

# Centrosome Amplification Can Initiate Tumorigenesis in Flies

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## SUMMARY

Centrosome amplification is a common feature of many cancer cells, and it has been previously proposed that centrosome amplification can drive genetic instability and so tumorigenesis. To test this hypothesis, we generated *Drosophila* lines that have extra centrosomes in ~60% of their somatic cells. Many cells with extra centrosomes initially form multipolar spindles, but these spindles ultimately become bipolar. This requires a delay in mitosis that is mediated by the spindle assembly checkpoint (SAC). As a result of this delay, there is no dramatic increase in genetic instability in flies with extra centrosomes, and these flies maintain a stable diploid genome over many generations. The asymmetric division of the larval neural stem cells, however, is compromised in the presence of extra centrosomes, and larval brain cells with extra centrosomes can generate metastatic tumors when transplanted into the abdomens of wild-type hosts. Thus, centrosome amplification can initiate tumorigenesis in flies.

## INTRODUCTION

Centrosomes are the main microtubule organizing centers in animal cells, and they comprise a pair of centrioles surrounded by an amorphous pericentriolar material (PCM) (Bornens, 2002; Kellogg et al., 1994). Centrosomes play an important part in organizing many cell processes, particularly during mitosis where they organize the poles of the mitotic spindle. The idea that centrosome amplification can contribute to tumorigenesis was first proposed by Theodor Boveri almost one hundred years ago (Boveri, 2008; Wunderlich, 2002). Boveri was aware that malignant cells often had an abnormal complement of chromosomes, and he had shown that the presence of extra centrosomes in sea urchin embryos invariably led to chromosome missegregation, as the chromosomes were randomly distributed among

the spindle poles formed by the multiple centrosomes. This elegant hypothesis, however, was largely ignored as the discovery of oncogenes led to the idea that tumorigenesis is a multistep process involving the accumulation of several mutations or epigenetic changes that ultimately give rise to a cancer cell. Nevertheless, it remains a fact that genetic instability is a common feature of many different types of cancer.

Centrosome amplification is a common feature of many cancer cells (D'Assoro et al., 2002a, 2002b; Nigg, 2006; Pihan et al., 1998, 2001). Moreover, levels of centrosome amplification are often correlated with levels of genetic instability (Brinkley, 2001; Ghadimi et al., 2000; Lingle et al., 2002). Thus, it is now widely assumed that centrosome amplification inevitably leads to genetic instability, and that this can be a significant factor in the generation of fully transformed cancer cells. In support of this possibility, it has recently been shown that inducing genetic instability in mice can increase the rates of tumor formation in some, but not all, tissues (Weaver et al., 2007).

Centrosome amplification, however, does not necessarily lead to spindle multipolarity (Quintyne et al., 2005; Ring et al., 1982). In at least some cell types, extra centrosomes can "cluster" together during mitosis, and the cells often ultimately divide in a bipolar fashion. Indeed, it is thought that many cancer cells in culture have evolved mechanisms to cluster their centrosomes during mitosis so they avoid generating high (and potentially lethal) levels of aneuploidy during every round of cell division (Brinkley, 2001). Thus, the consequences of amplifying centrosomes within the context of a normal developing organism are far from clear.

In flies and humans, the protein kinase SAK/PLK4 plays a critical part in initiating centriole duplication, and the overexpression of this protein can drive centriole overduplication in cells (Betten-court-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007). In this study, we have used stable *Drosophila* transgenic lines overexpressing SAK to drive centrosome amplification in ~60% of somatic cells. This has allowed us to assess the long-term consequences for an organism of having cells with too many centrosomes. Surprisingly, we find that cells with extra centrosomes invariably divide in a bipolar fashion in vivo, and the presence of extra centrosomes does not

generate large-scale genetic instability. The asymmetric division of the neural stem cells (neuroblasts), however, is perturbed, and ~10% of these cells ultimately divide symmetrically. Most importantly, we show that the transplantation of brain cells with too many centrosomes can induce the formation of metastatic tumors in normal hosts.

## RESULTS

### Flies Overexpressing GFP-SAK Have Too Many Centrosomes in ~60% of Their Somatic Cells but Are Viable and Fertile

It has previously been shown that the overexpression of the centriole replication protein SAK/PLK4 leads to the formation of extra centrosomes in cells (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Peel et al., 2007). To analyze the consequences of centrosome amplification within the context of a developing multicellular organism, we analyzed stable transformed *Drosophila* lines that expressed a GFP-SAK fusion protein under the control of the Ubiquitin promoter. This promoter is expressed at moderately high levels in all cells (Lee et al., 1988), and it leads to a dramatic overexpression of centriole duplication proteins, as these proteins are normally expressed at very low levels in cells (Peel et al., 2007). We generated several independent transformed lines, all of which showed similar degrees of centrosome amplification; we analyzed two of these lines in detail. Both lines behaved in an essentially indistinguishable manner in all the experiments reported here, so we simply refer to them as *SAKOE* lines, unless otherwise stated.

Quantification of centrosome number in *SAKOE* third-instar larval brain cells revealed that ~60% of these cells contained more than two centrosomes (Figures 1A–1C), and we obtained similar results in larval imaginal disc cells (data not shown). Note that in this and all subsequent experiments dots were only counted as centrosomes if they were stained by both centriolar and PCM or microtubule (MT) markers. Usually, 3 to 6 centrosomes were present in the cells with extra centrosomes, but cells with higher numbers were occasionally observed. To confirm that these structures were centrosomes, we performed an electron microscopy (EM) analysis of fixed whole-mount brains. In wild-type (WT) brains, two centrioles were identified at each spindle pole ( $n = 4$ ) (Figure 1D). In contrast, half of the spindle poles we examined in *SAKOE* cells contained multiple centrioles ( $n = 9/18$ ) (Figure 1E). Thus, the extra centrosomes we observe in the *SAKOE* cells contain morphologically recognizable centrioles.

We have previously shown that *DSas-4* mutant flies (that lack centrioles and centrosomes) are morphologically normal and are only slightly delayed in development compared to WT flies (Basto et al., 2006). Surprisingly, we found that *SAKOE* flies were also morphologically normal (Figure S1 available online), but they exhibited a much longer delay in development compared to WT and *DSas-4* mutants (Figure 1F). To test if this developmental delay was caused by the presence of extra centrosomes, we overexpressed GFP-SAK in a *DSas-4* mutant background. These flies contained no detectable centrioles (data not shown) and, like *DSas-4* mutants alone, they were only slightly delayed in development (Figure 1F). We conclude

that it is the presence of extra centrosomes in *SAKOE* flies that delays their development.

Despite the delay in development, adult *SAKOE* flies were viable and fertile, although a significant fraction of eggs laid by *SAKOE* females (~60%) died early in development due to an accumulation of mitotic errors (Peel et al., 2007). Nevertheless, transgenic fly lines containing extra centrosomes in ~60% of their somatic cells can be maintained in the laboratory as viable and fertile stocks for many generations (at present we have maintained these stocks for nearly 2 years).

