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Flies without Centrioles

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SUMMARY

Centrioles and centrosomes have an important role in animal cell organization, but it is uncertain to what extent they are essential for animal development. The *Drosophila* protein DSas-4 is related to the human microcephaly protein CenpJ and the *C. elegans* centriolar protein Sas-4. We show that DSas-4 is essential for centriole replication in flies. *DSas-4* mutants start to lose centrioles during embryonic development, and, by third-instar larval stages, no centrioles or centrosomes are detectable. Mitotic spindle assembly is slow in mutant cells, and ~30% of the asymmetric divisions of larval neuroblasts are abnormal. Nevertheless, mutant flies develop with near normal timing into morphologically normal adults. These flies, however, have no cilia or flagella and die shortly after birth because their sensory neurons lack cilia. Thus, centrioles are essential for the formation of centrosomes, cilia, and flagella, but, remarkably, they are not essential for most aspects of *Drosophila* development.

INTRODUCTION

Since their first description more than 100 years ago, centrosomes have been recognized as important organizers of animal cells. They consist of a pair of centrioles surrounded by an amorphous pericentriolar material (PCM), which nucleates and organizes microtubules (MTs). Through the MTs they organize, centrosomes are thought to have important roles in establishing cell polarity, positioning organelles within cells, directing intracellular traffic, and organizing cell division (Kellogg et al., 1994).

Although centrosomes are major organizers of animal cell division, they are not essential for mitotic spindle assembly. Some animal cells normally organize their spindles without canonical centrosomes, and cultured cells that have had their centrosomes removed by laser ablation or microsurgery can still form bipolar spindles (Hinchcliffe et al., 2001; Khodjakov et al., 2000). In these cases, the mitotic chromosomes appear to initiate the assembly of a bipolar spindle and thereby compensate for the lack of centrosomes. Acentrosomal cultured cells, however, often fail in cytokinesis, and, even if these cells complete cytokinesis successfully, they usually arrest in the following G1 phase (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). This has led to the suggestion that cells have a checkpoint that monitors centrosome integrity in G1 (Doxsey et al., 2005; Rieder et al., 2001).

Although centrosomes are dispensable for spindle assembly in many cell types, it is widely believed that they are essential for asymmetrical division because astral MTs directly contact cues in the cell cortex to position the mitotic spindle appropriately within the cell (Betschinger and Knoblich, 2004; Cowan and Hyman, 2004; Gonczy, 2002). Studies of *centrosomin* (*cnn*) and *asterless* (*asl*) mutants in *Drosophila*, however, suggest that centrosomes and astral MTs may not be essential for the asymmetric divisions of larval neuroblasts (Bonaccorsi et al., 2000; Giansanti et al., 2001; Megraw et al., 2001). These mutants appear to lack functional mitotic centrosomes, yet their neuroblasts have only subtle defects in aligning their spindles with cortical cues during early mitosis, and, by telophase, almost all neuroblasts appear to divide asymmetrically, just as in wild-type (wt) larvae. It remains controversial, however, whether *cnn* and *asl* mutants completely lack functional mitotic centrosomes (Raff, 2001).

Consistent with their many functions, centrosome dysfunction has been implicated in a wide variety of human diseases (Badano et al., 2005). Centrosome defects are believed to contribute to the genetic instability associated with many cancers (Nigg, 2002), and genetic studies have implicated centrosomes in microcephaly, a condition associated with a small brain size at birth (Woods et al., 2005). Of the four genes so far linked to microcephaly, three (ASPM, Cdk5Rap2, and CenpJ) encode centrosomal proteins (Bond et al., 2005; Kouprina et al., 2005). It has been postulated that the small brain size in these individuals may be caused by defects in asymmetric division in the neural precursor cells that generate neurons during early fetal development (Woods et al., 2005). In addition, the centrioles in many animal cells are thought to have important functions that are distinct from their function as organizers of the centrosome. They form the

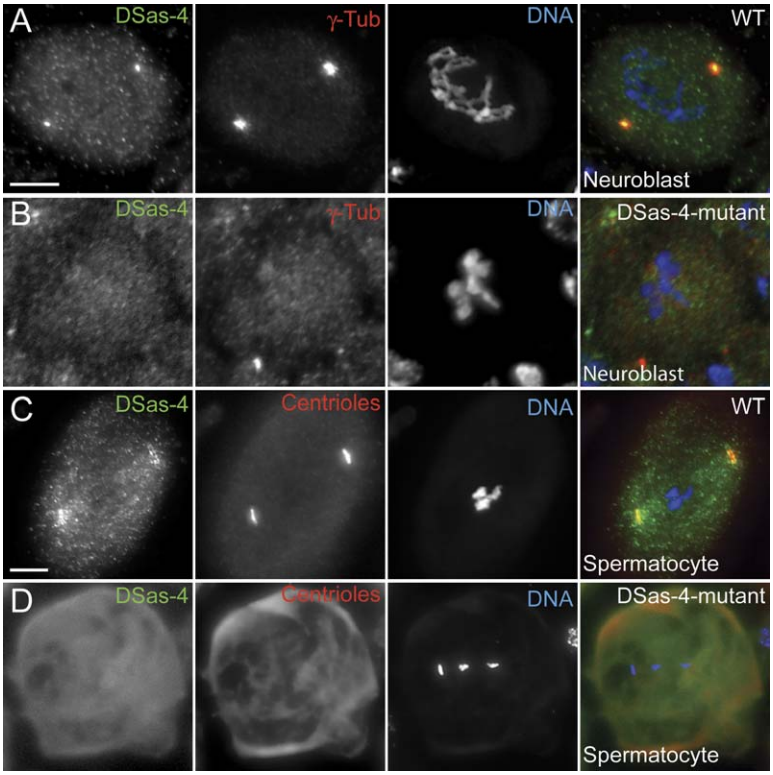

Figure 1. The DSas-4 Protein Is Associated with Centrioles

(A and B) The distribution of DSas-4, γ-tubulin, and DNA in wt (A) and *DSas-4* mutant (B) larval neuroblasts.

(C and D) The distribution of DSas-4, centrioles (stained with the GTU88* antibody [Martinez-Campos et al., 2004]), and DNA in wt (C) and *DSas-4* mutant (D) primary spermatocytes. Note that the cytoplasmic staining with the anti-DSas-4 antibodies in wt spermatocytes is likely to be real as it is absent in mutant spermatocytes, but this is less clear in neuroblasts. Scale bars = 5 μm.

basal bodies that nucleate the formation of cilia and flagella, and cilia defects contribute to a variety of human diseases (Eley et al., 2005; Pazour and Rosenbaum, 2002).

