

The SCF ubiquitin ligase protein Slimb regulates centrosome duplication in *Drosophila*

Edward J. Wojcik^{*†}, David M. Glover[‡] and Thomas S. Hays^{*}

The duplication of the centrosome is a key event in the cell-division cycle. Although defects in centrosome duplication are thought to contribute to genomic instability [1–3] and are a hallmark of certain transformed cells and human cancer [4–6], the mechanism responsible for centrosome duplication is not understood. Recent experiments have established that centrosome duplication requires the activity of cyclin-dependent kinase 2 (Cdk2) and cyclins E and A [7–9]. The stability of cyclin E is regulated by the ubiquitin ligase SCF, which is a protein complex composed of Skp1, Cdc53 (Cullin) and F-box proteins [10–12]. The Skp1 and Cullin components have been detected on mammalian centrosomes, and shown to be essential for centrosome duplication and separation in *Xenopus* [13]. Here, we report that Slimb, an F-box protein that targets proteins to the SCF complex [14,15], plays a role in limiting centrosome replication. We found that, in the fruit fly *Drosophila*, the hypomorphic mutation *slimb^{crd}* causes the appearance of additional centrosomes and mitotic defects in mutant larval neuroblasts.

Addresses: ^{*}University of Minnesota, Department of Genetics and Cell Biology, St. Paul, Minneapolis 55108, USA. [†]University of Cambridge, Department of Genetics, Cambridge CB2 3EH, UK.

Present address: [‡]Department of Biology, Virginia Tech University, Blacksburg, Virginia 24061, USA.

Correspondence: Edward J. Wojcik
E-mail: ewojcik@vt.edu

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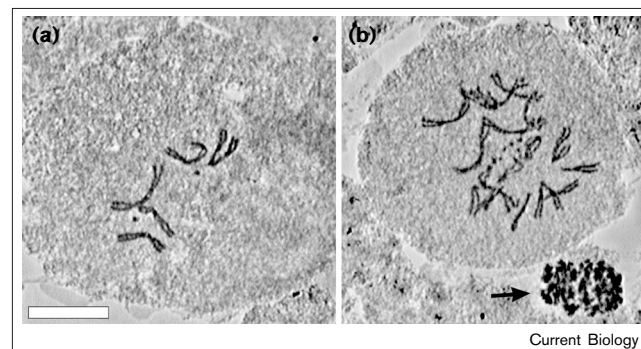
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Results and discussion

We first identified a mutant *Drosophila* line, initially named *centrosome replication defective* (*crd*), in a screen of late larval and pupal lethal mutants from a collection of third chromosome *P*-element insertion mutants [16]. Examination of larval neuroblast chromosome spreads revealed that homozygous *crd* mutants displayed two types of abnormal mitotic figures: metaphase figures comprising overcondensed chromosomes, and polyploid figures suggestive of defects in progressing through the mitotic cycle (Figure 1). The single *P* element in this line