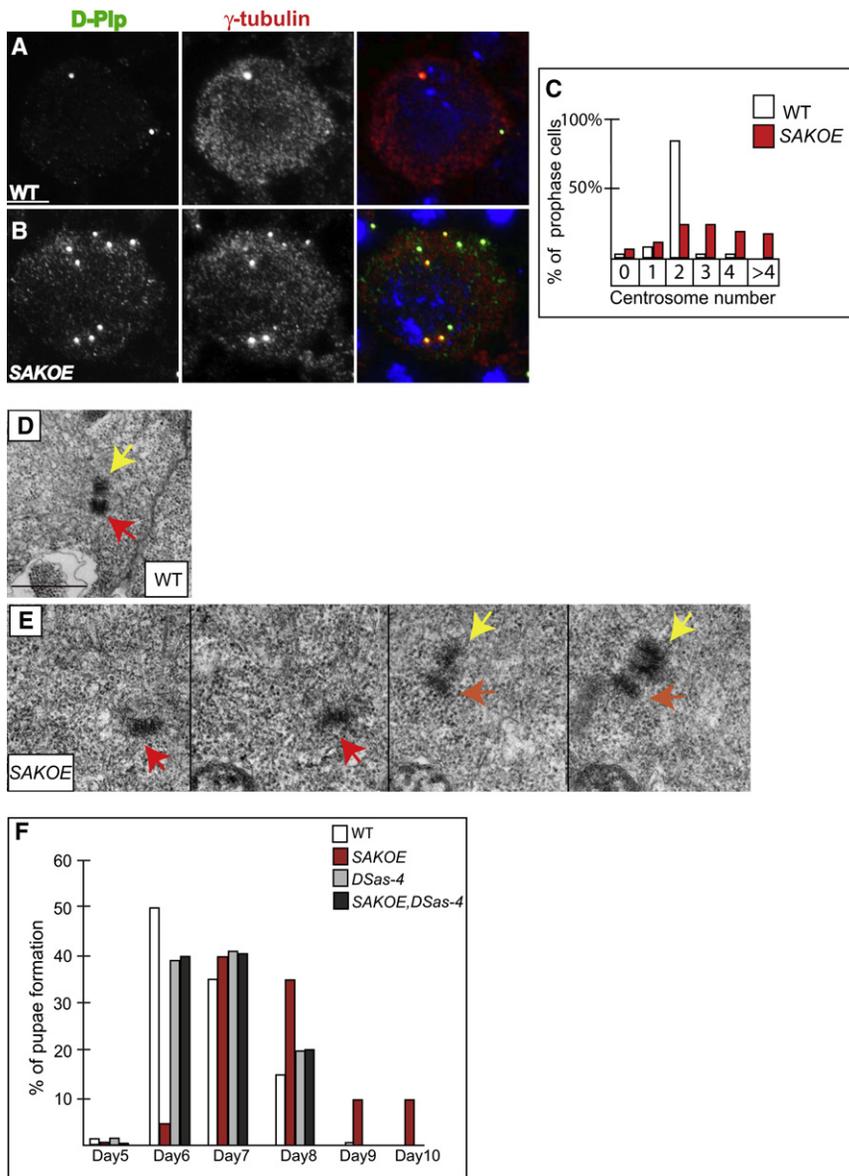
### Mitosis in Cells with Extra Centrosomes

These findings indicate that the presence of extra centrosomes in the majority of somatic cells in an organism is compatible with normal development and long-term survival. This suggests that extra centrosomes do not dramatically interfere with cell division and cell-cycle progression. To better understand how cells divide in the presence of extra centrosomes we examined fixed *SAKOE* third-instar larval brain cells.

It has previously been shown that centrosomes behave asymmetrically in WT neuroblasts, with one centrosome associated with more PCM and nucleating more MTs than the other throughout interphase and during the early stages of mitosis (Rebollo et al., 2007; Rusan and Peifer, 2007). This asymmetry was noticeable during early mitosis in WT neuroblasts (Figures 1A and 2A) but was often not apparent in *SAKOE* neuroblasts with extra centrosomes (Figures 1B and 2B). In these early mitotic cells, all centrosomes were associated with PCM and MTs and the centrosomes were often of different sizes, but it was usually not possible to identify a single “dominant” centrosome, either in terms of PCM recruitment or MT nucleation (Figure 2B). Thus, centrosome asymmetry appears to be disrupted in neuroblasts with extra centrosomes.

In WT brains, 98% of cells ( $n = 250$  cells) formed a bipolar spindle by metaphase (Figure 2C, see also Figure S2). Surprisingly, 93% of cells with extra centrosomes, ( $n = 500$  cells) had also formed a bipolar spindle by metaphase (Figures 2D and S2). Usually, several of the centrosomes were clustered at the poles of the spindle, but we also often observed centrosomes that were not associated with either pole (Figure 2D, see also Figure S3). In metaphase and anaphase cells, the non-pole-associated centrosomes usually contained less PCM than the centrosomes located at the poles, and they were usually not associated with robust asters of MTs (arrows, Figures 2D–2F, see also arrows in Figure 3E), suggesting that they were partially inactivated. Importantly, we made similar observations on the clustering and partial inactivation of extra centrosomes in living *SAKOE* brain cells (Figure S3 and Movies S1–S4).

Surprisingly, the frequency of multipolar and abnormal metaphase spindles was only slightly higher in *SAKOE* cells (2% and 5%, respectively,  $n = 500$  cells) than in WT cells (0% and 2%, respectively,  $n = 250$  cells) (Figure S2). And, by the time cells entered anaphase, the spindles were always bipolar in both WT and *SAKOE* brains ( $n = 200$  cells and  $n = 400$  cells, respectively), while the frequency of aneuploidy was only slightly higher in *SAKOE* brains (1.75%,  $n = 345$  cells) compared to WT (0.7%,  $n = 150$  cells) (Table 1). The mitotic index, however, was significantly higher in *SAKOE* brains ( $2.6\% \pm 0.5\%$ ,  $n = 7797$  cells from 4



**Figure 1. Overexpression of SAK Induces Centrosome Amplification and Delays Development**

(A and B) Immunostaining of G2 wild-type (WT) (A) and SAKOE (B) neuroblasts with the centriole marker D-PLP (left, green in merged panel), the PCM marker  $\gamma$ -tubulin (middle, red in merged panel), and DNA (blue in merged panel). In the WT neuroblast the two centrosomes are asymmetric (see text for details) and only one contains high levels of  $\gamma$ -tubulin. In the SAKOE neuroblast eight centrosomes are present and these contain varying amounts of  $\gamma$ -tubulin.

(C) Quantification of centrosome number in WT (white bars) and SAKOE (red bars) neuroblasts. Note that we only scored D-PLP dots as centrosomes if they also contained some PCM.

(D and E) EM micrographs of selected thin serial sections of WT (D) and SAKOE (E) neuroblast spindle poles. In the WT cell only two centrioles were detected at the spindle pole (red and yellow arrows). In this SAKOE cell three centrioles were detected at the spindle pole (red, yellow, and orange arrows).