Despite the likely importance of centrioles and centrosomes in so many cell processes, few experiments have addressed whether they have essential roles during animal development. The polo-like kinase Plk-4/Sak is essential for centriole replication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005), and *Drosophila sak* hypomorphic mutant third-instar larval brains appear to lack centrioles in ~20% of their cells, yet they develop into adults at rates that are only slightly slower than normal (Bettencourt-Dias et al., 2005). The centrioles that are present in *sak* mutants, however, appear to be fully functional, so it is difficult to infer whether centrioles or centrosomes have essential roles in *Drosophila* development. Centrioles and centrosomes are essential for the development of *C. elegans* embryos. Five *C. elegans* proteins, Sas-4, Sas-5, Sas-6, Spd-2, and Zyg-1 (the likely homolog of Plk-4/Sak) are required for centriole replication in worm embryos (Delattre et al., 2004; Kemp et al., 2004; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gonczy, 2003; O'Connell et al., 2001; Pelletier et al., 2004), and perturbing the function of any of them arrests embryonic development at the one or two cell stage. This early arrest, however, precludes an analysis of the importance of centrioles and centrosomes at later stages of worm development. In this report, we analyze a mutation in the *Drosophila DSas-4* gene and find that centrioles and centrosomes are not essential for most aspects of *Drosophila* development.

RESULTS

DSas-4 Is a Centriole-Associated Protein that Is Essential for Centriole Replication

The *Drosophila* protein encoded by the gene CG10061 shares a C-terminal domain with the human centrosomal protein CPAP/CenpJ and an N-terminal domain with the *C. elegans* centriolar protein Sas-4 (see Figure S1 in the Supplemental Data available with this article online). As described below, CG10061 appears to function in a similar manner to *C. elegans* Sas-4, and we hereafter refer to this protein as *Drosophila* Sas-4 (DSas-4).

We raised antibodies against the N-terminal region of DSas-4. Affinity-purified anti-DSas-4 antibodies recognized a small dot at the center of the centrosome at all stages of the cell cycle in both embryos (data not shown) and larval neuroblasts (Figure 1A). This staining was absent in *DSas-4* mutant larval neuroblasts (Figure 1B—the *DSas-4* mutant is described in more detail below). Such dot-like centrosomal staining is usually indicative of centriole staining, and DSas-4 colocalized with the *Drosophila* centriole markers GFP-PACT and GTU88* (Martinez-Campos et al., 2004) in fixed larval neuroblasts (data not shown). Moreover, anti-DSas-4 antibodies stained the very large centrioles found in spermatocytes (Figure 1C); this staining was absent in *DSas-4* mutant spermatocytes (Figure 1D). Thus, DSas-4 is closely associated with centrioles.

To test whether DSas-4 is required for centriole replication, we injected Texas red-labeled anti-DSas-4 antibodies into early embryos expressing GFP-Tubulin and

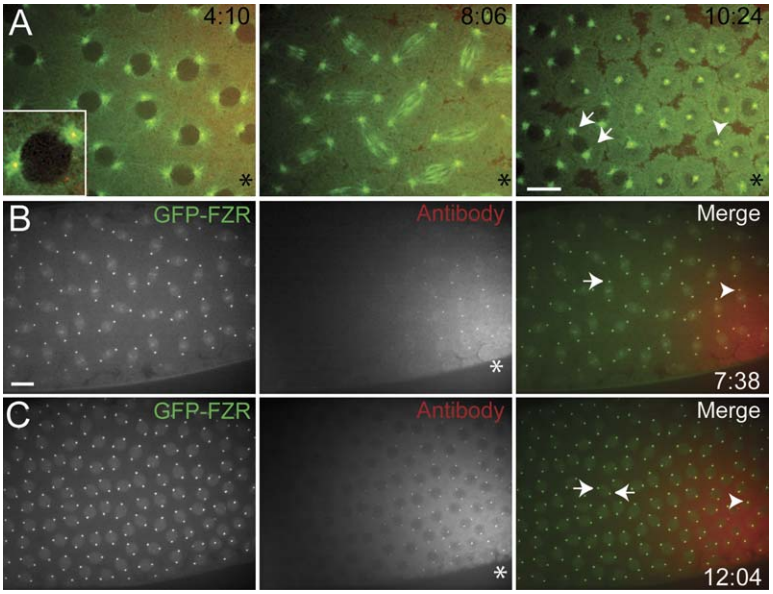

Figure 2. Anti-DSas-4 Antibodies Inhibit Centrosome and Centriole Replication in Embryos

Texas red-labeled antibodies were injected into syncytial embryos expressing either GFP-α-Tubulin (A) or GFP-Fzr (B and C), and the embryos were examined by time-lapse confocal microscopy—see Movies S1 and S2, respectively. The time (min:s) after antibody injection is indicated in each panel, and the asterisk marks the site of antibody injection. In both embryos, the injected antibodies bound to the centrioles closest to the injection site (see 2× magnified inset in [A]): antibodies are shown in red and the GFP-fusion proteins in green in all merged images.

(A) Shows the same embryo at three different time points. All of the nuclei in this embryo entered and exited mitosis normally, but at the end of mitosis (right panel), the centrioles and centrosomes nearest the injection site failed to divide and each nucleus is associated with a single centriole and centrosome (arrowhead). The centrioles further away from the injection site divided normally, and each nucleus is associated with two centrosomes (arrows).

(B and C) Shows the same embryo at two different time points. GFP-Fzr marks the centrioles and also weakly stains the spindle and chromosomes. This embryo entered (B) and exited (C) mitosis normally, but the centrioles nearest the injection site (arrowhead) failed to divide, while those further away from the injection site (arrows) divided normally.

Scale bars = 10 μm.

followed the embryos by time-lapse confocal microscopy. We injected the embryos just as they exited a round of mitosis, so that centriole replication would be just initiating at the time of antibody injection. In all eight embryos followed in this way, the injected antibody rapidly associated with the centrioles closest to the injection site. These embryos entered and exited mitosis normally, but, at the end of mitosis, the centrioles and centrosomes closest to the injection site failed to replicate (Figure 2A; Movie S1). We observed a similar failure in centriole replication when we injected these antibodies into embryos expressing the centriole marker GFP-Fzr (Figures 2B and 2C; Movie S2) or GFP-PACT (data not shown). (Note that we previously described GFP-Fzr as a centrosome marker [Raff et al., 2002], but we now believe it is associated with centrioles—see below). Thus, as is the case in *C. elegans*, perturbing DSas-4 function leads to a failure in centriole and centrosome replication.

Identification of a Mutation in the *DSas-4* Gene

The mutant stock l(3)S2214 has a P element inserted in the *DSas-4* coding region which is predicted to severely truncate the protein (see Figure S1). In Western blotting experiments, we were unable to detect DSas-4 protein in wt embryos or larval brains, presumably because it is present at very low levels, as is the case for many centriole components. We could detect the protein in wt cells by immunofluorescence, but we could not detect any DSas-4 protein associated with centrioles in *l(3)S2214* mutant larval brains (Figure 1B) or spermatocytes (Figure 1D). We therefore conclude that this mutation is at least a strong hypomorph, and we hereafter refer to this mutation as *DSas-4*^{S2214}. All the mutant phenotypes that we describe below can be reverted by the precise excision of this P element, demonstrating that it is the insertion in *DSas-4* that causes these phenotypes.

