Centrosome loss or amplification does not dramatically perturb global gene expression in Drosophila

Janina Baumbach¹, Mitchell P. Levesque²,* and Jordan W. Raff¹,‡

¹Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK
²Max Planck Institute for Developmental Biology, Spemannstrasse 35, 72076 Tübingen, Germany
*Present address: University of Zurich Hospital, Gloriastrasse 31, CH-8091 Zurich, Switzerland
‡Author for correspondence (jordan.raff@path.ox.ac.uk)

Summary
Centrosome defects are a common feature of many cancers, and they can predispose fly brain cells to form tumours. In flies, centrosome defects perturb the asymmetric division of the neural stem cells, but it is unclear how this might lead to malignant transformation. One possibility is that centrosome defects might also perturb cellular homeostasis: for example, stress pathways are often activated in response to centrosome defects in cultured cells, and stress contributes to tumourigenesis in some fly models. Here we attempt to assess whether centrosome loss or centrosome amplification perturbs cell physiology in vivo by profiling the global transcriptome of Drosophila larval brains and imaginal discs that either lack centrosomes or have too many centrosomes. Surprisingly, we find that centrosome loss or amplification leads to few changes in the transcriptional profile of these cells, indicating that centrosome defects are surprisingly well tolerated by these cells. These observations indicate that centrosome defects can predispose fly brain cells to form tumours without, at least initially, dramatically altering their physiology.

© 2012. Published by The Company of Biologists Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial Share Alike License (http://creativecommons.org/licenses/by-nc-sa/3.0).

Key words: Centrosome defects, Centrosome amplification, Tumours

Introduction
Centrosomes are the major microtubule (MT) organising centres (MTOCs) in many cell types and they are widely believed to have an important role in organising many cell processes such as cell division, the establishment and maintenance of cell polarity and the positioning of organelles within the cell. Centrosomes are also thought to act as “coordination centres” for various cellular pathways, as many cell cycle regulators and checkpoint proteins are concentrated at centrosomes (Hsu and White, 1998; Löffler et al., 2007; Tritarelli et al., 2004). Not surprisingly, centrosome defects have been implicated in a wide range of human diseases, most notably cancer, but more recently also in brain development and in conditions associated with defects in DNA damage repair (Alkuraya et al., 2011; Bakircioglu et al., 2011; Bond and Woods, 2006; Doxsey et al., 2005; Hsu and White, 1998; Lingle et al., 2002; Löffler et al., 2007; Pihan et al., 2003; Takada et al., 2007; Tritarelli et al., 2004).

Given their general importance, the recent demonstration that flies can proceed through the majority of development without centrosomes or with amplified centrosomes in the majority of their cells was very surprising (Basto et al., 2008; Basto et al., 2006). Somatic cell divisions were slowed when centrosomes were missing or amplified (as cells took longer than normal to assemble a bipolar spindle) but almost all cells appeared to ultimately divide normally in a bipolar fashion, although there was a small, but significant, increase in chromosome segregation errors in these cells. In contrast, asymmetically dividing larval neuroblasts (NBs) appeared to have considerable difficulty in dividing accurately when centrosomes were either absent or amplified. These large stem-cell-like progenitors normally divide asymmetrically to generate another self-renewing NB, and a smaller ganglion mother cell (GMC) that usually divides only a few more times before terminally differentiating into either a neuron or glial cell (Doe, 2008; Knoblich, 2008). In NBs that either lack centrosomes or have too many centrosomes this division is symmetric ~10–15% of the time (Basto et al., 2008; Basto et al., 2006). These symmetric divisions appear to give rise to two NB-like daughters (Basto et al., 2008; Lee et al., 2006a; Wang et al., 2006).

Intriguingly, brain cells that either lack or have too many centrosomes are predisposed to form tumours in abdominal transplantation assays (Basto et al., 2008; Castellanos et al., 2008). It is widely believed that this tumourigenesis is driven by the failure in asymmetric NB divisions (Castellanos et al., 2008; Gonzalez, 2007), and, in support of this possibility, mutations in genes encoding the asymmetrically distributed neural cell fate determinants Numb, Prospero and Brat also lead to tumourigenesis (Bello et al., 2006; Betschinger et al., 2006; Caussinus and Gonzalez, 2005; Lee et al., 2006a; Lee et al., 2006b; Wang et al., 2006). While a failure in asymmetric division
can clearly lead to an expansion of the NB pool and so to over-proliferation, it is unclear how centrosome malfunction promotes malignant transformation. One possibility is that the relatively mild chromosome segregation defects induced by centrosome loss or amplification could facilitate the accumulation of mutations (Basto et al., 2008; Basto et al., 2006); however, mutations causing large-scale genome instability do not lead to brain tumour induction (Castellanos et al., 2008). In addition, it has been suggested that transcriptional and/or epigenetic changes may be required to promote malignant transformation in these brains (Knoblich, 2010). Interestingly, centrosome defects are known to increase cellular stress in vitro (Mikule et al., 2007; Srsen et al., 2006; Uetake et al., 2007), and stress can promote tumorigenesis in at least some Droso phila tumour models (Rossi and Gonzalez, 2011; Wu et al., 2010).