(F) A graph showing the percentage of WT (white bars), SAKOE (red bars), *DSas-4* (light gray), and *DSas-4,SAKOE* (dark gray) pupae that formed between the 5th and 10th day of development. More than 95% of SAKOE pupae eclosed as adults, which is similar to WT controls. Scale bar (A and B) = 10  $\mu$ m; (D and E) = 0.5  $\mu$ m.

brains) compared to WT ( $1.9\% \pm 0.3\%$ ,  $n = 12,000$  cells from 4 brains) ( $p < 0.05$ ), indicating that mitosis takes longer than normal in cells that have extra centrosomes (Table 1). Thus, *Drosophila* somatic cells with extra centrosomes are delayed in mitosis but ultimately divide in a bipolar fashion.

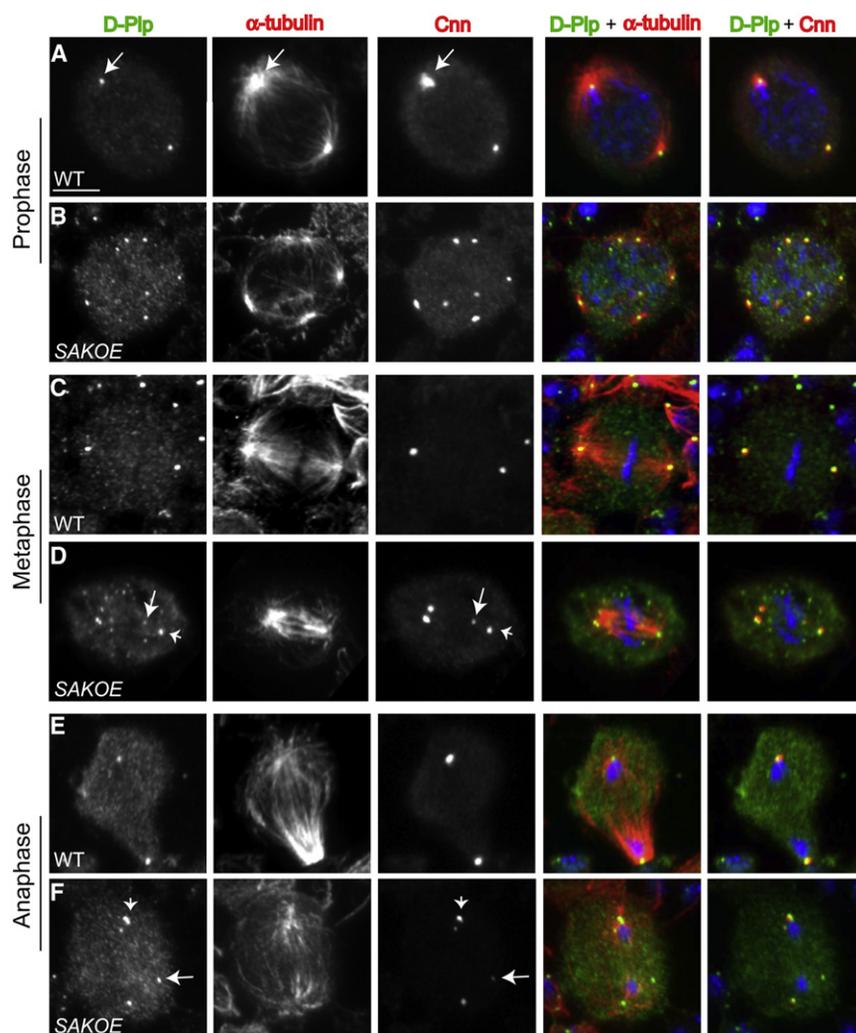
### The Spindle Assembly Checkpoint Is Essential in Flies with Extra Centrosomes

These observations suggested that the spindle assembly checkpoint (SAC) might delay mitosis in cells with extra centrosomes. To test this hypothesis, we crossed the SAKOE lines to *mad2* mutants. Although *Mad2* is an essential component of the SAC, *mad2* mutant flies are viable and fertile, indicating that the SAC is dispensable during unperturbed cell divisions in *Drosophila* (Buffin et al., 2007). The *mad2,SAKOE* flies were synthetically lethal and died as pupae, demonstrating that the SAC is absolutely essential for the viability of flies with extra centro-

somes. The *mad2,SAKOE* larvae developed slightly faster than WT flies, at a rate comparable to *mad2* mutants, and much faster than the SAKOE flies, demonstrating that it is the maintenance of an active SAC that causes the developmental delay in SAKOE flies (Figure 3F). As expected, the mitotic index in *mad2,SAKOE* brains ( $1.7\% \pm 0.5\%$ ;  $n > 2000$  cells) was lower than in WT or SAKOE brains (Table 1), confirming that the presence of extra centrosomes cannot delay mitosis in the absence of *Mad2*. In addition, we noticed a dramatic increase in the number of multipolar and defective spindles in *mad2,SAKOE* brain cells (Figures 3A–3C and S2), and the levels of polyploidy, aneuploidy, and lagging chromosomes during anaphase were also dramatically increased (Table 1). Thus, the SAC is essential in cells with extra centrosomes to prevent spindle multipolarity and the subsequent generation of large-scale genetic instability, presumably because it allows extra time for bipolar spindle formation.

### The Kinesin 14 Ncd Is Required for Efficient Centrosome Clustering

It has previously been shown that the minus-end-directed motor cytoplasmic Dynein plays an essential role in the clustering of supernumerary centrosomes (Quintyne et al., 2005). We tested whether another minus-end-directed motor, Ncd (HSET in



**Figure 2. Mitosis in Cells with Extra Centrosomes**

(A–F) Immunostaining of WT (A, C, and E) and SAKOE (B, D, and F) mitotic neuroblasts with D-PLP (left, green in merged panels),  $\alpha$ -tubulin (2nd panel, red in 4th panel), Cnn (3<sup>rd</sup> panel, red in 5<sup>th</sup> panel), and DNA (blue in merged panels). In the WT prophase cell (A), the arrow highlights the dominant centrosome that contains more PCM and nucleates more microtubules (MTs). In the SAKOE prophase cell (B), seven centrosomes are present, but there is no single dominant centrosome. In the WT metaphase (C) and anaphase (E) cells, a centrosome is located at each pole of the spindle. In the SAKOE metaphase (D) and anaphase (F) cells, several centrosomes are clustered at the poles of each spindle (arrowheads) while others are not (arrows). The centrosomes that are not clustered at the spindle poles appear to contain less Cnn and are not associated with robust astral MTs (compare this to the situation in the prophase cell shown in B). Scale bar = 10  $\mu$ m.