DSas-4 Mutant Third-Instar Larval Brain Cells Lack Centrioles and Centrosomes

As DSas-4 appears to have a role in centriole replication, its absence from centrioles in *DSas-4*^{S2214} mutants (Figures 1B and 1D) suggested that these cells might have no centrioles at all. To test this possibility, we examined the localization of the centriolar marker GFP-PACT (Martinez-Campos et al., 2004) in whole-mount preparations of wt and mutant third-instar larval brains. GFP-PACT robustly labeled centrioles in wt brains (Figure 3A), but we detected no centrioles in more than 1000 mutant cells scored from four different brains (Figure 3B). In addition, we examined larvae expressing a GFP-Fzr fusion protein, which has previously been shown to localize to centrosomes (Raff et al., 2002). We found that GFP-Fzr was associated with centrosomes in wt larval neuroblasts in interphase (Figure 3C); as these interphase centrosomes lack all known PCM markers (Martinez-Campos et al., 2004), this suggests that GFP-Fzr is closely associated with centrioles. Again, we could not detect any centrioles with GFP-Fzr in more than 1000 mutant cells scored from four different brains (Figure 3D).

These observations suggest that *DSas-4* third-instar mutant brains lack centrioles. To test whether this was the case, we performed an electron microscopic (EM)

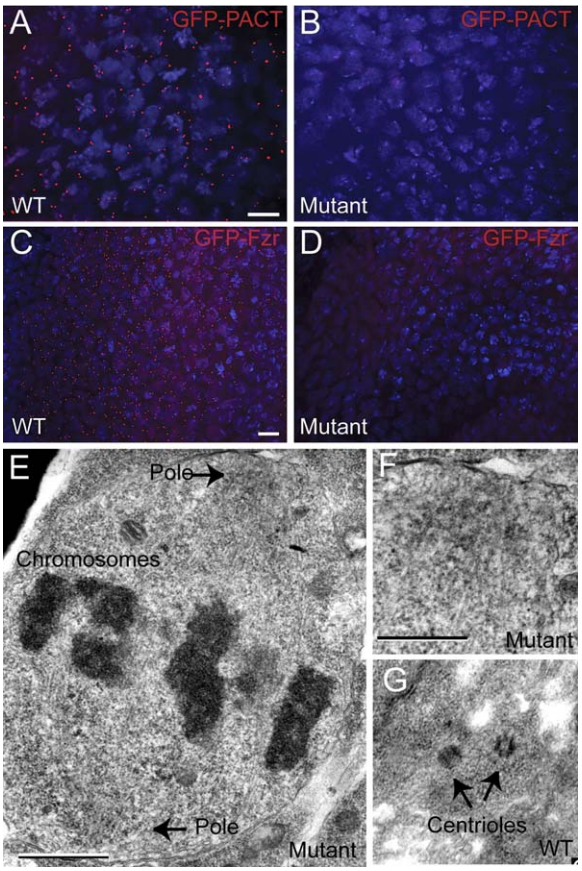

Figure 3. *DSas-4*^{S2214} Mutant Third-Instar Larval Brain Cells Lack Detectable Centrioles

(A–D) Whole mounts of third-instar larval brains from wt and *DSas-4* mutants that expressed the centriole markers GFP-PACT or GFP-Fzr (pseudocolored in red) were stained with Hoechst dye (blue) to visualize the DNA. No centrioles are detectable in mutant cells with either fusion protein.

(E–G) EM micrographs of selected thin sections of mutant (E and F) or wt (G) cells.

(E) No centrioles were found in serial sections of this mitotic cell.

(F) An enlargement of one of the spindle poles shown in (E); note that the spindle MTs converge at the acentriolar pole, which appears to be in close contact with the cell cortex (see Discussion).

(G) A centriole pair in a wt cell; these were readily detectable in all serially sectioned wt cells.

Scale bars (A–D) = 10 μm; (G) = 1 μm; (E and F) = 0.5 μm.

analysis of coded samples of either wt or *DSas-4* mutant whole larval brains. In serial reconstructions of eight cells from each sample (75 sections of 200 nm thickness, spanning a total of 15 μm), two or four clearly identifiable centrioles were detectable in each wt cell, while nothing that resembled a centriole was detectable in any of the mutant cells (Figures 3E–3G). Moreover, while randomly searching EM sections we typically identified one to two centrioles per 50 fields in wt preparations, but we failed to identify any centrioles in more than 800 fields of mutant cells. Thus, our failure to detect centrioles with GFP-PACT or GFP-Fzr almost certainly reflects the absence of centrioles in mutant cells.

We next stained mutant larval brain cells with the PCM markers γ-tubulin, Aurora A, Centrosomin (Cnn), D-TACC, Msps, CP190, and CP60. In all cases, we failed to detect centrosome staining at the poles of the mitotic spindles in >95% of mitotic cells (data not shown; see below). Thus, *DSas-4*^{S2214} mutant third-instar larval brain cells also lack centrosomes, suggesting that centrioles are essential for centrosome formation in flies.

DSas-4^{S2214} Mutants Gradually Lose Centrioles during Development

As described below, *DSas-4*^{S2214} mutant flies are viable but uncoordinated, and so they cannot mate to produce progeny. We therefore had to produce *DSas-4*^{S2214} mutant larvae from heterozygous mothers, which contribute some DSas-4 protein to the embryos. We presumed that centriole replication would start to fail in homozygous mutant embryos when the maternal supply of protein ran out. To determine when the mutant cells start to lose centrioles during development, we examined 0–20 hr collections of embryos laid by *DSas-4*^{S2214} heterozygous mothers (a quarter of which would be expected to be homozygous mutants for *DSas-4*). We readily detected centrioles with anti-DSas-4 antibodies and with GFP-PACT in all 0- to 3-hour-old embryos, presumably because maternal DSas-4 allowed centriole replication up to this stage of development in all embryos regardless of their genotype (data not shown). In 16/22 stage 15–16 embryos (~13–20 hr after fertilization), centrioles were detectable in all cells (Figure S2A); we presume these embryos were heterozygous for *DSas-4*^{S2214}. In 6/22 embryos, however, centrioles were no longer detectable in ~50%–80% of cells with either anti-DSas-4 antibodies or GFP-PACT (Figure S2B); we presume these embryos are homozygous for *DSas-4*^{S2214} and have started to run out of the maternal DSas-4 protein. This seems a reasonable presumption, as we never observed such a class of embryos in wt preparations.

We next examined the brains of homozygous mutant first-instar larvae (~22–46 hr after fertilization) and found that centrioles were detectable in ~10% of cells (Figure S2D). Thus, *DSas-4*^{S2214} mutants already lack centrioles in the vast majority of their brain cells after only 1–2 days of development.

