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### **► To cite this version:**

Dora Sabino, Nicholas H Brown, Renata Basto. Drosophila Ajuba is not an Aurora-A activator but is required to maintain Aurora-A at the centrosome. Journal of Cell Science, 2011, 124, pp.1156 - 1166. <10.1242/jcs.076711>. <hal-03455163>

**HAL Id: hal-03455163**

**<https://hal.science/hal-03455163v1>**

Submitted on 29 Nov 2021

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# *Drosophila* Ajuba is not an Aurora-A activator but is required to maintain Aurora-A at the centrosome

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Accepted 8 November 2010

Journal of Cell Science 124, 1156–1166

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doi:10.1242/jcs.076711

## Summary

The LIM-domain protein Ajuba localizes at sites of epithelial cell–cell adhesion and has also been implicated in the activation of Aurora-A (Aur-A). Despite the expected importance of Ajuba, Ajuba-deficient mice are viable, which has been attributed to functional redundancy with the related LIM-domain protein LIMD1. To gain insights into the function of Ajuba, we investigated its role in *Drosophila*, where a single gene (*jub*) encodes a protein closely related to Ajuba and LIMD1. We identified a key function in neural stem cells, where Jub localizes to the centrosome. In these cells, mutation in *jub* leads to centrosome separation defects and aberrant mitotic spindles, which is a phenotype similar to that of *aur-A* mutants. We show that in *jub* mutants Aur-A activity is not perturbed, but that Aur-A recruitment and maintenance at the centrosome is affected. As a consequence the active kinase is displaced from the centrosome. On the basis of our studies in *Drosophila* neuroblasts, we propose that a key function of Ajuba, in these cells, is to maintain active Aur-A at the centrosome during mitosis.

**Key words:** Ajuba, Aurora-A, Centrosome, Mitotic spindle, Tacc

## Introduction

The regulation of kinase activity in time and space is crucial for the coordination of cellular events (Pines and Rieder, 2001). Aurora-A (Aur-A), one of the three members of the Aurora family of kinases in mammals, is a serine/threonine kinase that functions as a key regulator of several events. The kinase Aur-A was first identified in *Drosophila* as a mitotic kinase (Glover et al., 1995). In flies, mutations in *aur-a* cause severe developmental defects and pleiotropic phenotypes, which include abnormal centrosome and spindle behavior, lack of astral microtubules (MTs), defects in chromosome segregation, spindle positioning, cortical targeting of cell fate determinants and neural stem-cell self-renewal (Berdnik and Knoblich, 2002; Wirtz-Peitz, 2008; Giet et al., 2002; Glover et al., 1995; Johnston et al., 2009; Lee et al., 2006a; Wang et al., 2006).

In vertebrate cells, Aur-A also plays a major role in mitosis, and recently an unexpected role for this kinase has been described in non-mitotic cells. Aur-A phosphorylates and activates the tubulin deacetylase HDAC-6 to promote disassembly of cilia and cell cycle re-entry (Pugacheva et al., 2007). The large spectrum of functions attributed to the kinase Aur-A is thought to be, at least in part, regulated by different cofactors or activators (Carmena et al., 2009). TPX2, a MT-associated protein (MAP), binds Aur-A, thereby promoting Aur-A autophosphorylation and targeting it to the mitotic spindle (Wittmann et al., 2000). Hef-1 (also known as Nedd9) binding and activation of Aur-A is required for HDAC-6 phosphorylation (Pugacheva et al., 2007). In *Drosophila*, a single

Aur-A activator, Bora, has been described so far. In *bora* mutants, defects in centrosome behavior and spindle assembly, together with defects in the asymmetric cell division of sensory organ precursors (SOPs), have been identified (Hutterer et al., 2006).

Ajuba (Jub) is a LIM-domain protein that localizes at the sites of cell–cell adhesion in epithelial cells and that has also been implicated in the activation of Aur-A (Hirota et al., 2003). Surprisingly, however, Jub-deficient mice are viable (Pratt et al., 2005); this has been attributed to functional redundancy with the related LIM-domain protein LIMD1. To gain insight into the function of Jub, we investigated its role in *Drosophila melanogaster*, where a single gene encodes a protein closely related to mouse Jub and LIMD1. We generated a mutation in *ajuba* (*jub*) and found that *jub* mutants die at the larval–pupal transition. We did not detect defects in cell adhesion or epithelial polarity. However, we identified a key function in neural stem cells, where Jub localized to the centrosome. In these cells, mutation of *jub* led to centrosome separation defects and abnormal mitotic spindles. Surprisingly, we found that in *jub* mutants Aur-A activity was not perturbed, but that Aur-A recruitment and maintenance at the centrosome was affected. As a consequence the active kinase was ectopically displaced into the cytoplasm, which resulted in abnormalities of the mitotic spindle. On the basis of our studies, we propose that a major function of Jub in *Drosophila* neuroblasts is to restrict active Aur-A to the centrosome during mitosis, but that Jub does not function as an Aur-A activator.

## Results

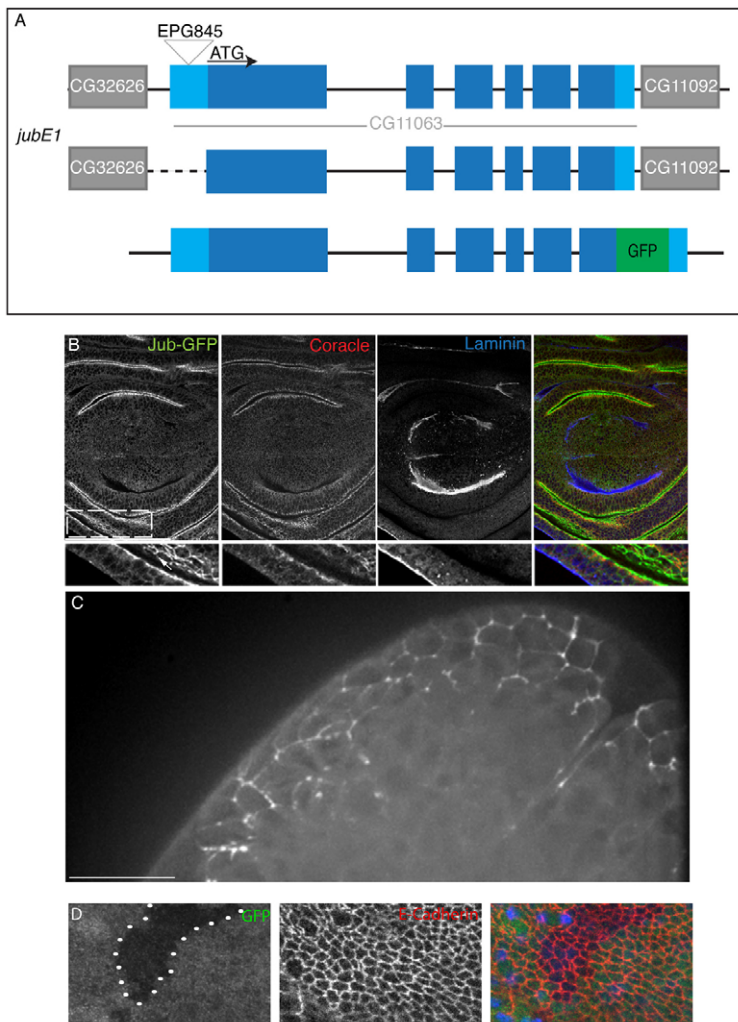
*Drosophila* contains a single ortholog of Jub and LIMD1 (CG11063). In order to investigate the function of *jub*, we analyzed a fly strain with an EP element (see the Materials and Methods) inserted into the 5'-untranslated region (UTR) of this gene, 163 bp

upstream of the ATG start codon (Fig. 1A). This insertion is lethal, so to confirm that it was the insertion that caused lethality, and to generate deletions, we mobilized the EP element. We obtained numerous viable excision lines, demonstrating that the lethality was caused by the insertion into the *jub* gene. We hypothesized that the insertion blocks *jub* transcription, and, given that the EP element is oriented correctly, that we could restore *jub* transcription by crossing the strain to Gal4 drivers; if a particular Gal4 line drives expression in the tissues that require Jub, then this should rescue the mutant, and this was indeed the case. Lines driving expression in both the epidermis (epithelia) and in the nervous system, such as *tubulin*-Gal4 and 69B-Gal4, rescued the allele to viability, showing that Jub function is required in these tissues. We also obtained one new allele within the *jub* locus, *jub<sup>E1</sup>*, consisting of a deletion of 1242 bp upstream of the ATG (Fig. 1A). We determined that this allele was of equivalent strength to that of the original EP insertion (homozygous *jub<sup>E1</sup>* flies died at the same stage of pupal development); we therefore chose to characterize the *jub<sup>E1</sup>* allele, hereafter simply referred to as the *jub* mutant.