#### Extra Centrosomes Lead to Abnormalities in the Asymmetric Divisions of Larval Neuroblasts

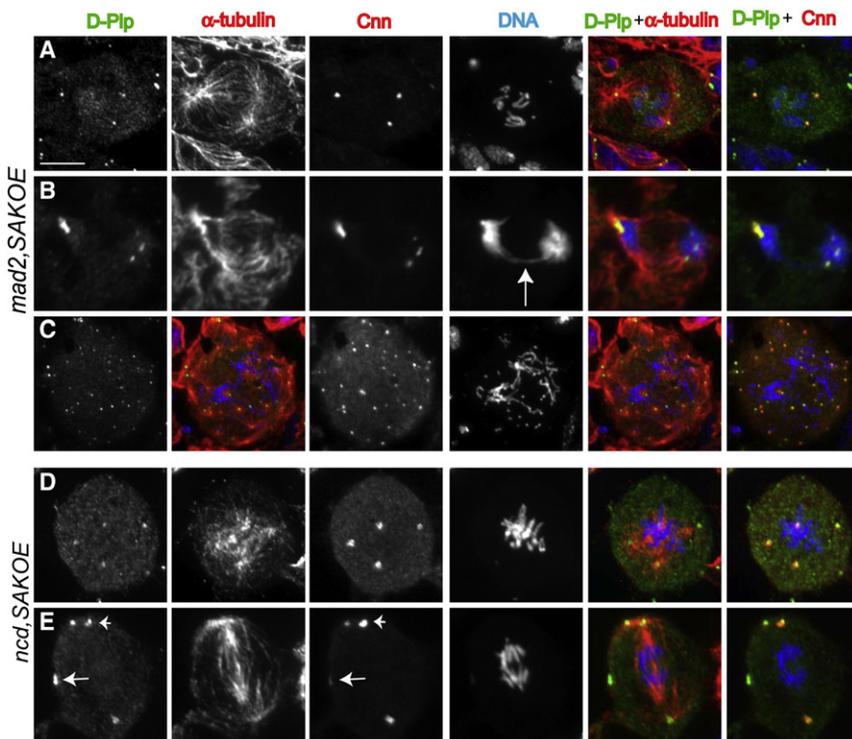
While centrosomes are not essential for somatic cell division (Basto et al., 2006; Hinchcliffe et al., 2001; Khodjakov et al., 2000; Uetake et al., 2007), the astral MTs generated by centrosomes seem to have a particularly important role during asymmetric divisions, when the spindles must align correctly with cortical polarity cues (Yu et al., 2006 and Siegrist and Doe, 2005). In *DSas-4* mutant neuroblasts (that completely lack centrosomes), the anastral spindles have great difficulty in aligning properly with cortical

vertebrates), could also be important for this process. Ncd is required for the efficient focusing of the spindle poles in fly cells (Goshima et al., 2005), but its activity is not absolutely essential in somatic cells and *ncd* mutants are viable (Endow and Komma, 1998; Skold et al., 2005). We expressed GFP-SAK in an *ncd* mutant background and found that the developmental rate of *ncd,SAKOE* flies was even slower than that of the *SAKOE* flies (Figure 3F), although the flies that hatched were all morphologically normal (data not shown). The *ncd,SAKOE* brains showed a dramatic increase in the rate of spindle multipolarity during prophase and metaphase (Figures 3D and S2), but we still never observed any multipolar spindles during anaphase (Figure 3E,  $n = 230$  cells). The levels of aneuploidy, polyploidy, and lagging chromosomes during anaphase were all slightly elevated in *ncd,SAKOE* brains compared to *SAKOE* or *ncd* mutant brains alone, while the mitotic index was much higher (Table 1). We conclude that cells with too many centrosomes have great difficulty in organizing a bipolar spindle in the absence of Ncd; nevertheless, these cells can still delay the exit from mitosis until bipolarity is eventually achieved.

cues and ~16% of neuroblasts ultimately divide symmetrically (Basto et al., 2006).

To test whether cells with extra centrosomes have defects in asymmetric cell division we initially examined the localization of the apical marker aPKC and the basal marker Miranda (Mira). In the majority of WT and *SAKOE* neuroblasts (91%,  $n = 80$  and 79%,  $n = 150$ , respectively) the aPKC and Mira crescents were correctly localized on opposite sides of the neuroblasts (Figures S4A–S4C and S4E). We noticed, however, that in some *SAKOE* neuroblasts the cortical proteins were either misaligned or delocalized from the cortex (Figures S4D and S4E), while the metaphase plate was also sometimes not oriented correctly with respect to the polarity axis (Figure S4C).

To characterize this alignment defect in more detail, we stained *SAKOE* third-instar larval neuroblasts with anti- $\alpha$ -tubulin antibodies and determined spindle orientation relative to the cortical aPKC crescent. In 95% ( $n = 50$ ) of WT neuroblasts the spindle was aligned within 30° of the center of the aPKC apical crescent (Figures 4A and 4E), but this was true in only 60% ( $n = 98$ ) of *SAKOE* neuroblasts that contained extra centrosomes (Figures

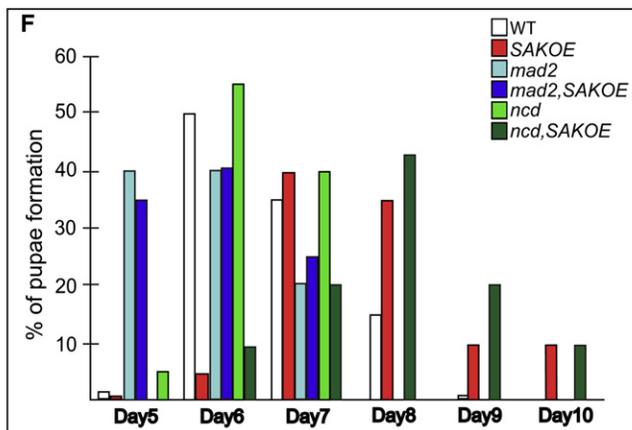


**Figure 3. Mad2 and Ncd Are Required for the Suppression of Spindle Multipolarity**

(A–C) Immunostaining of *mad2,SAKOE* mitotic neuroblasts with D-PLP (left, green in merged panels),  $\alpha$ -tubulin (2nd panel, red in 5th panel), Cnn (3rd panel, red in 6th panel), and DNA (blue in merged panels). For comparison with WT controls see Figures 2A and 2E. Tripolar anaphases (A), anaphases with lagging chromatids (arrow in B), and polyploid cells (C) are often seen in *mad2,-SAKOE* brains but very rarely in *SAKOE* neuroblasts (see Table 1). Note that *mad2,SAKOE* neuroblasts can also contain much larger numbers of extra centrosomes than is ever seen in *SAKOE* cells (~30 in the cell shown here).

(D and E) Immunostaining of *ncd,SAKOE* mitotic neuroblasts with D-PLP (left panel, green in merged panels),  $\alpha$ -tubulin (2nd panel, red in 4th panel), Cnn (3rd panel, red in 5th panel), and DNA (blue in merged panels). For comparison with WT control neuroblasts see Figures 2C and 2E. In *ncd,SAKOE* neuroblasts multipolar metaphases can be detected (D) but almost all anaphases are bipolar (E). Scale bar = 10  $\mu$ m.

