The *Drosophila* centrosome-associated protein CP190 is essential for viability but not for cell division

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Summary

The *Drosophila* CP190 and CP60 proteins interact with each other and shuttle between the nucleus in interphase and the centrosome in mitosis. Both proteins can bind directly to microtubules in vitro, and have been shown to associate with a specific pattern of loci on salivary gland polytene chromosomes, but their functions are unknown. Here we show that reducing the level of CP190 or CP60 by >90% in tissue culture cells does not significantly interfere with centrosome or microtubule organisation, with cell division, or with cell viability. However, CP190 is an essential protein, as flies homozygous for mutations in the *Cp190* gene die at late pupal stages of development. In larval brains of *Cp190* mutants, mitosis is not radically perturbed, and a mutated form of CP190 (CP190ΔM), that cannot bind to microtubules or associate with centrosomes, can rescue the lethality associated with mutations in the *Cp190* gene. Thus, CP190 plays an essential role in flies that is independent of its association with centrosomes or microtubules.

Key words: *Drosophila*, Centrosome, CP190, CP60, Mitosis

Introduction

Centrosome-nucleated arrays of microtubules are involved in numerous essential cellular processes in animal cells, including chromosome segregation, intracellular transport, cell movement and cytokinesis. In the fruit fly, *Drosophila melanogaster*, CP190 and CP60 are proteins that exhibit cell cycle-dependent localisation to the centrosome during mitosis and to the nucleus during interphase (Whitfield et al., 1988; Whitfield et al., 1995; Kellogg et al., 1989; Raff et al., 1993; Oegema et al., 1995). CP190 was originally identified using a monoclonal anti-centrosomal antibody (M. Frasch, Charakterisier ung chromatinassoziierter kerneproteine von *Drosophila melanogaster* mit hilfe monoklonaler antikörper, PhD thesis, University of Tübingen, Germany, 1985) (Frasch et al., 1986), which was subsequently employed to select the *Cp190* gene from a λgt11 expression library (Whitfield et al., 1988). CP190 was independently isolated by Kellogg et al. (Kellogg et al., 1989) using microtubule affinity chromatography, and thereafter, CP60 was identified as a CP190-associated protein by chromatography on columns constructed from anti-CP190 antibodies (Kellogg and Alberts, 1992). Despite the evident affinity of both CP190 and CP60 for microtubules (Kellogg et al., 1989; Kellogg et al., 1995) and the ability of CP190 to cause microtubule bundling in vitro (Oegema et al., 1995), microtubules are not required for the accumulation or maintenance of either CP190 or CP60 at the centrosome, leading to the suggestion that they, like γ-tubulin and pericentrin, are core components of the pericentriolar

matrix (Oegema et al., 1995). Centrosome-association of CP190 is dependent upon the presence of at least two other core components of the centrosome, Centrosomin (Megraw et al., 1999) and the *Drosophila* homologue of Spc98 (a spindle pole body component of *Saccharomyces cerevisiae*) encoded by *discs degenerate*-4 (Barbosa et al., 2000), and centrosomal localisation of CP190 is also disrupted in polo mutants, as is that of γ-tubulin (Donaldson et al., 2001).

CP190 begins to accumulate at the centrosome as soon as nuclear envelope breakdown occurs, whereas CP60 accumulates later and reaches maximal levels only in anaphase/telophase (Oegema et al., 1997). CP60 is extensively phosphorylated in vivo, and contains several cdc2 consensus phosphorylation sites (Kellogg et al., 1995). Intriguingly, when purified CP60 is phosphorylated by cdc2/cyclin B kinase in vitro, it loses its ability to interact with microtubules (Kellogg et al., 1995). These findings have led to the suggestion that the CP190-CP60 complex may be involved in regulating the interaction between centrosomes and microtubules during anaphase/telophase, when their levels at centrosomes are maximal, and when cdc2/cyclin B activity is in decline. However, despite widespread use of CP190 as a centrosomal marker in many avenues of *Drosophila* research, its centrosomal function and that of CP60 remains unknown.

Although both CP190 and CP60 were originally identified and characterised as a consequence of their association with the centrosome and with microtubules, during interphase they are both localised within the nucleus. Indeed, the amino acid

sequence of CP190 suggests that it is a C_2H_2 zinc-finger protein, and both CP190 and CP60 bind to specific chromosomal loci on salivary gland polytene chromosomes, leading to the suggestion that these proteins play a role in interphase nuclei (Whitfield et al., 1995). Subsequent work has indicated that both proteins are components of the nuclear matrix, as they remain insoluble after nuclei have been treated with DNAse I and extracted with high salt (Oegema et al., 1997). In the same paper, evidence from wide-field 3D microscopy studies was presented, showing that in diploid interphase nuclei of cycle 13 embryos, CP190 and CP60 do not extensively co-localise with each other or with DNA [in contrast to the observations of Whitfield et al. (Whitfield et al., 1995) on polytene chromosomes], suggesting that these proteins may be components of distinct extra-chromosomal nuclear domains (ENDs). In support of this possibility, overexpression of the EAST protein, a known END component, specifically recruits extra CP60 to an expanded END (Wasser and Chia, 2000). However, the nuclear roles of CP190 and CP60, whether chromosomal or extra-chromosomal, remain as obscure as their centrosomal functions.

Here we have used RNA-mediated interference to deplete the levels of CP190 and CP60 in *Drosophila* S2 cells, and we have identified mutations in the *Cp190* gene. Our studies demonstrate that CP190 is essential for fly viability, but suggest that neither CP190 nor CP60 are involved in regulating centrosome or microtubule behaviour during mitosis.

Materials and Methods

RNA interference (RNAi) treatment and analysis of S2 tissue culture cells

CP190 and CP60 cDNA templates were amplified by PCR using the primer pairs: for CP190, 5′-TAA TAC GAC TCA CTA TAG GGA GAC CAC ATG CCC AAC GAG TTC CAG GCG-3′, and 5′-TAA TAC GAC TCA CTA TAG GGA GAC CAC TGA TTC AGC TTG GCG TTG GAG-3′; for CP60, 5′-TAA TAC GAC TCA CTA TAG GGA GAC CAC ATG GCA ATC CAA CTG GAC AAG-3′, and 5′-TAA TAC GAC TCA CTA TAG GGA GAC CAC GTA CTC CTC CGA AAG TTT GCG-3′; the 5′ end of each primer also contained the T7 RNA polymerase promoter site (5′-TAA TAC GAC TCA CTA TAG-3′). PCR products (700 bp in length) were purified using the QIA quick Gel Extraction Kit according to the manufacturer's instructions. Purified PCR products (final concentration 100 μg/ml) were used to produce double-stranded RNA (dsRNA) using a Megascript T7 transcription kit (Ambion). The RNA was purified according to the manufacturer's instruction, heated at 65°C for 30 minutes and then placed in a beaker of water at 65°C and left on the bench to cool to room temperature. Each batch of RNA was analysed on an agarose gel to ensure the quality of dsRNA. S2 cells were grown in Schneider's Insect medium (Sigma) supplemented with 10% fetal calf serum (FCS, Gibco) and 50 μg/ml streptomycin and penicillin at 27°C. The RNAi treatment and subsequent viable cell count analysis of S2 tissue culture cells was performed essentially as described previously (Adams et al., 2001; Clemens et al., 2000; Giet and Glover, 2001). For immunofluorescence analysis, cells were fixed with cold (−20°C) methanol/3% EGTA and processed as described previously (Gergely et al., 2000).