DSas-4^{S2214} Mutant Flies Appear Morphologically Normal but Die Because They Lack Cilia in Their Sensory Neurons

The results described above indicate that *DSas-4*^{S2214} mutants proceeded through the ~4–5 days of larval development and ~4–5 days of pupal development without centrioles in the vast majority of their cells. Remarkably, development proceeded with near normal timing (Figure S3), and morphologically normal flies hatched at near-normal rates (Figures 4A and 4B). The hatched flies, however, were severely uncoordinated, and they usually

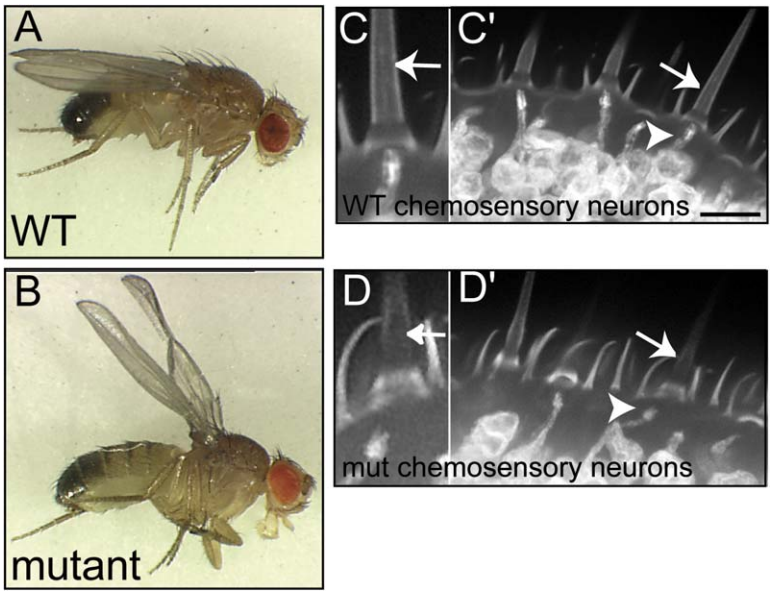

Figure 4. *DSas-4*^{S2214} Mutant Flies Are Morphologically Normal, but Lack Cilia

Picture of a wt (A) and *DSas-4*^{S2214} mutant (B) fly. Mutant flies were morphologically normal, but were uncoordinated and so could not hold their wings or legs in a normal position.

(C and D) Chemosensory neurons of the third-antennal segment were revealed in wt (C and C′) and *DSas-4*^{S2214} mutants (D and D′) by the expression of the membrane marker mCD8-GFP in all neurons. wt neurons extended dendrites (arrowhead in [C′]) to the base of the chemosensory bristles and cilia could be visualized as a thin line extending into the bristle (arrow in magnified view in [C]). The neuronal organization of the mutant antennal segment appeared normal, and neurons extended dendrites toward the chemosensory bristles (arrowhead in [D′]). No cilia could be detected in the bristles (arrow in magnified view in [D]), and the dendrites appeared to lose their connection with the bristles.

Scale bar = 10 μm.

got stuck in the food and died shortly after hatching. If they were allowed to hatch away from any food, however, they could survive for several days before they died from dehydration.

This uncoordinated phenotype is often associated with defects in the cilia of type I mechanosensory neurons (Dubruille et al., 2002). As mutant flies lack centrioles, we suspected that their uncoordination reflected a lack of cilia in their mechanosensory neurons, and we confirmed that this was the case (Figures 4C, 4D, and S4). Apart from these mechanosensory neurons, the only other cells in flies that have cilia or flagella are sperm (Kernan et al., 1994), and we confirmed that mutant sperm lacked centrioles and flagella (Figure S5). Thus, centrioles are essential for cilia and flagella formation in flies, but these structures are not essential for the development of flies from late embryos to adults.

Mitosis Is Slowed in *DSas-4*^{S2214} Mutant Flies

To examine how the mutant cells divide without centrosomes, we initially looked at cell division in fixed third-instar larval brains. In wt cells, centrosomes organized robust MT arrays at all stages of mitosis (Figure S6). In mutant cells, by contrast, centrosomes were not detectable, and there were few organized MT arrays at early stages of mitosis (Figure S6). As mitosis proceeded, however, MTs in the mutant cells appeared to polymerize around the mitotic chromatin, and these MTs became organized into bipolar spindles. The acentrosomal spindles appeared to segregate chromosomes normally, and we observed only a small increase in the proportion of aneuploid cells (~1% in wt versus ~3% in mutants; n = 200 and 231, respectively). The mitotic index was slightly increased in mutant brains (1.1 ± 0.3% in wt compared to 1.5 ± 0.6% in mutants), suggesting that the length of mitosis was extended by 30%–40% in mutant cells ($p > 0.02$).

DSas-4^{S2214} Neuroblasts Can Divide Asymmetrically, but Many Do Not

As described in the Introduction, the role of centrosomes in the asymmetric divisions of *Drosophila* neuroblasts is controversial. To examine asymmetric divisions in cells that completely lack centrosomes, we initially examined the distribution of the Inscuteable (Insc) and Miranda (Mira) proteins in fixed third-instar *DSas-4* mutant larval neuroblasts. Insc normally localizes to the apical cortex of neuroblasts prior to the entry into mitosis, and this induces the basal localization of Mira during mitosis (see Discussion). In wt metaphase neuroblasts, Insc and Mira formed crescents on opposite sides of the neuroblast in 87% of cells (n = 154; Figures 5A and 5G), and Mira segregated into the smaller ganglion mother cell (GMC) while Insc remained in the larger neuroblast during anaphase/telophase (98%, n = 96; Figure 5D). In *DSas-4* mutant metaphase neuroblasts, Insc was almost always in a cortical crescent (91%, n = 266), but Mira formed a cortical crescent in only 70% of cells, and this crescent was often mislocalized relative to the Insc crescent (~18% of cells; Figure 5G). In the cells where Mira failed to form a crescent, it was either cytoplasmic (21%; Figures 5B and 5G) or was enriched on the spindle (11%; Figures 5C and 5G). During anaphase/telophase, 30% (n = 104) of mutant cells appeared to partially missegregate Mira (Figure 5E), and some of these cells appeared to be dividing to produce two daughters of equal size (Figure 5F).