(F) A graph showing the percentage of WT (white bars), *SAKOE* (red bars), *mad2* (light blue), *mad2,-SAKOE* (dark blue), *ncd* (bright green), and *ncd,SAKOE* (dark green) pupae that formed between the 5th and 10th days of development. Note that the WT and *SAKOE* data shown here are the same as that shown in Figure 1A, as these experiments were performed at the same time.



4B and 4E). Moreover, a significant fraction of spindles in *SAKOE* neuroblasts with extra centrosomes (15%,  $n = 98$ ) were organized at right angles to the aPKC crescent (Figures 4C and 4E),

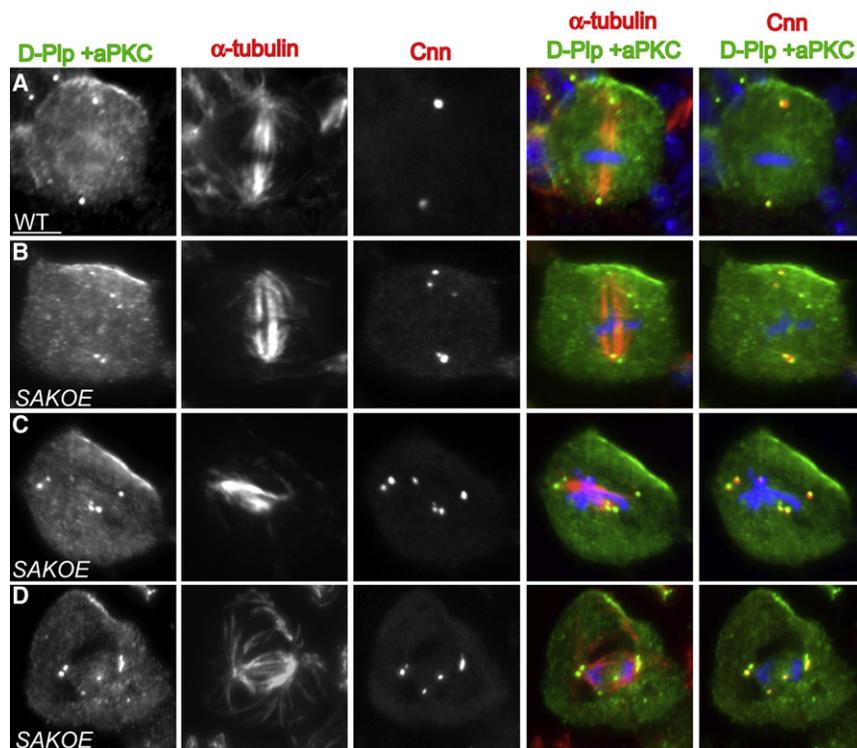
**Table 1. Quantification of Mitotic Defects**

	Mitotic Index	% Polyploidy	% Aneuploidy	%Anaphase with Lagging Chromatids
WT	1.9 ± 0.3	ND	0.7%	ND
SAKOE	2.6 ± 0.5	ND	1.75%	ND
<i>mad2</i>	1.6 ± 0.5	ND	0.6%	0.5%
<i>mad2,SAKOE</i>	1.7 ± 0.5	12%	10.0%	12.0%
<i>ncd</i>	2.2 ± 0.2	ND	1.0%	1.0%
<i>ncd,SAKOE</i>	5.2 ± 0.3	3.0%	4.5%	4.0%

ND: not detected.

more significant problems in aligning their spindles correctly with these cues.

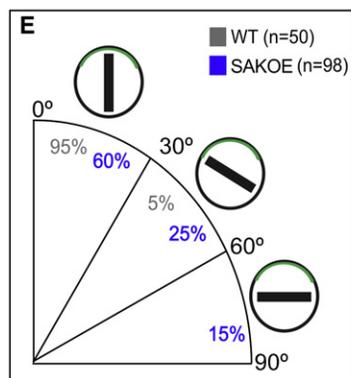
To investigate why neuroblasts with extra centrosomes have trouble properly aligning their spindles, we examined the distribution of Mud, the *Drosophila* homolog of NuMA. This protein has been implicated in spindle positioning, and it is normally concentrated at centrosomes, with the apical centrosome usually containing more Mud than the basal centrosome (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006) (Figure S6A). In neuroblasts with extra centrosomes, however, the asymmetric centrosome staining of Mud was usually not evident, and, in many *SAKOE* neuroblasts (20%,  $n = 90$ ), very little Mud could be detected at any of the centrosomes (Figures S6B and S6C). Thus, the centrosomal localization of Mud is perturbed in cells with extra centrosomes, potentially explaining, at least in part, why spindle orientation is perturbed.



**Figure 4. Asymmetric Cell Division Is Perturbed in Neuroblasts with Extra Centrosomes**

(A–D) Immunostaining of WT (A) and SAKOE (B–D) neuroblasts with D-PLP and aPKC (left panel, green in merged panels),  $\alpha$ -tubulin (2nd panel, red in 4th panel), Cnn (3rd panel, red in 5th panel), and DNA (blue in merged panels). In WT metaphase neuroblasts (A) the spindle is always aligned with the polarity axis (defined by the cortical aPKC crescent), as is the case in  $\sim$ 60% of SAKOE neuroblasts with extra centrosomes (B). In  $\sim$ 40% of SAKOE neuroblasts with extra centrosomes (C and D), however, the spindle fails to align properly with the aPKC crescent; spindle misalignment is also detected in some anaphase cells (D).

(E) Quantification of mitotic spindle alignment in WT and SAKOE neuroblasts that have extra centrosomes. In the schematic diagram of cells, the green crescent represent aPKC while the black bars represent the mitotic spindle. Scale bar = 10  $\mu$ m.