Fly strains

Flies were maintained on standard corn meal *Drosophila* medium at 25°C. The following strains were used in these studies: w^{67} was the

parental line used to generate all transformed lines; mwh^1 red^1 P{hsneo}l(3)neo43^1 e1/TM3, ry^{RK} Sb^1 Ser^1 was the P-element insertion stock used to generate Df(3R)P280^{NR27} by male recombination; $y*$ $w*$; CyO, H{w^{+mC}=PDelta2-3}HoP2.1/Bc^1 $Egfr^{E1}$ (derived by William Gelbart, Harvard University) was employed as the source of transposase for promoting male recombination; red^1 e^1 males were used for ethylmethane sulphonate (EMS) mutagenesis; ms(3)$K81$/ TM3 Sb^1 Ser^1 e^1 was used to balance mutagenised red^1 e^1 chromosomes prior to screening over Df(3R)P280^{NR27}. All stocks other than w^{67} and those whose derivation is described below, were obtained from Bloomington Stock Center, University of Indiana, USA.

Derivation of deficiencies uncovering the *Cp190* locus

Virgin females from the P-element insertion line mwh^1 red^1 P{hsneo}l(3)neo43^1 e^1/TM3, ry^{RK} Sb^1 Ser^1 were crossed with $y*$ $w*$; CyO, H{P{Delta2-3}}HoP2.1/Bc^1 $Egfr^{E1}$ males. CyO, H{P{Delta2-3}}HoP2.1/+; mwh^1 red^1 P{hsneo}l(3)neo43^1 e^1/+ males were selected from the progeny and crossed en masse with red^1 e^1 virgin females. Recombinant (red^+ e^1/ red^1 e^1 or red^1 e^+/ red^1 e^1) male progeny from this cross were individually mated to TM6B/TM3 $red*$ Ser^1 e^1 virgin females before extracting their genomic DNA. Candidate deficiencies were identified by electrophoretic analysis of PCR-amplified products from these DNA samples using 3 oligonucleotide primers: one complementary to the inverted repeat of the P-element [5′-CGA CGG GAC CAC CTT ATG TTA TTT C-3′], one complementary to a flanking genomic site 2.2 kb from the site of insertion (proximal to *Cp190*) [5′-ATG CCT ATG CAG CCT GCA AGA GCA GCG ATG-3′] and one complementary to a flanking genomic site 1.5 kb from the site of insertion (distal to *Cp190*) [5′-CTT GGA GAA CAT TTG CCA GTC CGA GGT TGG-3′]. Balanced stocks were established from lines corresponding to PCR products which showed absence of the 2.2 kb band but presence of the 1.5 kb band. Deficiency breakpoints of these stocks were identified by cloning the P-element (by means of its pUC insert) and sequencing of the associated genomic DNA using standard methods.

EMS mutagenesis screen for identification of *Cp190* mutants

Approximately 100 four day-old red^1 e^1 males were starved for 8 hours before feeding on 5% (w/v) sucrose containing 10 mM EMS for 15 hours. The EMS-treated males were transferred to freshly yeasted bottles and mated to ms(3)K81/TM3 Sb Ser e^1 virgin females at approximately 10 males and 50 females per bottle. After 4 days at 25°C the males were discarded, and the females transferred to fresh bottles every 2 days until they ceased to lay. Approximately 6000 red^1 e^1/ TM3 Sb Ser e^1 males were selected from the resulting progeny, and each mated in a separate yeasted vial with 5 red^+ Df(3R)P280^{NR27} e^1/ TM3 red Ser e^1 virgin females. Recessive lethal mutations uncovered by the deficiency were identified by screening the progeny from each vial for the absence of $red*e^1$/ red^+ Df(3R)P280^{NR27} e^1 flies, and stocks were established from their red^1 *e^1/ TM3 $red*$ Ser e^1 siblings. Candidate *Cp190* mutants were retested by mating males to red^1 Df(3R)P280^{NR27} e^+/TM3 red^1 Ser e^1 virgin females, and their status subsequently confirmed by rescue of both hemizygous and homozygous mutations by expression of a transgenic copy of *Cp190* under control of the polyubiquitin promoter.

Larval brain and testes squashes

Larval brains and testes were squashed and prepared for immunostaining as described previously (Williams and Goldberg, 1994). If brains were also to be stained to reveal the distribution of microtubules, then the protocol of Bonaccorsi et al. (Bonaccorsi et al., 2000) was followed. Incubation of slides with primary antibodies (diluted in PBT) was performed overnight in a humidified chamber at

4°C. After washing the slides 3 times in PBT, secondary antibodies were applied for 4 hours at room temperature. The slides were finally given 4×15-minute washes in PBT, before counterstaining for DNA with 0.5 µg ml⁻¹ Hoechst 33258 and mounting in 95% v/v glycerol in PBS containing 2.5% w/v n-propyl gallate.

Microtubule-spin downs, SDS-PAGE, and western blotting

Microtubule spin downs from embryo extracts expressing CP190ΔM, SDS-PAGE and western blotting were performed as described previously (Gergely et al., 2000; Laemmli, 1970)

Antibodies

The following antibodies were used in this study: the affinity-purified rabbit anti-CP190 and anti-CP60 have been described previously (Kellogg et al., 1995; Oegema et al., 1995), as has the rabbit anti-CNN anti-serum (Li and Kaufman, 1996), and the anti-D-TACC and anti-Msps affinity purified rabbit antibodies (Gergely et al., 2000; Lee et al., 2001). The mouse monoclonal DM1a (Sigma) was used to detect tubulin; the mouse monoclonal GTU88 (Sigma) was used to detect gamma-tubulin; an anti-phospho-histone H3 rabbit serum (Upstate Technology) was used to detect phospho-histone H3. All affinity-purified antibodies were used at 1-2 µg/ml in western blotting or immunofluorescence experiments. The DM1a, GTU88 and anti-phospho-histone H3 antibodies were used at a 1:500 dilution in western blotting and immunofluorescence studies.

