**Highlights**

- *Drosophila* Plk4 mutant NSCs show defects in centriole asymmetry and spindle positioning

- Apical centriole anchoring requires the PCM protein Spd-2 and the APC/C activator Fzr

- Movement of the centriole toward the basal side of the cell requires Plk4 activity

- At the mother centriole, Plk4 phosphorylates Spd2 to trigger PCM shedding and Fzr loss

**Authors**

Davide Gambarotto, Carole Pennetier, John M. Ryniawec, ..., Yuu Kimata, Gregory C. Rogers, Renata Basto

**Correspondence**

gcrogers@email.arizona.edu (G.C.R.), renata.basto@curie.fr (R.B.)

**In Brief**

Mitotic spindle orientation is tightly regulated during development and adulthood to maintain tissue organization and homeostasis. Spindle orientation requires the coordination between centrosomes and cortical cues. Gambarotto et al. report that the centrosome components Plk4 and Spd2 regulate centrosome asymmetry in interphase to influence spindle positioning in mitosis.
Plk4 Regulates Centriole Asymmetry and Spindle Orientation in Neural Stem Cells

Davide Gambarotto,1,5 Carole Pennetier,1 John M. Ryniawec,2 Daniel W. Buster,2 Delphine Gogendeau,1 Alix Goupil,1 Maddalena Nano,1 Anthony Simon,1 Damien Blanc,3 Victor Racine,3 Yuu Kimata,4 Gregory C. Rogers,2,* and Renata Basto1,6,*

1Institut Curie, PSL Research University, CNRS, UMR144, Biology of centrosomes and Genetic instability lab, Paris 75005, France
2Department of Cellular and Molecular Medicine, University of Arizona Cancer Center, University of Arizona, Tucson, AZ 85724, USA
3QuantaCell, 2 Allée du Doyen Georges Brus, Pessac 33600, France
4School of Life Science and Technology, ShanghaiTech University, 393 Middle Huaxia Road, Pudong, Shanghai, 201210, China
5Present address: University of Geneva, Department of Cell Biology, Sciences III, Geneva, Switzerland
6Lead Contact
*Correspondence: gcrogers@email.arizona.edu (G.C.R.), renata.basto@curie.fr (R.B.)
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SUMMARY

Defects in mitotic spindle orientation (MSO) disrupt the organization of stem cell niches impacting tissue morphogenesis and homeostasis. Mutations in centrosome genes reduce MSO fidelity, leading to tissue dysplasia and causing several diseases such as microcephaly, dwarfism, and cancer. Whether these mutations perturb spindle orientation solely by affecting astral microtubule nucleation or whether centrosome proteins have more direct functions in regulating MSO is unknown. To investigate this question, we analyzed the consequences of deregulating Plk4 (the master centriole duplication kinase) activity in *Drosophila* asymmetrically dividing neural stem cells. We found that Plk4 functions upstream of MSO control, orchestrating centriole symmetry breaking and consequently centrosome positioning. Mechanistically, we show that Plk4 acts through Spd2 phosphorylation, which induces centriole release from the apical cortex. Overall, this work not only reveals a role for Plk4 in regulating centrosome function but also links the centrosome biogenesis machinery with the MSO apparatus.

INTRODUCTION

*Drosophila* neural stem cells (NSCs; also called neuroblasts [NBs]) repeatedly divide asymmetrically to self-renew and to generate a committed progenitor, the ganglion mother cell (GMC). During interphase, a robust mechanism of centriole asymmetry controls mitotic spindle orientation (MSO) in the following mitosis (Rebollo et al., 2007; Rusan and Peifer, 2007) so that GMCs are always born at the same position relative to the NB (Figure 1A). Defects in polarity establishment or mutations in centrosome genes, which disrupt spindle positioning, interfere with asymmetric cell division and generate tumors (Basto et al., 2008; Basto et al., 2006; Castellanos et al., 2008; Caussin and Gonzalez, 2005), highlighting the importance of regulated stem cell division.

The stereotypical asymmetric centriole behavior in NBs described previously (Rebollo et al., 2007; Rusan and Peifer, 2007) largely contributes to the fidelity of asymmetric cell divisions. Within a centrosome, centrioles have different ages, and they can be structurally and/or functionally different (Conduit et al., 2015). This asymmetry is strongly visible during mitotic exit, just after disengagement of the mother-daughter centriole pair. The daughter or younger centriole retains microtubule (MT) nucleation activity, forming an aster that anchors the centriole to the apical cell cortex (hereafter called the apical centriole) (Rebollo et al., 2007; Rusan and Peifer, 2007; Conduit et al., 2010; Januschke and Gonzalez, 2010). In contrast, the mother or older centriole becomes inactivated and loses MT nucleation capacity, resulting in displacement away from the apical cortex toward the basal side (hence, referred to as basal centriole). Thus, the daughter centriole is retained in the NB, while the mother centriole is inherited by the GMC (Conduit et al., 2010; Januschke et al., 2011).

The discrepancy in the ability to nucleate MTs by the two centrioles can be explained by differences in pericentriolar material (PCM) retention (Conduit et al., 2010; Januschke et al., 2013). Maintenance of Polo kinase at the daughter centriole is crucial in retaining its PCM. Both positive and negative regulatory mechanisms control Polo localization on centrioles (Januschke et al., 2013; Ramdas Nair et al., 2016; Lerit and Rusan, 2013; Singh et al., 2014). The basal centriole is inactivated through “PCM shedding,” consisting of the rapid downregulation of the PCM (Rebollo et al., 2007; Rusan and Peifer, 2007), which is mediated by Pip and Bld10 (*Drosophila* orthologs of Pericentrin and Cep135, respectively) (Lerit and Rusan, 2013; Singh et al., 2014).

Although past studies have been instrumental in dissecting the molecular machinery responsible for asymmetric centriole behavior and the consequent impact on the MSO, the mechanisms responsible for centriole asymmetry establishment are not fully understood. Here, we uncover a function for Polo-like kinase 4 (Plk4), the master regulator of centriole duplication, in the establishment of this asymmetry. We show that Spd2 is a Plk4 substrate and that Spd2 phosphorylation triggers a basal-like centriole behavior. Furthermore, we found that the centriolar protein Fzr (the anaphase promoting complex [APC/C] activator
and the ortholog of Cdh1 in vertebrates) functions as a positive apical centriole retention factor in NBs. Our work provides evidence of a role for centrosomes in orchestrating centrosome asymmetry and MSO.

**RESULTS**

**Altering Plk4 Activity Perturbs the Centrosome Asymmetry Cycle and Causes Spindle Positioning Defects**

*Drosophila* NBs display a robust pattern of centriole asymmetry, which controls MSO and ensures that GMCs are always born at the same position relative to the NB (Figure 1A) (Rebollo et al., 2007; Rusan and Peifer, 2007) as confirmed by our time-lapse analysis of wild-type (WT) NBs (referred to as Control [Ctrl]) (n=20 Ctrl NBs from 8 brains) (Figures 1B and 1F; Video S1).

While analyzing the division of NBs expressing a GFP-tagged kinase-dead version of Plk4 (referred to as Plk4KD; Figure S1) in the Plk4 mutant (Plk4mut) background, we observed an unexpected behavior. The unduplicated centriole was preferentially maintained at the apical side in 88.5% of Plk4KD NBs (n = 23 out of 26 NBs from 7 brains). Of these, 46.2% contained an immobile centriole (Figures 1C and 1F; Video S2A) that remained apically anchored throughout interphase. In 46.3% of Plk4KD NBs, this centriole was maintained at an apical position even if it presented increased mobility. In this situation, the centriole movement was restricted to the apical hemisphere, the centriole moved from one side of the cell to the other (hereafter, referred to as apical mobile) (Figures 1D and 1F; Video S2A). Interestingly, in the example of Figure 1D and Video S2C, even if the apical cortex was deformed by a neighboring cell, the centriole was maintained at an apical position with little variation from one cell cycle to the following one. In the remaining 11.5% Plk4KD NBs, centrioles moved toward the basal side of the cell as described for basal centriole behavior (referred to as basal-like) (n = 3 out of 26 NBs from 7 brains) (data not shown; Figure 1F). Importantly, even if containing a single centriole, all Plk4KD NBs assembled a bipolar spindle. Initially, MT nucleation was noticed in the pole that contains the centriole; but rapidly, a bipolar array was generated, and cells invariably divided in a bipolar manner, similar to cells that lack centrioles (Basto et al., 2006; Moutinho-Pereira et al., 2009). These observations were very surprising because it is expected that as the unduplicated centriole ages, it should show a basal-like behavior, as shown by older mother centrioles (Figure S2A). The retention of the unduplicated centriole in NBs was also described in Plk4mut NBs where a significant number of NBs contain a single centriole (Bettencourt-Dias et al., 2005).

To ascertain the behavior of an unduplicated centriole that was generated using a different centriole duplication mutant background, we counted centrioles in NBs of Sas-4mut brains and compared these with Plk4KD and Plk4mut brains. *Drosophila* harboring mutations in key centriole genes are viable because of maternally provided centrosome assembly factors that ensure centriole duplication during early embryogenesis when centrosomes are essential (Stevens et al., 2007; Basto et al., 2006; Riparbelli and Caliaini, 2011; Bettencourt-Dias et al., 2005; Blachon et al., 2008). As development proceeds, centriole duplication ends around stage 15 or 16 (Basto et al., 2006) however, centrioles born prior to this stage are stably maintained. As cells continue to proliferate and increase in number throughout development, centrioles are detected in only a small number of cells. Indeed, a single centriole was detected in only 2.2% ± 1.4% of Sas-4mut NBs (n = 305 NBs from 7 brains) (Figure S2C). Moreover, we could not find centrioles in dividing NBs by live imaging of Sas-4mut brains (Figure S2D). In contrast, a single centriole was detected in 38.9% ± 5.6% of Plk4mut and 62.4% ± 15.4% of Plk4KD NBs (n = 187 NBs from 5 Plk4mut brains and n = 132 NBs from 5 Plk4KD brains) (Figure S2C). Importantly, the Plk4 mutant used in our study is a hypomorph (Bettencourt-Dias et al., 2005), explaining why some Plk4mut NBs contain centrioles at this developmental stage. Additionally, Plk4KD over-expression in the Plk4mut background might lead to a partial stabilization of the endogenous Plk4 protein, resulting in supernumerary centrosomes, which were detected at a low frequency (4.0% ± 5.2%) (Figure S2C). The presence of extra centrosomes, even in a small subset of cells, might contribute to an increased number of Plk4KD NBs with centrioles. Thus, our analysis of the behaviors of single centrioles in Plk4KD NBs suggests that loss of Plk4 activity promotes the apical cortical anchoring of an unduplicated centriole.