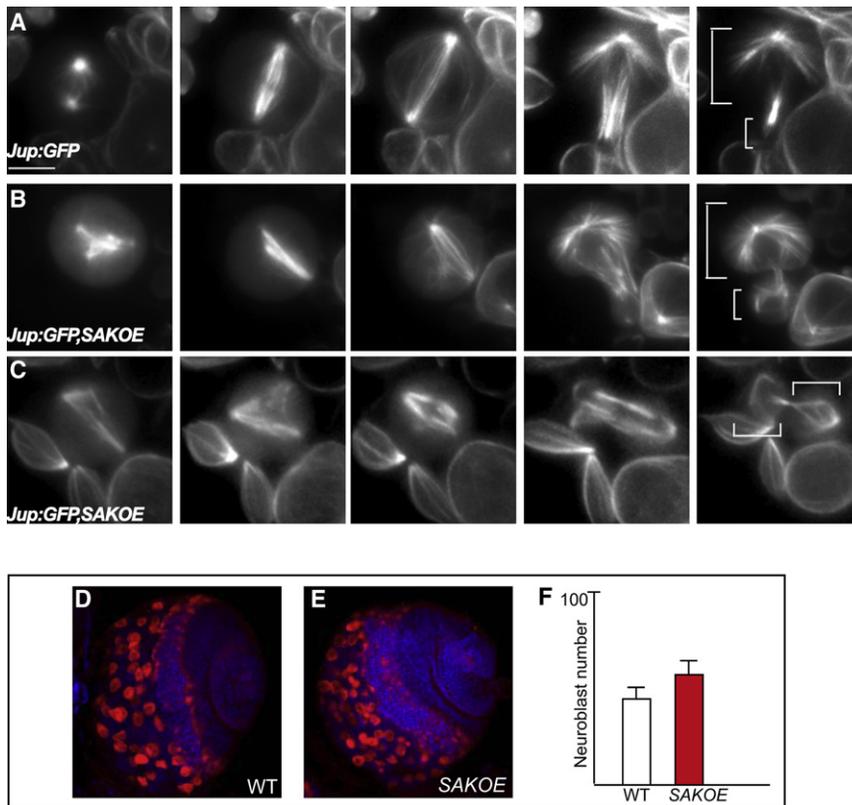
In *Drosophila* neuroblasts redundant mechanisms cooperate to ensure that the process of asymmetric division is extremely robust, and although many mutants have initial defects in aligning their spindles with cortical cues, most mutant cells ultimately divide asymmetrically (Bowman et al., 2006; Izumi et al., 2006; Siegrist and Doe, 2005; Siller et al., 2006). To test whether SAKOE neuroblasts ultimately divided asymmetrically we followed the behavior of the MT-associated protein Jupiter::GFP (Karpova et al., 2006) in living neuroblasts. As described above, multipolar spindles were often seen at early stages of mitosis (Figures 5B and 5C), but these usually resolved themselves into a bipolar spindle, and the cells divided asymmetrically ( $\sim$ 91%,  $n = 46$ , Figure 5B). In  $\sim$ 9% of cases, however, the cells with extra centrosomes divided symmetrically (Figure 5C), something we never observed in WT brains (Figure 5A,  $n = 30$ ) (Basto et al., 2006).

To test whether these asymmetric division defects could lead to an amplification of the neuroblast pool, we counted the number of central brain neuroblasts in WT and SAKOE brains (Figures 5D–5F). The number of neuroblasts in SAKOE brain lobes ( $55 \pm 10.4$ ,  $n = 40$  lobes from 20 animals) was slightly, but significantly ( $p < 0.01$ ), increased when compared to WT brains ( $45 \pm 9.4$ ,  $n = 40$  lobes, from 20 animals). Thus, the presence of extra centrosomes leads to an expansion in the number of stem cells in larval brains.

### Centrosome Amplification Can Initiate Tumorigenesis

A long-standing question in cancer biology has been whether the presence of extra centrosomes within a tissue can drive tumorigenesis. SAKOE adult flies showed no obvious evidence of tumor growth (data not shown), but this is not surprising as there is little cell division in adult flies and very few mutations give rise to tumors in adults. To overcome this potential problem, transplantation assays have been developed where larval imaginal discs or brains are transplanted into the abdomen of WT adult hosts (Caussinus and Gonzalez, 2005; Gonzalez, 2007; Woodhouse et al., 1998). Transplanted WT tissue can survive in adult hosts for several weeks without overproliferating or forming tumors. In contrast, the transplantation of tissue from several mutants leads to tissue overproliferation and the formation of metastatic tumors within the WT host.

To test whether SAKOE larval brains could form tumors when transplanted into WT hosts we expressed  $\alpha$ -Tubulin-GFP (*Tub-GFP*) in SAKOE flies so that we could follow the behavior of the transplanted tissue. We used *brain tumor (brat)* mutant brains as a positive control (Betschinger et al., 2006; Bello et al., 2006; Lee



**Figure 5. Symmetric Divisions in SAKOE Neuroblasts**

(A–C) The dynamics of Jupiter::GFP (Jup::GFP) in living WT (A) and SAKOE (B and C) neuroblasts. WT neuroblasts always divide asymmetrically to produce two cells of different sizes. In neuroblasts with extra centrosomes both asymmetric (B) and symmetric divisions (C) are observed.

(D–F) Quantification of the number of neuroblasts in the central brain region. Immunostaining of WT (D) and SAKOE (E) brain lobes with Miranda (red) and Hoechst for labeling DNA (blue). (F) A graph showing the average number of neuroblasts in WT (white bars) and SAKOE (red bars) central brain lobes. Scale bar (A–C) = 5  $\mu$ m. Error bars represent standard deviation (SD).

### Cell Division with Extra Centrosomes

Our observations reveal that, in vivo, the presence of extra centrosomes does not lead to large-scale genetic instability in somatic cells. As cells with extra centrosomes enter mitosis, most of the centrosomes are active and nucleate robust asters of MTs. As mitosis proceeds, however, many of the extra centrosomes become clustered together to form two

dominant poles that assemble a bipolar mitotic spindle. This phenomenon of centrosome clustering has been described in several systems (Murphy, 2003; Quintyne et al., 2005; Ring et al., 1982). In addition, however, we find that many extra centrosomes do not become clustered at the spindle poles. Instead, these extra centrosomes appear to be gradually inactivated, and they organize less PCM and nucleate fewer MTs as mitosis proceeds. We do not understand the mechanism of this inactivation, but it could result from a competition for limiting supplies of PCM components. Perhaps the centrosomes that cluster together can communally organize more PCM and so nucleate more MTs than isolated centrosomes. This would then provide a negative feedback loop as mitosis progresses so that, eventually, the isolated centrosomes are inactivated. Whatever its mechanism, the inactivation of isolated centrosomes ensures that they do not form extra spindle poles efficiently.

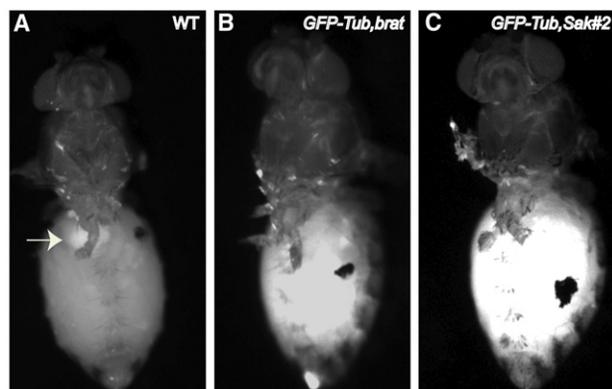
Cells with extra centrosomes are delayed in mitosis, and this delay is maintained by the SAC. It is possible that the presence of extra centrosomes somehow directly maintains the activity of the SAC, although previous reports suggest that this is not the case (Sluder et al., 1997). We suspect, therefore, that sister chromatids may be inefficiently aligned on multipolar spindles, and these improperly attached kinetochores ensure the maintenance of SAC activity until a bipolar spindle has formed.