We also stained neuroblasts for Insc, Mira, and tubulin, and measured the alignment of the spindle relative to the Insc crescents (Figures 5H–5K). In wt metaphase neuroblasts, 96% (n = 48) of spindles were properly aligned with Insc crescents while this proportion was only 50% (n = 78) in mutant metaphase neuroblasts (Figure 5H). We found a similar spindle misalignment relative to the Mira crescents (data not shown). Importantly, some

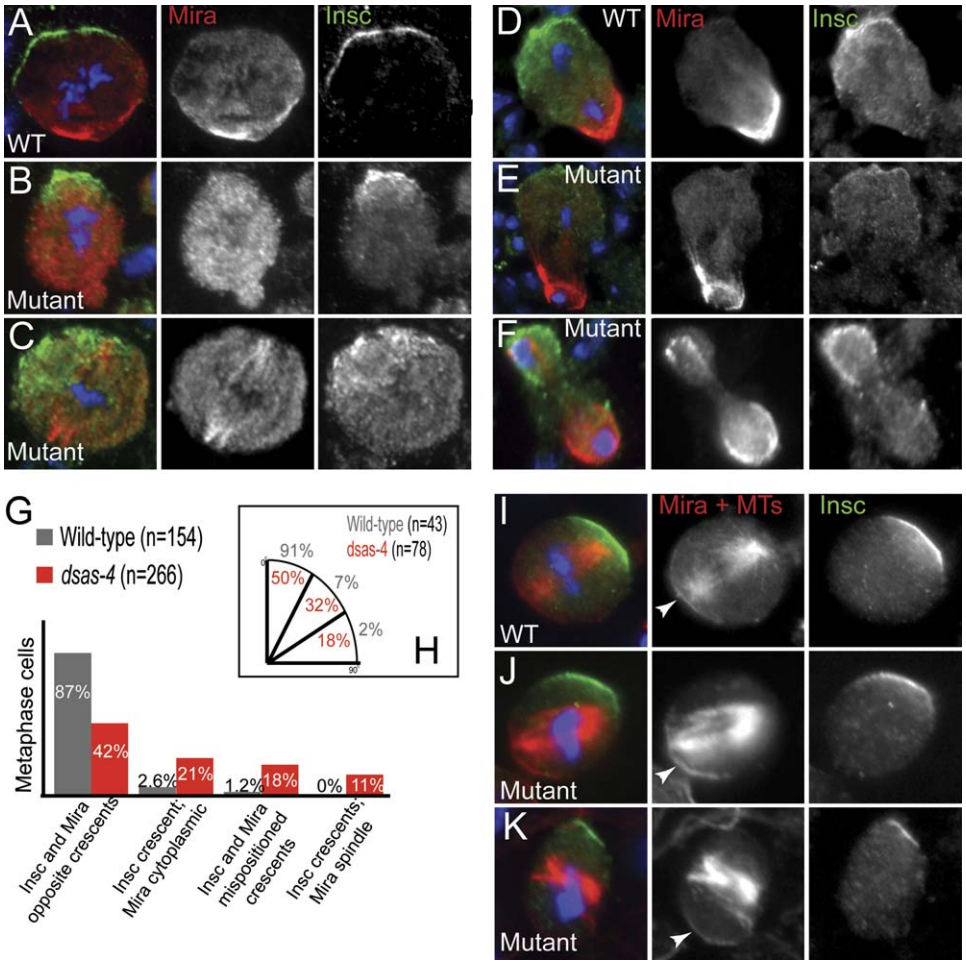

Figure 5. Asymmetric Division Defects in Fixed *DSas-4*^{S2214} Mutant Neuroblasts

(A–F) The distribution of Mira (red) and Insc (green) in wt (A and D) or mutant (B, C, E, and F) neuroblasts during metaphase (A–C) and anaphase (D–F).

(G) A bar chart quantifying the distribution of Insc and Mira proteins in wt and mutant metaphase neuroblasts. Note that ~6% and ~3% of both wt and mutant metaphase cells had no detectable Mira or Insc crescents or had Mira crescents but no Insc crescents, respectively; these classes are not depicted in the graph, which is why the percentages shown do not add up to 100%.

(H) A graphic illustration of the angle of spindle alignment relative to the Insc crescents in wt (gray) and mutant (red) metaphase neuroblasts.

(I–K) Examples of the localization of Mira and MTs (red) and Insc (green) in wt (I) and mutant (J and K) metaphase neuroblasts. Arrows highlight the position of the Mira crescent.

Scale bar = 5 μm.

spindles were misaligned even in mutant cells where robust Mira and Insc crescents had formed on opposite sides of the cell (Figures 5J and 5K). Thus, *DSas-4* mutant neuroblasts have significant problems in asymmetric division.

To better understand the process of asymmetric division in cells that lacked centrosomes, we used time-lapse video recording to examine living neuroblasts in larval preparations expressing either GFP-Tubulin (Figure 6) or the centrosomal marker Msps-GFP (Figure S7). In 27/27 wt neuroblasts, the spindle assembled from two prominent centrosomes, and the cells proceeded through an asymmetric division (Figures 6A and S7A; Movie S3). In mutant neuroblasts, acentrosomal spindles formed and the cells often exhibited unusual and erratic changes in cell shape throughout mitosis (Movies S4–S7). Despite these abnormalities, the majority of mutant cells (70/96) successfully divided asymmetrically (Figure 6B; Movie S4); in some cases, however, the size difference between the daughter cells was much less obvious than normal (Figure 6C; Movie S5) although these divisions were still scored as asymmetric. In 13/96 cases, the neuroblasts ultimately divided symmetrically (Figure 6D; Movie S6), while in a further 13/96 cases the cells initiated cytokinesis but ultimately failed to complete cell division (Figure 6E; Movie S7). Thus, ~30% of mutant neuroblast divisions were abnormal—either because they failed in cytokinesis or were symmetric.

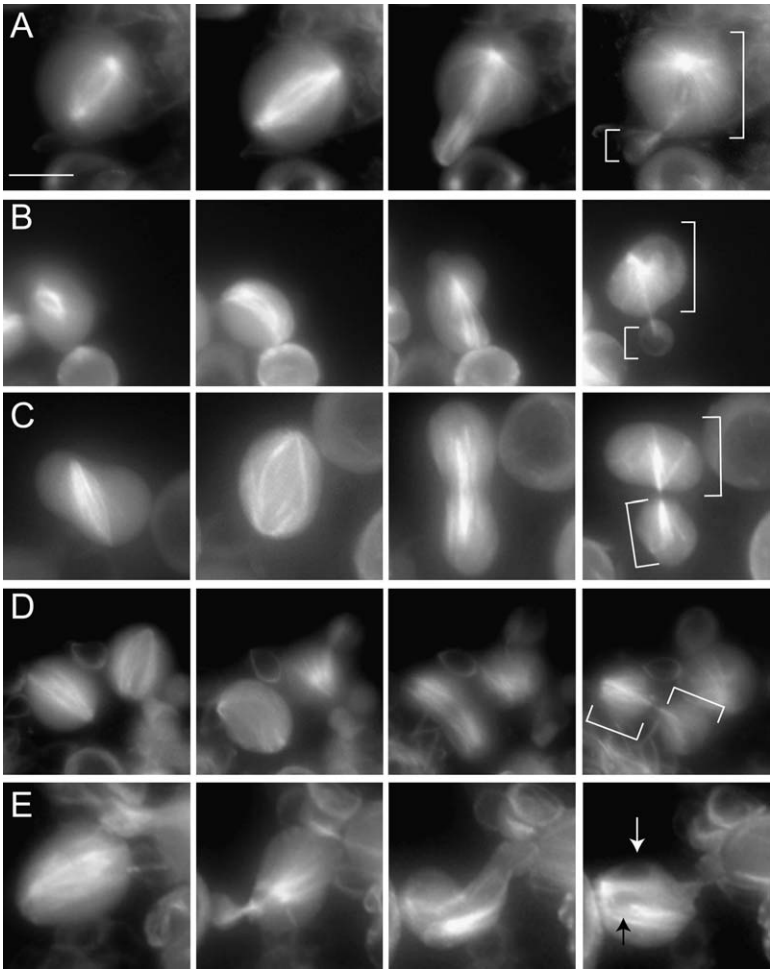

Figure 6. Asymmetric Cell Division Is Unreliable in *DSas-4*^{S2214} Mutant Larval Neuroblasts

The behavior of GFP-Tubulin in living wt (A) and *DSas-4*^{S2214} mutant (B–E) neuroblasts.