Figure 1



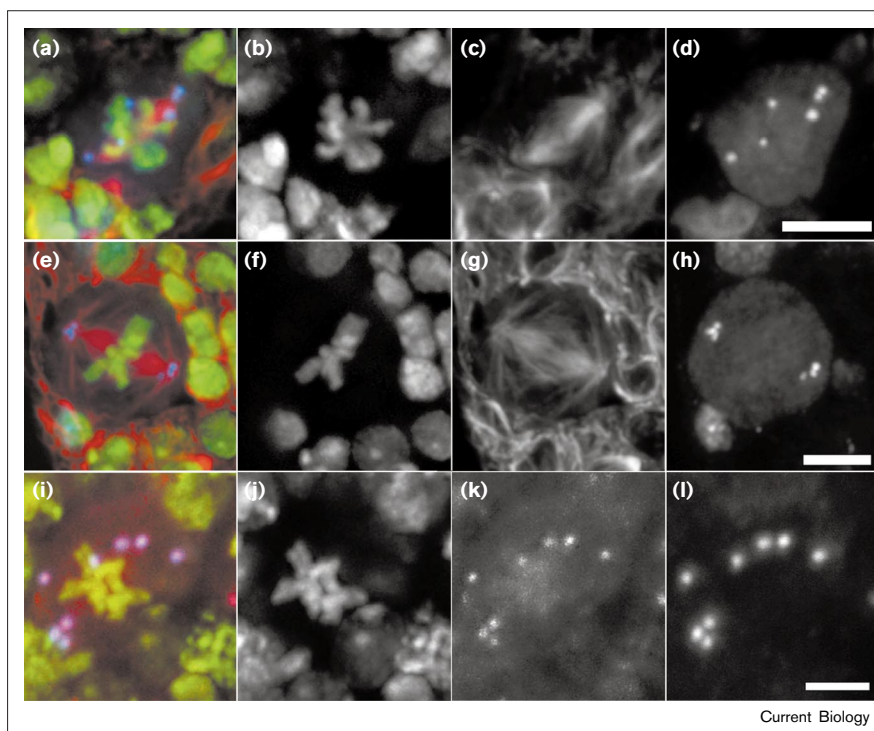
Polyploid *crd* mutant cells in mitotic chromosome spreads of larval third-instar neuroblasts. Orcein-stained brains of both wild-type and mutant *crd/Df(3R)e^{R1}* third-instar larvae were prepared using a standard protocol [29]. The resulting specimens were examined by phase-contrast microscopy. At least ten specimens of each genotype were examined, with a minimum of 50 optical fields each, to characterize the phenotype. Hemizygous *crd* animals had a reduced mitotic index (40 ± 28 per brain) compared with wild type (175 ± 54 per brain), and exhibited a partial metaphase block (metaphase to anaphase ratio of 10.0 ± 5.0 in *crd* mutants versus 2.9 ± 0.9 in the wild type). (a) Typical diploid wild-type neuroblast metaphase figure composed of eight chromosomes. (b) Two neighboring polyploid neuroblasts in a heteroallelic (*crd/slimb^{PZ(3)00295}*) mutant specimen. The smaller cell (arrow) also suffers from overcondensed mitotic chromosomes. Such overcondensed chromosomes in *Drosophila* neuroblasts are often an indication of a failure of mitotic progression [30]. Polyploid mitotic figures occurred at a frequency of 1 in 26 mitotic figures in the mutant, whereas they were never observed in wild-type specimens. Similar results to those listed here were seen in hemizygotes (*crd/Df(3R)e^{R1}*; data not shown). The scale bar represents 10 μ m.

mapped to position 93B_{10–13} by *in situ* hybridization to polytene chromosomes. The chromosomal deficiency *Df(3R)e^{R1}* uncovered the *crd* mutation and the resulting hemizygous animals also had a mitotic phenotype similar to the homozygous *crd* mutants. Whereas *crd* homozygotes died at the larval–pupal boundary, the mitotic defects and lethality of *crd* were reverted by excision of the *P* element.

Isolation of genomic DNA flanking the *crd* *P*-element insertion revealed that it was inserted 296 bp into the 5' untranslated region (UTR) of a previously identified locus, *supernumerary limbs* (*slimb*) [14,15]. Genetic analysis confirmed that *crd* and the *slimb* mutations were allelic and responsible for the observed centrosome replication defect. Accordingly, we renamed our allele *slimb^{crd}* (see Supplementary material).

To characterize further the *slimb^{crd}* mitotic phenotype, we examined the morphology of hemizygous *slimb^{crd}*

Figure 2



Confocal analysis of the *slimb^{crd}* mutant reveals excess centrosomes in diploid mitotic neuroblasts. The central nervous system from hemizygous wild-type and *slimb^{crd}* mutant larvae were dissected and prepared for indirect immunofluorescence (see Supplementary material and [31]). All panels show merged Z-series of diploid mutant neuroblasts (deduced by chromosome number) in metaphase. (a–d) Mutant neuroblast with six centrosomes triple-labeled for DNA (green), α -tubulin (red) and CP190 (blue) in (a), with the individual channels shown, respectively, in (b–d). Note the integrity of the bipolar spindle in the presence of off-axis centrosomes. (e–h) A second mutant neuroblast labeled as in (a–d). Note the bipolar spindle in this instance with three centrosomes clustered at both poles. (i–l) A *slimb^{crd}* mutant cell triple-labeled for DNA (green), γ -tubulin (red) and CNN (blue). The DNA channel is shown in (j), the γ -tubulin channel in (k) and the CNN channel in (l). The scale bars represent 5 μ m.