Transgenic lines that express CP190, CP190ΔM and CP60

To create transgenic lines that express CP190 and CP60, the full-length cDNAs were subcloned into the pWR-Pubq transformation vector that constitutively drives relatively high levels of expression throughout the organism (Lee et al., 1998; Gergely et al., 2000). To generate flies expressing CP190ΔM, the full-length CP190 cDNA was digested with BamHI and BssHII. The reaction was end-filled with klenow and re-ligated. This created an in-frame deletion of amino acids 311-541 of the CP190 coding sequence (thus deleting the previously identified centrosomal and microtubule targeting domain between amino acids 385-508). The resulting deleted cDNA was then subcloned into pWR-Pubq. Full cloning details are available upon request. Transformants were generated using standard P-element-mediated transformation (Roberts, 1986).

Image acquisition

The imaging of all brain and testes preparations was performed on a Zeiss Axioskop 2 microscope with a Photometrics CoolSnap HQ camera using MetaMorph software (Universal Imaging). The imaging of S2 tissue culture cells was performed on a Nikon E800 microscope with a Bio-Rad Radiance confocal system. All images were imported into Adobe Photoshop where the entire image was adjusted to use the full range of pixel intensities. In some images an Unsharp Mask filter was also applied to the entire image. In all cases, control and experimental images were treated in exactly the same way.

Results

CP190 and CP60 are not required for mitosis in *Drosophila* tissue culture cells

To test the potential function of CP190 and CP60 during mitosis we used double stranded RNA-mediated interference (RNAi) to reduce the levels of each protein in *Drosophila* S2 tissue culture cells. A western blot analysis revealed that both proteins were reduced to less than 50% of control levels after 24 hours of RNAi treatment (not shown), and to less than 5% of control levels after 96 hours (Fig. 1A). Surprisingly, cells depleted of either protein continued to grow with relatively normal kinetics throughout the 5-day time course of these experiments (Fig. 1B), and showed no significant difference in mitotic index in comparison with controls (Fig. 1C).

Immunofluorescence analysis of fixed cells at the 96 hour time point confirmed that both proteins were substantially depleted from cells (Fig. 2). The organisation of microtubules throughout mitosis appeared to be unaffected by the depletion of either CP190 or CP60 (Fig. 2A,B), and several centrosomal markers such as γ-tubulin, Centrosomin, D-TACC and Msps appeared to localise normally to centrosomes in CP190- or CP60-depleted cells (not shown, see below). The localisation of CP190 to nuclei in interphase and to centrosomes in mitosis was unaffected by the depletion of CP60 (Fig. 2C). In contrast, although the localisation of CP60 to nuclei in interphase was not affected by the depletion of CP190, the localisation of CP60 to mitotic centrosomes was strongly inhibited in the CP190-depleted cells (Fig. 2C). Thus, neither CP190 nor CP60 appear to be required to organise centrosomes or microtubules during cell division in *Drosophila* S2 tissue culture cells. However, the presence of CP190 appears to be necessary for the recruitment of CP60 to mitotic centrosomes.
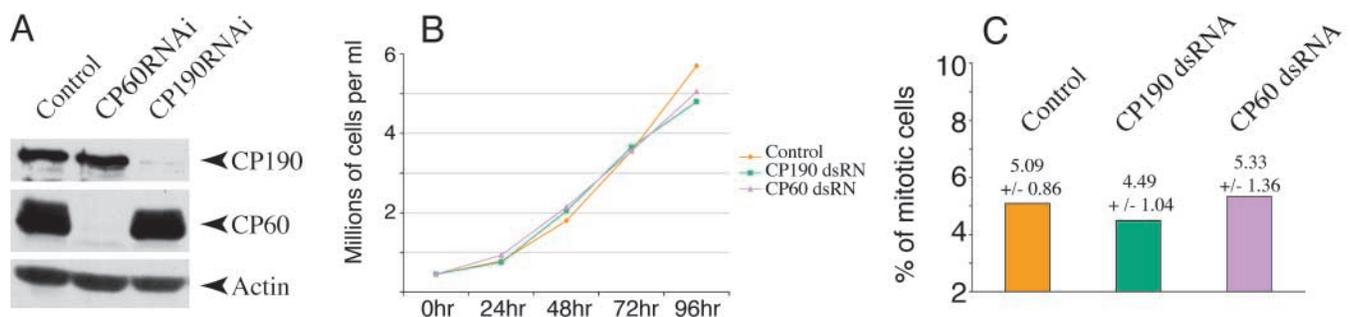

Fig. 1. RNAi depletion of CP190 or CP60 does not lead to growth or mitotic defects in tissue culture cells. (A) A Western blot showing the depletion of CP190 and CP60 in mock (lane 1), CP60 (lane 2) or CP190 (lane 3) RNAi-treated cells 96 hours after treatment. (B) Graph showing the total number of cells per ml during the 5-day time course of a typical RNAi-depletion experiment. (C) Graph showing the mitotic index (as judged by number of phospho-histone H3-positive cells) at the 96-hour time point of a typical RNAi-depletion experiment. The total numbers of cells counted were 811, 969, and 1052 for the control, CP60, and CP190 RNAi experiments, respectively. Similar results were obtained in two separate RNAi experiments (data not shown).

Isolation of mutations in the *Cp190* gene

To test whether CP190 has an essential function in flies, we performed a genetic screen to isolate mutations in the *Cp190* gene. Screening was performed in two stages: the first to generate a suitable deficiency to uncover the *Cp190* gene locus at 88E, and the second to exploit this deficiency to identify candidate *Cp190* mutations.

Starting with a P-element insertion P{hsneo}l(3)neo43[1] (Cooley et al., 1988), mapping approximately 4.5 kb upstream of the *Cp190* gene (Fig. 3A), P-element-mediated male recombination was used to generate deficiencies at or near the insertion site (Preston et al., 1996). Nearly 100 recombinants were isolated from a screen of over 2×10^5 flies, and from these, 4 fly-lines were identified that carried candidate *Cp190* deficiencies. Subsequent cloning and sequencing of the deficiency breakpoints revealed one deficiency, Df(3R)P280[NR27], with a breakpoint in the second exon of the *Cp190* gene. In addition to *Cp190*, Df(3R)P280[NR27] includes only three other gene loci, one encoding a homologue of the human chromodomain protein MRG15 (CG6363), the other two being uncharacterised genes (CG4338 and CG14865) (Fig. 3A).