(A) This wt neuroblast divided asymmetrically to produce another neuroblast and a smaller GMC (the size difference is indicated by white brackets—see Movie S3).

(B–E) The acentrosomal spindles in mutant neuroblasts often had problems positioning themselves in the cell, and many cells went through phases of irregular shape changes. The cell in (B) divided asymmetrically; the cell in (C) divided asymmetrically, although the size difference between the two daughter cells was not as large as normal; the cell in (D) divided symmetrically. See Movies S4, S5, and S6, respectively. The cell shown in (E) initially appeared to divide asymmetrically, but the late stages of cytokinesis failed and the daughter cells collapsed back together. Arrows indicate the position of the two nuclei in the cell. See Movie S7.

Scale bar = 10 μm.

The Distribution of Neurons, Neuroblasts, and Axons Are Grossly Normal in *DSas-4*^{S2214} Mutant Tissues

Although *DSas-4* mutant flies appeared to develop normally, we wondered whether the high rate of defective neuroblast divisions might lead to defects in neuronal development. We therefore analyzed wt and mutant whole-mount third-instar larval brains after staining them for neuronal and neuroblast markers. The size and morphology of wt and mutant brains were indistinguishable, and we could not detect obvious differences in the distributions of neurons (Figures 7A and 7B) or neuroblasts (Figures 7C and 7D). Moreover, it has recently been shown that the direction of axon outgrowth is dictated by the position of the centrosome in the neuronal cell body (de Anda et al., 2005). We therefore tested whether the direction of axon outgrowth was affected in *DSas-4* mutant third-instar eye discs, where the neurons uniformly extend their axons toward the optic lobes of the brain. Again, we found no significant differences between wt and *DSas-4* mutants in the organization of the neurons or in the direction of axon outgrowth (Figures 7E and 7F). Thus, it appears that the relatively high rate of failure of asymmetric divisions does not grossly perturb neuronal organization in *DSas-4* mutants.

DISCUSSION

In the present study, we show that the *Drosophila* DSas-4 protein is required for centriole replication. *DSas-4* mutant cells progressively lose centrioles during embryonic development as the maternally supplied DSas-4 protein is exhausted; by first-instar larval stages, ~90% of mutant brain cells lack detectable centrioles, and by third-instar larval stages, centrioles are essentially undetectable in these cells. We cannot detect any centrosomes, cilia, or flagella in mutant cells that lack centrioles, strongly suggesting that centrioles are essential for the formation of these structures in flies. Remarkably, these mutant flies develop at near-normal rates and are born at near normal Mendelian ratios, demonstrating that flies can proceed through the majority of development without centrioles, centrosomes, cilia, or flagella. Mutant adults, however, die shortly after birth because they lack cilia in type I sensory neurons. Thus, centrioles are essential for fly survival only because they are required for cilia formation.

In *C. elegans*, centrioles and centrosomes are essential for early development: mutant embryos that cannot replicate their centrioles arrest after only one or two rounds of

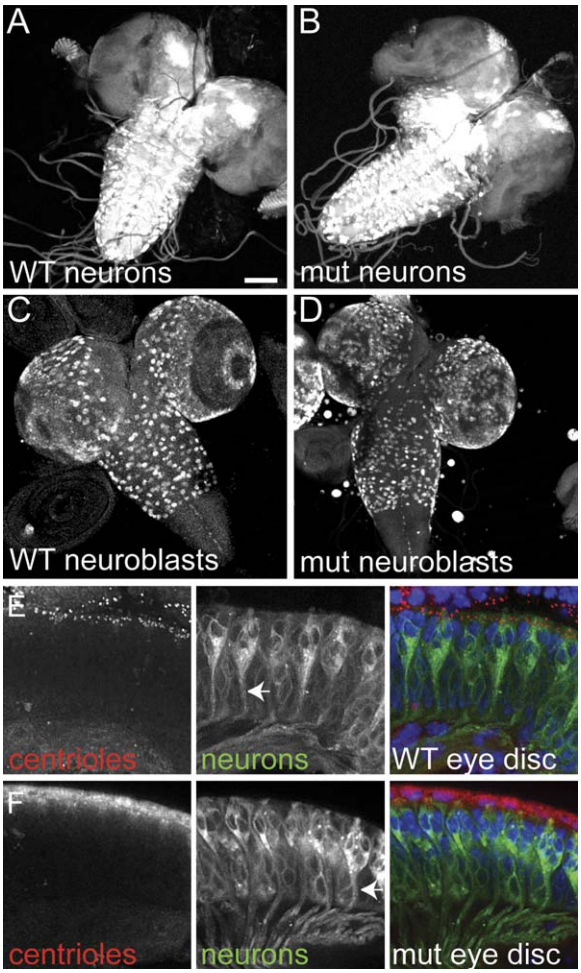

Figure 7. The Distribution of Neurons, Neuroblasts, and Axons Is Largely Unperturbed in *DSas-4*^{S2214} Mutant Tissues

Neurons in wt (A) and *DSas-4*^{S2214} mutant (B) third-instar larval brains were marked by the expression of an mCD8-GFP fusion protein in all neurons. Neuroblasts in third-instar brains of wt (C) and *DSas-4*^{S2214} mutant (D) were stained with anti-Mira antibodies. Note that the size and morphology of the brains and the distribution of neurons and neuroblasts are remarkably similar in the wt and mutant larvae.

(E and F) Neurons in third-instar eye-discs from wt (E) and *DSas-4*^{S2214} mutant (F) larvae were marked by the mCD8-GFP fusion protein (green); centrioles were marked with anti-D-PLP antibodies (red). Although there are no detectable centrioles in the mutant eye disc, the overall organization of the neurons is similar to that of wt, and the developing neurons extend axons (arrows) toward the optic lobes of the brain.

Scale bar (A–D) = 50 μm; (E and F) = 10 μm.

cell division. The same would probably be true for the earliest stages of *Drosophila* embryogenesis, as one would expect centrosomes to be especially important in early syncytial *Drosophila* embryos, in which hundreds of large spindles have to assemble and disassemble very quickly within a common cytoplasm (de Saint Phalle and Sullivan, 1998). In *DSas-4* mutants, however, the heterozygous mothers contribute DSas-4 to the early embryos, which therefore contain centrioles and centrosomes.