Thus, although flies with centrosome defects appear capable of progressing through most of development relatively normally, it remains possible that cell physiology could be significantly perturbed by these defects (Gonzalez, 2008). Here, we have attempted to address how centrosome loss or amplification might perturb cell physiology in vivo. We reasoned that any stress induced by these centrosome defects would likely induce physiological changes, at least some of which should be detectable as transcriptional changes. An analysis of global gene expression would, therefore, be a reasonable first readout of cell physiology. Indeed, many other Dro so phila tumour models such as l(3)mbt, brat, aurA, bgl and aPKC- 

Results

Centrosome defects in DSas-4 and DSas-6 mutant and SakOE larval brains lead to an increase in brain size

To compare the transcriptome of normal cells to cells that either lack centrosomes or have too many centrosomes we used DSas-4 or DSas-6 (Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007) or that have amplified centrosomes in the majority of their cells (due to the overexpression of Sak/Plk4) (Basto et al., 2008). Surprisingly, we found that these centrosome defects lead to very few changes to the global transcriptome, strongly suggesting that centrosome loss or amplification, per se, does not dramatically perturb Droso phila cell physiology in vivo.

Strategy for comparing the global transcriptome of cells that lack or have amplified centrosomes

We decided to analyse the global transcriptome from larval brain and imaginal disc tissues for three reasons: (1) It is easy to isolate these tissues in a highly reproducible manner from tightly staged wandering 3rd instar larvae; (2)brains and imaginal discs are morphologically complex tissues that are polarised and are mitotically active, so might be expected to be particularly sensitive to centrosome defects; (3) As described above, DSas-4 mutant and SakOE brains are capable of forming tumours in abdominal transplantation assays (Basto et al., 2008; Castellanos et al., 2008) demonstrating that centrosome defects can, at least eventually, lead to perturbations in the normal physiology of these cells.

In an attempt to minimise variations that might simply be due to inherent differences between different inbred Droso phila strains, we backcrossed the DSas-4 and DSas-6 mutant lines and the SakOE line to a w67 line (a wild type line carrying a point mutation in the white gene) that we used here as a wild type (WT) control. Our w67 control stock was isogenised as well, so that all flies in this stock contained nearly identical sets of chromosomes. As additional controls, we also backcrossed a different WT strain (Oregon-R – [Or-R]) and a WT strain containing a TM6 balancer chromosome to w67. All backcrossing was performed for at least 5 generations, and in each generation we selected for the Pw+ marker associated with the mutation or the transgene (or the endogenous w+ allele in the case of the WT Or-R stock, or the Tubby marker in the case of the TM6 balancer) and then backcrossed these flies to the original w67 line (supplementary material Fig. S1). This allowed us to compare tissues from different genetic backgrounds that would be largely isogenic with respect to w67 except for the region of the genome containing the P-element insertion that causes centrosome loss (in the case of the DSas-4 or DSas-6 mutation) or centrosome amplification (in the case of SakOE) (supplementary material Fig. S2). For each genotype to be tested, brains and imaginal discs were dissected from 3 independent batches of 10 wandering 3rd instar larvae and total RNA was prepared from each batch. We performed genome-wide expression profiling using Droso phila Genome 2.0 GeneChips (Affymetrix, Santa Clara, California, USA) and obtained lists of differentially expressed genes between WT and test strains that displayed at least 1.5-fold differential expression and adjusted P-values corrected for multiple hypothesis testing of  

The global transcriptome appears unaltered in DSas-6 mutant tissues

When we compared the transcriptome of w67 to that of the DSas-6 mutant strain only two genes had statistically significant
Fig. 1. Larval brains with extra centrosomes or without centrosomes are increased in size. (A) 3rd instar larval brain cells stained with antibodies against Asterless marking centrosomes (red) and tubulin (green). DNA is in blue. Wild type cells have two centrosomes at the spindle poles in metaphase, whereas D-Sas4 cells do not have centrosomes. SakOE cells have amplified centrosomes, which can lead to multipolar spindle formation in prometaphase. Scale bar is 5 μm. (B) Larval brains were stained with antibodies against Asterless and Centrosomin, and centrosomes were quantified in cells (n=295 cells in 12 brains for DSas-4; n=381 cells in 10 brains for DSas-6; n=246 cells in 12 brains for w67). (C) Centrosomes were quantified in SakOE brains as described in (B) (n=50 cells in 4 brains; n=89 cells in 6 brains for w67). (D) Brain lobe circumference was measured and brain lobe volume under the assumption of the lobes being spherical was calculated in DSas-4, DSas-6 and SakOE third instar larval brains. As WT controls, w67 larvae from the same food vial were measured for each strain (see Materials and Methods). A minimum of n=26 brains were measured per strain. (E) Wing disc size was analysed by calculating the product of width x height. Mutant wing discs were compared to WT wing discs of larvae grown in the same food vial. A minimum of n=22 wing discs were measured. A t-test was performed to test for significance of the differences between mutant tissues and corresponding WT control (two asterisks stands for a P-value of <0.01; one asterisk corresponds to a P-value of <0.05).
differences in their expression levels: DSas-6, which was down-regulated by ~16× (FDR-adjusted P-value 0.005) and CG8245, which was up-regulated by ~10× (FDR-adjusted P-value = 0.004). DSas-6 was also one of only two genes identified when the DSas-6 mutant strain was compared to the WT Or-R strain: it was down-regulated by ~16× (FDR-adjusted P-value = 0.001), while CG32795 was up-regulated by ~2× (FDR-adjusted P-value = 0.05) (Fig. 2A). We performed quantitative RT-PCR (qRT-PCR) to test the reliability of these results. This analysis confirmed that DSas-6 was down-regulated compared to both WT strains (~6 fold) (Fig. 2B). Interestingly, and in agreement with the chip data, CG8245 was significantly up-regulated (~40×) in DSas-6 mutants compared to w^67 (Fig. 2C), but not compared to Or-R, while CG327954 was slightly up-regulated (~1.8×) in DSas-6 mutants compared to Or-R, but not compared to w^67 (Fig. 2D). These results demonstrate that CG8245 and CG32795 are expressed at different levels in these strains, but this difference is unlikely to be caused by the lack of centrosomes in the DSas-6 mutant tissue as a similar difference is seen when the two WT strains are compared to each other. The different expression levels of CG8245 and CG32795 were also detected in the microarray comparison between w^67 and OregonR, although they were not scored as significant (data not shown).