A genomic rescue construct expressing *jub* tagged with GFP under its own promoter, completely rescued *jub* mutant lethality, showing that the *jub* phenotype is only due to the mutation in the *jub* gene. We detected Jub-GFP at adherens junctions in imaginal discs and in embryos (Fig. 1B,C), consistent with the localization

of mammalian Jub. Embryos lacking Jub survived to early pupal stages, even when the maternal contribution was removed by making germline clones. *jub* mutant clones within otherwise wild-type (WT) imaginal discs grew poorly and delaminated from the epithelia, consistent with the recently described role of *Drosophila* Jub in the Hippo pathway (Das Thakur et al., 2010), but we did not detect any clear loss of cell–cell adhesion or any polarity defects, as reflected by the normal distribution of cadherin (Fig. 1D).

We next turned our attention to whether *Drosophila* Jub activates Aur-A, and hence performs a function at the centrosome, as demonstrated by Hirota and colleagues (Hirota et al., 2003). In neural stem cells (neuroblasts; Nbs) from third-instar larval brains, which are rapidly dividing cells, Jub-GFP colocalized with Centrosomin (Cnn), a centrosomal protein associated with the pericentriolar material (PCM) (Fig. 2A). Jub-GFP was detected as a large dot colocalizing with Cnn exclusively in mitotic cells, suggesting that Jub-GFP is recruited during mitosis as a PCM component. Jub-GFP was not associated with centrosomes in other proliferating tissues, such as imaginal discs or the germline, nor in non-proliferating epithelia, such as the larval epidermis (data not shown); however, we did detect Jub-GFP at cell–cell contacts in these tissues (data not shown). Thus, we conclude that Jub is associated with the PCM, but only in some tissues, notably the mitotic cells within the larval brain.



**Fig. 1. Jub-GFP localizes at the apical cortex and in cell contacts, and *jub* mutant clones do not show cell–cell adhesion defects.**

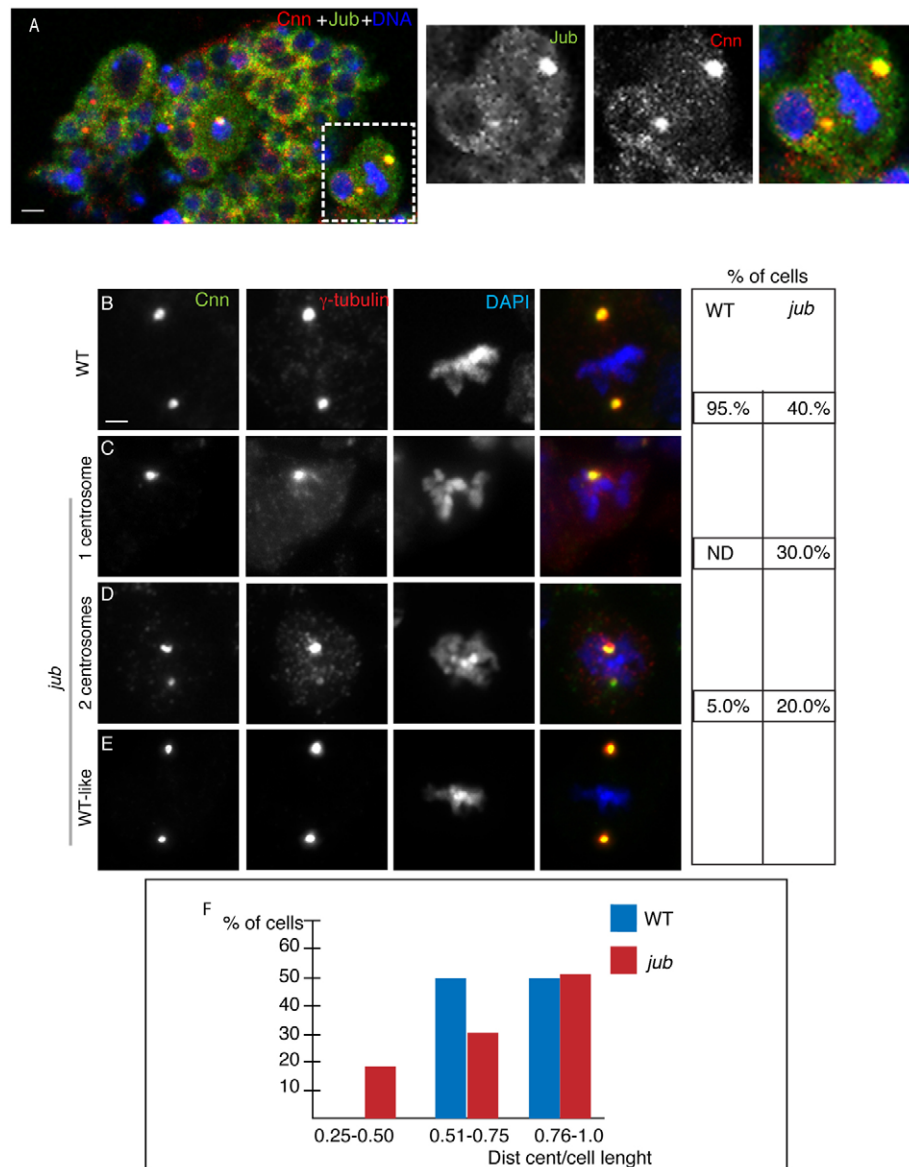
(A) Schematic representation of the *jub* genetic region (top). CG11063 contains six exons (dark blue boxes). It is an X chromosome gene, flanked by CG32626 and CG11092 (gray boxes). The EP-element EPG845 is located at the 5'-UTR of *jub*, 163 bp upstream of the translational start codon (ATG), as assigned by the Berkeley genome project release R5.26. Light blue boxes represent the *jub* 5'-UTR and 3'-UTR. Excision of this EP element resulted in the *jub<sup>E1</sup>* deletion. (B) A C-terminal fusion of *jub* and GFP under the control of the *jub* endogenous promoter (bottom diagram in A) rescues the *jub* mutant phenotype. In the wing disc epithelium, Jub-GFP (first panel, green in the merged picture) is localized to the apical cortex slightly above Coracle (second panel, red in the merged picture), which labels septate junctions. Jub is not detected at the basal cortex, stained by laminin (third panel, blue in the merged picture). Jub-GFP is also seen at cell–cell contacts (enlarged area in B, arrow). (C) In the embryo, Jub-GFP localizes at cell–cell contacts. The localization of Jub-GFP is mainly apical. (D) *jub* mutant clones (broken line) surrounded by WT cells, positive for GFP (left panel, green in the merged picture), labeled with E-Cadherin (second panel, red in the merged picture). DNA is shown in blue in the merged picture. Scale bars: 20  $\mu$ m (B,D); 100  $\mu$ m (C).

We next examined how loss of Jub affected centrosome function. Mitotic cells in the brains of *jub* homozygous mutant larvae had a range of defects: 10% lacked detectable centrosomes (data not shown), 30% had a single centrosome (Fig. 2C), 20% had two centrosomes that were unusually close to each other and mispositioned at the cell center (Fig. 2D,F), and the remaining 40% were WT-like (Fig. 2E). We measured the distance between centrosomes in well-established bipolar spindles, taking into account the overall cell length at metaphase (the distance between centrosomes over the cell length). We found that in 17% of *jub* mutant Nbs this ratio was very small (between 0.25–0.50), which was never the case in WT Nbs (Fig. 2F).

The centrosome defects suggest that Jub has a role in centrosome function. We tested the recruitment of several centrosome proteins in *jub* mutants and did not observe defects in the localization of either centriole or PCM proteins (Fig. 3). We quantified the intensity of the centriole- and PCM-associated protein Asterless (Asl) and the PCM component Cnn at the centrosome in both WT ( $n=20$  metaphase Nbs) and *jub* mutant ( $n=22$  metaphase Nbs) cells, and we did not detect significant differences [Asl intensity in WT

$129.4\pm28.8$  compared with that in *jub* mutants  $118.1\pm33.5$ ,  $P>0.2$ ; Cnn intensity in WT  $149.8\pm37.9$  compared with that in *jub* mutants  $148.9\pm39.8$ ,  $P>0.9$  (intensities are given as the mean gray value, as described in the Materials and Methods); Fig. 3E]. This demonstrates that the *jub* mutant phenotype is not caused by a failure to recruit these centriole- and PCM-associated proteins.