The SAC is not normally essential for fly development, as *mad2* mutant flies lack the SAC but are viable and fertile (Buffin et al., 2007). Flies with too many centrosomes, however, completely depend on the SAC as *mad2,SAKOE* flies exhibit high levels of spindle multipolarity and genetic instability and do not

et al., 2006b). We did not detect any overproliferation when *Tub-GFP*, WT brains were transplanted into WT hosts (Figures 6A and 6E,  $n = 90$ ), but 36% of *Tub-GFP*, *brat* brains formed tumors (Figures 6B and 6E,  $n = 50$ ) and several of the injected hosts went on to develop one or more GFP-positive metastases far from the original site of injection ( $n = 11/18$ ). We found that 14% of *Tub-GFP*, *SAKOE#2* brains ( $n = 104$ ) and 20% of *Tub-GFP*, *SAKOE#1* brains ( $n = 60$ ) formed tumors when transplanted into WT hosts (Figures 6C and 6E), and several of these hosts went on to develop one or more GFP-positive metastases far from the original site of injection (Figure 6D,  $n = 5/15$  for *SAKOE#2*, and  $n = 6/20$  for *SAKOE#1* brains). The overproliferation of either the *Tub-GFP*, *brat*, *Tub-GFP*, *SAKOE#2*, or *Tub-GFP*, *SAKOE#1* tissues invariably led to the premature death of the WT host within 10 to 15 days of injection.

### DISCUSSION

Here we have examined the long-term consequences of having too many centrosomes in a complex multicellular organism. Surprisingly, we find that flies with extra centrosomes are viable, fertile, and can be maintained in the laboratory as a stable diploid stock for many generations. We show that cells with extra centrosomes almost invariably divide in a bipolar fashion in vivo, and the presence of extra centrosomes does not generate large-scale genomic instability. Nevertheless, tissues with extra centrosomes have the ability to overproliferate and form tumors when transplanted into WT hosts. We conclude that centrosome amplification is sufficient to promote tumorigenesis in flies.

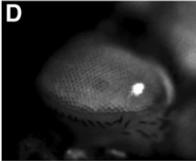


**Figure 6. Transplantation of SAKOE Brain Tissue Induces Tumor Formation in WT Hosts**

(A–C) Pictures of WT hosts transplanted with either *Tub-GFP,WT* (A), *Tub-GFP,brat* (B), or *Tub-GFP,SAKOE* (C) brains at 14 days post-injection. (A) The GFP fluorescence of the WT brain is still detectable in the host, but the brain has not detectably overproliferated (arrow). The GFP fluorescence of the *brat* (B) and SAKOE transplanted brains (C) has expanded to fill the abdomen of the host.

(D) Picture of a GFP-positive metastasis in the eye of a WT host.

(E) A table showing the frequency of tumor and metastases formation in WT hosts transplanted with the different types of brain tissue.



E	GFP-Tub WT	GFP-Tub <i>brat</i>	GFP-Tub <i>Sak#2</i>	GFP-Tub <i>Sak#1</i>
n° of tumors	0	18	15	12
% tumor formation	----	36%	14%	20%
Hosts injected	90	50	104	60

survive to adulthood. Thus, the SAC is essential to allow enough time for cells with extra centrosomes to organize bipolar spindles. Interestingly, while SAKOE flies are severely delayed in development, the *mad2,SAKOE* flies develop faster than WT flies, indicating that the SAC-dependent delay in mitosis also slows the development of SAKOE flies.

It has previously been shown that centrosome clustering in cells with extra centrosomes is dependent on the activity of dynein (Quintyne et al., 2005). Here we show that another minus-end-directed motor, Ncd (HSET in vertebrates), also plays a role in this process. Ncd is not essential for *Drosophila* development, and *ncd* mutants develop at normal rates with few mitotic defects (Endow and Komma, 1998; Skold et al., 2005; this work). We found that *ncd,SAKOE* flies were severely delayed in development, exhibited elevated levels of spindle multipolarity during early mitosis, had a dramatically increased mitotic index, but ultimately divided in a bipolar fashion. We conclude that Ncd enhances the efficiency of bipolar spindle formation in cells with extra centrosomes, but it is not absolutely essential, and the SAC ensures that these cells do not exit mitosis until they have formed a bipolar spindle.

### Extra Centrosomes in Nonsomatic Tissues

The development of flies with extra centrosomes in their somatic tissues is delayed but otherwise appears to proceed normally. It is known, however, that the role of the centrosome differs between embryonic and somatic tissues in *Drosophila*. While somatic fly cells can tolerate the absence of centrosomes, these organelles are essential for early embryonic development in flies (Dix and Raff, 2007; Stevens et al., 2007; Varmark et al., 2007). Although SAKOE flies are viable and fertile, we note that ~60% of SAKOE embryos accumulate mitotic defects and die during early embryonic development (Peel et al., 2007). Moreover, the mechanisms that ensure bipolar spindle formation in the presence of extra centrosomes may be absent in male germ cells; the presence of extra centrioles or centriole frag-

ments in these cells leads to the formation of multipolar spindles and to male sterility (Dix and Raff, 2007; Martinez-Campos et al., 2004). The overexpression of SAK does not lead to centrosome amplification in male germ cells (Peel et al., 2007), presumably explaining why

SAKOE male flies are fertile. Interestingly, we could not detect any uncoordinated behavior in flies with extra centrosomes, suggesting that cilia assembly and function are unaffected by the presence of extra centrioles (Baker et al., 2004; Basto et al., 2006; Martinez-Campos et al., 2004).

### Centrosome Amplification and Tumor Formation

We find that brain cells with extra centrosomes can form tumors when injected into WT adult flies. The pathways that lead to tumor formation are complex, and the events that initiate this process remain controversial. Our work shows, however, that centrosome amplification is sufficient to initiate tumorigenesis in the fly.

It is not clear how centrosome amplification initiates tumor formation. Boveri originally hypothesized that extra centrosomes might promote tumorigenesis by promoting genetic instability. The nature of the link between aneuploidy and cancer, however, remains controversial. Recently, it has been shown that increased levels of aneuploidy in mouse models can promote tumor formation in certain tissues at later stages in life but suppress tumor formation upon exposure to certain carcinogens or upon the loss of particular tumor suppressor genes (Sotillo et al., 2007; Weaver et al., 2007). Thus, aneuploidy does not invariably lead to cancer formation. In SAKOE brains the rate of aneuploidy is low, although it is higher than that observed in WT brains (1.75% compared to 0.7%, respectively). It is possible that this modest increase in aneuploidy could allow cells with extra centrosomes to initiate tumor formation in flies.

Alternatively, previous studies in flies have shown that there is a correlation between defects in the asymmetric divisions of larval neural stem cells (neuroblasts) and the ability of injected mutant brain tissue to form tumors when transplanted into WT hosts (Caussinus and Gonzalez, 2005; Woodhouse et al., 1998). Defective asymmetric divisions can result in the expansion of the neuroblast population, which ultimately leads to overproliferation (Bello et al., 2006; Betschinger et al., 2006;

Lee et al., 2006a, 2006b; Yu et al., 2006). We find that the asymmetric division of neuroblasts is perturbed in *SAKOE* brains, and this leads to an expansion of the neuroblast population—a defect that could allow *SAKOE*-injected brains to overproliferate and form tumors. Indeed, there is much interest in the idea that mutations in stem cells could be central to the generation of cancer (Al-Hajj and Clarke, 2004; Gonzalez, 2007). Importantly, although the increase in aneuploidy and the defects in asymmetric division are only seen in *SAKOE* cells that have extra centrosomes, we cannot rule out the possibility that SAK overexpression induces tumors via some other mechanism that is unrelated to centrosome amplification. Indeed, mice that are heterozygous for SAK have a variety of cell-cycle defects and have an increased incidence of spontaneous tumor formation (Swallow et al., 2005).