We next examined the consequence of over-expressing active Plk4 (referred to as a Plk4WT) on centriole positioning in NBs (Figure S1A). Over-expression of Plk4 generated a large number of NBs with extra centrosomes (78.1%; n = 274 NBs from 6 brains) (Figure S2C) (Basto et al., 2008; Hagedanck et al., 2005; Matthiens et al., 2013). Surprisingly, however, and in contrast to Plk4KD NBs, centrioles in the majority of Plk4WT NBs displayed basal-like movement (70%; n = 14 of 20 NBs from 7 brains) (Figures 1E and 1F; Video S2C). Additionally, these centrioles did not produce detectable interphase MT asters, similarly to basal centrioles in Ctrl NBs. In the remaining 30% of Plk4WT NBs, centrioles presented apical mobile behavior with either a reduced MT aster (10%) or without a noticeable MT aster (20%) (n = 2 and n = 4 of 20 NBs from 7 brains, respectively). The lack of an apical centriole in Plk4WT NBs was unforeseen as at least

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**Figure 1. Plk4 Regulates Centriole Dynamics in Interphase, Impacting Spindle Orientation**

(A) Schematic drawing representing two consecutive cell cycles of a *Drosophila* NB depicting centrosome behavior.

(B–E) Images from time-lapse movies of Ctrl (B), Plk4KD (C and D), and Plk4WT (E) larval NBs. Tubulin in red, RFP-Sas-6 (B), GFP-Plk4KD (C and D), and GFP-Plk4WT (E) in green. See also Figures S1 and S2. The blue arrow denotes the centrosome (or centriole in the case of Plk4WT) inherited by the NB at the end of mitosis in the first column but, in all other images, marks the centriole that was localized at the apical cortex (apical centriole) after disengagement. White arrows point to the centriole that moves basally in Ctrl NBs. The yellow arrow points to the centrosome positioned at the spindle pole at the end of mitosis in Plk4WT NBs. Time, minutes. Scale, 4 μm. Diagrams on the right illustrate centriole behavior in early interphase.

(F) Graph shows the percentage of centriole behavior categories during interphase in the indicated genotypes. Centriole behavior was categorized as apical-like in (B) or (C), apical-mobile-like in (D), when the centrosome moved laterally even if remained localized within the apical hemisphere, or basal-like in (E).

(G) Quantification of the angle between two consecutive mitoses in Ctrl, Plk4KD and Plk4WT. Statistical significance (SS) was assessed by unpaired t test.
Figure 2. The Single Centriole of Plk4KD NBs Does Not Behave as in Ctrl NBs after MT Depolymerization

(A) Schematic drawing of NBs with a hypothetic apical basis length (dashed line). Position zero was considered the apical cortex, while position 100 at the basal cortex was determined by its connection with GMCs.

(B) Immunostaining of Ctrl (top) and Plk4KD (bottom) NBs and GMCs with (right) or without (left) colcemid treatment with antibodies against aPKC and Prospero in green and red, respectively. The centrosome is labeled with Cnn antibodies (white) and DNA in blue. The apical centrosome in Ctrl NBs was recognized by containing a higher Cnn signal (white arrowhead). Scale: Ctrl, 3 μm; and Plk4KD, 4 μm. See also Figure S3.

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one of the young centrioles is predicted to maintain an apical localization during interphase.

Since the asymmetric centriole cycle controls MSO over several NB divisions (Rebollo et al., 2007; Rusan and Peifer, 2007), we investigated the consequences of Plk4KD or Plk4WT expression in MSO. We filmed Ctrl, Plk4KD, and Plk4WT NBs over at least two consecutive mitoses and found that spindles in Plk4KD NBs maintained a fixed position, similar to Ctrl NBs (average angle = 13.5 ± 3.8°; variation between 0° and 22°; n = 15 NBs from 8 brains for Plk4KD and average angle = 12.0 ± 2.2°; variation between 0° and 25°; n = 14 NBs from 3 brains for Ctrl; p = not significant (ns); Figure 1G). In contrast, Plk4WT NBs displayed a more variable MSO through consecutive cycles (average angle = 25.0 ± 4.0°; variation between 0° and 75°; n = 23 from 10 brains; p = 0.026; Figure 1G). Characterization of MSO relative to the polarity axis using atypical protein kinase C (aPKC) as a marker (Lee et al., 2006; Homem and Knoblich, 2012) showed that in Ctrl NBs, spindles were oriented along the polarity axis (average angle = 9.9 ± 0.7°; variation between 2.3° and 45°; n = 48 NBs from 4 brains) (Figures S3A and S3B). In Plk4KD, the average angle was slightly increased to 15.3 ± 1.2° (variation between 5.4° and 60.9°; n = 62 NBs from 5 brains; p = 0.013) showing that even if the unduplicated centriole tends to maintain apical localization during interphase, mitotic spindles do not orient as correctly as Ctrl NBs. Importantly, in Plk4WT NBs, the average angle was increased to 23.5 ± 2.1° (variation between 3.9° and 89.6°; n = 96 NBs from 7 brains; p < 0.0001) (Figures S3A and S3B), confirming that increased Plk4 activity influences apical centrosome positioning in interphase and, thus, MSO. Defects in MSO can lead to defects in asymmetric cell division leading to the generation of two NBs instead of one NB and one GMC (Albertson and Doe, 2003; Basto et al., 2006; Basto et al., 2008; Castellanos et al., 2008; Homem and Knoblich, 2012). Using Dead pan (Dpn) (San-Juan and Baonza, 2011) as an NB marker, we determined the average number of NBs in the central brain lobe of Ctrl brains (48.9 ± 0.9; n = 9 brain lobes), similarly to previous studies (Basto et al., 2008; Gogendeau et al., 2015). Importantly, an increase in the number of NBs was noticed in Plk4WT central brain (55.2 ± 1.0; n = 9 brain lobes; p = 0.0003). Taken together, our results indicate that Plk4 activity must be tightly regulated not only to ensure the formation of a single procentriole per mother but also to control MSO and asymmetric cell division in NBs.

An MT-Independent Mechanism Contributes to Apical Centriole Maintenance in Plk4 KD NBs

While characterizing Plk4KD NBs, we realized that the apical aster appeared to have decreased MT nucleation when compared to Ctrl NBs (Figures 1C and 1D; Videos 1 and 2), suggesting that a yet unidentified mechanism might contribute to centriole anchoring in this cell type. To further test this possibility, we treated Ctrl and Plk4KD brains with colcemid to depolymerize MTs for 1 h. After fixation, we used two different conditions to label NBs and centrosomes. First, NBs were identified by aPKC and GMCs by Prospero. In this case, only a centrosome marker (Cnn) was used (Figure 2A). In the second method, we used two centrosomal antibodies, Plp and Cnn, which is important to unambiguously identify centrosomes, in particular in centrosome mutants. NBs and accompanied GMCs were labeled with phalloidin, and their respective identity was determined by their size (Figure S3C). In Ctrl NBs treated with colcemid, centrosomes were closer to the basal axis as expected (Januschke and Gonzalez, 2010), reaching positions similar to the ones found in Plk4WT NBs (Figures 2B, 2C, S3D, and S3E). Although centrosomes were further away from the apical axis in colcemid-treated Plk4KD NBs, they were not positioned closer to the basal hemisphere as observed in Ctrl NBs. This was also the case in Plk4mut NBs, suggesting that the absence of active Plk4 delays or inhibits movement toward the basal side of the cell. Consistent with these findings, time-lapse imaging revealed that centrosomes in Plk4KD NBs treated with colcemid appeared less mobile than centrosomes from colcemid-treated Ctrl brains (n = 16 NBs from 3 brains for each condition) (Figures 2D and 2E; Videos S3A and S3B). These results indicate that an alternative mechanism, which seems to be MT-independent, contributes to centriole apical anchoring in Plk4KD NBs.

Fzr Contributes to Anchoring of Apical Centrioles

In order to explain the apical localization of unduplicated centrioles in Plk4KD NBs, we next immunostained NBs for centriole (Asterless [Aasl] and PCM (γ-tubulin, Spd2, and Cnn) proteins and used three-dimensional structural illumination microscopy (3D SIM) to characterize potential structural changes. Of these, Spd2 localization in Plk4KD NBs (Figure 3A) (data not shown); Spd2 is the Drosophila ortholog of Cep192, a major PCM component (Dix and Raff, 2007; Conduit et al., 2014). During interphase, Spd2 appeared as a ring in Ctrl NBs (Figure 3A) (Fu and Glover, 2012; Meghini et al., 2016; Menella et al., 2012). In Plk4KD NBs, Spd2 appeared as a larger ring, while centrioles from Plk4WT NBs displayed a smaller Spd2 diameter (Figure 3A).

Spd2 was recently shown to recruit the APC/C activator Fizzy-related (Fzr) to the centrosome at the end of mitosis (Meghini et al., 2016). We thus investigated whether the differences in Spd2 localization in Plk4KD or Plk4WT NBs impacted Fzr levels on interphase centrioles. We generated flies expressing Red Fluorescent Protein (RFP)-Fzr (referred to as Fzr). 3D SIM images showed that Fzr occupied the internal region of the Spd2 ring (Figure 3A), as previously described (Meghini et al., 2016). In Plk4KD NBs, Spd2 formed an enlarged ring, and Fzr occupied
Figure 3. Maintenance of Apical Anchoring in Plk4KD NBs Is Fzr Dependent

(A) 3D SIM images showing Spd2 (red) and Fzr (green) localization on interphase centrioles in Ctrl, Plk4KD and Plk4WT NBs. Scale, 400 nm.

(B) Dot plot showing Fzr fluorescent intensity levels at the centrosome (Ctrl apical, 0.20 ± 0.02; Ctrl basal, 0.04 ± 0.01; Plk4 KD, 0.24 ± 0.03; Plk4 WT, 0.07 ± 0.02). Error bars represent means ± SD from at least 3 independent experiments. SS was assessed by unpaired t test. See also Figure S4.

