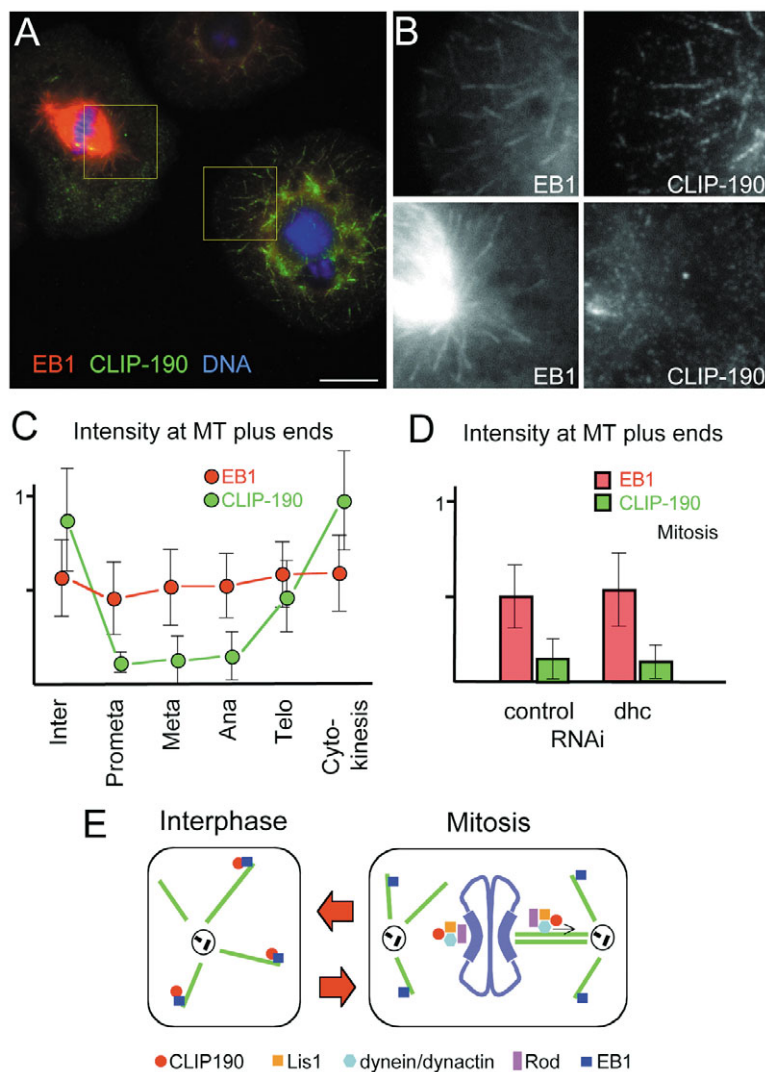
terminal region consisting of two CAP-Gly domains (the microtubule-binding domain), the C-terminal region containing zinc finger motifs (the putative cargo binding domain), and the long coiled-coil region which connects these two domains (the linker domain). The N-terminal domain (CLIP-190-N), C-terminal domain (CLIP-190-C) and part of the coiled-coil region (CLIP-190-cc) were expressed in bacteria as proteins fused with GST (glutathione-S transferase). After purification, each GST fusion and also GST alone was incubated with bacterially produced EB1 fused with MBP (maltose-binding protein) or MBP alone. MBP-EB1 and MBP bands were weakly present in all pull-down assays but a large amount of MBP-EB1 was pulled down only by GST-CLIP-190-N (Fig. 4D). These results demonstrate that the CAP-Gly domain of CLIP-190 directly interacts with EB1.

#### Localisation of CLIP-190 to microtubule plus-ends depends on EB1

The physical interaction between these two proteins prompted us to examine their localisation dependencies. For this purpose, EB1 or CLIP-190 were depleted from S2 cells by RNAi and immunostained with antibodies against EB1, CLIP-190 and  $\alpha$ -tubulin. Immunoblots indicated that more than 90% of the target proteins were depleted 6 days after the addition of dsRNA. In both cases the levels of the other protein were not altered (data not shown). Depletion of CLIP-190 did not affect EB1 localisation to microtubule ends (data not shown). However, depletion of EB1 abolished the localisation of CLIP-190 to plus-ends of interphase microtubules (Fig. 4E,F), although a substantial proportion of microtubules were still growing under these conditions. By contrast, mitotic localisation of CLIP-190 to kinetochores was not affected by EB1 depletion (Fig. 4G), consistent with their distinct localisation during mitosis. In summary, CLIP-190 physically interacts with EB1 and localises to microtubule plus-ends in an EB1-dependent manner.