A standard EMS mutagenesis screen (Ashburner, 1989) was then performed to isolate mutations that were either lethal or female sterile over the Df(3R)P280[NR27] deficiency chromosome. From approximately 6×10^3 mutated chromosomes, four candidate *Cp190* mutants were isolated as recessive lethals over Df(3R)P280[NR27]. All four mutants were fully rescued as hemizygotes over Df(3R)P280[NR27] by a second chromosome insertion of the full-length *Cp190* cDNA expressed under control of the polyubiquitin promoter (Fig. 3B). Two of the four mutants, *Cp190*[1] and *Cp190*[2] were also fully rescued as homozygotes, demonstrating unequivocally that (at least for these two alleles) the lethality must be because of mutations at the *Cp190* locus and that *Cp190* is an essential gene.

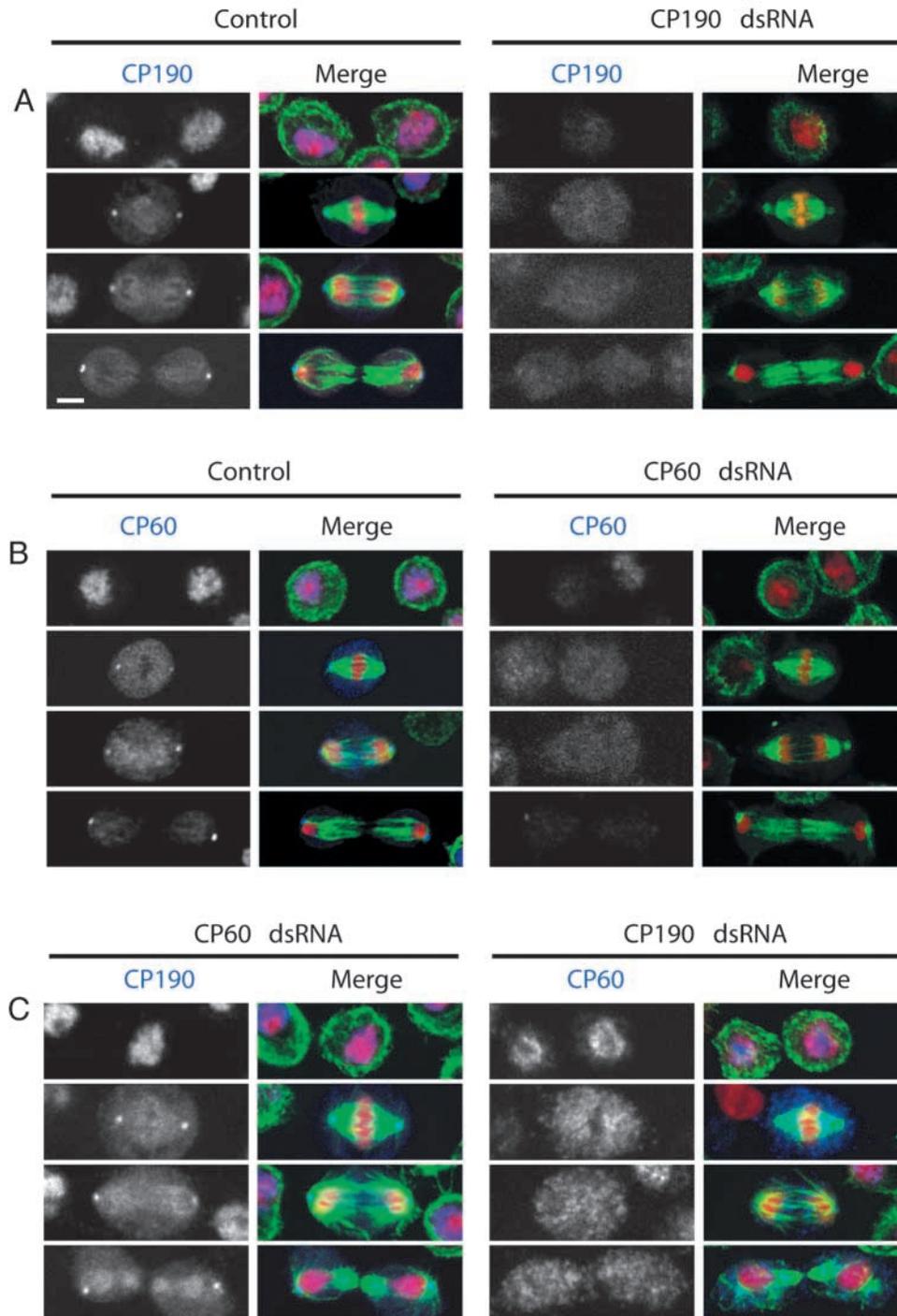Animals homozygous for *Cp190*[1] and *Cp190*[2] (or

Fig. 2. Microtubule organisation is not disrupted during mitosis in CP190 or CP60 RNAi-depleted cells. (A) The localisation of CP190 (black and white panels, blue in merged panels), microtubules (green in merged image), and DNA (red in merged image) in mock (left set of panels) and CP190 (right set of panels) RNAi-treated cells at various stages of the cell cycle. Interphase, top row; metaphase, second row; anaphase, third row; telophase, bottom row. (B) The localisation of CP60, microtubules and DNA in mock and CP60-depleted cells at various stages of the cell cycle [all labelling as in (A)]. (C) The localisation of CP190 in CP60-depleted cells (left panels) and CP60 in CP190-depleted cells (right panels) [all labelling as in (A)]. Scale bar: 5 μm.
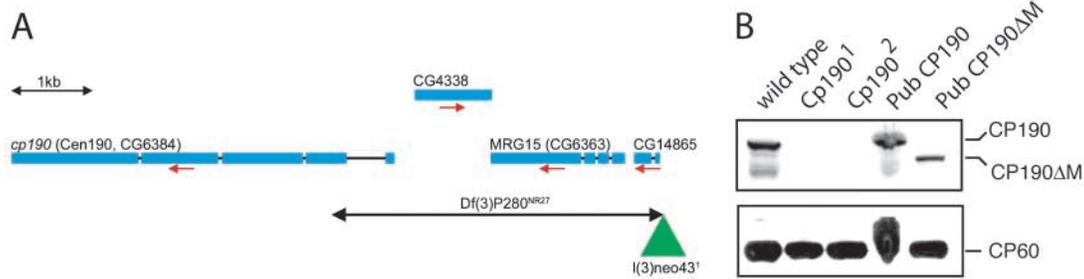
Fig. 3. The generation of mutations in the *Cp190* gene. (A) A schematic representation of the *Cp190* locus. Genes are highlighted in blue, and the position of the l(3)neo43 P-element insertion is shown in green. The extent of the Df(3R)P280[NR27] is indicated by the black line. (B) A western blot showing the levels of CP190 and CP60 in wild type, and *Cp190*[1] and *Cp190*[2] homozygous mutant brains, and in *Cp190*[2] homozygous mutant brains that also express either a Pubq-CP190 or Pubq-190ΔM transgene (as indicated above each lane). Note that both CP190 and CP60 appeared to migrate slightly abnormally in the pUbq-CP190 over-expressing lane; this was because of a defect in this gel and it was not seen when this sample was re-run on a different gel.