(C) Graph shows the percentage of centriole behavior categories in interphase (compare with Figure 1F for Ctrl and Plk4KD).

(D) Quantification of the angle between two consecutive mitoses in Fzrmut and Plk4KD, Fzrmut. SS was assessed by unpaired t test. See also Figure S5.

(E) Immunostaining of Fzrmut and Plk4KD, Fzrmut NBs and GMCs with or without colcemid treatment labeled with antibodies against aPKC and Prospero in green and red, respectively. The centrosome was labeled with Cnn antibodies (white) and DNA in blue. The apical centrosome in Fzrmut NBs was recognized by containing higher Cnn signal (white arrowhead). Scale for Ctrl, 4 μm. Note that on the Fzrmut panel with colcemid, the two centrosomes are very close to each other. See also Figure S5.

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a broader area, while in Plk4\(^{KD}\) NBs, Fzr occupied a smaller area. Using images from confocal microscopy, we quantified Fzr fluorescent intensity levels at the centrioles of interphase NBs. Apical centrioles from Ctrl NBs and centrioles from Plk4\(^{KD}\) NBs contained higher Fzr levels than basal centrioles from Ctrl NBs and Plk4\(^{WT}\) centrioles (Figure 3B).

We confirmed these observations by analyzing Fzr localization using time-lapse microscopy. At the end of mitosis, Fzr was recruited to the centrosome in Ctrl NBs but exclusively remained associated with the apical centriole (Figure S4A). Fzr was also recruited to the single centriole at the end of mitosis in Plk4\(^{KD}\) NBs and to Plk4\(^{WT}\) centrioles (Figure S4A) however, its levels diminished on Plk4\(^{WT}\) interphase centrioles. These results demonstrate not only that Fzr displays an asymmetric centriolar distribution in interphase NBs but also that Plk4 activity contributes to the removal of Fzr from centrioles during interphase.

To investigate whether Fzr plays a role in establishing centrosome asymmetry, we used a hypomorphic Fzr mutant (Fzr\(^{mut}\)), which presents an overall reduction in Fzr levels (Jacobs et al., 2002). Using time-lapse microscopy of GFP-tubulin-expressing NBs in Fzr\(^{mut}\) brains, we analyzed the position of the apical MT aster. 80% of Fzr\(^{mut}\) NBs displayed apical centriole positioning (n = 16 out of 20 NBs from 4 brains) (Figure 3C), and a near-normal MSO was maintained over consecutive cycles (average angle = 19.2 ± 3.1°; variation between 6.1° and 30.9°; n = 9 NBs from 3 brains; Figure 3D). Fzr\(^{mut}\) NBs displayed normal centrosome numbers (n = 70 NBs from 4 brains) (Figure S2C). Interestingly, 20% of Fzr\(^{mut}\) NBs displayed an apical mobile behavior (Figures 3C and S4D). We also analyzed Fzr\(^{mut}\) brains after colcemid treatment and found that centrosomes were positioned closer to the basal hemisphere (Figures 3E, 3F, S3E, and S3F). It is noteworthy that in untreated conditions, Fzr\(^{mut}\) centrosomes were positioned further away from the apical cortex than Ctrl centrosomes (compare Figures 2C and 3F with Figures S3D and S3F). Taken together, our results suggest that Fzr may contribute to apical centrosome anchoring in NBs by acting in synergy with the MT nucleation pathway.

We next reasoned that decreasing Fzr levels in Plk4\(^{KD}\) NBs, which have reduced MT nucleation, should induce basal-like behavior. Importantly, very few Plk4\(^{KD}\), Fzr\(^{mut}\) flies reached the third instar larvae stage, suggesting that combining these two mutations perturbs development. Nevertheless, we were able to obtain larvae for these studies. Remarkably, decreasing Fzr levels in Plk4\(^{KD}\) NBs induced movement of the unduplicated centriole toward the basal side in 61.1% of NBs (n = 11 of 18 NBs from 5 brains; p = 0.0009 compared to Plk4\(^{KD}\)) (Figures 3C and 3G). Additionally, 33.3% of Plk4\(^{KD}\), Fzr\(^{mut}\) NBs (n = 6 of 18) fell into the apical mobile category, and only one Plk4\(^{KD}\), Fzr\(^{mut}\) NB maintained an apical centriole at the apical cortex during interphase (Figure 3C). Moreover, the average angle of Plk4\(^{KD}\), Fzr\(^{mut}\) NBs increased (Figure 3D) when compared to Fzr\(^{mut}\) and, more importantly, when compared to Plk4\(^{KD}\) NBs, (average angle = 30.5° ± 3.7°; variation between 0° and 90°; n = 32 NBs from 8 brains; p = 0.0013 relative to Plk4\(^{KD}\), and p = 0.124 relative to Fzr\(^{mut}\)). Strikingly, colcemid treatment of Plk4\(^{KD}\), Fzr\(^{mut}\) NBs caused centrosomes to reposition more toward the basal hemisphere (Figures 3E, 3F, S3E, and S3F). Thus, our findings suggest that Fzr participates in an MT-independent apical centriole anchoring mechanism, which is particularly active with reduced functional Plk4.

Fzr is an activator of APC/C, an E3 ubiquitin ligase that promotes mitotic exit and progression through G1 (Sivakumar and Gorbsky, 2015). Our results indicate that Fzr regulates centrosome asymmetry and, consequently, MSO. However, it remains to be investigated whether this function requires proteasome-mediated degradation of an APC/C ubiquitination target. To investigate this question, we characterized the localization of Cdc27, a core APC/C subunit, in Ctrl NBs (Huang and Raff, 2002). Although GFP-Cdc27 was slightly enriched at the centrosome at the end of mitosis, this localization was rather transient and, during interphase, GFP-Cdc27 appeared evenly distributed throughout the cytoplasm (Figure S4E). These observations reveal that APC/C is not at the centrosome at the right time to control apical anchoring. To rule out a role for APC/C in promoting apical anchoring, we tested whether inhibiting proteasomal degradation had an effect on centriole behavior by incubating Ctrl brains with Bortezomib (BZ), a proteasome inhibitor (Adams et al., 1999). Importantly, all NBs analyzed (n = 8 brains) maintained a stably anchored apical centriole, similar to controls (Figures S4F and S4G). The conditions we used increased the percentage of prometaphase-arrested NBs (41.4%; n = 7 brains) when compared to Ctrl brains incubated with DMSO (22.6%; n = 8 brains; p = 0.0433), validating the use of BZ as a proteasome inhibitor in Drosophila brains. Taken together, these results suggest that Fzr contributes to apical centriole anchoring and centrosome asymmetry independent of APC/C activity. Thus, retention of Fzr at the apical centriole constitutes a distinct mechanism that contributes to asymmetric cell division independent of MTs and APC/C activity.

We also asked whether an actin-based structure could contribute to apical centriole anchoring. Indeed, actin filament nucleation at the centrosome has recently been shown to occur in certain cell types (Farina et al., 2016; Obino et al., 2016). However, even if fine sub- apical actin structures could be detected, these were not co-localizing with centrosomes, and so we think that they could not be responsible for apical centriole anchoring in Ctrl or Plk4\(^{KD}\) NBs (Figure S5A). Further, we depolymerized filamentous actin (F-actin) using cytochalasin D. This treatment resulted in cytokinesis inhibition, confirming the effect of actin depolymerization. Interestingly, both Ctrl and Plk4\(^{KD}\) NBs showed a modification of the MT cytoskeleton where ectopic cytoplasmic nucleation sites were noticed (Figures S5B). Nevertheless, the apical centriole remained apically localized at the cortex throughout interphase. Thus, actin does not seem to play a role in maintaining the centriole at the apical cortex during interphase.

(F) Dot plot showing the position of centrosomes in Fzr\(^{mut}\) and Fzr\(^{mut}\),Plk4\(^{KD}\) NBs with (+) and without (-) colcemid (Fzr\(^{mut}\) – 19.26 ± 0.9; Fzr\(^{mut}\),Plk4\(^{KD}\) – 43.1 ± 1.8; Fzr\(^{mut}\),Plk4\(^{KD}\) – 36.6 ± 2.3; Fzr\(^{mut}\),Plk4\(^{KD}\) + colcemid – 57.4 ± 2.2). At least 27 NBs were analyzed for each condition from 8 different brains. Error bars represent SD. SS was assessed by unpaired t test. See also Figure S3.

(G) Images from time-lapse movies of Plk4\(^{KD}\), Fzr\(^{mut}\) NBs. Tubulin (red) and GFP-Plk4\(^{KD}\) (green). Blue arrow marks the centriole initially positioned in the NB, but later inherited by the GMC (right). Diagram illustrates centriole behavior in interphase. Time, minutes. Scale, 4 μm.
Plk4 Phosphorylates Spd2 to Promote a Basal-like Behavior

To understand how Plk4 could promote centriole asymmetry, we next focused on Spd2 because of its unique redistribution on centrosomes in response to Plk4 activity. Measurements of Spd2 fluorescence intensity levels on interphase centrosomes in Ctrl NBs revealed that its distribution appeared asymmetric, with high levels on the apical centriole and lower levels on the basal (Figures 4A and 4B). In contrast, Spd2 levels were extremely reduced in the majority of Plk4WT centrioles, displaying basal-like values.