#### Cell-cycle regulation of CLIP-190 localisation

Simultaneous visualisation of CLIP-190 and EB1 allowed us to examine the cell-cycle regulation of CLIP-190 localisation to microtubule plus-ends. We noticed that CLIP-190 localisation to microtubule plus-ends is greatly attenuated during mitosis (cells on the left in Fig. 5A; Fig. 5B lower panels) compared with interphase (cells on the right in Fig. 5A; Fig. 5B upper panel). By contrast, EB1 localisation to microtubule plus-ends during mitosis was comparable to that observed in interphase (Fig. 5A). For further analysis, we quantified the intensity of the signals on microtubule plus-ends at each cell-cycle stage (Fig. 5C). The graph shows that CLIP-190 levels at plus-ends decreased dramatically at the beginning of mitosis but started to recover during



**Fig. 5.** The cell-cycle regulation of microtubule plus-end localisation. (A,B) S2 cells were stained simultaneously for EB1 and CLIP-190. (A) The CLIP-190 signal at microtubule plus-ends (either interphase or astral microtubules) is strong in interphase but very weak in mitosis. By contrast, the EB1 signal is constant in both mitosis and interphase. Bar, 10  $\mu$ m. (B) Higher magnification images of interphase (upper panels) and mitotic cells (lower) marked by the squares in A. (C) Cell-cycle change of CLIP-190 and EB1 localisation to microtubule plus-ends. The plus-end signal-intensity relative to background was measured for each cell-cycle stage (a total of ten microtubules from three different cells were scored for each mitotic stage) and is shown as a circle with the standard deviation represented by vertical bars. CLIP-190 dissociates from microtubule plus-ends during mitosis. (D) Levels of EB1 and CLIP-190 at microtubule plus-ends in mitotic cells after control ( $n=30$  microtubules from three different cells) and Dhc ( $n=18$  microtubules from three different cells) RNAi. In Dhc-depleted cells, CLIP-190 does not localise to kinetochores, but still dissociates from microtubule plus-ends during mitosis. (E) Cell-cycle regulation of CLIP-190 localisation. Molecular requirements for CLIP-190 localisation during the cell cycle are illustrated. During interphase, CLIP-190 localises to microtubule plus-ends in an EB1-dependent manner. The association of CLIP-190, but not EB1, to microtubule ends is greatly reduced during mitosis. Instead, CLIP-190 is localised to unattached kinetochores in mitosis. This localisation depends on dynein-dynactin and Lis1. Dynein-dynactin and Lis1 localisation are mutually dependent on each other, and both depend on Rod. Upon attachment to microtubules, CLIP-190 is removed from kinetochores by the motor-activity of dynein.

telophase. By contrast, EB1 levels remained unchanged during the cell cycle. Therefore, the association of CLIP-190 to microtubule plus-ends appears to be cell-cycle-regulated.

However, this cell-cycle-dependent localisation might be mediated simply by sequestration of CLIP-190 by kinetochores during mitosis. The two following experimental results excluded this possibility. First, CLIP-190 localisation to microtubule plus-ends was not recovered until telophase, although CLIP-190 kinetochore localisation was lost at metaphase (Fig. 5C). Secondly, the depletion of dynein-dynactin, Rod or Lis1 abolished CLIP-190 localisation to kinetochores, but did not restore CLIP-190 localisation to plus-ends of astral microtubules during mitosis (Fig. 5D). EB1 localisation to the plus-ends of astral microtubules was not affected by depletion of these proteins. We therefore conclude that CLIP-190 association to microtubule plus-ends is cell-cycle-regulated.