These qRT-PCR experiments indicate that our chip analysis likely provides a robust and reliable comparison of the transcriptomes from these different strains. Remarkably, therefore, we conclude that there are few, if any, significant changes to the transcriptome of these DSas-6 mutant tissues when compared to WT.

A small number of genomic regions are aberrantly expressed in DSas-4 mutant tissues

We next compared the transcriptome of the DSas-4 mutant strain to w^67. We identified 19 genes that were up-regulated and 8 genes...
that were down-regulated in the DSas-4 mutant tissue (supplementary material Table S1). Again, we also compared DSas-4 mutant tissue to the other WT control, Or-R, and identified 15 genes that were up-regulated and 7 that were down-regulated (supplementary material Table S2). We assessed the overlap of these two lists and identified 14 genes that were differentially expressed when DSas-4 was compared to both WT controls (Table 1; Fig. 3A). Although these 14 genes are strong candidates for genes that are differentially expressed in DSas-4 mutant cells, they did not obviously cluster into any large

### Table 1. Genes significantly up- or down-regulated in DSas4 mutant cells compared to both WT controls (≥1.5-fold, adj.P-value ≤ 0.05).

| Gene     | Map          | Fold Change^a | Function^b          |
|----------|--------------|---------------|---------------------|
| Hsc70-2  | 87D10-87D10  | 141.10        | protein folding, response to heat |
| CG31157  | 87D10-87D10  | 23.52         | unknown              |
| CG31287  | 89B7-89B7    | 16.92         | protein folding      |
| CG9279   | 76B6-76B6    | 8.81          | microtubule-based movement |
| miple    | 61B3-61B3    | 4.56          | unknown              |
| Ugt86Di  | 86D5-86D5    | 3.45          | metabolic process    |
| GstD3    | 87B8-87B8    | 2.25          | unknown              |
| lig3     | 87B9-87B9    | 2.08          | DNA ligation, DNA replication, DNA recombination |
| Rbp4     | 86C6-86C6    | 1.88          | mRNA processing      |
| CG3634   | 77E8-77E8    | 1.61          | unknown              |
| TFIIHbeta| 86C6-86C6    | -2.07         | transcription from RNA polymerase II promoter |
| MED7     | 86C5-86C5    | -2.13         | transcription from RNA polymerase II promoter |
| wnd      | 76B9-76B9    | -2.34         | protein phosphorylation |
| DSas-4   | 84C6-84C7    | -6.82         | centriole duplication |

^aFold change compared to w67 is given
^bGO Term for Biological Function

Fig. 3. Gene expression in DSas-4 cells. (A) Venn diagram of significant genes in DSas-4 cells (third instar larval brains and wing discs) compared to w67 and OregonR WT controls found in microarray analysis (≥1.5-fold, FDR-adj.P-value ≤ 0.05). (B–F) Validation of up- and down-regulation by quantitative PCR. Fold change found in microarray analysis is shown above, and fold change found by qPCR analysis is given below the blue bar indicating the transcript. Red (up-regulation), green (down-regulation) and black (no change) bars indicate the position where microarray probes and qPCR primers bind to the transcript. (B) An unannotated transcript (dark blue), spanning the 5’ end of Hsc70-2, the gene CG31157 and the genomic region between the two genes, appears to be up-regulated in DSas-4 cells.
functionally related group (Table 1). Moreover, it seems unlikely that these differences are directly caused by the lack of centrosomes in the DSas-4 mutant cells, as none of these genes were identified as being differentially regulated in DSas-6 mutant cells that also lack centrosomes. In addition, we noticed that several of the genes that are most differentially regulated in DSas-4 mutant cells were located very close to one other in the genome. For example, the two most up-regulated genes, Hsc70-2 and CG31157, are located directly adjacent to each other, while two of the most statistically significantly down-regulated genes (after DSas-4 itself and wallenda), Transcription factor TFIIIBeta and Mediator complex subunit 7 (MED7), are separated by only one gene, perhaps indicating that chromatin architecture may be altered in these regions in the DSas-4 mutant cells.

To confirm whether these genes were differentially expressed in DSas-4 mutant tissues we performed qRT-PCR experiments with the top 4 up-regulated genes (Hsc70-2, CG31157, CG31287, and CG9279) and the two most down-regulated genes (DSas-4 itself and wallenda). We designed qRT-PCR primers that would amplify across the region of each gene that was probed in our microarray experiments, as well as primers that would detect expression from a different region of each gene. As noted above, the two most up-regulated genes lie right next to each other in the genome and are transcribed in the same direction. To our surprise, we found that the 5’ end of HSC70-2 was not overexpressed, but the 3’ end (the region probed in our chip analysis) was strongly overexpressed by ~185x, while both the 5’ and 3’ ends of CG31157 were overexpressed by ~1400x and ~130x, respectively, in DSas-4 mutant tissue (Fig. 3B). We also analysed the expression levels of the small intergenic region between these genes and found that this was also overexpressed by ~260x in DSas-4 mutant tissue. We conclude that an unusual transcript that starts in the second half of HSC70-2 and reads through the CG31157 gene is highly overexpressed in DSas-4 mutants (Fig. 3B). We observed a similar phenomenon for CG9279 (Fig. 3C) and wallenda (Fig. 3D), as our qRT-PCR analysis revealed that the expression of the regions of the genes probed in our chip analysis were indeed mis-regulated, but the expression levels of a different region of each transcription unit were not. In contrast, the transcript levels of CG31287 (Fig. 3E) were not detectably altered in qRT-PCR experiments, suggesting that this is a false positive in our chip analysis. Down-regulation of DSas-4 mRNA in the DSas-4 mutant was also confirmed by qRT-PCR (Fig. 3F).