We next explored whether Plk4 regulates centrosome asymmetry through Spd2 by testing whether Spd2 is a Plk4 substrate. We found that Plk4 phosphorylated Spd2 in vitro, and, using tandem mass spectrometry, 28 phosphorylated Ser and Thr residues within Spd2 were identified (Figures S6A–S6D). To test the functional relevance of these modifications in vivo, we generated flies expressing RFP-tagged Spd2 as either WT (Spd2WT) or phosphomutant transgenes, including phosphomimetic Spd2 harboring 28 aspartic acid or glutamic acid substitutions (Spd2DS) and a non-phosphorylatable alanine mutant (Spd2AS) (Figure S1B), all under the control of the same promoter and inserted on the same chromosome locus. The expression of Spd2 transgenes seemed to be comparable in brain extracts (Figure S6E). We then analyzed the behavior of Spd2WT and Spd2 phosphomutant-expressing NBs by time-lapse microscopy in order to observe centriole behaviors and analyze MSO over consecutive mitoses. As expected, over-expression of Spd2WT produced proper centrosome asymmetry, whereby an apical aster appeared after centriole disengagement (Figure 4C; Video S4A). As with endogenous Spd2, Spd2WT was not detected at the basocentral centriole soon after disengagement until just before the following mitosis, while it remained associated with the apical centrosome. Interestingly, we also observed reduced MT nucleation, suggesting that over-expression of Spd2 might change the nucleation capacity of interphase centrosomes. Importantly, MSO was maintained with relatively small variation (average angle = 13.8° ± 7.5°; variation between 7° and 28.0°; n = 10 NBs from 4 brains) (Figure 4F), indicating that over-expressing Spd2WT does not impact spindle positioning through consecutive cell cycles.

Despite localizing to the centrosome at the end of mitosis, Spd2DE levels decreased in a similar manner from both centrioles soon after their disengagement (Figure 4D; Video S4B). Interestingly, a fixed apical centrosome was not detected throughout most interphase, and both centrioles recruited Spd2DE with similar kinetics during late G2 and prophase. Importantly, in Spd2DE NBs, we measured a highly variable MSO over two consecutive mitoses (average angle = 41.8° ± 20.6°; variation between 7° and 69°; n = 11 NBs from 4 brains; p < 0.0007 when compared to Spd2WT) (Figure 4F).

We next examined the distribution of Spd2AA. Similar to Spd2WT and Spd2DE, Spd2AA localized to centrosomes at the end of mitosis. However, unlike Spd2WT, Spd2AA was maintained on both centrioles throughout a period of interphase (Figure 4E; Video S4C). Centrosome asymmetry was nevertheless established but much later, supporting the view that establishment of Spd2 asymmetry on centrioles relies, at least initially, on its phosphorylation state. In some NBs, the apical centriole was maintained at the apical cortex but frequently displayed an apical mobile behavior, while the other centriole also remained in the apical hemisphere (Figure 4E). Spd2AA expression caused slightly more variation in MSO than Spd2WT (average angle = 21.2° ± 8.9°; variation between 7° and 47°; n = 13 NBs from 5 brains; p = 0.08; ns, when compared to Spd2WT), but not as dramatic as Spd2DS (p = 0.0051 when compared to Spd2DE) (Figure 4F).

We investigated whether replacement of the 28 phospho-residues within Spd2 in both Spd2 phosphomutants (Spd2DE and Spd2AA) was affecting its ability to homodimerize or interact with Cnn (Galletta et al., 2016; Conduit et al., 2014). We depleted endogenous Spd2 from S2 cells by targeting its 3’ UTR and then transiently co-expressed various combinations of Spd2WT, Spd2DE, and Spd2AA tagged with either GFP or V5 (Figure S6E). Anti-GFP immunoprecipitations (IPs) from S2 cell lysates showed that V5-tagged Spd2DE and Spd2AA phosphomutants self-associate (Figure S6G). Moreover, V5-Cnn co-IPed with each of the GFP-Spd2 phosphomutants (Figure S6H), showing that the 28-amino-acid substitutions in the non-phosphorylatable and phosphomimetic Spd2 mutants do not cause protein misfolding or affect Cnn binding.

We also investigated whether Cnn centrosomal localization was influenced by the Spd2 phosphorylation state. A marked asymmetry in Cnn levels was observed between the apical and basal centrioles in Spd2WT NBs (Figures 5A and 5B). Although quite variable, Cnn was still recruited to interphase centrosomes regardless of the Spd2 phosphorylation status. We also measured the levels of Plp. Plp was still preferentially enriched in the basal centrioles of Spd2WT NBs and in one of the two centrioles in the Spd2 phosphomutants, even if displaying higher levels in Spd2AA than in Spd2WT (Figures 5A and 5C).

We next measured centrosomal levels of Spd2 using anti-Spd2 antibodies (Dix and Raff, 2007). Notably, in both Spd2DE and Spd2AA NBs, Spd2 levels on centrosomes appeared more symmetric than centrioles in Spd2WT NBs (Figures 5D and 5E). In addition, Spd2 levels on Spd2AA centrosomes were comparable with Spd2WT apical centrosomes, while they were decreased in Spd2DE (Figure 5D).

Since Spd2 recruits Fzr to the centrosome (Meghini et al., 2016) and Fzr participates in centriole apical anchoring, we hypothesized that Fzr localization and/or levels might be altered in Spd2 phosphomutant NBs. Fzr showed asymmetric localization soon after centriole disengagement in Spd2WT NBs (Figures 5D and 5F; noticeable by the short distance between the two centrioles). Strikingly, Fzr levels were decreased in both centrioles of Spd2DE NBs, suggesting that Spd2 phosphorylation impacts Fzr recruitment or maintenance in interphase centrosomes. Interestingly, in Spd2AA NBs, Fzr was present on both centrioles even when they were positioned far apart from one another (Figures 5D), suggesting that Fzr is maintained at both centrioles long after disengagement. Fzr asymmetry between even the two centrioles was noticeable although less pronounced than in Spd2WT NBs (Figure 5F). Our findings suggest that Plk4 phosphorylation of Spd2 downregulates Spd2 and Fzr recruitment to centrioles, thereby controlling centriole asymmetry and dynamics and, consequently, MSO in the following cell cycle.
Figure 4. Spd2 Phosphomutant NBs Display Centrosome Asymmetry and MSO Defects

(A) Ctrl and Plk4WT interphase NBs immunostained for tubulin (red) and Spd2 (green). DNA in blue. The blue arrow marks the apical centrosome in Ctrl NBs where Spd2 is detectable. White arrows denote centrosomes with low Spd2 levels. Scale, 4 μm.

(B) Dot plot showing Spd2 fluorescent intensity levels at the centrosome in the indicated genotypes (Ctrl apical, 1.6 ± 0.12; Ctrl basal, 0.6 ± 0.09; Plk4WT, 0.4 ± 0.04). Error bars represent means ± SD from at least 3 independent experiments. SS was assessed by unpaired t test.

(C–E) Images from time-lapse movies of GFP-Spd2 WT (C), GFP-Spd2 DE (D), and GFP-Spd2 AA (E) larval NBs. Tubulin (red). See also Figures S1 and S6. Blue arrows mark the centrosome inherited by the NB at the end of mitosis. In the interphase panels, blue arrows mark the centriole that was localized at the apical cortex (apical centriole in Spd2WT) or the centrosome that was maintained at the apical hemisphere for longer periods of time after disengagement. White arrows mark the non-apical centrioles. (Right) Diagrams illustrate centriole behavior in each genotype after disengagement during early interphase. Time, minutes. Scale, 4 μm.

(F) Quantification of the angle between two consecutive mitosis in Spd2WT, Spd2DE, and Spd2AA. SS was assessed by unpaired t test.

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Figure 5. Spd2 Phosphomutants Influence Spd2 and Fzr Centriolar Recruitment without Impacting on Cnn or Plp

(A) Immunostaining of Spd2 WT, Spd2 DE, and Spd2 AA early interphase NBs for Cnn (red) and Plp (green). DNA in blue. Insets show higher magnifications of each centriole. Scale, 4 μm. See also Figures S1 and S6.

(B) Graph showing Cnn fluorescent intensity (FI) in arbitrary units (A.U.) for Spd2 WT, Spd2 DE, and Spd2 AA in apical, basal, and centriolar regions.

(C) Graph showing Plp fluorescent intensity (FI) in arbitrary units (A.U.) for Spd2 WT, Spd2 DE, and Spd2 AA in apical, basal, and centriolar regions.

(D) Immunostaining of Spd2 WT, Spd2 DE, and Spd2 AA early interphase NBs for Fzr (red) and DNA (blue).

(E) Graph showing Spd2 fluorescent intensity (FI) in arbitrary units (A.U.) for Spd2 WT, Spd2 DE, and Spd2 AA in apical, basal, and centriolar regions.

(F) Graph showing Fzr fluorescent intensity (FI) in arbitrary units (A.U.) for Spd2 WT, Spd2 DE, and Spd2 AA in apical, basal, and centriolar regions.

(legend continued on next page)
DISCUSSION

Here, we show that Plk4 plays a new role in the establishment of centrosome asymmetry and MSO in Drosophila NSCs. Our findings are consistent with a model where upon centriole disengagement at mitotic exit, the mother centriole inherits Plk4, which triggers centriole movement towards the NB basal side by disrupting microtubule-organizing center (MTOC) activity (Figure 6). Our data suggest that Plk4 needs to be removed or inactive at the apical centriole, in order to ensure maintenance of MTOC activity. Although the model predicts an unequal distribution of Plk4 between the two NB centrioles, we are unable to test this aspect of the model. Endogenous Plk4 protein levels are extremely low and, despite several attempts to raise antibodies, we were unable to detect endogenous Plk4. Expression of GFP-tagged Plk4 under control of endogenous or weak promoters resulted in the stabilization of the protein, which invariably leads to increased activity and the unwanted supernumerary centrosomes (Basto et al., 2008; Aydogan et al., 2018).

In this model, active centriole-bound Plk4 targets Spd2, triggering Spd2 displacement and promoting, most likely, loss of additional PCM proteins from the basal centriole. It is thus conceivable that mother centrioles lacking Spd2 lose MT nucleation capacity, which induce their movement toward the basal side. It has been previously shown that asymmetric loss of PCM in the basal centriole was sufficient to trigger movement toward the basal side of the NB (Rebollo et al., 2007; Rusan and Peifer, 2007). Further, Spd2 removal also results in the loss of Fzr and consequently disables a previously undescribed second mechanism that contributes to maintaining centrosomes at the apical cortex or hemisphere. It will be important to investigate whether Spd2 mutants, which did not show defects in MSO during mitosis (Dix and Raff, 2007), display altered centriole behavior during interphase.