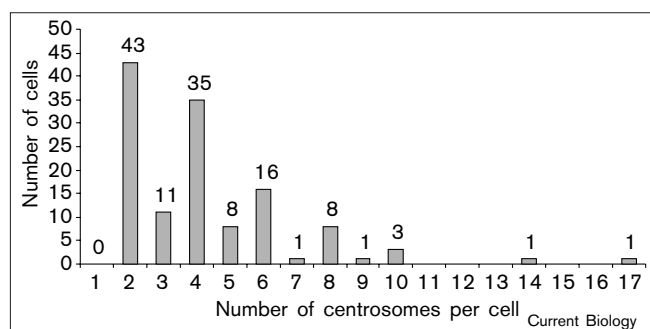
neuroblasts by confocal microscopy. Most striking of the abnormalities in mutant mitotic cells was the excessive number of centrosomes revealed by the distribution of the centrosomal antigen CP190. Diploid cells were observed that contained more than two and as many as seventeen centrosomes (Figures 2 and 3). Polyploid giant neuroblasts were also observed in mutant brain discs and contained far greater numbers of centrosomes than predicted by a failure in cleavage alone (see Supplementary material).

To confirm that the CP190-positive structures identify centrosomes, we tested for the presence of the majority of the known intrinsic components of *Drosophila* microtubule-organizing centers, including γ -tubulin, Centrosomin (CNN) [17] and Abnormal spindle (ASP) [18]. Each component was present at the putative centrosomes (Figure 2). Double-label experiments using antibodies against γ -tubulin, CP190, CNN or ASP (Figure 2i–l and see Supplementary material) showed that signals from all these antigens always coincided with the putative centrosome foci in both wild-type and *slimb^{crd}* mutant mitotic neuroblasts. In addition, the size and shape of the multiple foci in *slimb^{crd}* neuroblasts were uniform and comparable to centrosomes in wild-type cells. Taken together, these observations are most consistent with the presence of excess numbers of centrosomes in diploid *slimb^{crd}* mutant cells, and are not readily explained by the aberrant aggregation of centrosomal antigens or the fragmentation of a single pair of centrosomes (see, for example, [19–21]).

Analysis of centrosome number in mutant diploid neuroblasts revealed that a majority of cells (66%) contained excess centrosomes (Figure 3). The aberrant numbers of centrosomes often exceeded the four foci that would be expected if the defect arose from the precocious separation of the centriole pair associated with each centrosome (for example, see [13]). Instead, the aberrant centrosome numbers suggest that repeated rounds of centrosome duplication occurred during individual cell cycles in *slimb^{crd}* cells. Significantly, the increase in number of centrosomes was not random, with 74% of cells, excluding normal cells with two centrosomes, containing even numbers of centrosomes (Figure 3). This result suggests that not all of the extant centrosomes are licensed to replicate, but is more consistent with the continuous replication of the starting pair of centrosomes. This phenotype is distinct from an assembly/fragmentation defect in centrosome morphogenesis recently reported for a *Drosophila Hsp90* mutation [20].

It is striking that, despite the presence of excess centrosomes in *slimb^{crd}* cells, the spindles were bipolar (Figure 2c,g) and showed no indication of branching or multipolarity as seen in other mitotic mutants, for example, *PP187B* [22]. For centrosomes considerably displaced from the poles (for example, see Figure 2c,d), no substantial or stable microtubule-organizing activity was apparent, further suggesting that the additional centrosomes are ‘immature’ or functionally distinct. Still, the low incidence of hyperploid