Taken together, these data strongly suggest that a small number of genomic regions are genuinely mis-expressed in DSas-4 mutant cells, but at least some of this mis-expression is due to the production of aberrant transcripts that are not normally found in WT cells. The significance of this is unclear (see Discussion), but, as these regions are not mis-expressed in DSas-6 mutant cells, it seems unlikely that these changes are a direct result of the lack of centrosomes in DSas-4 mutants.

No genes appear to be consistently mis-expressed in DSas-6 and DSas-4 mutant tissues

We reasoned that our failure to identify any genes that are consistently mis-expressed in tissues lacking centrosomes might be due to a high rate of false negatives, perhaps because we were setting our statistical cut-off for significance at too high a level. To test if this was the case, we compared the overlap between the 100 most significantly differentially regulated genes in DSas-4 and DSas-6 mutant tissue, even though most of these genes had an adjusted P-value of more than 0.05. Only 4 genes were differentially regulated in both mutants, strongly suggesting that most of these genes are unlikely to be consistently mis-expressed in cells that lack centrosomes (Table 2). Of the 4 genes that did overlap, two seemed poor candidates for genes that might be differentially regulated in cells lacking centrosomes: CG13822 is up-regulated in DSas-4 mutants but down-regulated in DSas-6 mutants, while CG2541 is only down-regulated by ~1.22 and ~1.4 fold in DSas-4 and DSas-6 mutants, respectively. CG13857 and pathetic were both up-regulated in DSas-6 and DSas-4 mutant tissues (Table 2), but a qRT-PCR analysis again suggested that only the expression of the 3’ region of each gene (the region probed in our chip analysis) was affected, while the 5’ region was not. This analysis strongly supports our conclusion that a lack of centrosomes in these cells leads to the significant mis-expression of very few, if any, genes.

A small number of genes are differentially expressed in SakOE cells

To assess the effect of having too many centrosomes in cells we compared the transcriptome of SakOE cells to cells from the WT w67 and Or-R strains. We found 55 and 57 genes, respectively, to be up- or down-regulated in the SakOE tissues (supplementary material Table S3, S4), and 32 of these genes were present in both comparisons (Table 3; Fig. 4A). No large functionally related group of genes appeared to be particularly enriched in this geneset (supplementary material Table S5, S6).

To test whether some of these genes were really differentially expressed in SakOE cells we again performed qRT-PCR experiments with several of the most up- (CG32055, B32, CG14687, CG9279 and Gram-negative bacteria binding protein 2 (GNBP2)) or down-regulated (Braf, CG11999 and CG7900) genes. Five of these eight genes were confirmed by the qRT-PCR analysis, which revealed that both their 5’ and 3’ regions were mis-expressed in the SakOE cells compared to the WT strains.

| Table 2. Genes that are differentially regulated in both DSas-4 and DSas-6 among the 100 most significant genes in each genotype. |
|-----------------|---------------|---------------|---------------|
| Gene            | CG13157       | CG32541       | CG13822       |
| Fold Change DSas-4 | 8.71 (0.02*)  | 6.99 (0.024*) | −1.26 (0.055*) |
| Fold Change DSas-6 | 5.31 (0.45*)  | 5.31 (0.55*)  | −1.40 (1*)     |
| DSas-4 5’ qPCR  | 8.5 fold ↑    | no change     | n.d.          |
| DSas-4 3’ qPCR  | no change     | no change     | n.d.          |
| DSas-6 5’ qPCR  | 4 fold ↑      | 2.6 fold ↑    | n.d.†         |
| DSas-6 3’ qPCR  | no change     | no change     | n.d.†         |
| *(adjusted P-value) |               |               |               |
Table 3. Differentially regulated genes in SakOE cells compared to both WT controls (≥1.5-fold, adj.P-value ≤ 0.05).