It is clear from previous studies that centrosomes are not required for spindle assembly, as mitotic chromosomes and MT-dependent motor proteins can organize the assembly of bipolar spindles. It is nonetheless surprising that centrioles and centrosomes are dispensable for cell division during most stages of *Drosophila* development. Although a *Drosophila* cell line that lacks centrioles has been identified (Debec and Abbadie, 1989), these cells often fail to divide normally (Piel et al., 2001). Cultured mammalian cells that have had their centrosomes removed also often fail to complete cytokinesis, and those cells that do divide often then arrest in G1 of the next cycle, suggesting that centrosomes are required for both efficient cytokinesis and cell-cycle progression (Doxsey et al., 2005; Rieder et al., 2001). One might expect, therefore, that an animal lacking centrosomes would, at the very least, be at a severe growth disadvantage compared to a normal animal. This seems not to be the case in *Drosophila*. Although spindle assembly is slowed in acentrosomal *DSas-4* mutant cells, once assembled, these spindles make few chromosome-segregation errors. Moreover, the ~30%–40% increase in the duration of mitosis in mutant cells does not significantly delay development, probably because mitosis occupies such a small fraction of the total cell cycle. Thus, in flies at least, centrioles and centrosomes are not essential for any aspect of somatic cell-cycle progression or cell division; unlike cultured mammalian cells, fly cells do not arrest in G1 if they have no centrioles or centrosomes.

We do find, however, that centrioles or centrosomes have an important role in asymmetric division in *Drosophila*. This contrasts with previous studies on *cnn* and *asl* mutants, which also appear to lack functional mitotic centrosomes. The cortical cues that guide asymmetric division are localized normally in these mutants, although spindles fail to align efficiently with these cues at early stages of mitosis (Bonaccorsi et al., 2000; Giansanti et al., 2001; Megraw et al., 2001). By telophase, however, ~90% of the mutant cells have properly aligned spindles, and the cells appear to divide asymmetrically (Giansanti et al., 2001). In *DSas-4* mutants, by contrast, at least two aspects of asymmetric division are perturbed. First, the localization of Miranda to a basal cortical crescent occurs unreliably, suggesting that centrosomes play an important part in establishing and/or maintaining cortical Miranda during asymmetric division (see below). Second, ~30% of mutant neuroblasts either divide symmetrically or fail to complete cytokinesis. We suspect that the explanation for the differences between the *DSas-4* mutants and the *cnn* and *asl* mutants is that the centrosomes in *cnn* and *asl* mutants are partially functional, whereas they are completely absent in *DSas-4* mutants. *Cnn* mutants, for example, have centrioles (Megraw et al., 2001), and we can detect astral MTs in at least some *cnn* mutant neuroblasts using live-cell imaging techniques, which are more sensitive than those used in previous studies (Figure S8).

Our observations fit well with recent evidence for two partially redundant mechanisms that ensure the fidelity

of asymmetric division in embryonic fly neuroblasts (Siegrist and Doe, 2005). The first mechanism is MT independent and is initiated prior to the entry into mitosis by Insc/Par protein complexes concentrated at the apical cortex. These complexes recruit Pins/Gαi complexes, which then help drive the redistribution of proteins like Miranda to the basal cortex. In the absence of Insc/Par complexes, a MT-dependent mechanism can recruit Pins/Gαi complexes to a cortical region adjacent to one of the spindle poles. Presumably, these two mechanisms normally cooperate to ensure that the forming spindle efficiently aligns with preexisting cortical cues. If one mechanism is perturbed, however, the other is apparently sufficient to allow neuroblasts to divide asymmetrically. This redundancy presumably explains why neuroblasts carrying mutations that affect asymmetric division usually have misaligned spindles at metaphase but are "rescued" by telophase and so ultimately divide asymmetrically. Our analysis emphasizes that these two mechanisms are only partially redundant: Miranda does not consistently localize to the basal cortex in *DSas-4* mutant cells, even though the Insc/Par complex almost always localizes correctly. Moreover, telophase rescue is inefficient in these cells, and ~30% of cells either fail in cytokinesis or divide symmetrically. Thus, *Drosophila* neuroblasts apparently have great difficulty in compensating for a lack of centrosomes.

Despite these difficulties, ~70% of acentrosomal *DSas-4* mutant larval neuroblasts divide asymmetrically. How can cells that lack centrosomes and astral MTs align their spindle with cortical cues so as to divide asymmetrically? Our live-cell analysis provides a potential explanation. Many acentrosomal spindles extend across the full length of the cell, so that the spindle poles are in close contact with the cortex, and this was also a noticeable feature of the mutant spindles we analyzed by EM (see Figure 3E). This may allow the acentrosomal spindles to interact directly with cortical cues even in the absence of astral MTs, perhaps explaining how the majority of these spindles ultimately align correctly.

It is even more surprising that flies in which ~30% of the brain neuroblasts fail to divide properly seem to have so few developmental defects. The brain seems grossly normal in size, morphology, and histology. Moreover, the neuronal axons in the developing eye disc seem to be oriented correctly, which is unexpected, as previous studies have suggested that the initial direction of axon outgrowth in these cells is defined by the position of the centrosome (de Anda et al., 2005). *DSas-4* mutants may well have subtle defects in neuronal development such as mild proliferation defects which would require lineage trancing experiments to be detected. However, it is clear that the developing fly brain has a remarkable ability to compensate for large-scale abnormalities in neuroblast divisions. It is unclear how the brain manages this. In humans, mutations in *CenpJ*, the homolog of *DSas-4*, results in microcephaly, which has been proposed to be caused by abnormalities in neural precursor cell divisions during fetal development (Woods et al., 2005). Our finding that neuroblast divisions are frequently abnormal in *DSas-4* mutant flies provides the first direct support for this proposal, although the brains of *DSas-4* mutants appear no smaller than wt brains. Perhaps the developing human brain cannot compensate for abnormalities in neural precursor cell divisions in the way that the developing fly brain can.

It will be of great interest to determine whether centrioles and centrosomes are largely dispensable for much of development in other organisms. This may be difficult to address in other systems. In flies, only type I mechanosensory neurons and sperm have cilia and flagella, respectively, so the lack of centrioles produces only an uncoordinated phenotype. By contrast, many types of vertebrate cells have a primary cilium, which, in some cells at least, is required for the cell to respond to certain extracellular signals (Corbit et al., 2005; Huangfu and Anderson, 2005; Schneider et al., 2005). Moreover, cilia in vertebrates have crucial roles in the development of organs such as the kidney (Eley et al., 2005; Pazour and Rosenbaum, 2002). Thus, a lack of centrioles is likely to have a more devastating effect on vertebrate development than on fly development, which might make it difficult to assess whether developing vertebrates can compensate for the lack of centrioles and centrosomes in cell division in the way that *Drosophila* apparently can.