How Fzr promotes apical anchoring remains to be determined. Our findings support the idea that Fzr promotes centriole apical localization in an APC/C-independent manner. Although we have not identified a mechanism that displaces or inhibits Plk4 on the apical centriole, it is still possible that Fzr promotes apical anchoring by regulating, either directly or indirectly, the levels or activity of Plk4 at the apical centriole. However, we did not detect an interaction between Plk4 and Fzr in Drosophila brain extracts (D.G. and R.B., unpublished data). The observation that Fzr is absent from mitotic centrosomes in Ctrl NBs suggests that dynamic centrosome movements (typical of mitosis to allow efficient MT interactions with the cortex and chromosomes) require Fzr removal away from the centrosome. Moreover, the asymmetric maintenance of Fzr at the apical centriole, soon after disengagement, correlates with the low mobility typical of this centrosome. It is thus possible that maintenance of Fzr at the

Figure 6. Model of Centriole Symmetry Breaking and Spindle Orientation in Drosophila NBs
At the end of mitosis, the mother-daughter centrioles of the NB disengage. The mother (or basal) centriole retains Plk4 activity, which phosphorylates Spd2, causing (1) PCM shedding and, thus, loss of MT nucleation and (2) Fzr displacement, which inhibits apical anchoring. As yet undiscovered mechanism, Plk4 is inactive on the daughter (or apical) centriole, resulting in the stable maintenance of a centriole-bound population of non-phosphorylated Spd2, which promotes both MT nucleation and Fzr-dependent cortical anchoring.
apical centriole functions as a barrier to mobility. We speculate that such an effect could be direct (Fzr itself can assemble in high-order structures to inhibit centriole mobility) or indirect (through a yet unknown interactor). Importantly, our work shows that maintenance of Spd2 at the apical centriole serves two major functions: PCM retention, MT nucleation and Fzr recruitment to inhibit mobility.

The functions of Plk4 and Spd2 uncovered and described here establish an unexpected association between the centrosome biogenesis machinery (centriole duplication and PCM recruitment) and centrosome asymmetry and spindle positioning apparatus. Additionally, we have identified a mechanism of apical centriole anchoring that appears to compensate for centriole duplication defects in NSCs by promoting apical retention. It will be interesting to investigate whether, the partial loss-of-function mutations in Plk4 described in humans, which cause microcephaly and dwarfism, support centriole retention at the apical cortex of NSCs (Martin et al., 2014; Tsutsumi et al., 2016; Shacheil et al., 2014). This might be beneficial not only by conferring the capacity to assemble primary cilia but also to ensure stem cell viability due to the presence of a centriole (Lambrus and Holland, 2017). Further, it will be important to investigate whether centrosome repositioning described during epithelial mesenchymal transition (EMT) or at mitotic exit (Burute et al., 2017; Piel et al., 2001) also relies on these mechanisms.

STAR METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

D.G. and R.B. conceived the project, analyzed the data, and, together with G.C.R., wrote the manuscript. D.G. performed most of the experimental procedures. C.P. generated tools and most of the transgenic fly stocks. J.M.R., D.W.B., and G.C.R. conceived and performed kinase assays, mass spectrometry, and S2 cell experiments. D.G. generated Plk4WT transgenic fly stocks. A.S. helped with the maintenance of fly stocks, crosses, and dissection, and D.-B. and V.R. identified the methodology used to track centrosome or centriole behavior in time-lapse movies and the representation of their dynamics. Y.K. shared unpublished data. A.G. and M.N. helped R.B. to set up the live imaging of some fly strains. R.B. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-Spd2 | (Dix and Raff, 2007) | RRID: AB_2587456 |
| Rabbit polyclonal anti-Plp | (Martinez-Campos et al., 2004) | N/A |
| Rabbit polyclonal anti-aPKC | Santa Cruz | Cat# SC116 |
| Guinea pig polyclonal anti-Spd2 | This paper | N/A |
| Guinea pig polyclonal anti-Cnn | (Lucas and Raff, 2007) | N/A |
| Mouse monoclonal anti-α-Tubulin (clone DM1A), purified antibody | Sigma-Aldrich | Cat# T6199; RRID: AB_477583 |
| Mouse monoclonal anti-α-Tubulin (clone DM1A), ascites fluid | Sigma-Aldrich | Cat# T9026; RRID: AB_477593 |
| Mouse monoclonal anti-prospero | DSHB | MR1A; RRID: AB_528440 |
| RFP-Booster (Atto 594) | Chromotek | Cat# rba594; RRID: AB_2631390 |
| Mouse anti-GFP (clone JL-8) | Clonetech Laboratories | Cat# 632381; RRID: AB_2313808 |
| Mouse anti-HA (clone HA-7) | Sigma-Aldrich | Cat# H3663; RRID: AB_262051 |
| Mouse anti-V5 | Invitrogen | Cat# 46-0705; RRID: AB_2556564 |
| Alexa-Fluor 488 goat anti-rabbit | Thermo Fisher Scientific | A11008 |
| Alexa-Fluor 546 goat anti-mouse | Thermo Fisher Scientific | A11031 |
| Alexa-Fluor 633 goat anti-guinea pig | Thermo Fisher Scientific | A21105 |
| **Fluorescent molecules** |        |            |
| 546-conjugated Phalloidin | Thermo Fisher Scientific | A22283 |
| 647-conjugated Phalloidin | Thermo Fisher Scientific | A22287 |
| ATTO 488-Booster | ChromoTek | AD 488-21 |
| ATTO 594-Booster | ChromoTek | AD 594-21 |
| **Bacterial and Virus Strains** |        |            |
| DH5α™ Competent Cells | Thermo Fisher Scientific | Cat# 18258012 |
| One Shot™ BL21DE3 Competent Cells | Thermo Fisher Scientific | Cat# C600003 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Demecolcine | Sigma-Aldrich | Cat# D7385; CAS: 477-30-5 |
| Cytochalasin D | Sigma-Aldrich | C8273 |
| Bortezomib (PS-341) | Selleck Chemicals | Cat# S1013; CAS: 179324-69-7 |
| *Pfu Ultra* HF DNA polymerase | Agilent Technologies Genomics | Cat# 600380 |
| Schneider’s *Drosophila* medium | Gibco | Cat# 21720-024 |
| SF-900 II serum free medium | ThermoFisher | Cat# 10902096 |
| Nucleofector II | Amaxa (Lonza) | Cat# AAD-1001S |
| Fetal bovine serum | Gibco | Cat# 10500 |
| Penicillin-streptomycin | Gibco | Cat# 15140 |
| Acetic acid | VWR | Cat# 20103 295 |
| Protein-A conjugated Dynabeads | Invitrogen | Cat# 10001 D |
| Dimethyl pimelimidate dihydrochloride | Sigma-Aldrich | Cat# D8388 |
| Sodium azide (Na₃N₅) | Fisher | Cat# S2271 |
| Dithiothreitol (DTT) | Fisher | Cat# BP172 |
| Phenylmethylsulfonyl fluoride (PMSF) | Sigma-Aldrich | Cat# 78830 |
| Soybean trypsin inhibitor, type II-S (SBTI) | Sigma-Aldrich | Cat# T9128 |
| SIGMAFAST protease inhibitor cocktail, EDTA-free | Sigma-Aldrich | Cat# S8830 |

(Continued on next page)
### Reagents or Resources

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| MBP-Spd2-NT1 (a.a. 1-84) | This paper | N/A |
| GST-Spd2-N2 (a.a. 85-340) | This paper | N/A |
| GST-Spd2-M (a.a. 341-662) | This paper | N/A |
| GST-Spd2-C (a.a. 663-1146) | This paper | N/A |
| Plk4[a.a.1-317]-FLAG-His6 | (Brownlee et al., 2011) | N/A |

### Experimental Models: Organisms/Strains

| Organism/Strain | Source |
|-----------------|--------|
| D. melanogaster: UAS-GFP-Plk4KD (Plk4KD) | This paper |
| D. melanogaster: UAS-GFP-Plk4WT (Plk4WT) | This paper |
| D. melanogaster: Ubq-RFP-Fzr | This paper |
| D. melanogaster: Ubq-GFP-Cdc27 | (Huang and Raff, 2002) |
| D. melanogaster: WorGAL4 | (Lee et al., 2006) |
| D. melanogaster: AseGAL4 | (Zhu et al., 2006) |
| D. melanogaster: ActGAL4 | Bloomington Drosophila Stock Center (Ahmad and Henikoff, 2001) |
| D. melanogaster: UAS-RFP-Spd2 constructs generation: F 5'-cgcgcgACTAGTGGCGGCACCGGGCGGCACC-3' and R 5'-cgcgcgCCGCGGTTAAAACTAATCGGGAC-3' | This paper |
| RFP cloning from pURW: F 5'-cgcgcgGATATCAGCTCTCCGAGGACGTCATC-3' and R: 5'-cgcgcgGGATCCGGCGCCGGTGGAGTGGCGGCCCTC-3' | This paper |