The resolution of our images was not sufficient to resolve whether the ‘single’ centrosome found in many *jub* mutant Nbs contained one or two centrosomes as the single centrosome could have arisen from defects in either duplication or separation mechanisms. We favor a defect in separation as, in several cells, two ‘dots’ close to each other could be distinguished (Fig. 3A,D, arrowheads). However, to completely rule out a possible function in centrosome duplication, we tested whether Jub was required for the increased centriole replication caused by overexpression of the centriole replication kinase Sak (also known as Plk4) (SakOE) (Basto et al., 2008). In 60% of SakOE Nbs, supernumerary centrosomes were detected (supplementary material Fig. S1B). The generation of extra centrosomes was blocked by *Sas-4* mutations (supplementary material Fig. S1C), but not in the *jub* mutant (supplementary material



**Fig. 2. Jub-GFP is associated with the centrosomes in mitotic neuroblasts, and *jub* mutants display centrosome defects.** (A) Jub-GFP (green) and Cnn (red) colocalize at the centrosome in third-instar larval mitotic Nbs. DNA is shown in blue. (B–E) In *jub* mutant third-instar larval brains, centrosome separation defects (C,D) can be detected. In the majority of WT mitotic cells (B), two centrosomes, revealed by Cnn (first panel, green in the merged picture) and  $\gamma$ -tubulin (second panel, red in the merged picture) staining, can be detected. In 30% of *jub* mutant mitotic cells (C), we only detected what seems to be a single centrosome. In 20% of *jub* mutant mitotic cells (D), two centrosomes close to each other can be detected. In 40% of *jub* mutant mitotic cells (E), centrosomes behave as in the WT cells. (F) Bar graph quantifying the ratio of the distance between the centrosomes and the cell length at metaphase in WT (blue bar) and *jub* mutant Nbs (red bar). Scale bars: 20  $\mu$ m (A); 10  $\mu$ m (B). ND, not detected.

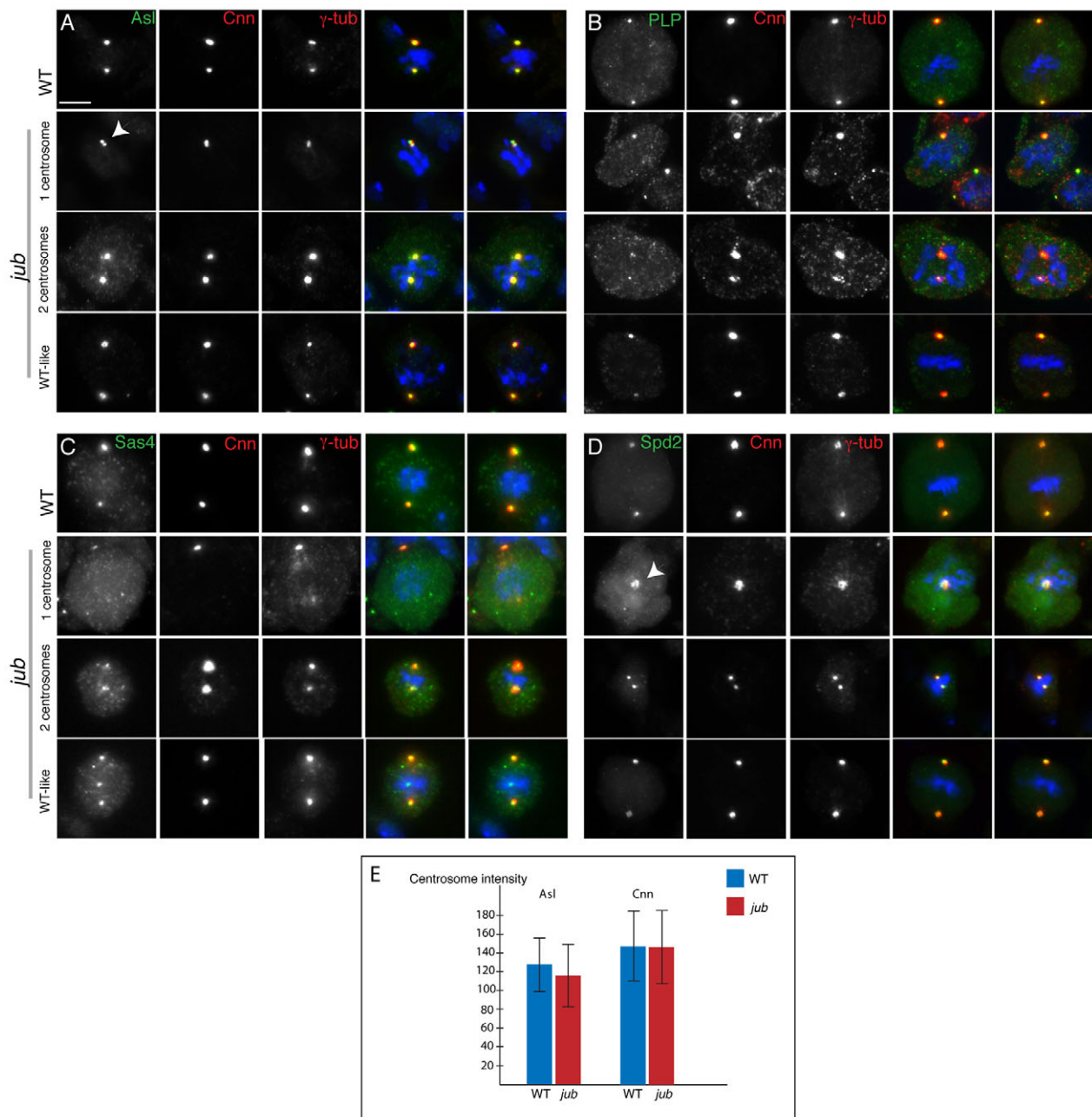


Fig. S1D). This demonstrates that Jub is not required for multiple rounds of centriole duplication and thus suggests that it is not required for centriole duplication. Therefore, the defects in *jub* mutant Nbs, including the appearance of 'single' centrosomes, probably arise from a failure in centrosome separation.

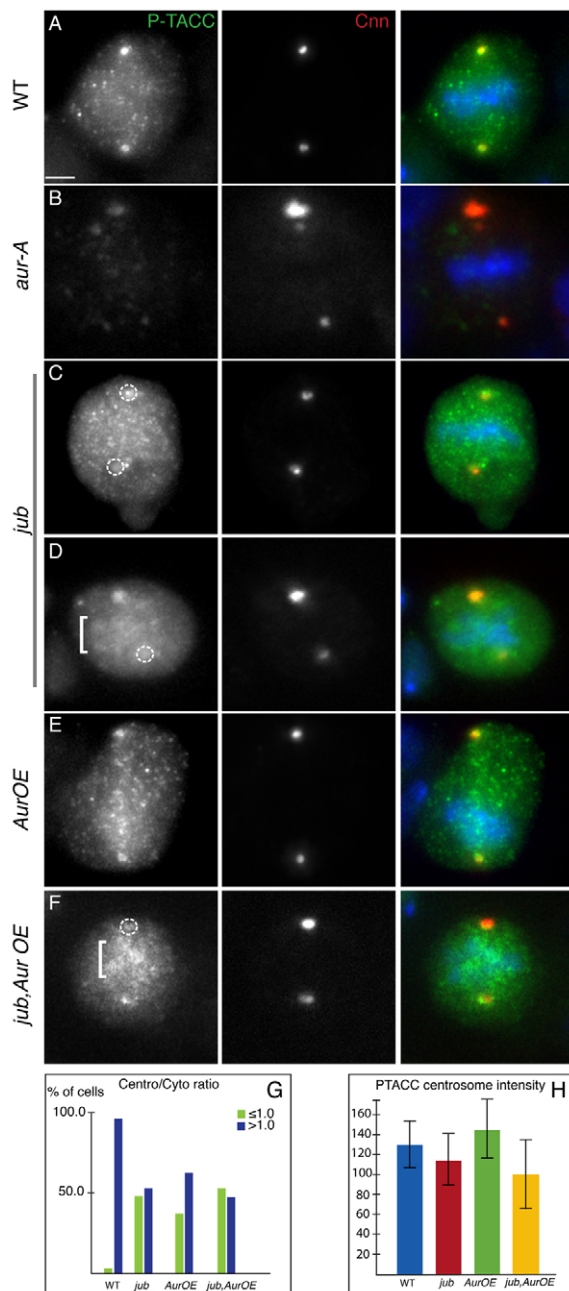
Aur-A, a major mitotic kinase, is known to regulate several mitotic events at the centrosome level, such as separation and maturation (Barr and Gergely, 2007). Consistent with evidence in human cells showing that Jub activates Aur-A (Hirota et al., 2003), it is known that *Drosophila* Nbs lacking Aur-A also have defects

in centrosome separation (Glover et al., 1995), which we were able to confirm in our observations (data not shown). However, the phenotypes of mutations in *jub* and *aur-A* are not identical; *aur-A* mutants have additional defects (data not shown) (Glover et al., 1995), demonstrating that the presence of Jub cannot be a requirement for all Aur-A activity, and therefore is unlikely to be the sole activator of this kinase, even in Nbs.