Figure 3



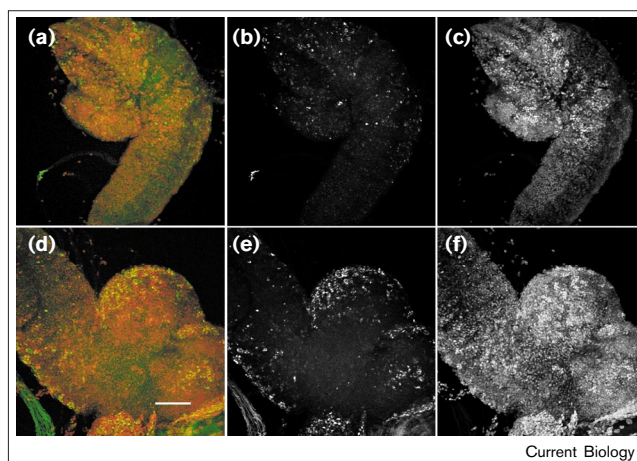
Frequency of centrosomes within diploid *slimb^{crd}* mutant neuroblasts ($n = 128$). Whole-mount fixed larval brains were labeled with either anti- γ -tubulin or anti-CP190 antibodies to identify centrosomes, and Sytox Green (Molecular Probes) for DNA. Centrosomes were counted within Z-series of well-stained mitotic neuroblasts (as in Figure 2), from eight brains from two different experiments, and plotted. Hyperploidy cells were not included in this analysis.

cells associated with the *slimb* mutations suggests that, despite the lack of severe spindle defects, excess centrosomes may disrupt the downstream events associated with cytokinesis or cleavage.

The failure of homozygous *slimb⁻* clones to proliferate in mosaic animals [15] is consistent with the cell-cycle defects we observe. In *Drosophila* embryos, *Xenopus* and sea-urchin extracts, as well as mammalian cells, centrosome duplication is closely tied to known mitotic regulators [7,9,23]. Therefore, it is possible that *slimb* mutations affect centrosome replication indirectly by generally modulating progression through mitosis. Because it has previously been shown that centrosome replication is coupled to S phase *in vitro*, and that an abnormally prolonged S phase can result in centrosome over-replication [9,24], we determined whether the duration of S phase was increased in *slimb^{crd}* cells. If S phase was lengthened in the *slimb* mutant cells, then we would expect to observe an increase in the total number of S-phase cells seen at any given moment in mutant, compared with wild-type, tissues. Instead, we found a decrease in the number of S-phase cells in *slimb* mutant brains (Figure 4). Therefore, we propose that Slimb is not likely to lengthen the progression of mitosis and, instead, acts more directly to stop centrosome replication.

The *slimb* gene was first identified as a negative regulator of the Hedgehog (Hh) and Wingless (Wnt/Wg) signaling pathways in *Drosophila* [14,15]. It was recognized as a member of the F box/WD40 class of proteins that can act as targeting factors for the SCF complex, an E3 ubiquitin ligase first identified from cell-cycle studies in yeast (reviewed in [25–27]). SCF activity is also required to degrade cell-cycle regulatory proteins in metazoans, as is

Figure 4



Third-instar *slimb^{crd}* larval brains exhibit fewer cells in S phase of mitosis. S-phase cells were labeled as described in the Supplementary material. Images are merged Z-series of entire brains. Representative examples of (a–c) *slimb^{crd}* and (d–f) wild-type brains. Replicating DNA (green channel) and nuclei (propidium iodide, red channel) are shown in (a,d), which are pseudocolored. The green channel from (a,d) is shown alone in (b,e), respectively. The red channel from (a,d) is shown alone in (c,f), respectively. The scale bar represents 40 μ m.

evident from the accumulation of cyclin E in mouse knockouts deficient for its Cullin 1 component [12]. We have now found that, in *Drosophila* neuroblasts, Slimb is required to restrict centrosome duplication during the cell cycle. The target whose presumed degradation is regulated by Slimb is not known. One possibility is that the target is cyclin E, as this Cdk2 subunit is known to be degraded by the SCF complex and is also required for centrosome duplication [7,9]. This may in part explain why antibodies to either the Skp1 or Cullin1 components of SCF can block the initial separation of replicating centrioles *in vitro* [13]. Nevertheless, because individual F-box proteins, such as Slimb, can interact with more than one target protein, and multiple F-box proteins localize at centrosomes during mitosis [13,28], other centrosomal targets and distinct steps in centrosome replication are likely to be involved.