| Gene      | Map       | Fold Changea | Functionb                  |
|-----------|-----------|--------------|----------------------------|
| w         | 3B6-3B6   | 74.81        | eye pigment biosynthetic process |
| CG32055   | 67D11-67D11 | 43.82        | nuclear mRNA splicing via spliceosome, mRNA splice site selection |
| BS2       | 87F7-87F7 | 14.38        | unknown                     |
| CG14687   | 86C6-86C6 | 8            | unknown                     |
| CG9279    | 76B6-76B6 | 7.57         | microtubule-based movement  |
| CG13032   | 73B6-73B6 | 7.3          | carboxylic acid metabolic process, proteolysis |
| CG5618    | 77B5-77B5 | 4.48         | unknown                     |
| CG31495   | 87F15-87F15 | 3.63      | unknown                     |
| mple      | 61B3-61B3 | 3.47         | unknown                     |
| CG7433    | 76D8-76E1 | 3.09         | cellular amino acid metabolic process, gamma-aminobutyric acid metabolic process |
| Past1     | 87C6-87C6 | 3.07         | endocytosis, germline development, imaginal disc-derived wing margin development |
| GNB2      | 75D6-75D6 | 3.02         | carbohydrate metabolic process, defense response |
| CG32939   | 8E4-8E4   | 2.32         | nuclear mRNA splicing via spliceosome, mRNA splice site selection |
| Rbp1      | 86C6-86C6 | 2.3          | unknown                     |
| CG2004    | 8A2-8A2   | 2.27         | nuclear mRNA splicing via spliceosome, mRNA splice site selection |
| P58IPK    | 85D27-85D27 | 2.24      | protein folding             |
| SrpRheta  | 66D11-66D11 | 2.17       | neurogenesis, larval chitin based cuticle development |
| CG18542   | 85E4-85E4 | 1.99         | unknown                     |
| p24-1     | 10F1-10F1 | 1.86         | neurogenesis, post-Golgi vesicle-mediated transport |
| CaBP1     | 35F12-35F12 | 1.75    | cell redox homeostasis, glycerol ether metabolic process |
| CG6951    | 77A3-77A4 | 1.71         | neuron homeostasis, neuron projection development |
| Manf      | 89B13-89B13 | 1.67      | protein retention ER lumen |
| KDEL      | 31E1-31E1 | 1.6          | transcription from RNA polymerase II promoter |
| MED 7     | 86C5-86C5 | −2.07        | unknown                     |
| CG5830    | 72C1-72C2 | −2.12        | transcription from RNA polymerase II promoter |
| TFIIHbeta | 86C6-86C6 | −2.15        | nuclear mRNA splicing via spliceosome |
| scaf6     | 73E5-73E5 | −2.23        | unknown                     |
| CG32158   | 73A3-73A3 | −2.74        | unknown                     |
| CG32027   | 75E2-75E2 | −4.91        | Regulation of transcription from RNA polymerase III promoter |
| Brf       | 90A3-90A5 | −7.59        | unknown                     |
| CG11999   | 82F10-82F10 | −15.43    | unknown                     |
| CG7900    | 84E11-84E12 | −44.92    | unknown                     |

aFold change compared to w67 is given
bGO Term for Biological Function

(Fig. 4B–F). The 3’-region of CG9279 was up-regulated (in agreement with the microarray data) but the 5’ region was not (Fig. 4G) and if anything appeared to be down-regulated) suggesting that an aberrant transcript may be produced from this genomic region in the SakOE cells, as we observed in several genomic regions in the Dsas-4 mutant cells. The genes CG14687 and B52 appeared to be false positives in our microarray analysis as either no, or only a very small, change in expression levels was detected by qRT-PCR (Fig. 4H,I). These results indicate that at least some of these 32 genes are genuinely mis-expressed in SakOE cells, although, as these genes do not cluster into any obvious functional pathway, it is unclear whether this mis-expression is a direct consequence of the centrosome amplification in these cells (see Discussion). Nevertheless, it is clear from this analysis that centrosome amplification does not lead to a large-scale perturbation of the transcriptome and that only a relatively small number of genes, if any, are consistently mis-regulated in cells as a result of centrosome amplification.

**Discussion**

We show here that centrosome loss or amplification does not dramatically alter the global transcriptome of Drosophila brain or imaginal disc cells. While we have identified a small number of transcripts (~10–15) whose expression appears to be genuinely mis-regulated in Dsas-4 mutant cells, several of these appear to be aberrant transcripts that do not encode normal proteins, and few of these genes appear to be linked by any known common function. We currently do not understand why these transcripts are mis-expressed in Dsas-4 cells, but it seems unlikely that these transcripts are components of specific pathways that are activated or inactivated in response to the lack of centrosomes, as none of these transcripts are mis-expressed in Dsas-6 mutant cells, which also lack centrosomes. Similarly, we identified a slightly larger subset of genes (~30) that appear to be mis-expressed in cells that overexpress Sak and so have too many centrosomes. Again, however, few of these genes appear to be linked by any known common function. Unfortunately, we have no independent way of efficiently driving centrosome amplification in brain and imaginal disc cells (independent of Sak overexpression), to test whether centrosome amplification is directly responsible for this transcriptional mis-regulation or whether it is due to some other change in the SakOE cells, as appears to be the case in Dsas-4 cells. The human homologue of Sak, Plk4, has been reported to phosphorylate Hand1, a transcription factor that controls cell fate (Martindill et al., 2007), perhaps explaining why more genes are differently expressed in SakOE cells than in cells that lack centrosomes.

While we cannot rule out that centrosome defects lead to significant post-transcriptional changes without affecting the transcription of many genes, our findings strongly suggest that
centrosome defects do not induce a major stress response in these cells. This finding is important, as centrosome defects appear to be a source of stress to cells in vitro (Mikule et al., 2007; Srsen et al., 2006; Uetake et al., 2007), where they have been shown to activate a stress response via the p38 and p53 pathways. It is not known what effect the activation of these pathways might have on gene expression in Drosophila, but genome wide expression studies in mammalian cells have shown that p38 stress-activated protein kinase activation leads to changes in expression of many genes, some of which are transcription factors (Ferreiro et al., 2010). Our GO enrichment analysis does not show enrichment of any known stress response genes in response to centrosome defects. Thus, although cellular stress has been linked to tumourigenesis in some Drosophila models (Rossi and Gonzalez, 2011; Wu et al., 2010), it appears unlikely to be an important driver of tumourigenesis in flies with centrosome defects.