To test whether the kinase activity of Aur-A was perturbed in *jub* mutants, we used antibodies that specifically recognized the phosphorylated form of 'transforming acidic coiled-coil protein'



**Fig. 3. Centriole and PCM markers are recruited in *jub* mutant brain cells.** The centriole- and PCM-associated proteins Asl (A) and Plp (B), the centriolar protein Sas-4 (C) and the PCM protein Spd2 (D) (left panels, green in the merged picture) are recruited to the centrosome, together with Cnn (second panel, red in the first merged picture) and  $\gamma$ -tubulin ( $\gamma$ -tub) (third panel, red in the second merged picture) in WT (top row) and *jub* mutant cells, independently of the behavior of the centrosomes. In some cases, two dots close to each other can be distinguished (arrowheads in A,D). DNA is shown in blue. (E) Bar graph plotting the intensity of the centriole- and PCM-associated protein Asl (left) and the PCM component Cnn (right) at the centrosome in both WT (blue bars) and *jub* mutant cells (red bars). Scale bar: 10  $\mu$ m.



(Tacc), a MT-associated protein (Gergely et al., 2000a; Gergely et al., 2000b). In *Drosophila* embryos, Tacc is associated with centrosomes and spindle MTs during mitosis, but the phosphorylated form, P-Tacc, is only found at centrosomes (Barros et al., 2005). Examination of the localization of P-Tacc in WT Nbs showed that it was concentrated at the centrosomes in mitotic cells (Fig. 4A), although noticeable levels were also detected in the cytoplasm, which suggests that active Aur-A is also present in the cytoplasm. We measured the intensity of P-Tacc at the centrosome and in the cytoplasm, and calculated the ratio between the centrosome and cytoplasm intensities (Fig. 4G,H, centro/cyto ratio). In the majority of WT Nb cells (96%;  $n=34$  cells), this ratio was higher than 1, suggesting that there was an enrichment at the centrosome, with a mean centrosome intensity of  $130.2 \pm 25.6$  (Fig. 4G,H). As expected, in *aur-A* mutants P-Tacc levels were

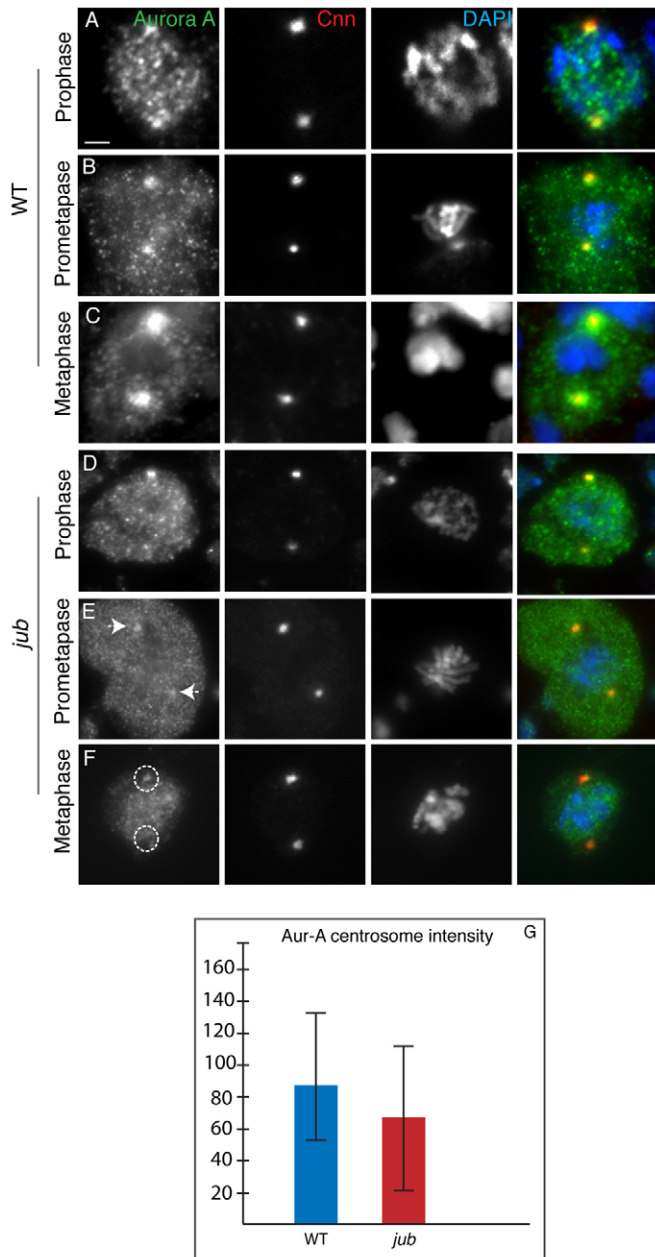
**Fig. 4. Aur-A kinase is active in *jub* mutant cells but displaced from the centrosome.** (A) In WT metaphase cells, P-Tacc (P-TACC; first panel, green in the merged picture) is detected strongly at the centrosome (revealed by Cnn staining, second panel, red) and in the cytoplasm. DNA is shown in blue. (B) In *aur-A* mutants, P-Tacc levels are extremely reduced both at the centrosome and in the cytoplasm. (C,D) In *jub* mutants, the P-Tacc distribution is different from WT. P-Tacc levels are reduced at the centrosome (white dotted circles in C and D) but increased in the cytoplasm, and P-Tacc could even be seen accumulating around the chromatin region (brackets in D). (E) In *AurOE* cells, P-Tacc levels are increased at the centrosome and cytoplasm. (F) In *jub* mutant *AurOE* cells, P-Tacc levels are increased in the cytoplasm and around DNA (square brackets) but decreased at the centrosome (highlighted by the white dotted circle). (G) Quantification of the ratio between the average intensity of P-Tacc at the centrosome and cytoplasm (centro/cyto ratio). 96% of WT cells have a centro/cyto ratio greater than 1 (blue column). 47% of *jub* mutant cells have a centro/cyto ratio lower than 1 (green column). 63% of *AurOE* cells display a centro/cyto ratio greater than 1; in 53% of *jub* mutant *AurOE* cells, this ratio is also lower than 1. (H) Quantification of the intensity of P-Tacc (PTACC) at the centrosome. *jub* mutant and *jub* mutant *AurOE* cells show a significant reduction in the P-Tacc signal compared with WT cells, whereas *AurOE* cells have higher levels than WT cells. Scale bar: 10  $\mu$ m.

substantially decreased (Fig. 4B), both at the centrosome and cytoplasm. Loss of Jub, however, did not result in the disappearance of P-Tacc but rather an alteration in its distribution within the cell. A diminished mean intensity of P-Tacc at the centrosome ( $114.7 \pm 26.0$ ) could be detected, and this was accompanied by an increase in the cytoplasmic level, which resulted in a centro/cyto ratio lower than 1 in 47% ( $n=58$ ) of the Nbs (Fig. 4C,D,G,H).

As we detected a difference in P-Tacc distribution rather than in its amount in *jub* mutants, we next examined Aur-A distribution directly by antibody staining. In WT cells, Aur-A was first recruited to the centrosomes before nuclear envelope breakdown, and the amount at the centrosomes increased as mitosis progressed (Fig. 5A–C). In *jub* mutants, the initial recruitment occurred, although at a decreased level (Fig. 5D), but, unlike in WT cells, Aur-A did not accumulate at the centrosome as mitosis progressed (Fig. 5D–F). Quantification of the Aur-A intensity at the centrosome in metaphase cells revealed a significant decrease in Aur-A in *jub* mutant cells ( $68.8 \pm 44.8$ ;  $n=86$ ) when compared with that in WT cells ( $88.5 \pm 39.1$ ;  $n=38$ ;  $P<0.02$ ). This suggests that Jub is required to recruit and/or maintain Aur-A at the centrosome throughout mitosis.