hemizygous over Df(3R)P280[NR27]), and *Cp190*[1]/*Cp190*[2] heterozygotes show some larval mortality, but approximately half the mutants survived until late pupal stages of development, dying as pharate adults. Western blotting analysis revealed that the CP190 protein, although readily detectable in brains from wild-type 3rd instar larvae, was not seen in samples from either *Cp190*[1] or *Cp190*[2] homozygotes, suggesting that both lesions may be null or are at least strong hypomorphs (Fig. 3B).

Cp190 mutants do not have obvious mitotic or meiotic defects

Analysis of the eyes, wings and cuticle of pharate adults homozygous for either *Cp190*[1] or *Cp190*[2] revealed no obvious defects in tissue organisation, suggesting that these animals were not dying as a consequence of major defects in mitosis (data not shown). This conclusion was confirmed by a detailed analysis of mitosis in brains from homozygous mutant 3rd instar larvae. In mutant cells there were no dramatic differences in the organisation of the spindle at any stage of mitosis (Fig.

4). Astral microtubules were readily detectable in mutant spindles, even though CP190 was not detectable at centrosomes (Fig. 4). In addition, we observed many mutant neuroblasts undergoing morphologically normal asymmetric divisions (not shown). In agreement with our results using RNAi in *Drosophila* S2 cells, the localisation of CP60 at centrosomes was severely disrupted in *Cp190* mutant larval brain cells (Fig. 5A), whereas the localisation of several other centrosome-associated proteins was not dramatically altered (Fig. 5C). Finally, the mitotic index was not significantly altered in mutant brains (data not shown), indicating that microtubule organisation was relatively normal and that the spindle assembly checkpoint was not being triggered in these cells.

We also assayed whether meiosis occurred normally in *Cp190* mutant larval testes. In fixed mutant testes, the distribution of microtubules appeared to be normal and the localisation of γ-tubulin and centrosomal protein centrosomin (CNN) was not perturbed during meiosis I or II (not shown). In living mutant testes, an analysis of onion stage spermatids by phase contrast microscopy revealed no obvious defects in chromosome segregation (not shown). Taken together, these data strongly suggest that although CP190 function is essential, it is not required for centrosome or microtubule function during mitosis in larval brains or meiosis in larval testes.
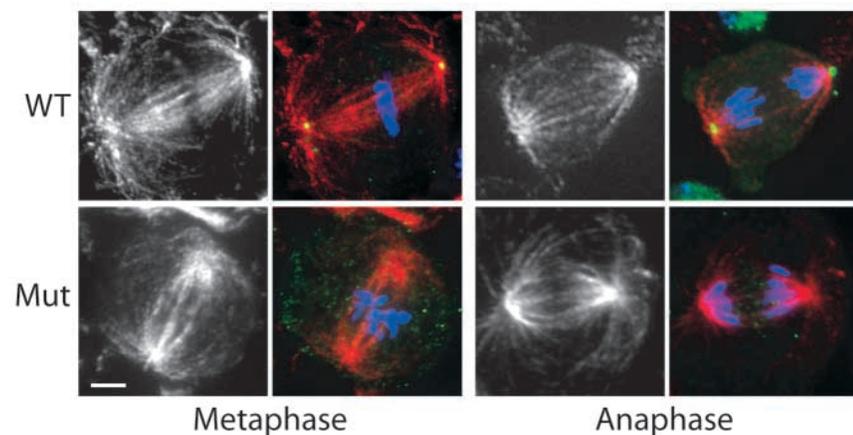
The ability of CP190 to interact with centrosomes and microtubules is not essential for its function

The observation that spindle formation and function is not disrupted in *Cp190* mutants, raises the intriguing question of why CP190 can bind directly to microtubules and is recruited to centrosomes during mitosis if it has no function in regulating microtubule or centrosome behaviour. To address whether the ability of CP190 to bind to centrosomes and microtubules is essential for its function, we expressed a form of CP190 in flies that cannot bind to centrosomes or microtubules.
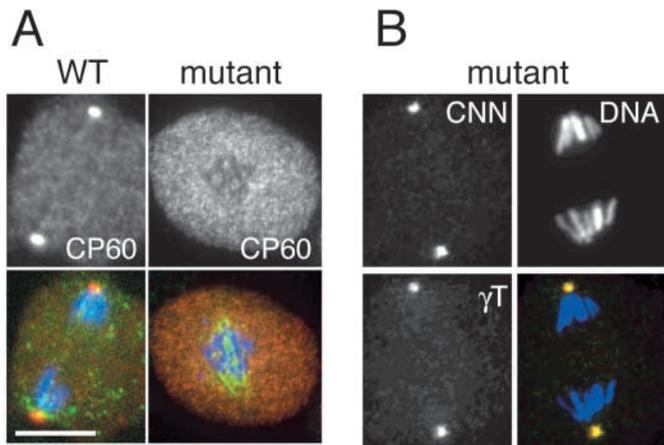

Fig. 4. Mitosis is not disrupted in *Cp190* mutant larval brain cells. The distribution of microtubules (black and white panels, red in merged images), CP190 (green), and DNA (blue) in typical wild type (WT) and *Cp190*[2] mutant larval brain cells in metaphase (left panels) and anaphase (right panels). Mitotic spindle organisation appears normal in the mutant cells even though CP190 is no longer detectable at centrosomes.

Fig. 5. The centrosomal localisation of CP60 is specifically disrupted in *Cp190* mutant cells. (A) The distribution of CP60 (black and white panels, red in merged images), microtubules (green), and DNA (blue) in typical wild type (WT) (left panels) and *Cp190* mutant (right panels) brain cells in anaphase. CP60 is not detectable at the centrosomes of *Cp190* mutant cells. (B) The centrosomal localisation of CNN (red in merged image) and γ-tubulin (green in merged image) is not disrupted in *Cp190* mutant cells. DNA is shown in blue in the merged image. Scale bar: 10 μm.