If centrosome defects per se do not result in dramatic changes in cell physiology, then why are Drosophila brain cells without centrosomes or with amplified centrosomes predisposed to form...
tumours (Basto et al., 2008; Castellanos et al., 2008)? It is known that spindle assembly is slow in cells that have lost their centrosomes or have extra centrosomes, and this leads to a relatively modest increase in chromosome segregation errors (Basto et al., 2008; Basto et al., 2006; Ganem et al., 2009). Aneuploidy has long been thought to contribute to tumorigenesis (Boveri, 2008) and although mutations that lead to large-scale chromosomal instability do not seem to drive tumour formation in flies (Castellanos et al., 2008), it is possible that the low-level of aneuploidy induced by centrosome defects is actually a more effective driver of cancer (Weaver et al., 2007). Alternatively, although most fly cells with centrosome defects appear to divide relatively normally, it has previously been shown that such asymmetrically dividing NBs divide symmetrically ~10–15% of the time, as the astral MTs generated by centrosomes in these cells help the spindle to efficiently align with cortical cell fate determinants (Basto et al., 2008; Basto et al., 2006). This appears to lead to an expansion of the NB pool, consistent with the increase in brain size that we report here.

Our transcriptional profiling experiments, however, show that this brain overgrowth is likely to be benign as it leads to few, if any, changes in gene expression. The NBs that accumulate in these brains are therefore unlikely to be malignant – whereas the tumours formed from these brains in abdominal transplants are clearly malignant, as they show immortality, aneuploidy and the ability to metastasise (Basto et al., 2008; Castellanos et al., 2008; Caussinus and Gonzalez, 2005), all of which almost certainly require large-scale changes in gene expression (Ramaswamy et al., 2003; Rhodes et al., 2004; Scott et al., 2011). We conclude that in brain tissues with centrosome defects, an additional step must lie between the generation of overgrowing, but physiologically normal brains, and the tumours that can eventually be formed from these brains in transplantation experiments. An attractive possibility is that the overproliferation of NBs leads to a bigger stem cell pool and larger brains, but that the low level of chromosomal missegregation facilitates the generation of mutations that occasionally lead to the generation of a malignant stem cell, which ultimately drives tumour formation.

This model has obvious similarities to the cancer stem cell (CSC) theory. A correlation between CSC and chromosomal instability has been suggested (Lagasse, 2008; Li et al., 2009) and recent studies have indicated that aneuploidy and CSCs models of cancer are not mutually exclusive (Conway et al., 2009; Liang et al., 2010). It will be interesting to test whether low level aneuploidy in cells without centrosomes or with too many centrosomes leads to the accumulation of mutations in NBs in vivo. Transcriptional profiling experiments on single cells could answer this question in the future.

### Materials and Methods

#### Fly strains

The following mutant alleles were used in this study: *D Sas-4<sup>Δ2214</sup>* (Basto et al., 2006) *D Sas-6<sup>Δ2062</sup>* (Rodrigues-Martins et al., 2007). Overexpression of Sak in the *SakOE* strain is caused by the ubiquitin-promoter driven expression of GFP::Sak (Basto et al., 2008). *Laboratory w<sup>1118</sup>* and *Oregon-R w<sup>1118</sup>* strains were used as wild type controls.

#### Immunofluorescent staining and quantification of centrosome numbers in larval brains

For immunofluorescent staining, brains of third instar larvae were dissected in PBS and fixed in 4% Formaldehyde in PBS for 20 minutes. Brains were transferred to 45% acetic acid for 15 seconds and then to 60% acetic acid on a coverslip for 3 min. Then the brains were squashed between a slide and the coverslip and flash frozen in liquid nitrogen. Coverslips were removed, and the slides were placed in 100% methanol for 5 minutes. Samples were rehydrated in PBS for 1 h and then incubated with primary antibodies under a mounted coverslip in a moist chamber overnight at 4°C. On the next day the slides were washed in PBS and incubated with the secondary antibodies for three hours. After incubation the slides were washed in PBS, stained for 10 minutes in Hoechst33258 (Life Technologies, Carlsbad, California, USA) and then mounted in mounting medium (85% glycerol and 2.5% n-propylgallate). Slides were observed on a Zeiss Axioskop 2 microscope (Carl Zeiss, Ltd., Welwyn Garden City, Hertfordshire, UK) with a CoolSNAP HQ camera (Photometrics, Tucson, Arizona, USA), using a 63x/1.40 oil objective. Images were acquired using Metamorph software (Molecular Devices, Sunnyvale, California, USA), imported into Photoshop CS2 (Adobe, San Jose, California, USA), and adjusted to use the full range of pixel intensities. Neuroblasts and ganglion mother cells were scored in prophase in order to ensure that centrosomes were duplicated but extra centrosomes would not be clustered at the spindle poles. These cells were identified using DNA morphology and dots were scored as centrosomes only if they co-stained for Cnn and Asl.

#### Antibodies

For quantification of centrosomes in brain cells, the following primary antibodies were used at a 1:50 dilution: rabbit anti-Asl (Conduit et al., 2010) and guinea pig anti-Cnn (Lucas and Raff, 2007). Alexa488 anti-guinea pig and Alexa568 anti-rabbit secondary antibodies were used at a 1:100 dilution (Molecular Probes, Life Technologies, Carlsbad, California, USA). For stainings of spindle and centrosome, rabbit anti-Asl and mouse monoclonal anti-a-tubulin (DM1a, Sigma-Aldrich, St. Louis, Missouri, USA) were used. *Laboratory w<sup>1118</sup>* and *Oregon-R w<sup>1118</sup>* strains were used as wild type controls.