Aur-A plays a role in the recruitment of Tacc and 'Mini spindles' (MSPs) to the centrosome, which promotes astral MT stabilization (Giet et al., 2002). The decrease in Aur-A levels at the centrosome in *jub* mutant cells during metaphase could therefore result in a decrease in the recruitment of Tacc to the centrosome. To investigate this question, we stained WT and *jub* mutant Nbs with anti-Tacc antibodies. In prophase cells, Tacc was recruited to the centrosome in *jub* mutant cells in the same manner as in WT cells (Fig. 6A,C; data not shown). By metaphase, however, even if an obvious difference could not be seen, the intensity of Tacc at the centrosome was significantly different between WT ( $n=42$ ) and *jub* mutant cells ( $n=56$ ) (Fig. 6B,D,E;  $148.8 \pm 39.1$  in WT compared with  $122.4 \pm 41.8$  in *jub* mutants;  $P<0.002$ ). The lack of Tacc recruitment or maintenance at the centrosome in metaphase *jub* mutant Nbs is probably a consequence of not maintaining Aur-A at the centrosome.

We next sought to address whether, in the absence of Jub, it was the loss of active Aur-A from the centrosome or the elevation of



**Fig. 5. The recruitment and/or maintenance of Aur-A to the centrosome during mitosis is impaired in *jub* mutants.** (A–C) In WT brain cells, Aur-A (left panel, green in the merged panel) localizes at the centrosome (left panel, Cnn, red in the merged panel) during prophase (A) (DNA staining, third panel, blue in the merged panel). During prometaphase (B), Aur-A intensity increases both at the centrosome and cytoplasm. By metaphase (C), Aur-A intensity is even higher at the centrosome and cytoplasm but is excluded from the DNA region. (D–F) In *jub* mutants, the initial recruitment during prophase (D) still takes place, albeit to a lower level when compared with that in WT. During prometaphase (E) and metaphase (F), Aur-A does not accumulate at the centrosome (arrows in E and dashed circles in F) but is still increased in the cytoplasm. (G) Quantification of the intensity of Aur-A signal at the centrosome in WT (blue bar,  $n=36$ ) and *jub* (red bar,  $n=86$ ) metaphase cells. Scale bar: 10  $\mu$ m.

active Aur-A in the cytoplasm that was causing the cytoplasmic accumulation of P-Tacc. To test this, we overexpressed Aur-A, surmising that this would increase the amount in the cytoplasm

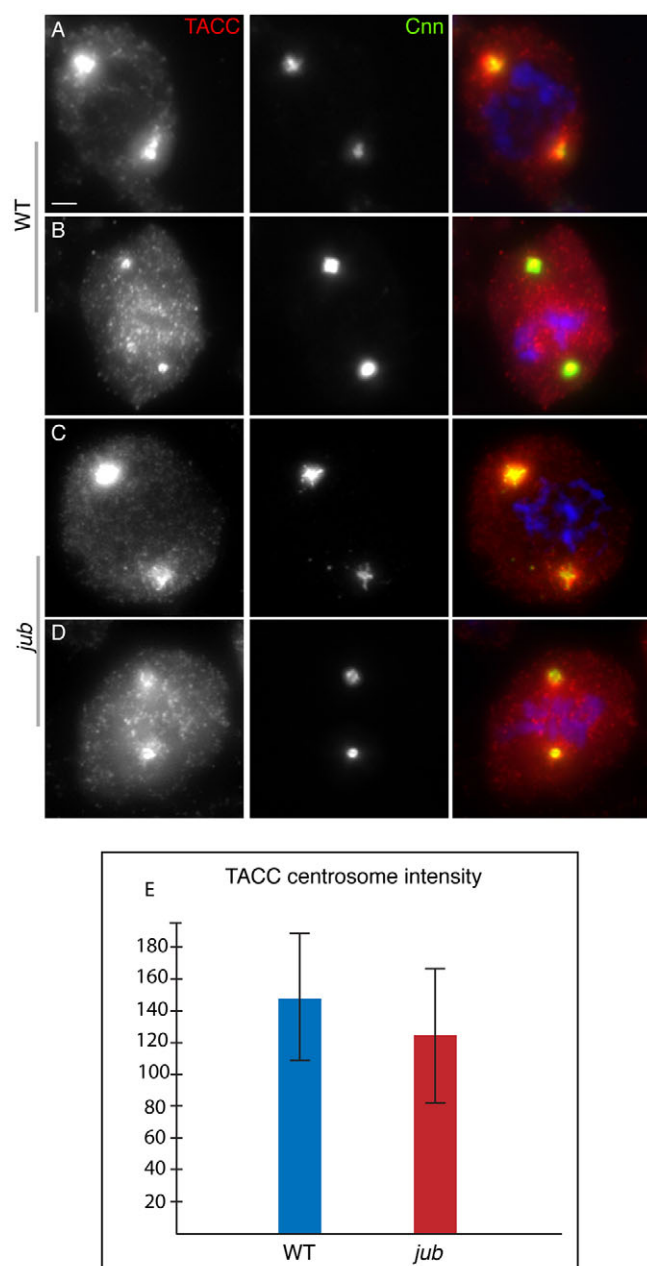
without reducing the amount at the centrosome. We used flies that moderately overexpressed GFP–Aur-A under the control of a ubiquitous promoter (Ubi) in all cells (Lee et al., 1988). Aur-A-overexpressing flies (AurOE) developed slower than WT flies, but we did not detect an increase in lethality or any developmental defect (data not shown).

In AurOE Nbs, we detected increased levels of P-Tacc at both the centrosome (average intensity of  $143.8 \pm 28.7$ ;  $n=38$ ) and in the cytoplasm (Fig. 4E). However, the centro/cyto ratio was higher than 1 (Fig. 4G,H) in only 63% of the cells compared with 96% of WT cells, indicating that Aur-A overexpression results in a greater degree of P-Tacc accumulation in the cytoplasm. In the absence of Jub, overexpressed Aur-A did not increase the levels of P-Tacc at the centrosome (average intensity of  $100.3 \pm 34.3$ ;  $n=38$ ) but did cause even higher levels in the cytoplasm (Fig. 4F), with 53% of *jub* mutant AurOE cells having a centro/cyto ratio lower than 1 (Fig. 4G,H), which is similar to the *jub* mutation alone. Our results therefore suggest that Jub is not required to activate Aur-A but, rather, to recruit or maintain the active kinase at the centrosome during mitosis. It seems probable that Aur-A is activated at the centrosome in Nbs and in other cell types (Portier et al., 2007; Hachet et al., 2007), and that Jub maintains active kinase at the centrosome; in the absence of Jub, Aur-A is released into the cytoplasm in an untimely fashion.

In addition to the centrosome separation defects, *jub* mutant Nbs have mitotic spindle defects, even in Nbs with two well-separated centrosomes. These include bipolar anastral spindles (Fig. 7B; compare the spindle with the WT spindle shown in Fig. 7A) (20%;  $n=40$ ) and completely disorganized spindles, which appear to be nucleated by the chromatin pathway (Fig. 7C; 25%;  $n=40$ ). This latter type of disorganized spindle was also found when Aur-A was overexpressed (Fig. 7D; 52%;  $n=45$ ). The occurrence of these spindle abnormalities thus correlates with the elevation in active Aur-A in the cytoplasm. To assess the relationship between Aur-A and spindle abnormalities, we examined how elevation or reduction of Aur-A affected the *jub* mutant spindle phenotype. In *jub* mutant AurOE Nbs, the proportion of disorganized spindles was even higher (61%;  $n=37$ ; Fig. 7E) than that in *jub* mutants or AurOE cells alone. Furthermore, mitosis in *jub* mutant AurOE cells was highly perturbed, as revealed by an increased mitotic index (MI); these cells showed severe mitotic abnormalities, such as aneuploidy, polyploidy and anaphases with lagging chromosomes (data not shown). These more severe defects were not present in *jub* mutants, where the MI was not significantly different from that of WT (WT MI  $1.66 \pm 1.0$ ,  $n=6807$ , compared with the *jub* mutant MI  $1.88 \pm 0.9$ ,  $n=11197$ ;  $P>0.4$ ). This is consistent with the lack of Jub resulting in an even greater fraction of the overexpressed and active Aur-A becoming misplaced in the cytoplasm and causing these defects. Consistent with this view, the percentage of abnormal spindles in *jub* mutants was suppressed by removing one copy of Aur-A because in *jub*, *aur*<sup>+/-</sup> mutant cells the total number of spindle defects was decreased from 45% to 34% ( $n=106$ ) (Fig. 7F).