It has previously been shown that amino acid residues 385-508 of CP190 can bind directly to microtubules in vitro, and can target a bacterially expressed fusion protein to centrosomes when injected into embryos (Oegema et al., 1995). We therefore made a P-element-transformation construct that deleted this region of CP190 (CP190ΔM – Fig. 6A), and used it to derive several transgenic fly-lines that express CP190ΔM under the control of the polyubiquitin promoter (Lee et al., 1998; Gergely et al., 2000).

In extracts made from pUbq-CP190ΔM embryos, although the endogenous CP190 strongly interacted with microtubules in microtubule spin-down experiments, CP190ΔM did not (Fig. 6B). In mutant larval brains that expressed the pUbq-CP190 transgene (and so contain the full-length CP190 protein), anti-CP190 antibodies strongly stained mitotic centrosomes (Fig. 6C), whereas in mutant larvae expressing the pUbq-CP190ΔM transgene, anti-CP190 antibodies no longer stained centrosomes during mitosis (Fig. 6C). Taken together, these data confirm that CP190ΔM cannot interact with microtubules or centrosomes.

To our surprise, the pUbq-CP190 and Pubq-CP190ΔM transgenes rescued the lethality associated with *Cp190* mutations with equal efficiency (Fig. 7). This demonstrates that CP190ΔM is at least partially functional, and that the ability of CP190 to interact with centrosomes and microtubules is not absolutely essential for the viability of the fly. However, the homozygous *Cp190* mutants rescued by the CP190ΔM transgene were unhealthy and lived for only a few days, implying that the ability of CP190 to bind to centrosomes may be of some functional significance (see Discussion).

Overexpression of both CP190 and CP190ΔM is lethal

During the course of our experiments, we noticed that three out of seven transgenic Pubq-CP190 lines and all seven of our
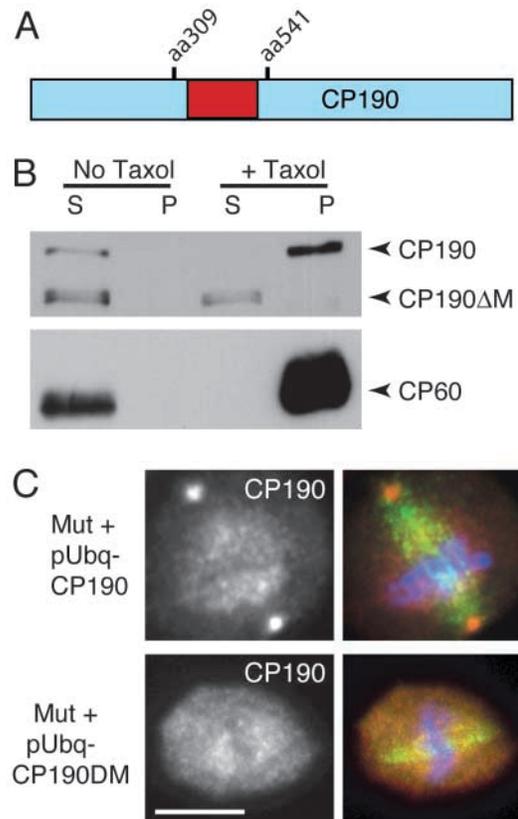

Fig. 6. CP190ΔM does not interact with centrosomes or microtubules. (A) Schematic representation of the CP190 protein. The previously identified centrosomal and microtubule targeting domain is shown in red, whereas the region deleted in CP190ΔM is indicated by the black bars above the protein (aa309-aa541). (B) A western blot of a microtubule spin-down experiment probed with anti-CP190 and anti-CP60 antibodies. In the absence of taxol in the embryo extract, microtubules do not form. Under these conditions, when the extract is centrifuged on a sucrose cushion, CP190, CP190ΔM and CP60 all remain in the supernatant (S) and are not detectable in the pelleted material (P). In the presence of taxol, microtubules are polymerised in the extract. Under these conditions, both CP190 and CP60 co-sediment with microtubules through the sucrose cushion and are found in the pellet. CP190ΔM, however, does not co-sediment with the microtubules and is not detectable in the pellet. (C) The localisation of CP190 (black and white panel, red in merged image), microtubules (green in merged image) and DNA (blue in merged image) in *Cp190* mutant brains that also contain a transgene expressing either full-length CP190 (pUbq-CP190 – top panels) or CP190ΔM (pUbq-CP190ΔM – bottom panels). Only the full-length CP190 is concentrated at centrosomes. Scale bar: 10 μm.

Pubq-CP190ΔM lines were homozygous lethal at late-pupal stages of development. Indeed, even in the four Pubq-CP190 lines that were homozygous viable, there was a noticeable increase in the level of pupal mortality. When we examined the levels of CP190 and CP190ΔM protein in these flies we found that CP190ΔM was overexpressed to a greater extent than CP190 (~10-fold compared with ~3-5-fold) in all of the transgenic lines (Fig. 8A). Because the mRNAs for both proteins were expressed from the same promoter and contained the same 5′ and 3′ UTRs, it seems probable that the consistently higher levels of CP190ΔM overexpression could
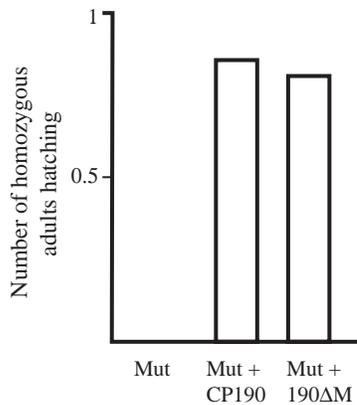
Fig. 7. CP190 and CP190ΔM can both rescue the lethality associated with mutations in the Cp190 gene. A graph showing the number of hatching homozygous adults from crosses of *Cp190* mutant flies on their own (Mut), or of mutant flies carrying a single copy of the pUbq-CP190 or pUbq-CP190ΔM transgene (as indicated under the graph). The expected number of eclosing adults from each cross is normalised to 1. Thus, ~85% and ~80% of mutant flies expressing the CP190 or CP190ΔM transgene, respectively, survive to adulthood. The graph shows the average figure from 3 independent experiments in which more than 100 surviving flies were counted for each genotype.