## Discussion

The CLIP family of proteins is implicated in regulating microtubule dynamics and linking microtubule plus-ends with other cellular structures (Galjart and Perez, 2003). To understand these functions, it is crucial to elucidate where and how these proteins localise within cells. Here, we studied the molecular mechanisms of CLIP-190 localisation using RNAi in *Drosophila* cells, rather than using the expression of dominant proteins, to gain a clearer and more comprehensive view. The study revealed that distinct, cell-cycle-dependent mechanisms localise CLIP-190 to microtubule plus-ends and unattached kinetochores.

### CLIP-190 localisation to microtubule plus-ends in interphase

CLIP and EB1 proteins are two major families of microtubule plus-end-tracking proteins. This study is the first demonstration of both a physical interaction and localisation-dependency between CLIP and EB1 proteins in higher eukaryotes. We show that CLIP-190 requires EB1 to localise to microtubule plus-ends, and that it directly interacts with EB1 through its CAP-Gly domain, which also binds to microtubules (Pierre et al., 1992). Considering that this has previously been reported for fission yeast homologues (Busch and Brunner, 2004), our findings demonstrate that this interaction and dependency are conserved among eukaryotes. The most obvious interpretation would be that EB1 simply bridges microtubule plus-ends and CLIP-190. However, we think this may not be the case. First, CLIP-170 has been shown to bind directly to microtubule plus-ends in vitro (Diamantopoulos et al., 1999). Secondly, our localisation studies show incomplete overlapping of the two proteins on microtubule plus-ends. Also, other EB1 interacting proteins, such as RhoGEF2 and the spectraplakins Short stop, which both require EB1 for their localisation to microtubule plus-ends (Rogers et al., 2004; Slep et al., 2005), show distinct localisation from that of EB1. Therefore, it is more likely that EB1 acts as a loading factor for these proteins, rather than a simple bridge. In addition, it seems that multiple microtubule plus-end-binding proteins, such as CLIPs, CLASPs, p150<sup>Glued</sup>, EB1, Lis1, Dynein, Short stop, APC and RhoGEF2, directly interact with each other and with microtubules (Su et al., 1995;

Akhmanova et al., 2001; Coquelle et al., 2002; Tai et al., 2002; Ligon et al., 2003; Subramanian et al., 2003; Lansbergen et al., 2004; Rogers et al., 2004; Mimori-Kiyosue et al., 2005). It is an exciting challenge to understand the regulatory network acting on microtubule plus-ends.

In addition, we found that the association of CLIP-190 to microtubule plus-ends is greatly reduced during mitosis. This is in contrast to EB1, which is associated with plus-ends throughout the cell cycle. This cell-cycle-regulation has not been described in other systems, possibly because of a lack of co-examination with EB1, allowing us to conclude that it is not the consequence of a change in microtubule dynamics. It might be possible that CLIP-190 is modified during the cell cycle. Since EB1 is essential for CLIP-190 localisation to microtubule ends, EB1 activity or interaction between EB1 and CLIP-190 might also be regulated. Alternatively, other inhibitory proteins might be activated during mitosis to attenuate CLIP-190 association with microtubule ends. Phosphorylation of the EB1 homologue mal3p and its inhibitory effects on the interaction with the CLIP-190 homologue tip1p have been shown in fission yeast (Busch and Brunner, 2004). It remains to be examined whether this phosphorylation is cell-cycle-regulated. Cell-cycle regulation might be important for releasing CLIP-190 for kinetochore function or preventing the plus-end-binding activity from interfering with CLIP-190 function at kinetochores. This report is the first to describe the cell-cycle regulation of the plus-end-binding of CLIP proteins. Elucidation of the precise mechanism and significance of this regulation may lead to further understanding of the temporal and spatial regulation of microtubules in cells.

### CLIP-190 localisation to unattached kinetochores

CLIP-190 localises to unattached kinetochores in mitosis. The localisation of CLIP proteins to kinetochores has been shown in mammalian, *Drosophila* and budding-yeast cells (Dujardin et al., 1998; Lin et al., 2001; Maiato et al., 2002). Studies of CLIP-170 localisation to kinetochores in mammalian cells suggest an intricate physical and functional relationship with the dynein-dynactin complex. CLIP-170 binds directly to and requires Lis1 for kinetochore localisation. In turn, Lis1 interacts with multiple subunits of dynein-dynactin and is displaced from kinetochores when the motor complex is disrupted (Coquelle et al., 2002; Tai et al., 2002). Most of these studies relied on the overexpression of dominant-negative proteins.