Our observations have important implications for understanding the potential link between centrosomes and cancer. In the literature it is often stated as fact that the presence of extra centrosomes in cells generates genetic instability. This assumption is based on the observation that extra centrosomes clearly lead to spindle multipolarity and genetic instability in some systems (Brinkley and Goepfert, 1998; Wunderlich, 2002) and the strong association between these two phenotypes in many cancer cells (D'Assoro et al., 2002a, 2002b; Fukasawa, 2005; Lingle et al., 1998, 2002; Lingle and Salisbury, 2000; Nigg, 2006; Pihan et al., 1998, 2001, 2003; Saunders, 2005). Our data demonstrate, however, that the presence of extra centrosomes does not inevitably lead to genetic instability *in vivo*, at least in a relatively simple organism like *Drosophila*. Instead, extra centrosomes are reasonably well tolerated in flies because several pathways cooperate to ensure that these cells ultimately divide in a bipolar fashion. Only when one or more of these pathways is compromised is large-scale genetic instability generated.

These findings highlight the possibility that the presence of extra centrosomes could prove to be an “Achilles heel” for many different cancers. Fly cells with too many centrosomes are viable, but they are much more reliant on certain pathways (such as the SAC) or proteins (such as Ncd) for their survival than normal cells. It seems plausible that inhibiting these pathways in cancer patients could effectively kill the cancer cells, while leaving normal cells relatively unharmed.

## EXPERIMENTAL PROCEDURES

### Generation of Transgenic Lines

*P*-element-mediated transformation vectors were generated by amplifying the complete SAK coding region from a full-length cDNA with *att* sites at either end for Gateway cloning (Invitrogen). PCR products were inserted into the Gateway pDONR Zeo vector and sequenced. This vector was recombined with the pUbq-GFPNT Gateway vector (R.B., unpublished data) to place full-length SAK with GFP at its N terminus under the control of the Ubiquitin (Ubq) promoter.

### Fly Stocks

We used *w<sup>67</sup>* and *wf* stocks as controls in our experiments. The majority of experiments described here were performed with two transgenic lines: Ubq-GFP-SAK#1 and Ubq-GFP-SAK#2. We recombined Ubq-GFP-SAK#2 with the *DSas-4<sup>S2214</sup>* (Basto et al., 2006), *mad2<sup>C</sup>* (Buffin et al., 2007), and *ncd<sup>1</sup>* (Endow and Komma, 1998) mutations by standard recombination methods. For

live-cell imaging we crossed the following markers into the appropriate genetic backgrounds: Ubq-RFP- $\alpha$ -Tubulin (a gift from Saskia Suijkerbuijk and Jeroen Dobbelaere), Ubq-GFP-*DSas-4* (Peel et al., 2007), and a MT-associated protein tagged to GFP, Jupiter:GFP (Karpova et al., 2006). Measurements of growth and survival rates were performed as previously described (Basto et al., 2006).

### Antibodies

The following antibodies were used in this study: rabbit anti-*DSas-4* (Basto et al., 2006), rabbit anti-D-PLP (Martinez-Campos et al., 2004), rabbit anti-Cnn (Lucas and Raff, 2007) (all at 1–2  $\mu$ g/ml final concentration), guinea pig anti-Cnn (1:500, E. Lucas and J.W.R., unpublished data), rabbit anti-Mud (1:250) (Izumi et al., 2006), mouse anti-Miranda (1:20) (Ikeshima-Kataoka et al., 1997), rabbit anti-aPKC (1:500, SC-216, Santa Cruz Biotechnology, inc.), mouse anti- $\gamma$ -tubulin (1:1000; GTU88, Sigma), mouse anti- $\alpha$ -tubulin (1:1000; DM1a, Sigma), rabbit anti-phospho-Histone3 (1:2000, Upstate Biotechnology). All fluorescent secondary antibodies were obtained from Molecular Probes (Invitrogen).

### Immunofluorescence Analysis of Brains

Brains were dissected and fixed as described previously (Martinez-Campos et al., 2004). Preparations were examined using either a Zeiss Axioskop II microscope with a CoolSnapHQ camera (Photometrics) with Metamorph software (Molecular Devices Corp.), or a Zeiss LSM 510 Meta scanning confocal system mounted on a Zeiss Axiophot II microscope, or 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. Spindles were classified as multipolar when more than two centrosomes (revealed by costaining of D-Plp and Cnn) organized  $\alpha$ -tubulin foci. Mitotic spindles were classified as abnormal when it was not possible to identify clearly spindle poles and/or when the morphology of the spindle was very disorganized.

The levels of aneuploidy and polyploidy were calculated in third-instar larval brain squashes as described previously (Basto et al., 2006). The mitotic index was calculated by fixing and squashing four brains of the appropriate genotype and then staining them with anti-phospho-histone H3 antibodies and Hoechst. Pictures of 5–10 fields of cells (typically containing 200–400 total cells per field) were obtained from each brain, and the ratio of mitotic (phospho-histone H3 positive) to nonmitotic cells was calculated for each field. Each field analyzed was counted as an “event” and thus used to calculate the standard deviation and the significance of the difference between the different datasets (p value) using the two-tailed t test function in Excel.

Analysis of spindle position relative to the apical aPKC crescent position was performed by staining fixed brains with anti-aPKC, anti-DPLP, anti- $\alpha$ -tubulin, and anti-Cnn antibodies. For *SAKOE* neuroblast analysis, we only scored mitotic cells with more than two centrosomes and where an aPKC crescent could be clearly distinguished. The angle between the spindle axis and the aPKC crescents was determined using the measurement tool in Metamorph. The data were analyzed for statistical significance using the two-tailed t test function in Excel.

Previous studies with anti-Mud antibodies described Mud localization at centrosomes and also at the apical cortex (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). In our hands, we could not detect Mud at the apical cortex, presumably because we used different fixation conditions designed to allow us to quantify centrosome number in the *SAKOE* cells.

Neuroblast numbers were obtained by counting the number of Miranda-positive central brain neuroblasts in 40 WT and 40 *SAKOE* brain lobes. From the 40 WT brain lobes we found a minimum and maximum number of 30 and 69 neuroblasts, respectively, with an average of 45 (SD  $\pm$  9.4). From the 40 *SAKOE* brain lobes we found a minimum and maximum number of 41 and 78 neuroblasts, respectively, with an average of 55 (SD  $\pm$  10.4). The significance of the difference between the two datasets was assessed using the two-tailed t test function in Excel.

## SUPPLEMENTAL DATA

Supplemental Data include Experimental Procedures, six figures, and four movies and can be found with this article online at <http://www.cell.com/cgi/content/full/133/6/1032/DC1/>.

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