#### Measurement of brain and imaginal disc size

*DSas-4, DSas-6* and *SakOE* strains were recombined with a Hand-GFP transgene that is expressed in the larval cardiac tissue (Han et al., 2006) with *w<sup>67</sup>* to generate new flies and *SakOE* w<sup>67</sup> was crossed with *Hand-GFP, DSas-4,Hand-GFP/TM6C* and *DSas-6,Hand-GFP/TM6C* females and males and females and males were allowed to lay eggs for a maximum of 12 hours. A few days later, brains and wing discs of wandering third instar larvae were dissected and pictures were taken with a Nikon DS-F1 camera mounted on a Nikon SMZ900 dissecting microscope (Nikon, Kingston Upon Thames, Surrey, UK); the circumference of the brain lobes and the width and height of the wing discs were then measured in ImageJ (Abramoff et al., 2004). After making these measurements, the genotype of the tissue that had been measured was checked by examining the larval carcass for Hand-GFP expression. In this way we ensured that the analysis was performed blind, and that the mutant (or *Sak* overexpressing) and WT larvae were grown under identical conditions in the same vial. Brain and wing disc sizes of at least 20 animals were measured per data-point and the average and standard error were calculated.

### Microarray analysis

#### Transcriptional profiles of wild type flies

*DSas-4* flies, *DSas-6* flies and *SakOE* flies were generated. Total RNA of brains and imaginal discs from 10 third instar larvae per sample was isolated using TRIzol (Life Technologies, Carlsbad, California, USA) chloroform extraction and isopropanol precipitation. For each sample three independent samples were analysed. Purity and integrity of the purified RNA was assessed on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, California, USA). Concentration was determined with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA). 500 ng RNA per sample was processed to labelled cRNA using the Affymetrix 3' IVT Express kit and hybridized to the manufacturer's instructions and hybridised to Affymetrix Drosophila 2.0 GeneChips for 16 hrs at 45°C. Gene chips were washed and stained with streptavidin-phycerothrin using the Affymetrix Fluidics Station 450 and scanned on an Affymetrix GeneChip 3000 scanner (all Affymetrix, Santa Clara, California, USA). Quality control of microarray expression data was performed using the Bioconductor package AffyPLM (Gautier et al., 2004). Probe intensities from Affymetrix image files (*.CEL* files) were normalised using quantile normalisation (Bolstad et al., 2003), and expression signals of all genes (probesets) were calculated using GCRMA (Guanosine Cytidine robust multarray analysis) (Wu and Irizarry, 2007). Differentially expressed genes between WT and *SakOE* samples were identified using the Bioconductor package limma (Golubovskiy et al., 2010). Probe intensities from Affymetrix image files (*.CEL* files) were normalised using quantile normalisation (Bolstad et al., 2003), and expression signals of all genes (probesets) were calculated using GCRMA (Guanosine Cytidine robust multarray analysis) (Wu and Irizarry, 2007). Differentially expressed genes between WT and *SakOE* samples were identified using the Bioconductor package limma (Golubovskiy et al., 2010).
Quantitative PCR analysis

For qPCR analysis RNA was isolated as described for the microarray experiments. 1 μg RNA was treated with DNaseI (Invitrogen life technologies) and used for reverse transcription with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK) using oligo(dT) primer. cDNA was diluted 1:2 and 1 μl diluted cdNA was used for qPCR analysis using the SensiMix SYBR No-ROX Kit (Bioline Reagents Ltd, London, UK) in a MJ Research PTC-200 thermal cycler with a Chromo4 detector (both BioRad, Hercules, California, USA). Gene specific primers for qPCR analysis were designed using PeriPrimer (Marshall, 2004) (supplementary material Table S7). Expression of the tested genes was measured in triplicates and gene expression levels for each individual sample were normalised expression of EF1α. At least 2 independent samples were measured per gene. Relative gene expression was determined and expressed as

\[ 2^{-\Delta\Delta CT} = (CT_{\text{gene}} - CT_{\text{EF1α}}) \times 10000 \]

(Livak and Schmittgen, 2001).

Competing Interests

The authors have no competing interests to declare.

References

Abraham, M. D., Magalhães, P. J. and Ram, S. J. (2004). Image processing with ImageJ. Bioinformatics 20, 3065-3072.

Alkouraya, F. S., Cai, X., Emery, C., Mochida, G. H., Al-Dosari, M. S., Feliz, J. M., Hill, R. S., Barry, B. J., Partlow, J. N., Gascon, G. G. et al. (2011). Human mutations in NDE1 cause extreme microcephaly with lissencephaly. Am. J. Hum. Genet. 88, 536-547.

Bakircioglu, M., Carvalho, O. P., Khurshid, M., Cox, J. J., Tuysuz, B., Barak, T., Yilmaz, S., Caglayan, O., Dinser, A., Nicholas, A. K. et al. (2011). The essential role of centrosomal NDE1 in human cerebral cortex neurogenesis. Am. J. Hum. Genet. 88, 523-531.

Basto, R., Lau, J., Vinogradova, T., Gardiol, A., Woods, C. G., Khodjakov, A. and Raff, J. W. (2006). Flies without centrosomes. Cell 125, 1375-1386.

Basto, R., Brunk, K., Vinadogrova, T., Peel, N., Franz, A., Khodjakov, A. and Raff, J. W. (2006). Centrosome amplification can initiate tumorgenesis in flies. Cell 133, 1032-1042.

Bello, B., Reichert, H. and Hirth, F. (2006). The brain tumor gene negatively regulates neuronal progenitor cell proliferation in the larval central brain of Drosophila. Development 133, 2639-2648.

Benjamini, Y. and Hochberg, Y. (2005). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. A Stat. Soc. 57, 289-300.

Betshnger, J., Mechtri, K. and Knoblich, J. A. (2006). Asymmetric segregation of the tumour suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell 124, 1241-1253.

Bolstad, B. M., Irizarry, R. A., Astrand, M. and Speed, T. P. (2003). Analysis of microarray gene expression data: a tutorial. BMC Bioinformatics 5, 97.

Bond, J. and Woods, C. G. (2006). Cytokinetin genes regulating brain size. Curr. Opin. Cell Biol. 18, 95-101.