RNAi in *Drosophila* culture cells has been demonstrated to be very efficient and robust (Clemens et al., 2000). In our and others' experiences, RNAi has been shown to knock down target protein expression in all cases where depletion was checked. In most experiments of this study, we confirmed effective depletion by immunoblotting or through the induction of specific cellular defects (see supplementary material Fig. S2). Therefore, we are confident of efficient depletion by RNAi in most, if not all, of our cases. The ease of RNAi enabled us to study the requirement for CLIP-190 localisation in a more systematic manner. Reassuringly, our results using RNAi generally confirmed the previous observations deduced from overexpression of dominant-negative proteins.

However, we found a previously unreported dependency,



namely the requirement of Lis1 for dynein localisation to kinetochores. In mammalian cells, dynein is required for Lis1 localisation, whereas the overexpression of full-length or truncated Lis1 does not prevent dynein localisation to kinetochores (Tai et al., 2002). These results lead to the idea that Lis1 might be an auxiliary protein that bridges the dynein complex to cargo proteins. Our RNAi results clearly indicate that Lis1 is required for dynein localisation to kinetochores. Combined with previous results, Lis1 and dynein seem to depend on each other for their localisation. This is the first report of such dependency in any eukaryote, and it gives the Lis1 protein a more integral part in dynein function.

Our results also suggest that microtubule attachment directly removes CLIP-190 from kinetochores rather than through spindle-checkpoint signalling. Dynein seems to be responsible for the removal of CLIP-190 from kinetochores in addition to its role in localising CLIP-190 to kinetochores. It has been shown that dynein removes several kinetochore proteins along microtubules upon the attachment of microtubules (Howell et al., 2001; Wojcik et al., 2001). Our study provides the first evidence that a member of the CLIP family also utilises dynein-motor-activity to leave attached kinetochores. Interestingly, we found that unlike in interphase, EB1 is not required for the mitotic localisation of CLIP-190 to unattached kinetochores. This is intriguing in the light of recent evidence that EB1 associates with attached kinetochores when the kinetochore microtubules are polymerizing (Tirnauer et al., 2002).

In conclusion, our results indicate that CLIP-190 localisation is regulated during the cell cycle and requires distinct mechanisms in mitosis and interphase (Fig. 5E). Spatial and temporal regulation of CLIP-190 localisation probably play crucial roles in the regulation of microtubule dynamics and their interaction with other cellular structures.

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## RNAi primer sequences:

For 2<sup>nd</sup>:

Both 5'- and 3'- primers:

PCR-T7 minimum promotor sequence:  
gaattaatacgaactcactatagggaga

For NudE:

5' – cgactcactatagggagactcaagttggaatcgc  
3' – cgactcactatagggagatcttaagcgaatgctgct

For  $\beta$ -Lactamase:

5'–cgactcactatagggagattcctgttttgcacc  
3'–cgactcactatagggagaagtgaggcacctatctca

For Dic:

5'–cgactcactatagggagacgctgtactcctttgagg  
3'–cgactcactatagggagactatcgatttcattgaa

For CLIP-190:

5'–cgactcactatagggagaagctcagcaagtgaaca  
3'–cgactcactatagggagattgaagagtcctctcct

For p150<sup>Glued</sup>:

5'–cgactcactatagggagaccaccagcgattcagtg  
3'–cgactcactatagggagattcttctccgctagatcc

For EB1:

5'–cgactcactatagggagaatggctgtaaacgtctac  
3'–cgactcactatagggagatgcccgctgttggcac

For BubR1:

5'–cgactcactatagggagatgggctttgatatacacg  
3'–cgactcactatagggagaccatgtcaagtcaccat

For Mad1:

5'–cgactcactatagggagaaactgagcgaatatcgcc  
3'–cgactcactatagggagaattcaaatgcttggtatc

For Rod:

5'–cgactcactatagggagacggcgctcagcaagagcgc  
3'–cgactcactatagggagagagggcgctgatgctgc

For CENP-meta:

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For Dhc:

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For Lis1:

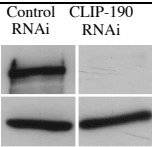
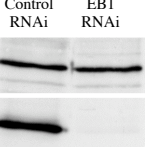
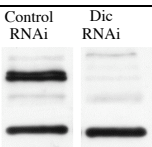
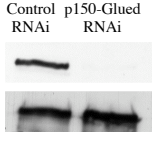
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3'–cgactcactatagggagactatggaggtgcagaaat

For NudC:

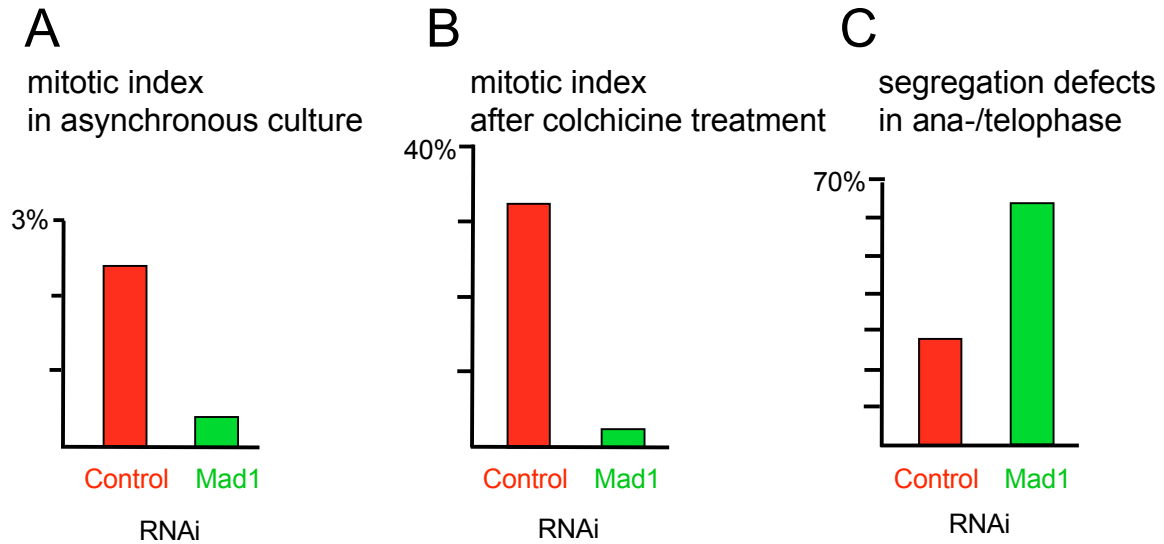
5'–cgactcactatagggagagcaatcgctagtaaggg  
3'–cgactcactatagggagaccagctgcgatcgtgct

For NudC-like

5' – cgactcactatagggagaattgttatcgatagacgt  
3' – cgactcactatagggagagctatgcttgctgctcac