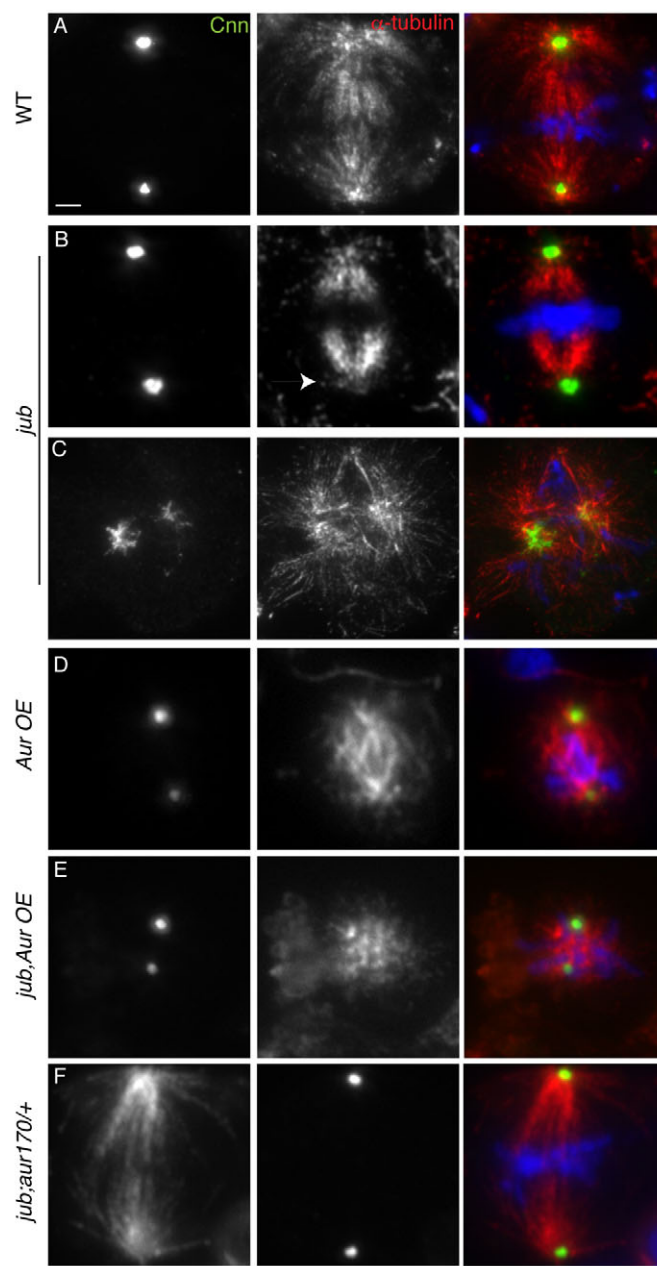
In conclusion, it is clear that, in *Drosophila*, Jub is not required to activate Aur-A, but that it plays an essential role in regulating its spatial function. We attempted to find a biochemical interaction between Aur-A and Jub in *Drosophila* brain extracts using two different approaches but we were not successful (supplementary material Fig. S2). This raises the possibility that in *Drosophila* Jub does not bind Aur-A directly and, therefore, that it maintains Aur-A at the centrosome through an unidentified partner.





**Fig. 6. Tacc localization is perturbed in *jub* mutant cells.** In WT (A,B) and *jub* mutant Nbs (C,D), Tacc (TACC; first panel, red in the merged picture) is recruited to the centrosomes (second panel, green in the merged picture) during prophase (top row). By metaphase (bottom row), a weaker Tacc signal is detected at the centrosome. (E) Quantification of the intensity of Tacc signal at the centrosome in WT (blue bar,  $n=42$ ) and *jub* (red bar,  $n=56$ ) metaphase cells. Scale bar: 20  $\mu\text{m}$ .

Our results are consistent with Jub having an essential job in keeping the active kinase at the centrosome, which is important for at least three reasons. First, Aur-A activity is required at the centrosome to trigger centrosome separation; this probably occurs by Aur-A phosphorylating a yet unidentified substrate within the centrosome. Second, Aur-A activity on the centrosome might be required to produce sufficient levels of P-Tacc on the centrosome; the lack of astral MTs in *jub* mutant Nbs might result from reduced centrosomal P-Tacc given that loss of astral MTs also occurs in

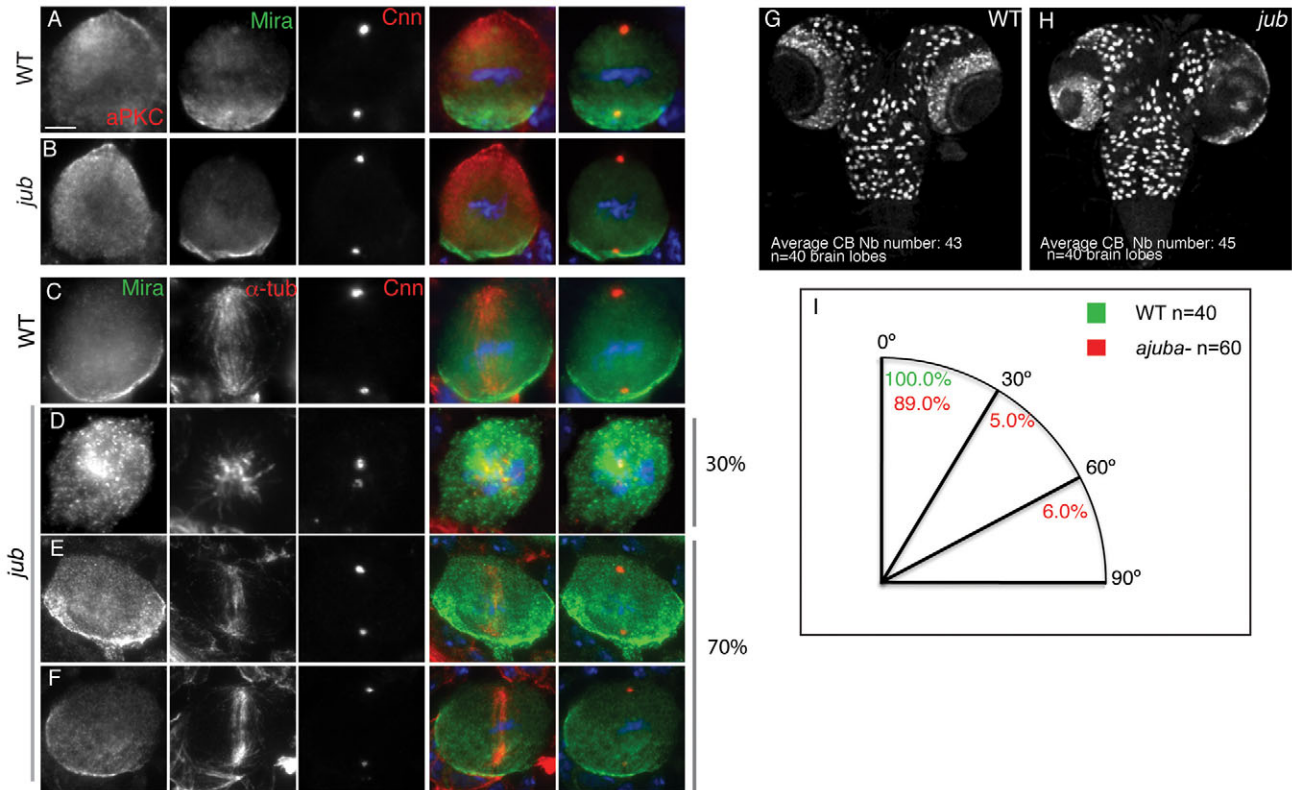


**Fig. 7. Defects in spindle organization can be found in *jub* mutants.**

(A) The distribution of centrosomes (Cnn, left panel, green in the merged panel), MTs (second panel, red in the merged panel) and DNA (blue) in WT cells, shows a well-organized bipolar mitotic spindle that displays a robust array of astral MTs. (B) 20% of *jub* mutant cells display a weak array of astral MTs (arrowhead). In 25% of *jub* mutant cells (C), 52% of AurOE cells (D) and 61% of *jub* mutant AurOE cells (E), the spindle is disorganized and, even when centrosomes are present, seems to be acentrosomal. (F) Reduction of Aur-A levels in the *jub* mutant background (*jub;aur170/+*) decreases the proportion of cells with disorganized spindles to 34%. Spindle morphology was only quantified in cells that contained two centrosomes. Scale bar: 10  $\mu\text{m}$ .