Centrosomes are thought to influence many aspects of cell behavior, including cell migration and cell polarity. Our findings suggest that centrosomes are not essential for any of these processes during most of fly development. It remains unclear, however, whether these processes do not depend on centrosomes in *Drosophila* or whether they are normally dependent but can compensate for the absence of centrosomes. *DSas-4* mutants should provide a useful model to explore the importance of centrosomes in many cell processes.

EXPERIMENTAL PROCEDURES

Fly Stocks

The P-element line, *P{lacW}l(3)s2214*, was obtained from Bloomington stock center (Indiana University, Bloomington, IN). We used either w^{67} or *Dsas4*/TM6C as controls for all our experiments. P-element excision was performed using standard genetic methods and precise excisions were confirmed by sequencing. The GFP-PACT (Martinez-Campos et al., 2004), GFP-Fzr (Raff et al., 2002), and Msps-GFP (Lee et al., 2001) transgenic lines contain GFP-fusions driven from the pUbq promoter that is expressed in all tissues. The mCD8-GFP (Lee and Luo, 1999) and GFP-α-Tubulin (Grieder et al., 2000) transgenic lines contain GFP fusions linked to the UAS promoter. We drove their expression in neurons and neuroblasts using either a pan-neuronal elav-Gal4 driver, or the Gal4 line MZ1407 that drives expression in all brain cells.

Antibodies

Antibodies were raised in rabbits against an MBP-DSas-4 fusion protein containing the first 260 amino acids of the DSas-4 protein. Serum production was performed by Eurogentec; antibodies were affinity purified and stored as described previously (Gergely et al., 2000). Antibodies that were to be injected into embryos were labeled with NHS-Texas red (Molecular Probes) and concentrated to ~5 mg/ml. For immunostaining, affinity-purified anti-DSas-4, D-PLP

(Martinez-Campos et al., 2004), Cnn (R.B., unpublished data), D-TACC (Gergely et al., 2000), Msps (Lee et al., 2001), CP190, CP60 (Kellogg and Alberts, 1992), and Aurora A (Barros et al., 2005) antibodies were used at 1–2 μg/ml final concentration. The following antibodies were also used (final dilutions indicated in parentheses): mouse anti-Miranda (1:20) (Ikeshima-Kataoka et al., 1997), rabbit anti-Inscuteable (1:500) (Yu et al., 2000), mouse anti-γ-tubulin (1:1000; GTU88, Sigma), mouse anti-α-tubulin (1:1000: DM1a, Sigma), rabbit anti-phospho-Histone3 (1:2000, Upstate Biotechnology). All fluorescent secondary antibodies were obtained from Molecular Probes.

Immunofluorescence

Zero to twenty hour Oregon R embryos were fixed in methanol and processed for immunostaining as described previously (Huang and Raff, 1999). Whole-mount brains and eye disks were dissected in PBS and fixed for 20 min in 4% formaldehyde followed by three washes in PBS. Brains were either stained with antibodies at 4°C O/N, or, if they expressed GFP markers, mounted directly in mounting media (80% glycerol + 1% N-propylgallate) with 0.5 μg/ml Hoechst. Whole-mount antennae were dissected from pupae and fixed in 4% formaldehyde for 20 min. They were washed briefly three times in PBS and then mounted in mounting media. Squashed brain and testes preparations were prepared and stained as described previously (Martinez-Campos et al., 2004). Fixed preparations were examined using either a Zeiss Axioskop II microscope with a CoolSnapHQ camera (Photometrics) with Metamorph software (Molecular Devices Corp.), on a Zeiss LSM 510 Meta scanning confocal system mounted an a Zeiss Axiophot II microscope, or on a Perkin Elmer ERS Spinning Disc confocal system using ERS software mounted on a Zeiss Axiovert 200M microscope. All images were processed with Adobe Photoshop software: all images were adjusted using the same procedures that were applied to the whole image.

Analysis of spindle position relative to Insc crescent position was performed by triple staining fixed brains with anti-Mira and anti-α-tubulin mouse antibodies and anti-Insc rabbit antibodies. We only scored metaphase cells where an Insc crescent could be clearly distinguished. The angle between the spindle axis and the Insc crescents was determined by the measurement tool using Metamorph software.

Live Analysis

Live embryos were injected with Texas red-labeled antibodies as described previously (Gergely et al., 2000). Embryos were then followed by time-lapse confocal microscopy using the Perkin Elmer ERS Spinning Disc confocal system described above. Live testes analysis was performed as described previously (Martinez-Campos et al., 2004) and live analysis of third-instar larval neuroblasts was performed as described (Buffin et al., 2005). Samples were analyzed on the Zeiss Axioskop II widefield microscope system described above. For the analysis of Msps-GFP or GFP-Tubulin in neuroblasts, nine focal planes spaced by 0.5 μm were acquired every 25 s. All images shown are maximum intensity projections. All images were processed with Volocity (Improvision) software and all control and experimental images were adjusted using the same procedures applied to the whole image.

Electron Microscopy

Testes of wt and *DSas-4* mutant adults or pupae were dissected and processed for electron microscopy as described previously (Martinez-Campos et al., 2004). Isolated brains were fixed with 2.5% glutaraldehyde, postfixed with 1% OsO_4 and embedded en bloc according to standard EM procedures. Wild-type and *DSas-4* samples were coded so that EM operators did not know whether the sample under evaluation was from the mutant or control animals. Two full series of 200 nm sections spanning 15 μm (75 sections/series) were cut from each sample on Leica UltraCut UCT ultramicrotome. The sections were mounted on slot grids and stained with lead citrate according to standard EM protocols. Images were recorded on film and then scanned on a flatbed scanner at 600 dpi. Contrast was adjusted in Adobe PhotoShop CS. Profiles of individual cells were evaluated by an experienced EM operator at 10–12.5 K magnification. Each structure that potentially resembled a centriole was further analyzed at higher 20–25 K magnification.

Measurements of Fly Growth and Survival Rates

Fly stocks were generated that contained either a wild-type (+) third chromosome or *DSas-4*^{S2214} mutant third chromosome heterozygous with a TM6B balancer that contained the dominant marker Tubby (Tb). +/TM6B, Tb or *DSas-4*^{S2214}/TM6B, Tb flies were self crossed and allowed to lay ~100 eggs in a vial over the course of 12 hr. The development rates of the +/+ and *DSas-4*^{S2214}/ *DSas-4*^{S2214} larvae were measured by counting the number of larvae that pupated or the number of adults that eclosed of each genotype from each vial each day.

Analysis of Mitotic Defects in Fixed Cells

The mitotic index of fixed cells was obtained by staining fixed preparations of larval neuroblasts with Hoechst and anti-phosphohistone H3 antibodies and counting the ratio of phosphohistone H3 positive to negative cells. The levels of aneuploidy and polyploidy were calculated in third-instar larval brain squashes as described previously (Basto et al., 2000).

Supplemental Data

Supplemental Data include eight figures and seven movies and can be found with this article online at http://www.cell.com/cgi/content/full/125/7/1375/DC1/.

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