The requirement for an E3 ubiquitin ligase targeting component to regulate both signaling pathways and centrosome duplication offers one means of coordinating the regulation of developmental processes, and signals for cell proliferation, with the mechanics of cell-cycle progression. It is not difficult to imagine how competition for rate-limiting levels of Slimb protein might regulate the division of cells within a particular developmental program. Similar regulatory networks may also be of relevance in the significant number of human cancers in which the degradation of β -catenin is dysregulated and in human tumor cells with the known occurrence of excess centrosomes.

Supplementary material

Additional methodological detail and two figures showing that off-axis centrosomes in the *slimb^{crd}* mutant contain ASP, and that polyploid *slimb^{crd}* neuroblasts have bipolar spindles and excess centrosomes, are available at <http://current-biology.com/supmat/supmatin.htm>.

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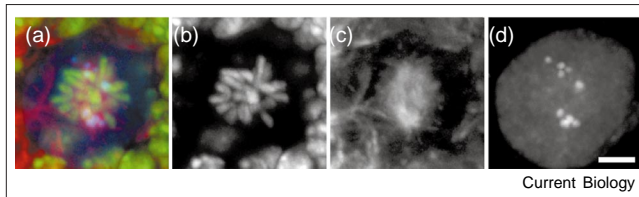
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Figure S1



Off-axis centrosomes in the *slimb^{crd}* mutant contain ASP. (a) Adjacent metaphase *slimb^{crd}* mutant neuroblasts (arrowheads) triple-labeled for DNA (green), γ -tubulin (blue) and ASP (red). The ASP channel from (a) is shown alone in (b). The lower arrowhead points to the ASP signal at a cluster of off-axis centrosomes; the upper arrowhead points to an off-axis extra centrosome. (c) The γ -tubulin channel from (a). The scale bar represents 5 μ m.

Figure S2



Polyloid *slimb^{crd}* neuroblasts can be seen with bipolar spindles and excess centrosomes. (a) Merged Z-series of an aberrant polyloid neuroblast fluorescently labeled for DNA (green), tubulin (red) and CP190 (blue). (b–d) The DNA, tubulin and centrosome channels, respectively, from (a). The scale bar represents 5 μ m.

and anti-ASP (at 1:200, [S5]). The fluorescent DNA stains ToPro-3 or Sytox Green (both at 1:2000; Molecular Probes) were interchangeably used as required. Fluorescently conjugated secondary antibodies used were anti-mouse Texas Red conjugate (Jackson ImmunoResearch Labs) and an anti-rabbit Alexa-488 conjugate (Molecular Probes). Z-series data were collected on a Bio Rad MRC 1024 confocal system. Sequential image acquisition prevented bleed-through artifacts from both double- and triple-labeled samples. Projections of Z-series were created with NIH image, and final images adjusted for printing using Adobe PhotoShop.

Labeling of S-phase cells in larval brains

We followed the procedure of Millset al. [S6] with the following modifications. Wild-type and mutant brains were isolated intact and carried through the entire protocol as whole mounts. Both wild-type and mutant brains from each replicate experiment were performed simultaneously and in the same buffers. Replicating DNA was labeled *in situ* with Alexa 488 dUTP (Molecular probes). Samples from several independent experiments were analyzed by confocal microscopy as above.

Supplementary references

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Supplementary materials and methods

Fly stocks

Animals that are trans-heterozygous for *slimb^{crd}* and a previously identified recessive lethal *slimb* allele, *slimb^{PZ00295}* [S1], failed to survive beyond mid-larval development. Cells in the central nervous system of these larvae displayed a mitotic phenotype indistinguishable from that of *slimb^{crd}* hemizygotes (not shown). The *slimb^{crd}* homozygotes die at the larval–pupal boundary; animals hemizygous for *slimb^{crd}* exhibited lethality at an earlier stage of the third-larval instar, suggesting that the P allele is hypomorphic. Furthermore, the *slimb^{PZ00295}* mutation was both viable and fertile in combination with the *slimb^{crd}* revertant chromosomes. Amorphic *slimb* alleles have previously been shown to result in embryonic lethality [S1].

Immunofluorescence and confocal microscopy

Whole-mount larval brains were prepared for immunofluorescence as described [S2]. Triple-labeled specimens were generated by using combinations of the following antibodies: anti- γ -tubulin (Sigma, DM1A at 1:200); anti-CP190 (at 1:500; [S3]); anti-CNN (at 1:500 [S4]);