### Oligonucleotides

| Oligonucleotide | Source |
|-----------------|--------|
| Pkl4KD construct generation: F 5'- GTCAAGATAGCCAACTTTGGACTGGCC-3' and R 5'- GGCCAGTCCAAAGTTGGCTATCTTGAC-3' | This paper |
| Ubq-RFP-Fzr construct generation: F 5'-GGGGACAAAGTTTGTACAAAAAAGCAGGCTTCATGTTTAGTCCCGAGTACGAGAAG-3' and R 5'-GGGGACCACTTTGTACAGAAAGCTGGGACTTTATCTGATATTGGCAAACAGATT-3' | This paper |
| UAS-RFP-Spd2 constructs generation: F 5'-cgcgcgACTAGTGGCGGCACCGGGCGGCACC-3' and R 5'-cgcgcgCCGCGGTTAAAACTAATCGGGAC-3' | This paper |
| RFP cloning from pURW: F 5'-cgcgcgGATATCAGCTCTCCGAGGACGTCATC-3' and R: 5'-cgcgcgGGATCCGGCGCCGGTGGAGTGGCGGCCCTC-3' | This paper |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| MBP-Spd2-NT1 constructs generation: | F 5'-gcgcgcGGATCCATGGACAGTAGCAGTGGAAGCCAA-3' and R 5'-gcgcgcaACGTGTTTACTGGAGGCCAGTGCTTCTTGTGTTG-3' | This paper N/A |
| GST-Spd2-NT2 constructs generation: | F 5'-gcgcgcGGATCCATGGACAGTAGCAGTGGAAGCCAA-3' and R 5'-gcgcgctCGAGTTATGGCTGTTGCTTCTCGCCACAC-3' | This paper N/A |
| GST-Spd2-M constructs generation: | F 5'-gcgcgcGGATCCGACAATAAAACATACACTAAAACG-3' and R 5'-gcgcgctCGAGTTATGTGAAATCCGCTGGTGGAACTGGC-3' | This paper N/A |
| GST-Spd2-C constructs generation: | F 5'-gcgcgcGGATCCGCGAGTGGAAGACGTGGGTTGGGA-3' and R 5'-gcgcgctCGAGTTAAAATTTAAAACTAAAGGGGACACT-3' | This paper N/A |
| GFP-Spd2 constructs generation: | F 5'-GCTACCAGGTTAATGGTGAGCAAGGGCAGGAG-3' and 5'-ACTAGTCTTGTACAGCTCGTCCATGCC-3' | This paper N/A |
| Spd2-UTR dsRNA synthesis: | F 5'-TAATACGACTCACTATAGGGTTTTCGCGTTCGCACTGCAAACTGTAACGTGTTTAAGGACAAAGCGGATTTGTTTTATTTGCTTC-3' and R 5'-TAATACGACTCACTATAGGGCTTTTAGGAAACAAGCG-3' | This paper N/A |
| V5-Spd2 constructs generation: | F 5'-GGGGGATTCTAGCTGACATGGACAGTAGCAGTGGAAGCCAA-3' and R 5'-GGCTTCCACTGCTACTGTCCATCGTAGATCG-3' | This paper N/A |
| V5-Cnn construct from EST: | F 5'-GGGGGATTCTAGCTGACATGGACAGTAGCAGTGGAAGCCAA-3' and R 5'-GGCTTCCACTGCTACTGTCCATCGTAGATCG-3' | This paper N/A |

Recombinant DNA

| attB-Placman-Ap′ plasmid | DGRC | GenBank EF106980 |
|--------------------------|------|-----------------|
| attB-pUAST-GFP-Plk4WT-SV40 | This paper | N/A |
| attB-pUAST-GFP-Plk4KD-SV40 | This paper | N/A |
| attB-pUAST-GFP-Plk4PACT-SV40 | This paper | N/A |
| pET28a-Plk4(a.a.1-317)-FLAG-His6 | Brownlee et al., 2011 | N/A |
| pMal-C2X | Addgene | Cat# 75286 |
| pGEX-6P-2 | Addgene | Cat# 27-4598-01 |
| pMal-C2X-Spd2-NT1 (a.a. 1-84) | This paper | N/A |
| pGEX-6P-2-Spd2-NT2 (a.a. 85-340) | This paper | N/A |
| pGEX-6P-2-Spd2-M (a.a. 341-662) | This paper | N/A |
| pGEX-6P-2-Spd2-C (a.a. 663-1146) | This paper | N/A |
| pUbq-RFPNT | Basto et al., 2008 | N/A |
| pUbq-RFPNT-Fzr | This paper | N/A |
| attB-pUAST-RFP-Spd2WT-Sv40 | This paper | N/A |
| attB-pUAST-RFP-Spd2KD-Sv40 | This paper | N/A |
| attB-pUAST-RFP-Spd2PACT-Sv40 | This paper | N/A |
| pOT2 Spd2 EST (Clone ID LD24702, DGC EST Library 1.0) | DGRCCG17286 |
| pMT/5His B | ThermoFisher | Cat# V412020 |
| pEGFP C1 | Addgene | Catalog #6084-1 |
CONTACT FOR REAGENT AND RESOURCES SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Renata Basto (renata.basto@curie.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Animals
Species: *Drosophila melanogaster*. Flies were raised on *Drosophila* culture medium (0.75% agar, 3.5% commeal, 5% yeast, 5.5% sugar, 2.5% methyl, 1% penicillin-streptomycin, 0.4% proionic acid). Flies stocks were maintained at 18°C in plastic vials or plastic bottles. Crosses were maintained at 25°C in plastic vials. Brains were collected from 3rd instar larvae (which were staged as the number of days after egg laying and confirmed with developmental landmarks such as size, mouth hook and position within the tube). For all experiments except *FzrrapG0418* (*Fzrmut*) and in combination with Plk4 KD, we analyzed male and female since we did not notice any difference between the two sexes. In the case of Fzrmut, we analyzed only males. In all experiments, Plk4 KD, Plk4 WT and Plk4 PACT were recombined with the Plk4 mutant-*Sakc06612* (*Bettencourt-Dias et al., 2005*) (BDSC#17774). Controls (Ctrl) were used accordingly to the experiments (please see Key Resources Table and below for detailed analysis).

Fly Stocks
UAS lines: UAS-GFP-Plk4 KD (Plk4 KD, this study), UAS-GFP-Plk4 WT (Plk4 WT, this study).
Reportor lines: Ubq-α-Tubulin-RFP and Ubq-α-Tubulin-GFP (*Dobbelaire et al., 2008*), Ubq-RFP-Sas-6 (*Peel et al., 2007*), Ubq-RFP-Fzr (this study), Ubq-GFP-Cdc27 (*Huang and Raff, 2002*).
Mutant alleles: *Sakc06612* (BDSC#17774, *Bettencourt-Dias et al., 2005*), *Sas-4d2214* (BDSC#12119, *Basto et al., 2006*), *FzrrapG0418* (BDSC#12297, *Jacobs et al., 2002*).
Driver lines: WorGAL4 (provided by C. Doe, *Albertson et al., 2004*), AseGAL4 (provided by T. Lee, *Zhu et al., 2006*), ActGAL4 (BDSC#25374), GAL80ts (BDSC#7108). *wf* was used as a control strain.

Cell Lines
Female *Drosophila* S2 cells (*Zhang et al., 2010*) (Invitrogen) were cultured in SF-900 II (Life Technologies) + Pen/Strep (Gibco) and split every 3-4 days.
METHOD DETAILS

Generation of Drosophila Transgenic Lines

The Plk4WT transgene was synthetized by GenScript (GenScript®, NJ, USA) in the pUC57 plasmid, using the coding DNA sequence (CDS) of Drosophila Plk4. The Plk4KD allele was generated by site-directed mutagenesis (Plu Ultra HF DNA polymerase, 600380, Agilent Technologies Genomics) from the Plk4WT allele with primers F (5'- GTCAAGATAGCCAACCTTGAGCTGCGCC-3') and R (5'- GGCCAGTCCAAATGTGCTATCGACG-3'). Underlined is the triplet coding for D156, mutated into N. All Plk4 versions were initially cloned into a pDONR™/Zeo plasmid (Invitrogen, CA, USA) to have the GFP at their N-termius, spaced by linker of three triplicets (GGCGACCCGGCGGCACC), which allows for high expression and stabilization of the Plk4 sequences. To generate all Plk4 transgenic stocks, the PhiC31 integrase-mediated transgenesis system was used. The different Plk4 constructs, flanked by NotI and Ascl, were cloned into a p[acman] plasmid containing the attB site (DGR, GenBank EF106980) and injections were performed by BestGene (BestGene Inc, CA, USA). The Bloomington stock BI9738 (y¹w¹1118;PbAc (y¹-attp-9A)VK00020) was used as attP-containing docking site strain. All transgenes were inserted in the third chromosome in the insertion site 99F8.

To generate the Ubq-RFP-Fzr stock, the genomic sequence of Fzr was first amplified by PCR with primers F: (5'-GGGGACAGTTTTGATACAAAAAGCAGGCTTC ATGTTTAGTCCCGAGTACGAGAAG-3') and R: (5'-GGGGACACTTGTATTTTGCAAAACATCCATTGTT-3'). Fzr specific sequences are underlined. The PCR product was then recombined using BP Gateway reaction (Gateway® Technology, Invitrogen, ThermoScientific) into pDONR™/Zeo plasmid. After sequence validation through Sanger sequencing, the Fzr construct was recombined, using LR Gateway reaction in the pUbq-RFPNT vector (Basto et al., 2008), which allows for moderate expression of the tagged protein (Lee et al., 1988). BestGene performed injections in the w¹1118 stock. With this system the transgene insertion occurred randomly. For all experiments, stocks with transgenes injected in the second chromosome were used.

For UAS- RFP-Spd2 stocks, the Spd2 cDNA was cloned in phase with RFP on its N-terminus with the primers F: (5'- cgcgcgcACT AGTGGGCCAGGCCGACCGCACGTGGAAGCCAA) and R: (5'- cgcgcgcCCGCGGTTAAAACTAATCGGGAC cgcgcgcATTGGTGAGCAAGGGCAGGAG) and Sp40 (sequence described above) at its C-terminus with the enzymes Spe1 andSacII in the Bluescript vector. RFP was cloned from the pJRW vector (DGRC #1282) with primers F: (5'- cgcgcgcGATATCATGGCCTCCTCCGAGGACGTCATC-3') and R: (5'- cgcgcgcGATATCATGGCCTCCTCCGAGGACGTCATC-3') in the Bluescript vector with the enzymes EcoRV and BamHI, spaced by a linker composed of GGGTTG. Restriction enzymes are in italic. Spd2DE and Spd2AA constructs were synthetized by GeneART (ThermoFisher). In Spd2DE construct, all serines (Ser) and threonines (Thr) identified were mutated to aspartate (D) with the exception of Thr371, Thr384, Thr458, Thr493, Thr706, Thr754, which were mutated to alanines (A). After fusion to RFP and SV40 sequence validation through Sanger sequencing, all constructs comprising RFP-Spd2-SV40 was subsequently cloned in the pUAS promoter which allows for high expression and stabilization of the tagged protein (Lee et al., 1988). BestGene performed injections in the w¹1118 stock. With this system the transgene insertion occurred randomly. For all experiments, stocks with transgenes injected in the second chromosome were used.

For Spd2-NT1 construct, one T was added between the KpnI site and GFP sequence to maintain the frame in the pMT/V5 HsB vector. For Spd2-NT2, Spd2-M and Spd2-C were cloned into the pGEX-6P-2 vector with the enzymes HindIII, BamHI, XhoI, with GST on their N-terminus. After sequence validation through Sanger sequencing, all constructs comprising RFP-Spd2-SV40 was subsequently cloned in the pUAS promoter which allows for high expression and stabilization of the tagged protein (Lee et al., 1988). BestGene performed injections in the w¹1118 stock. With this system the transgene insertion occurred randomly. For all experiments, stocks with transgenes injected in the second chromosome were used.