Fig. 8. The overexpression of CP190 or CP190ΔM is lethal, but this lethality can be rescued by the co-overexpression of CP60. (A) A western blot showing the levels of CP190 or CP190ΔM in wild-type adults (lane 1), in three independent homozygous transgenic Pubq-CP190 lines (lanes 2-4), and in three independent heterozygous Pubq-CP190ΔM transgenic lines. All three Pubq-CP190 transgenic lines are homozygous viable, but exhibit high levels of pupal mortality, whereas all three Pubq-CP190ΔM lines are homozygous lethal. (B) A western blot showing the levels of CP190, CP190ΔM or CP60 in wild-type adults (lane 1), or in adults that are homozygous for independent Pubq-CP190 insertions and a Pubq-CP60 insertion (lanes 2,3), or in adults homozygous for independent Pubq-CP190ΔM insertions and a Pubq CP60 insertion (lanes 4,5). Note that both the Pubq-CP190 insertions and both the Pubq-CP190ΔM insertions used in this experiment are normally homozygous lethal. The homozygous adults appear to be viable because they also overexpress CP60. The asterisk highlights a background band that is recognised by the anti-CP60 antibodies and is shown here as a loading control.

be because of intrinsic differences in stability between the two proteins. Whatever the underlying reason, the pupal mortality in these lines appears to be directly related to the level of overexpression of CP190 or CP190ΔM as we were unable to generate any combination of transgenic lines that contained one copy of Pubq-CP190ΔM and one copy of Pubq-CP190, or any combination of transgenic lines that contained more than two copies of Pubq-CP190. These findings strongly suggest that the overexpression of CP190 or CP190ΔM is lethal, and that both proteins probably cause pupal lethality by the same mechanism. Analysis of larval brains and larval testes, however, revealed no obvious defects in mitosis or meiosis in larvae overexpressing either CP190 or CP190ΔM (not shown).

In view of the apparent toxicity associated with overexpression of CP190, we wondered if the relative levels of CP190 and CP60 in the fly might be important. We therefore tested whether overexpression of CP60 could rescue the pupal lethality caused by the overexpression of CP190. We found that flies carrying multiple copies of a Pubq-CP60 transgene had no detectable mitotic defects in larval brains and were perfectly viable, even though they overexpressed CP60 by >20-fold (not shown, see Fig. 8B). Moreover, several lines carrying two copies of the Pubq-CP190ΔM transgene or two copies of a lethal Pubq-CP190 transgene (that were normally homozygous lethal) were viable as adults if they also carried a copy of the Pubq-CP60 transgene (Fig. 8B). Thus, the overexpression of CP60 appears to rescue the lethality associated with the overexpression of both CP190 and CP190ΔM.

Discussion

Several lines of evidence have led to the suggestion that CP190 and CP60 are involved in regulating the behaviour of centrosomal microtubules. Both proteins associate with the
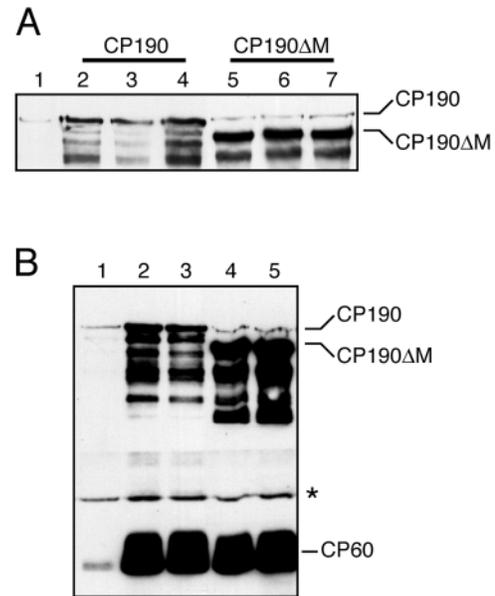
centrosome in a cell cycle-regulated manner, bind directly to microtubules in vitro, and CP60 is a phosphoprotein whose microtubule-binding properties are regulated by cdc2/cyclin B kinase in vitro. We find, however, that in tissue culture cells depleted of either CP190 or CP60, or in cells from *Cp190* mutant larvae that have no detectable CP190 or CP60 at centrosomes, there is no defect in centrosome or microtubule behaviour during mitosis. Moreover, *Cp190* mutant flies have none of the disorganised eye, cuticle or bristle phenotypes that are usually associated with mitotic mutants. We conclude that, although CP190 is required to recruit CP60 to centrosomes, neither protein plays a crucial role in organising centrosomal microtubules during mitosis.

Although CP190 does not appear to be required for mitotic spindle function, the protein is essential, and *Cp190* mutants invariably die as pharate adults. Surprisingly, this essential function of CP190 does not depend on its localisation to centrosomes, or on its ability to bind to microtubules. The transgenic expression of a form of CP190 that can no longer interact with centrosomes or microtubules (CP190ΔM) rescued

the lethality of the *Cp190* mutant almost as efficiently as the transgenic expression of full-length CP190. CP190 and CP60 are both concentrated in nuclei during interphase, and appear to be components of an extra-chromosomal nuclear domain (END). The two proteins do not extensively co-localise in the nucleus, and we show here that although CP190 is required to localise CP60 to centrosomes during mitosis, it is not required to localise CP60 to nuclei. Our RNAi experiments suggest that CP60 is also not required to localise CP190 to nuclei. Thus, both proteins appear to be recruited independently to separate ENDs within the nucleus. An attractive possibility is that the essential function of CP190 is to influence events within the nucleus.

In support of this possibility, CP190 has several domains common to proteins that influence nuclear events. CP190 contains four classical C_2H_2 zinc-finger domains and an N-terminal Broad complex/Tramtrack/Bric-a-brac (BTB) domain – a domain often found in zinc-finger-containing proteins that bind to DNA and regulate transcription or chromatin structure. Interestingly, CP190ΔM retains the BTB domain, one of the four zinc-fingers, and it can still localise to interphase nuclei, presumably explaining how CP190ΔM could still perform its putative nuclear function. Moreover, our data suggests that even relatively moderate (5-10-fold) overexpression of CP190 is lethal to flies. Again, the lethality caused by the overexpression of CP190 does not require that the protein binds to centrosomes or microtubules, as the overexpression of CP190ΔM also leads to the same pupal lethality that is associated with the overexpression of CP190. Interestingly, the overexpression of CP60 by >20-fold does not appear to be deleterious to flies, but the co-overexpression of CP60 can rescue the lethality associated with the overexpression of CP190 or CP190ΔM. This suggests that the relative levels of CP190 and CP60 in the nucleus may be important. Clearly, however, more work will be required to understand the function of CP190 in the nucleus.