Boveri, T. (2008). Concerning the origin of malignant tumours by Theodor Boveri. Boveri, T.

Boveri, T. and Zeng, Y.-X. (2006). Centrosome defects in Drosophila. Dev. Cell 11, 178-190.

Braekevelt, A. K., Ohtsuka, K., Takahashi, H., Otomo, K., Yamanouchi, K., Watanabe, N. and Kato, T. (2007). DNA damage-induced accumulation of centrosomal Chk1 contributes to its checkpoint function. J. Cell Biol. 178, 725-732.

Desplan, C. and Haendel, S. A. (2007). The essential roles of centrioles and the centriolar matrix in Drosophila centrosome organization. J. Cell Biol. 178, 725-732.

Doe, C. Q. (2012). Functional genomics identifies neural stem cell sub-type in the mammalian brain. Curr. Biol. 22, 1854-1864.

Doe, C. Q., Deniset-Besseau, B., Kockelkoren, G. and Martin, P. (2007). Mechanisms of asymmetric stem cell division. Curr. Opin. Cell Biol. 19, 185-193.

Doe, C. Q., Lohn, G., Urmann, J., Farzaneh, M., Kothe, A., Wernig, M., Bruns, A., Krämer, A. and Rehmsmeier, M. (2012). Loss of centrosome integrity induces p38-p53-p21-dependent G1-S arrest. Nat. Cell Biol. 14, 160-170.

Eck, Z., Yi, P., Li, X. and Olson, E. N. (2006). Hand, an evolutionarily conserved transcription factor, is required for dorsal-ventral axis formation. Dev. Biol. 299, 129-143.

Gautier, L., Cope, L., Bolstad, B. M. and Irizarry, R. A. (2004). Affy—analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20, 375-377.

Gonzalez, C. (2008). Spindle orientation, asymmetric division and tumour suppression in Drosophila stem cell systems. Nat. Rev. Genet. 9, 179-191.

Gonzalez, C. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.

Hediger, K., Delaval, B., Kaldis, P., Jurczyk, A., Hergert, P. and Doxsey, S. J. (2008). Centrosome function during stem cell division: the devil is in the details. Curr. Opin. Cell Biol. 20, 444-447.
Ramaswamy, S., Ross, K. N., Lander, E. S. and Golub, T. R. (2003). A molecular signature of metastasis in primary solid tumors. Nat. Genet. 33, 49-54.

Rhodes, D. R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A. and Chinnaiyan, A. M. (2004). Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. Proc. Natl. Acad. Sci. USA 101, 9309-9314.

Rodrigues-Martins, A., Bettencourt-Dias, M., Riparbelli, M., Ferreira, C., Ferreira, I., Callaini, G. and Glover, D. M. (2007). DSAS-6 organizes a tube-like centriole precursor, and its absence suggests modularity in centriole assembly. Curr. Biol. 17, 1465-1472.

Rossi, F. and Gonzalez, C. (2011). Synergism between altered cortical polarity and the PI3K/TOR pathway in the suppression of tumour growth. EMBO Rep. 13, 157-162.

Scott, K. L., Nogueira, C., Heffernan, T. P., van Doorn, R., Dhakal, S., Hanna, J. A., Min, C., Jaskelioff, M., Xiao, Y., Wu, C. J. et al. (2011). Proinvasion metastasis drivers in early-stage melanoma are oncogenes. Cancer Cell 20, 92-103.

Smyth, G. K. (2005). Limma: linear models for microarray data. In Bioinformatics And Computational Biology Solutions Using R And Bioconductor (ed. R. Gentleman, V. Carey, S. Dudoit, R. Irizarry and W. Huber), pp. 397-420. New York: Springer.

Srøen, V., Gnadt, N., Dammermann, A. and Merdes, A. (2006). Inhibition of centrosome protein assembly leads to p53-dependent exit from the cell cycle. J. Cell Biol. 174, 625-630.

Takada, S., Kwak, S., Koppetsch, B. S. and Theurkauf, W. E. (2007). grp (chk1) replication-checkpoint mutations and DNA damage trigger a Chk2-dependent block at the Drosophila midblastula transition. Development 134, 1737-1744.

Tritarelli, A., Oricchio, E., Ciciarello, M., Mangiacasale, R., Palena, A., Lavia, P., Soddu, S. and Cundari, E. (2004). p53 localization at centrosomes during mitosis and postmitotic checkpoint are ATM-dependent and require serine 15 phosphorylation. Mol. Biol. Cell 15, 3751-3757.

Uetake, Y., Loncarek, J., Nordberg, J. J., English, C. N., La Terra, S., Khodjakov, A. and Sluder, G. (2007). Cell cycle progression and de novo centriole assembly after centrosomal removal in untransformed human cells. J. Cell Biol. 176, 173-182.

Wang, H., Somers, G. W., Bashirullah, A., Heberlein, U., Yu, F. and Chia, W. (2006). Aurora-A acts as a tumor suppressor and regulates self-renewal of Drosophila neuroblasts. Genes Dev. 20, 3453-3463.

Weaver, B. A. A., Silk, A. D., Montagna, C., Verdier-Pinard, P. and Cleveland, D. W. (2007). Aneuploidy acts both oncogenically and as a tumor suppressor. Cancer Cell 11, 25-36.

Wu, M., Pastor-Pareja, J. C. and Xu, T. (2010). Interaction between RasV12 and scribbled clones induces tumour growth and invasion. Nature 463, 545-548.

Wu, Z. and Irizarry, R. A. (2007). A statistical framework for the analysis of microarray probe-level data. Ann. Appl. Stat. 1, 333-357.