For Spd2-NT2 construct, one T was added between the KpnI site and GFP sequence to maintain the frame in the pMT/V5 HsB vector. For Spd2-NT2, Spd2-M and Spd2-C were cloned into the pGEX-6P-2 vector with the enzymes HindIII, BamHI, XhoI, with GST on their N-terminus. After sequence validation through Sanger sequencing, all constructs comprising RFP-Spd2-SV40 was subsequently cloned in the pUAS promoter which allows for high expression and stabilization of the tagged protein (Lee et al., 1988). BestGene performed injections in the w¹1118 stock. With this system the transgene insertion occurred randomly. For all experiments, stocks with transgenes injected in the second chromosome were used.

Generation of Spd2 Fragments

Spd2 fragments were amplified by PCR from the Spd2 cDNA, using the following primers: for Spd2-NT1 (a.a. 1-84) F 5' - gcgcgcgACT AGTGGGCCAGGCCGACCGCACGTGGAAGCCAA - 3' and R 5' - gcgcgcgTTTTGATACAAAAAGCAGGCTTC ATGTTTAGTCCCGAGTACGAGAAG - 3', for Spd2-NT2 (a.a. 85-340) F 5' - gcgcgcgGGATCCATGGCCTCCTCCGAGGACGTCATC - 3' and R 5' - gcgcgcgATATCATGGCCTCCTCCGAGGACGTCATC - 3', for Spd2-M (a.a. 341-663) F 5' - gcgcgcgATATCATGGCCTCCTCCGAGGACGTCATC - 3', and R 5' - gcgcgcgATATCATGGCCTCCTCCGAGGACGTCATC - 3', for Spd2-C (a.a. 664–1146) F 5' - gcgcgcgCCGCGGTTAAAACTAATCGGGAC cgcgcgcATTGGTGAGCAAGGGCAGGAG - 3' and R 5' - gcgcgcgATATCATGGCCTCCTCCGAGGACGTCATC - 3'. Spd2 specific sequences are underlined. Restriction enzymes are in italic. Spd2-NT1 was cloned into the pMal-C2X vector with the enzymes BamHI and HindIII, with MBP on its N-terminus. Spd2-NT2, Spd2-M and Spd2-C were cloned into the pGEX-6P-2 vector with the enzymes BamHI and XhoI, with GST on their N-terminus. After sequence validation through Sanger sequencing, Spd2 fragments were used for in vitro kinase assay.

Generation of Spd2 Phosphomutant Transgenes for S2 Experiments

Generation of the GFP-Spd2 phosphomutant transgenes was obtained as follows. GFP was amplified by PCR from the pEGFP C1 vector, flanked with KpnI and Spel restriction sites, using the following primers: F 5' – cgcgcgcGATATCATGGCCTCCTCCGAGGACGTCATC – 3' and 5' – cgcgcgACTAGTGTGCTACAGCTTCGTCATGCC – 3'. GFP specific sequences are underlined. Restriction enzymes are in italic. In the F primer, one T was added between the KpnI site and GFP sequence to maintain the frame in the pMT/V5 HsB vector. Next, Spd2 phosphomutant sequences were recovered from the Bluescript vector (already used for the generation of RFP-Spd2 stocks, see above) by digestion with the enzymes Spel and SacII and cloned into the pMT/V5 HisB vector with GFP on their N-terminus.
The V5-Spd2 phosphomutants were generated from the GFP-Spd2 phosphomutant (in the pMT/V5 HisC vector) by a megaprimer PCR strategy. GFP sequences were replaced with V5 sequence using the megaprimer F 5’ – GGGGATCTAGATTTAATTAATTAGCTGTTCCGGCCGCTTCCGGTACCATGggtaagcctatccctaaccctctcctcggtctcgattctacgATGGACCAGTCTAAA CGGGGTACCATGggtaagcctatccctaaccctctcctcggtctcgattctacgATGGACAGTAGCAGTGGAAGCC - 3’. The V5-tag is in lower case and Spd2 specific sequences are underlined. Insert sequences were verified by sequencing.

Expression of UAS-transgenes was carried out using either the UAS/GAL4 system (Brand and Perrimon, 1993) or the temporal and regional gene expression targeting (TARGET) method (McGuire et al., 2004). Worniu (Wor) GAL4 (Albertson et al., 2004) and Asense (Ase) GAL4 (Zhu et al., 2006) were used to induce transgene expression exclusively in NBs to perform live imaging experiments. WorGal4 and AseGal4 were recombined with either Ubq-α-Tubulin-RFP or Ubq-α-Tubulin-GFP transgenes (Dobbelaere et al., 2008), localized on the 2nd chromosome. As control for live imaging experiments, to analyze centriole behavior we used the Ubq-Sas-6 RFP line, which was crossed with Ubq-α-Tubulin-GFP, WorG4 or Ubq-α-Tubulin-GFP, AseGal4. As control for live imaging experiments, to analyze centriole behavior we used the Ubq-Sas-6 RFP line was crossed with Ubq-α-Tubulin-GFP, or Ubq-α-Tubulin-GFP, WorG4 or Ubq-α-Tubulin-GFP, AseGal4. All these lines showed similar centriole behavior and cell cycle timings. We referred to them as Ctrls in the text. A recombinant comprising the ActGal4 (BDSC#25374) and GAL80ts (BDSC#7108) on the 2nd chromosome was used to induce the expression of the transgenes in a temporal manner to be used in immunostaining experiments. At 18°C, GAL80ts binds to and inhibits the transcriptional activation domain of GAL4. The fly crosses were established and fly crosses were maintained at 18°C–20°C. Second instar larvae were then moved to 29°C in order to inhibit the binding between GAL80ts and GAL4. This leads to the expression of the UAS-transgenes within 16–18 hours. Two days after, mid third instar larval brains were dissected for immunostaining. Ctrls were performed with this stock, where the ActGal4/GAL4 80ts recombinants were allowed to develop between 18°C–20°C, before being placed in the 29°C incubator.

Drosophila genotypes and crosses

Figure 1
Control: GFP-Tub, AseGal4/Cyo-GFP; U X Ubq RFP-Sas6
Plk4KD: RFP-Tub, AseGal4/Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb
Plk4WT: RFP-Tub, AseGal4/Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4WT, Plk4 mut/ TM6 Tb

Crosses were maintained at 25°C

Figure 2
Control: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb
Plk4KD: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb
Plk4mut: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb
Plk4WT: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4WT, Plk4 mut/ TM6 Tb

Crosses were maintained at 18°C for 4 days after egg laying and transferred to 29°C for 48 hrs.

Figure 3
Control: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb
Plk4KD: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb
Plk4WT: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4WT, Plk4 mut/ TM6 Tb

Crosses were maintained at 18°C for 4 days after egg laying and transferred to 29°C for 48 hrs.

Figure 4
Control: Fzrmut: Fzr/Fm7 Kr-GFP; If/Cyo-GFP X GFP-Tub/Cyo-GFP
Fzrmut, Plk4KD: Fzr/Fm7 Kr-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb X RFP-Tub, AseGal4/Cyo-GFP; Plk4mut /TM6 Tb
Fzrmut, Plk4mut: Fzr/Fm7 Kr-GFP; If/Cyo-GFP
Fzrmut, Plk4KD: Fzr/Fm7 Kr-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb X ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb

Crosses were maintained at 18°C for 4 days after egg laying and transferred to 29°C for 48 hrs.

Figure 5
Control: Spd2WT: If/Cyo-GFP; Spp2WT/TM6Tb X GFP-Tub, AseGal4/Cyo-GFP; Plk4mut /TM6 Tb
Spd2DE: If/Cyo-GFP; Spd2DE/TM6Tb X GFP-Tub, AseGal4/Cyo-GFP; Plk4mut /TM6 Tb
Spd2AA: If/Cyo-GFP; Spd2DE/TM6Tb X GFP-Tub, AseGal4/Cyo-GFP; Plk4mut /TM6 Tb
Crosses were maintained at 25°C

Figure 5
Spd2WT: If/Cyo-GFP; Spd2WT/TM6Tb X ActGal4, Gal80ts/Cyo-GFP; Sb/TM6Tb
Spd2DE: If/Cyo-GFP; Spd2DE/TM6Tb X ActGal4, Gal80ts/Cyo-GFP; Sb/TM6Tb
Spd2AA: If/Cyo-GFP; Spd2DE/TM6Tb X ActGal4, Gal80ts/Cyo-GFP; Sb/TM6Tb
Crosses were maintained at 18°C for 4 days after egg laying and transferred to 29°C for 48 hrs.

Figure 2
Control: ActGal4, Gal80ts, /Cyo-GFP
Plk4KD: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb
Plk4WT: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4WT, Plk4 mut/ TM6 Tb
Crosses were maintained at 18°C for 4 days after egg laying and transferred to 29°C for 48 hrs.

Figure 3
Control: ActGal4, Gal80ts, /Cyo-GFP
Plk4KD: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4KD, Plk4 mut/ TM6 Tb
Plk4WT: ActGal4, Gal80ts, /Cyo-GFP; Plk4mut /TM6 Tb X +/Cyo-GFP; GFP- Plk4WT, Plk4 mut/ TM6 Tb
Crosses were maintained at 18°C for 4 days after egg laying and transferred to 29°C for 48 hrs.