Finally, it is worth considering why CP190 and CP60 may have evolved an ability to interact with centrosomes and microtubules when this apparently plays no role in their function. One possibility is that these proteins do play a role in some aspect of centrosome/microtubule function, but this function is only essential during early embryogenesis. The *Drosophila* CNN, for example, is essential for mitosis in early embryos, but appears to be dispensable for all other cell divisions in the organism. In the case of CNN it seems that the organisation of centrosomal microtubules is perturbed to some extent in larval neuroblasts, but this centrosomal disorganisation only causes lethal errors in mitosis during early syncytial development (Megraw et al., 1999; Megraw et al., 2001). In contrast, we find no evidence to suggest a role for CP190 in mitosis in larval neuroblasts. Nevertheless, to analyse the potential function of CP190 in early embryos, we have recently made germ line clones (Chou and Perrimon, 1996) with the *Cp190*[1] and *Cp190*[2] mutations. We find that mitosis is largely unperturbed in these embryos, but that they fail in axial expansion, an actin/myosin-dependent process that normally spreads the nuclei evenly throughout the early embryo. Clearly, more work is needed to assess the role of CP190 in axial expansion, but it is possible that this function may require that CP190 can interact with centrosomes and/or microtubules.

References

Adams, R. R., Maiato, H., Earnshaw, W. C. and Carmena, M. (2001). Essential roles of *Drosophila* inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation. *J. Cell Biol.* **153**, 865-880.

Ashburner, M. (1989). Drosophila*, a Laboratory Handbook*. New York: Cold Spring Harbor Laboratory Press.

Barbosa, V., Yamamoto, R. R., Henderson, D. S. and Glover, D. M. (2000). Mutation of a *Drosophila* gamma tubulin ring complex subunit encoded by *discs degenerate*-4 differentially disrupts centrosomal protein localization. *Genes Dev.* **14**, 3126-3139.

Bonaccorsi, S., Giansanti, M. G. and Gatti, M. (2000). Spindle assembly in *Drosophila* neuroblasts and ganglion mother cells. *Nat. Cell Biol.* **2**, 54-56.

Chou, T. B. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. *Genetics* **144**, 1673-1679.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **97**, 6499-6503.

Cooley, L., Kelley, R. and Spradling, A. (1988). Insertional mutagenesis of the *Drosophila* genome with single P elements. *Science* **239**, 1121-1128.

Donaldson, M. M., Tavares, A. A., Ohkura, H., Deak, P. and Glover, D. M. (2001). Metaphase arrest with centromere separation in polo mutants of *Drosophila*. *J. Cell Biol.* **153**, 663-676.

Frasch, M., Glover, D. M. and Saumweber, H. (1986). Nuclear antigens follow different pathways into daughter nuclei during mitosis in early *Drosophila* embryos. *J. Cell Sci.* **82**, 155-172.

Gergely, F., Kidd, D., Jeffers, K., Wakefield, J. G. and Raff, J. W. (2000). D-TACC: a novel centrosomal protein required for normal spindle function in the early *Drosophila* embryo. *EMBO J.* **19**, 241-252.

Giet, R. and Glover, D. M. (2001). *Drosophila* aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis. *J. Cell Biol.* **152**, 669-682.

Kellogg, D. R. and Alberts, B. M. (1992). Purification of a multiprotein complex containing centrosomal proteins from the *Drosophila* embryo by chromatography with low-affinity polyclonal antibodies. *Mol. Biol. Cell* **3**, 1-11.

Kellogg, D. R., Field, C. M. and Alberts, B. M. (1989). Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early *Drosophila* embryo. *J. Cell Biol.* **109**, 2977-2991.

Kellogg, D. R., Oegema, K., Raff, J., Schneider, K. and Alberts, B. M. (1995). Cp60 a microtubule associated protein that is localized to the centrosome in a cell cycle specific manner. *Mol. Biol. Cell* **6**, 1673-1684.

Laemmli, U. K. (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, M. J., Gergely, F., Jeffers, K., Peak-Chew, S. Y. and Raff, J. W. (2001). Msps/XMAP215 interacts with the centrosomal protein D-TACC to regulate microtubule behaviour. *Nat. Cell Biol.* **3**, 643-649.

Li, K. J. and Kaufman, T. C. (1996). The homeotic target gene Centrosomin encodes an essential centrosomal component. *Cell* **85**, 585-596.

Megraw, T. L., Kao, L. R. and Kaufman, T. C. (2001). Zygotic development without functional mitotic centrosomes. *Curr. Biol.* **11**, 116-120.

Megraw, T. L., Li, K., Kao, L. R. and Kaufman, T. C. (1999). The centrosomin protein is required for centrosome assembly and function during cleavage in *Drosophila*. *Development* **126**, 2829-2839.

Oegema, K., Marshall, W. F., Sedat, J. W. and Alberts, B. M. (1997). Two proteins that cycle asynchronously between centrosomes and nuclear structures: *Drosophila* CP60 and CP190. *J. Cell Sci.* **110**, 1573-1583.

Oegema, K., Whitfield, W. G. F. and Alberts, B. (1995). The cell cycle dependent localization of the Cp190 centrosomal protein is determined by the coordinate action of 2 separable domains. *J. Cell Biol.* **131**, 1261-1273.

Preston, C. R., Sved, J. A. and Engels, W. R. (1996). Flanking duplications and deletions associated with P-induced male recombination in *Drosophila*. *Genetics* **144**, 1623-1638.

Raff, J. W., Kellogg, D. R. and Alberts, B. M. (1993). *Drosophila* gamma-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* **121**, 823-835.

Roberts, D. (1986). Drosophila*, A Practical Approach*. Oxford: IRL Press.

Wasser, M. and Chia, W. (2000). The EAST protein of *Drosophila* controls an expandable nuclear endoskeleton. *Nat. Cell Biol.* **2**, 268-275.

Whitfield, W. G., Chaplin, M. A., Oegema, K., Parry, H. and Glover, D. M. (1995). The 190 kDa centrosome-associated protein of *Drosophila melanogaster* contains four zinc finger motifs and binds to specific sites on polytene chromosomes. *J. Cell Sci.* **108**, 3377-3387.

Whitfield, W. G., Millar, S. E., Saumweber, H., Frasch, M. and Glover, D. M. (1988). Cloning of a gene encoding an antigen associated with the centrosome in *Drosophila*. *J. Cell Sci.* **89**, 467-480.

Williams, B. C. and Goldberg, M. L. (1994). Determinants of *Drosophila* zw10 protein localization and function. *J. Cell Sci.* **107**, 785-798.