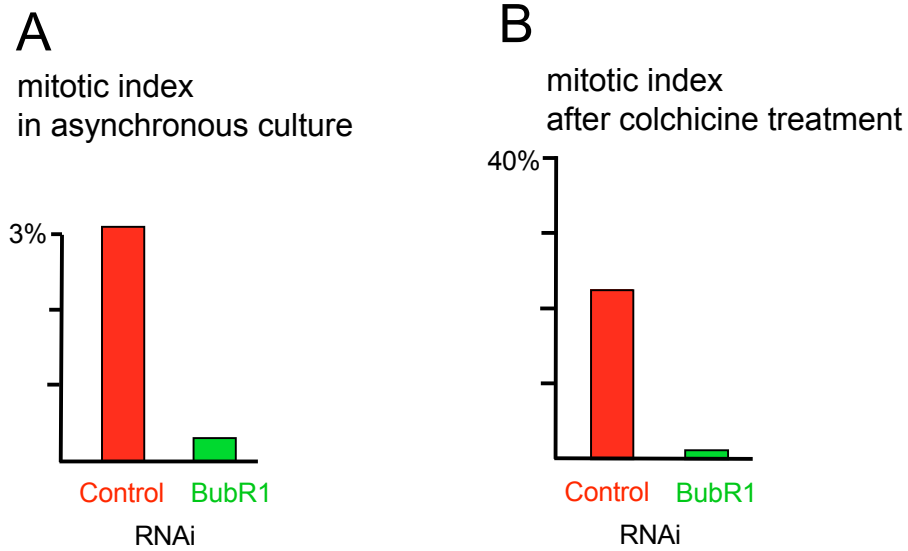
Protein depleted	Western blot	Effect on CLIP-190 localization	Other phenotype observed
CLIP-190	 <p>Control RNAi    CLIP-190 RNAi</p> <p>CLIP-190</p> <p>Tubulin</p> <p>6 Days RNAi</p>	CLIP-signal disappears	-
EB1	 <p>Control RNAi    EB1 RNAi</p> <p>Tubulin</p> <p>EB1</p> <p>6 Days RNAi</p>	Removes CLIP-190 From interphase MT-plus-ends	EB1 signal decreases; Small and unastral mitotic spindles
Rod	-	Removes CLIP-190 from unattached kinetochores	Low mitotic index (MI; 1.6%, compared to 2.4% in control) Failure to arrest in colchicine (3.9% MI, compared to 22.1% in control)
Dhc	-	Removes CLIP-190 from unattached kinetochores	High MI (7.2%, compared to 2.4% in control); Inefficient arrest in colchicine (12.9% MI, compared to 22.1% in control)
Dic	 <p>Control RNAi    Dic RNAi</p> <p>Dic</p> <p>Tubulin</p> <p>5 Days RNAi</p>	Removes CLIP-190 from unattached kinetochores	High MI (8.1%, compared to 4.6% in control); Inefficient arrest in colchicine (15% MI, compared to 27% in control)
p150-Glued	 <p>Control RNAi    p150-Glued RNAi</p> <p>p150-Glued</p> <p>Tubulin</p> <p>5 Days RNAi</p>	Removes CLIP-190 from unattached kinetochores	High MI (7.5%, compared to 4.6% in control); Inefficient arrest in colchicine (11% MI, compared to 27% in control)
Lis1	-	Removes CLIP-190 from unattached kinetochores	High MI (11.7%, compared to 2.4% in control); Inefficient arrest in colchicine (19.5% MI, compared to 22.8% in control)
Mad1	-	-	Low MI (0.4%, compared to 3.1% in control); Failure to arrest in colchicine (1.7% MI, compared to 16.3% in control)
BubR1	-	BubR1 signal disappears	Low MI (0.3%, compared to 3.2% in control); Failure to arrest in colchicine (0.5% MI, compared to 22.1% in control)
CENP-meta	-	-	Chromosome misalignment; Low MI (2%, compared to 3.5% in control); Inefficient arrest in colchicine (11.5% MI, compared to 15.4% in control)
NudE	-	-	High MI (5.8%, compared to 3.1% in control); Inefficient arrest in colchicine (15.8% MI, compared to 20% in control)
NudC	-	-	Cells quite sick Low MI (0.8%, compared to 3.1% in control); Failure to arrest in colchicine (6.7% MI, compared to 20% in control)
NudC-like	-	-	-

All counts for mitotic index (MI) were done for at least 1000 cells per sample.



**Supplementary Figure 3. Checkpoint defect induced by Mad1 RNAi.**

**(A)** Low mitotic index in Mad1 RNAi. The mitotic index of asynchronously growing S2 cells after control (red bar) or Mad1 RNAi (green bar; n=1000 for both). **(B)** Failure to arrest in overnight colchicine treatment after Mad1 RNAi. The mitotic index of S2 cells, treated overnight with colchicine, after control (red bar) or Mad1 RNAi (green bar; n=1000 for both). **(C)** Segregation defects in Mad1 RNAi. The percentage of S2 cells in late anaphase/telophase displaying chromosome bridges or lagging chromosomes after control (red bar) or Mad1 RNAi (green bar; n=100 for both).



**Supplementary Figure 4. Checkpoint defect induced by BubR1 RNAi.**

**(A)** Low mitotic index in BubR1 RNAi. The mitotic index of asynchronously growing S2 cells after control (red bar) or BubR1 RNAi (green bar; n=1000 for both). **(B)** Failure to arrest in overnight colchicine treatment after BubR1 RNAi. The mitotic index of S2 cells, treated overnight with colchicine, after control (red bar) or BubR1 RNAi (green bar; n=1000 for both).