*tacc* mutant embryos (Barros et al., 2005) and Nbs (supplementary material Fig. S3). Third, maintaining active Aur-A at the centrosome prevents its abnormal accumulation in the cytoplasm. Aur-A is known to phosphorylate several MT-associated proteins, such as Msp5 (the ChTog and XMap215 homolog), Eg5, XMCAK, ASAP





**Fig. 8. In *jub* mutant Nbs, cortical targeting of Mira is delayed and spindle misorientation can be detected, but *jub* mutants do not display defects in Nb self-renewal.** In WT (A) and *jub* mutant (B) cells, aPKC (first panel, red in the merged picture) accumulates at the apical cortex, opposite to Mira (second panel, green in the merged picture). (C) In WT cells, the mitotic spindle (second panel, red in the merged first panel) and the two centrosomes (third panel, red in the merged second panel) are correctly positioned perpendicularly to the Mira crescent (first panel, green in the merged panels). (D) In 30% of metaphase *jub* mutants, Mira cortical targeting is delayed. Mira is either in the cytoplasm or associated with the centrosome and spindle MTs. (E,F) In 70% of metaphase *jub* mutants, Mira is associated with the basal cortex. In 11% of metaphase *jub* mutants (F), the mitotic spindle is not aligned along the polarity axis (I). (G,H) Nb number, revealed by Mira staining in WT cells (G) is comparable to that in *jub* mutant cells (H). CB, central brain. Scale bar: 10  $\mu$ m.

(Carmena et al., 2009) and p150 (glued) (Rome et al., 2010). It is therefore possible that higher levels of active kinase in the cytoplasm result in abnormal phosphorylation of these proteins (or even other unidentified substrates), resulting in abnormal mitotic spindles.

We next tested whether Jub contributes to the recently described role for Aur-A in controlling polarity during Nb asymmetric cell division. Aur-A inhibits Nb self-renewal by regulating cortical targeting of cell-fate determinants and spindle orientation (Lee et al., 2006a; Wang et al., 2006; Wirtz-Peitz et al., 2008). Cortical atypical protein kinase C (aPKC) is essential for promoting self-renewal of Nbs, and normally becomes concentrated at the apical cortex in WT Nbs undergoing mitosis (Knoblich, 2008; Lee et al., 2006b; Gönczy, 2008). This occurred normally in *jub* mutant Nbs (Fig. 8A,B); by contrast, in *aur-A* mutants, aPKC is uniformly distributed in 26% of the cases (Lee et al., 2006a). At the opposite side of the cell, cell-fate determinants, such as Prospero, Numb, Brat and their adaptor proteins Miranda (Mira) and Pon, localize to the basal cortex, forming a crescent that is inherited by the ganglion mother cell (GMC). In *jub* mutant Nbs at metaphase, Mira was within a crescent in only 70% of the cells (Fig. 8E,F), compared with 98% WT Nbs ( $n=40$ ) (Fig. 8C); 30% of *jub* mutant cells had Mira distributed throughout the cytoplasm or associated with spindle MTs at this stage (Fig. 8D). In *Sas-4* mutants, where centrosome duplication is affected, Mira is also mislocalized at

anaphase and telophase, resulting in its segregation to both daughter cells (Basto et al., 2006). However, this was not seen in *jub* mutant Nbs, where Mira concentrated exclusively in the daughter GMC at anaphase (100%;  $n=201$ ), suggesting that there was only a delay in cortical Mira targeting (data not shown).

In addition to having defects in the asymmetric distribution of proteins, *aur-A* mutant Nbs fail to position the spindle correctly (Hutterer et al., 2006; Johnston et al., 2009; Lee et al., 2006a; Wang et al., 2006; Wirtz-Peitz et al., 2008). This is also the case with mutations that specifically affect centrosome behavior, causing the spindle to be aligned orthogonally to the polarity axis (Basto et al., 2008; Basto et al., 2006; Cabernard and Doe, 2009; Lucas and Raff, 2007; Rusan and Peifer, 2007). In metaphase Nbs, the mitotic spindle was normally oriented along the polarity axis in 100% of WT cells ( $n=40$ ) (Fig. 8C), but in *jub* mutants 11% ( $n=60$ ) had an abnormal spindle orientation, with 89% showing a WT orientation (Fig. 8E,F,I). The mispositioned spindles invariably had a poor astral MT array, suggesting that the failure in alignment results from the lack of astral MTs. To test whether these defects resulted in supernumerary Nbs, we quantified Nb numbers in the presence and absence of Jub. We did not observe any major difference (Fig. 8G,H), suggesting that the defects in spindle positioning and cortical targeting of cell-fate determinants are corrected as the cells exit mitosis, as is the case with other *Drosophila* mutations (Cai et al., 2001; Peng et al., 2000).

## Discussion

In this study, we generated mutations in the *jub* gene in *Drosophila* in order to examine its functions within an intact animal without the complications of potential redundancy with closely related genes. Unexpectedly, we discovered that Jub had an essential role in just a subset of cells within the animal, namely the neural stem cells. Although we have not exhaustively examined all cell types, cell cycles that are normally very sensitive to centrosome or MT perturbation, such as the nuclear divisions of the early embryo (Lucas and Raff, 2007; Stevens et al., 2007) and the meiotic divisions of the male germline (Martinez-Campos et al., 2004), occurred normally in the absence of Jub. Thus, Nbs are especially dependent on Jub to generate normal centrosomes and spindles, consistent with the clearly detectable levels of Jub-GFP on the centrosomes in these cells, but not in other cell types.

Within the Nbs lacking Jub, we have detected three related, but distinct, phenotypes: defects in the separation of centrosomes following mitosis, defects in spindle assembly, and defects in cortical targeting of determinants and orientation of the mitotic spindle. These phenotypes are shared by Nbs lacking Aur-A, consistent with previous work demonstrating that Jub and Aur-A proteins bind to each other and function together (Hirota et al., 2003). However, we were unable to detect a biochemical interaction between these two proteins in *Drosophila* brains. In addition, the loss of Aur-A causes a number of additional defects that we did not observe in *jub* mutant Nbs, such as defects in centrosome maturation (Berdnik and Knoblich, 2002; Giet et al., 2002) and increased levels of genomic instability (Castellanos et al., 2008; Glover et al., 1995), demonstrating that Jub is not required for the majority of Aur-A functions.

It is worth mentioning that we failed to identify Nbs with supernumerary centrosomes in *jub* mutants. Defects in centrosome separation should result in the generation of daughter cells without centrosomes (which we see in 10% of the cells in *jub* mutant brain cells) and in those with two centrosomes, which should undergo duplication during the following cell cycle to produce extra centrosomes. Future work will be required to explain the absence of Nbs with supernumerary centrosomes.

Our results show that, in the absence of Jub, Aur-A is not as concentrated at the centrosome, and hence Tacc recruitment is affected. However, even in the absence of Jub, Tacc can be phosphorylated by Aur-A, which further supports the idea that Jub is not an Aur-A activator, at least in *Drosophila*. Furthermore, it appears that loss of Jub results in a displacement of Aur-A from the centrosome. Thus, the key question is whether the defects caused by loss of Jub are due to diminished Aur-A activity on the centrosome, elevation of activity in the cytoplasm or both. The defects in centrosome separation, just after cell division, might be due to diminished levels of Aur-A on the centrosome, whereas the loss of astral MTs might be explained by diminished levels of P-Tacc or other MAPs. Our manipulation of Aur-A levels in the presence or absence of Jub suggests that it is the elevated cytoplasmic Aur-A activity that is causing the defects in spindle assembly. Elevated cytoplasmic Aur-A is also likely to account for the defects in spindle positioning during asymmetric cell division.

One possible explanation for the Nb-specific requirement for Jub is that the centrosome cycle is significantly different in these cells (Rebollo et al., 2007; Rusan and Peifer, 2007). In Nbs, just after centrosome duplication and migration to the apical cortex, one of the centrosomes moves away from the other. This dynamic centrosome continues to move throughout the S and G2 phases,

which means that centrosome separation in Nbs takes place substantially before mitosis, in contrast with the timing in other cell types (Rebollo et al., 2007; Rusan and Peifer, 2007). It is therefore possible that Jub is only required for centrosome separation in cells where centrosomes separate earlier in the cell cycle, substantially before the following mitosis, when Aur-A activity is still present.

Finally, we have not obtained evidence to support a role for Jub as an Aur-A activator (Hirota et al., 2003), as we did not see a reduction in the phosphorylation of the Aur-A substrate Tacc. Many cell types require Aur-A function, including embryos and male spermatocytes. We did not detect Jub-GFP at the centrosome in these cells and did not see *jub* mutant phenotypes in the early embryo or male germline (data not shown). In addition, we were unable to co-immunoprecipitate Jub and Aur-A in brain extracts. We therefore propose that the main function of Jub is to bind Aur-A at the centrosome, not to activate the kinase, but rather to restrict its activity in time and space. Too much active Aur-A in the cytoplasm during mitosis seems to perturb astral MT nucleation and centrosomal spindle assembly. Alternatively, Jub might also help to recruit and/or maintain Aur-A at the centrosome, so that it can be activated by another protein concentrated there, and, most crucially in Nbs, 'hold' the active Aur-A away from the cytoplasm. Unfortunately, such a candidate protein has not yet been identified in flies. Flies do not have an obvious TPX2 orthologue and the only Aur-A activator identified so far lacks a function in the fly brain (Hutterer et al., 2006). The failure of Jub to regulate Aur-A in *Drosophila* could also just reflect differences in the way Aur-A is regulated between vertebrates and invertebrates. In human cells, Jub is also associated with kinetochores and spindle MTs, and it has been shown that Jub, together with BubR1 and Aurora B, plays a role in the regulation of the metaphase-to-anaphase transition (Ferrand et al., 2009). However, the lack of a mitotic phenotype in Jub-knockout mice also strongly suggests that it might not play an essential role in Aur-A activation.

## Materials and Methods

### Fly stocks

The EP-element insertion in the *jub* gene, *EPG845*, was generated by GenExel, Korea. The insertion was located in the 5'-UTR of *jub*, 163 bp upstream of the translational start codon (ATG). We generated a *jub* excision mutant by EP-element mobilization with the transposase source *P{Δ2-3}99B*, provided by the stock *TM2, ry P{Δ2-3}99B/MKRS, P{Δ2-3}99B* (Bloomington Stock Center). Mobilization was performed using standard procedures. In this study we characterize the *jub<sup>EL</sup>* line that resulted from a deletion of 1.3 kb of the X chromosome (spanning from 13724163 to 13725403 bp). *jub* mutants died at the larval-pupal transition and the *jub<sup>EL</sup>* mutation is probably an hypomorph, as other *jub* mutations die earlier in development (Das Thakur et al., 2010). *jub* mutant lethal chromosomes were balanced with FM7c marked with GFP to allow distinguishing between WT and mutant embryos. Different Jub-GFP transgenic lines were combined with the *jub* mutation, both for imaging and for rescue experiments. The following stocks were also used in our experiments: *SakOE* and *Sas-4;SakOE*, (Basto et al., 2008), *aur<sup>170</sup>* and *aur<sup>190</sup>* (Glover et al., 1995), GFP-tubulin, *tacc<sup>1</sup>* and *tacc<sup>stella592</sup>* (Lee, M. J. et al., 2001). We used *wf* flies as the control line for our experiments.

### Live imaging

Dechorionated embryos were aligned on coverslips, mounted in halocarbon oil and were examined on a spinning disc confocal system (Nikon Eclipse TE2000-E), with a 63× objective, equipped with Metamorph software and a CoolSnapHQ2 camera.

### Immunostaining

For most of the immunostaining procedures, we used the following protocol: third-instar larvae brains were dissected, immediately fixed in 4% formaldehyde for 20 to 30 minutes at room temperature, then placed in 45% acetic acid for 5 seconds and in 60% acetic acid for 3 minutes. The brains were squashed between a coverslip and a glass slide, immersed in liquid nitrogen, placed in ice-cold methanol for 7 minutes, rehydrated in PBT (PBS with 0.1% Triton X-100) three times for 5



minutes and washed in PBS twice. For P-Tacc and Aur-A stainings, we blocked the sample twice in PBS with 5% BSA, for 30 minutes each, before adding the primary antibodies.

Whole-mount brains were immunostained using the following protocol: third-instar larvae brains were dissected and fixed in 4% paraformaldehyde for 40 minutes at room temperature, washed three times for 10 minutes, incubated with primary antibodies, washed three times for 10 minutes, incubated with secondary antibody for 2 hours at 25°C, washed three times and stained with 0.5 mg/ml Hoechst 33258 for 20 minutes (all washes and incubations were performed in PBT). This protocol was adapted to the brains from the *jub* mutant expressing Jub-GFP by substituting PBT for PBS in all steps of the procedure.

Third-instar wing imaginal discs were dissected and immunostained using the following protocol: the tissue was fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed three times for 10 minutes with PBS and three times for 10 minutes with PBT, incubated with primary antibodies overnight at 4°C, washed three times for 10 minutes with PBS, incubated with secondary antibody overnight at 4°C and washed three times with 0.5 mg/ml Hoechst 33258 in PBT for 20 minutes.

Primary antibodies were against the following proteins: P-Tacc (rabbit, 1:500) (Barros et al., 2005), Cnn (guinea-pig, 1:500) (Lucas and Raff, 2007), DM1a ( $\alpha$ -tubulin, mouse, 1:1000; Sigma), GTU88 ( $\gamma$ -tubulin, mouse, 1:1000; Sigma), Aur-A (rabbit, 1:500) (Barros et al., 2005), Ast (rabbit, 1:500) (Stevens et al., 2009), Plp (rabbit, 1:500) (Martinez-Campos et al., 2004), Sas-4 (rabbit, 1:500) (Basto et al., 2006), Spd2 (rabbit, 1:500) (Dix and Raff, 2007), aPKC (rabbit, 1:500; SC-216, Santa Cruz Biotechnology), Mira (mouse, 1:20) (Ikeshima-Kataoka et al., 1997), Mira (rabbit, 1:200) (Shen et al., 1997), Tacc (mouse, 1:500) (Gergely et al., 2000a), *Drosophila* E-Cadherin (rat, 1:70) (Hybridoma Bank). Secondary antibodies were purchased from Molecular Probes (Invitrogen).

Preparations of squashed brains were examined using either a Nikon Eclipse 90i Upright Microscope with a Piezo Flexure Objective Scanner and a 100 $\times$  lens, or a Leica DM6000B Microscope and a 63 $\times$  or 100 $\times$  lens, using a charge-coupled-device camera (CoolSNAP HQ2). Whole-mount brains were imaged with an Eclipse Ti inverted microscope with a 40 $\times$  objective. All image acquisitions were performed with Metamorph software. All images were processed using the Adobe Photoshop software.

#### Quantification of centrosome, spindle defects and spindle positioning

Quantification of centrosome number in WT and *jub* mutant brain cells was restricted to mitotic cells where both centrosomal markers (Cnn and  $\gamma$ -tubulin) colocalized. Classification of spindle morphology was restricted to cells that contained two centrosomes. The alignment of the mitotic spindle relative to the axis of polarity was calculated using the angle measurement tool in the Metamorph software. We only quantified metaphase cells that contained two centrosomes.

#### Average intensity measurements and statistical analysis

We used the software ImageJ to obtain the average intensity of the P-Tacc, Tacc, Ast and Cnn signal. We measured the average intensity of the background signal in two different spots of the slide (outside the cell area) and averaged them. This value was subtracted from the average intensity at the centrosome (centro). We subtracted equally the background to the cytoplasm average intensity (cyto). When appropriate, we calculated the ratio of the two intensities (centro/cyto). To avoid any variation owing to the mitotic phase, we only quantified cells that were in metaphase. The intensity of the protein signal was measured using the ImageJ measure function. The units are the average gray value within the selection (sum of the gray values of all the pixels in the selection divided by the number of pixels). We used the software SigmaStat to perform one-way ANOVA analysis on the raw data and defined statistical significance at  $P < 0.05$ .

#### Immunoprecipitations in the brain

For immunoprecipitations, 200 larval brains of each genotype (WT and *jub* mutants expressing *Jub-GFP*) were extracted with lysis buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl and 1% Triton X-100, supplemented with protease inhibitors], and cleared by centrifugation at 16,000 *g* for 15 minutes. The supernatants were incubated with either GFP-trap beads (Chromotek) or with magnetic Protein-A beads crosslinked to anti-Aur-A antibodies (Dynabeads; Invitrogen), prepared according to the manufacturer's instructions for two hours, with gentle rotation at 4°C. Beads were washed twice with washing buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA and 150 mM NaCl) and re-suspended in sample buffer. Primary antibodies against Aur-A (rabbit) (Barros et al., 2005) and GFP (rat) (Chromotek) were diluted 1:1000. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research) were diluted at 1:10,000.

We are grateful to Jordan Raff for providing reagents essential for this study and for allowing us to use GFP-Aur-A flies before their publication. We thank J. Overton for generating Jub-GFP transgenic flies, C. Penetier for technical help, C. Barriere, D. Gogendeau, V. Marthiens, T. Maia and M. Rujano for critical reading of the manuscript and helpful discussions, and P. Paul-Gilloteaux and V. Frasier from the

Imaging platform UMR144 for help with image analysis. We acknowledge the Nikon Imaging Centre (Institut Curie). This work was supported by a FCT PhD grant, a FEBS short-term fellowship and a JCS Company of Biologists short-term fellowship to D.S. Work in the Brown laboratory was supported by Wellcome Trust grant 069943. Work in the Basto laboratory is supported by the Institut Curie, an ATIP grant from the CNRS, an installation grant from Fondation pour la Recherche Medicale and an ERC Starting grant. Deposited in PMC for immediate release.

Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/7/1156/DC1>

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