Live Imaging
WorderGAL4 or AseGAL4 drivers were used to induce the transgene expression only in NBs. In general, the over-expression of any GFP-Plk4 or RFP-Spd2 proteins leads to the formation of large green/red aggregates that are frequently segregated into the GMCs. Other smaller aggregates or green particles can often be seen in NBs and GCMCs. In all the movies analyzed and presented in this article, the centrosome or centriole were distinguished by its capacity to nucleate MTs, or through its position at spindle poles or association with the spindle during mitosis in the the period of time-lapse (even if not included in the stills). In certain cases, the timeframe where it is possible to distinguish a centriole or centrosome is not included in the Figures shown, but we carefully characterize centrosome/centriole behavior and dynamics to identify the same centrosome/centriole in different time frames. Mid third instar larval brains were dissected in Schneider’s Drosophila medium (21720-024, Gibco, ThermoScientific) supplemented with 10% heat-inactivated fetal bovine serum (10500, Gibco, ThermoScientific), penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹) (penicillin–streptomycin 15140, Gibco, ThermoScientific) (hereafter referred to as live imaging medium). Brains were placed on a glass-bottom 35 mm dish (P35G-1.5-14-C, Mat Tek Corporation, MA, USA) with 10 μl of medium, covered with a permeable membrane (Standard membrane kit, YSI, OH, USA) and sealed around the membrane borders with Voltalef oil 10S (WVR BDH Prolabo). One or two brain lobes were recorded using a Yokagawa CSU-X1 spinning head mounted on a Nikon TIE inverted microscope. The microscope was equipped with an EMCCD Evolve 512 x 512 (Photometrics, AZ, USA) and controlled by the Metamorph software 7.7 (Molecular devices). Four-dimensional z-stacks of 18–26 μm at 0.75-μm intervals were acquired every 30 or 60 s using an x60, NA 1.4 oil-immersion objective. The same laser power and acquisition settings were kept for all time lapse acquisitions. Images were processed with Fiji (NIH) and Adobe Photoshop.

Immunohistochemistry and Antibodies
Third instar larval brains were dissected in PBS and fixed in 4% formaldehyde in PBS for 30 min. After fixation, brains were transferred to 45% acetic acid (diluted in water) for 15 sec and then to 60% acetic acid (diluted in water) for 3 min. Brains were then mounted onto a slide, squashed and immediately flash-frozen in liquid nitrogen, followed by a further fixation with ice-cold methanol, at -20°C for 7 min. Next, brains were rehydrated in PBS + 0.1% Triton X-100 (T9284, Sigma), 3 times for 15 min. Once dried, brains were...
incubated overnight at 4C with the primary antibody solution diluted in PBS + 0.1% Triton, in a humid chamber. Brains were then rehydrated in PBS + 0.1% Triton, 3 times for 15 min, allowed to dry and incubated for 2h at 25C with the secondary antibody solution in PBS + 0.1% Triton, in the dark, in a humid chamber. Next, brains were rehydrated in PBS + 0.1% Triton, 3 times for 15 min and incubated 15 min with Hoechst 33342 (Invitrogen), at 0.5ug/ml in PBS + 0.1% Triton. Finally, once dried, brains were mounted in mounting medium (1.25% N-propyl gallate, 75% glycerol, 25% H2O).

For 3D SIM imaging or confocal imaging, whole mount third instar larval brains were dissected in PBS and fixed in 4% paraformaldehyde diluted in PBS + 0.1% Triton for 30 min. Brains were then permeabilized with 3 washes in PBS + 0.3% Triton. Next, a blocking step followed, by incubation in PBS + 10% NGS for 30 min. Next, brains were incubated with primary antibody solution (PBS + 0.3% Triton), first a few hours at room temperature on gentle agitation, and then overnight at 4C. Brains were then washed 3 times in PBS + 0.3% Triton, followed by incubation with secondary antibody solution, overnight at 4C which also included a phalloidin conjugated probe to label the cell membrane. After 2 washes in PBS + 0.3% Triton (5 min each), brains were incubated with Hoechst 33342 (0.5ug/ml in PBS +0.3% Triton) for 30 min before a final wash in PBS. Finally, brains were rapidly washed 3 times in PBS. They were mounted in mounting medium as described above. For the 3D SIM analysis, the appropriate NBs in interphase were chosen based on the presence of a large intact nucleus. The apical cortex was identified by the presence of a centrosome/centriole in Ctrl and Plk4KD brains and by its position relative to GMCs. In Plk4 WT NBs, centrioles are positioned towards the basal hemisphere.

Primary antibodies used: rabbit anti-Spd2 (1:500; (Dix and Raff, 2007), guinea pig anti-Cnn (1:1000, (Lucas and Raff, 2007), rabbit anti-PPHC (Plp) (Martinez-Campos et al., 2004), mouse anti-z-Tubulin (DM12) (1:500, Sigma Aldrich), rabbit anti-aPKC (1:100, SC116, Santa Cruz) and mouse anti-Prospero (1:20, DSHB). Secondary antibodies used: Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 647 (Molecular Probes, ThermoScientific), Phalloidin conjugated probes (R415) for F-actin labelling in red, from molecular probes (Thermo Fisher) were used to label the cell membrane, RFP and GFP Boosters (Atto 594 and Atto 488) (1:100, Chromotek).

**Drug Treatments**

For analysis of centrosome position in NBs, third instar brains were placed in PBS or PBS supplemented with colcemid -demecolcine (D7385, Sigma Aldrich) (50uM final concentration) for exactly 1h at 25C. Brains were then fixed as described above for whole mount preparations.

To inhibit the proteasome, Bortezomib (PS-341, Selleck Chemicals) was added to the live imaging medium at a concentration of 50uM, a concentration that led to mitotic arrest in NBs. Brains were fixed after 2 hours of incubation in the medium supplied with the drug. Control brains were incubated in live imaging medium with 1% DMSO. Brains were then fixed as described above for whole mount preparations.

For live imaging, third instar larval brains were dissected in live imaging medium and then incubated in live imaging medium implemented with the appropriate drug. To depolymerize MTs, demecolcine (D7385, Sigma Aldrich) was added to the medium at a concentration of 50 μM as described previously (Januschke and Gonzalez, 2010). We noticed that fluorescence intensity of centriolar tagged proteins used in this study- RFP-Sas6 and GFP-Plk4KD decreases at the centriole, indicating a possible instability of these proteins in the absence of centriolarMTs. For actin depolymerization, cytochalasin D (C8273, Sigma Aldrich) was added to the medium at a concentration of 50 μM. After dissection, brains were placed on the glass-bottom dish in 10 μl of live imaging medium with demecolcine or cytochalasin D. Brains were filmed immediately after.

**Western Blot of Spd2 Transgenic Lines**

Twenty third instar larval brains of each genotype were dissected in cold PBS supplied with 1% protease inhibitor cocktail (P8340, Sigma) and 1mM PMSF (P7626, Sigma) and collected in a tube. 20 ml of sample buffer was added to the brains and the tissues were stripped with the help of blunt forceps on ice to induce mechanical dissociation. Samples were boiled at 70 C for 10min. Samples were run in 10% Bis/Tris gel in MOPSSDS buffer at 180V (Np0301 and NP0001 from NuPAGE-ThermoFisher) and transferred fir 1h at 100V in the cold using 0.2 μm NC nitrocellulose (GE Healthcare Life Science). Membranes were then blocked in PBS supplemented with 0.1% Tween20 (PBST) and 10% dried milk powder for 30 min followed by incubation O/N 4C with Spd2 primary antibody at 1:500 (Dix and Raff, 2007), diluted in PBST with 3% dried milk powder. Membranes were washed 4 times for 10min in PBST and then incubated for 2h at room-temperature with a Rabbit secondary antibody conjugated to Horseradish peroxidase (HRP) (# G21234, Life Technologies) diluted in PBST with 3% dried milk powder. The secondary antibody solution was then removed and membranes washed 5 times for 10min in PBST. Finally, membranes were incubated with SuperSignalTM West Pico Chemiluminescent Substrate (34080, Thermo ScientificTM) and revealed using the BioRad Chemidoc MP system. Images were analyzed using ImageLab software.

**In Vitro Kinase Assays and Mass Spectrometry**

Bacterially-expressed constructs of Drosophila Plk4 (amino acids 1–317) C-terminally tagged with FLAG-Hisa and Drosophila Spd2 N-terminally tagged with either Glutathione S-Transferase (GST) or Maltose-binding protein (MBP) were purified on HisPur resin (ThermoFisher), glutathione resin (NEB) and amylose resin (NEB), respectively, according to manufacturer’s instructions. Prior to assay, purified proteins were resolved by SDS-PAGE and scans of the Coomassie-stained gels analyzed by densitometry (ImageJ, NIH) to determine protein purity. Total protein concentrations of the same reagents were measured by Bradford assay (BioRad). The total protein and purity measurements were used to calculate the concentration of each protein reagent. (Contaminants and proteolytic fragments are excluded by this calculation.). In vitro phosphorylation assays were performed by incubation with
100 μM ATP for 60-90 min at 24°C in reaction buffer [40 mM Na HEPES (pH 7.3), 150 mM NaCl, 5 mM MgCl₂, 0.5 mM MnCl₂, 1 mM DTT, 10% (by volume) glycerol]. Samples were resolved by SDS-PAGE, and proteins visualized by Coomassie staining. Phosphorylation of protein substrates was evaluated by including γ-32P-ATP in assays and, subsequently, the presence of radiolabeled substrates detected by autoradiography of dried gels. Phosphorylated residues within proteins were identified by tandem mass spectrometry (Table S1) of purified bacterially-expressed proteins phosphorylated in vitro (described above in ‘Generation of Spd2 fragments’) in the presence of non-radioactive ATP and performed at the Arizona Proteomics Consortium (University of Arizona). Samples of Spd2 were reduced (10 mM dithiothreitol, 55°C, 1 hr), alkylated (55mM iodoacetamide, 24°C, 45min), and trypsin digested (~1μg trypsin, 37°C, 12hrs) in-gel, and then extracted. Peptide samples were desalted using ZipTip 0.6xL C₁₈ resins (EMD Millipore, Billerica, MA). The peptides were then separated by HPLC on a C₁₈ analytical column, ionized by electrospray ionization (ESI) in positive mode, and analyzed on a LTQ Orbitrap Velos (Thermo Electron Corp., San Jose, CA) mass spectrometer. All LC MS analyses were carried out in “data-dependent” mode in which the top 6 most intense precursor ions detected in the MS1 precursor scan (m/z 300-2000) were selected for fragmentation via collision induced dissociation (CID). Precursor ions were measured in the Orbitrap at a resolution of 60,000 (m/z 400) and all fragment ions were measured in the ion trap.

RNAi of Drosophila S2 Cells
Cells were plated at 50% confluency and treated with 10 μg/day dsRNA for 7 days. Cells were passaged before they reached ~90% confluency. On day 5, cells were transfected with 2 μg of plasmid and 10 μg dsRNA using the Nucleofector II (Lonza). Transgenes were expressed by treating with 0.5 mM CuSO4 for 24 hours.

Co-Immunoprecipitation and Immunoblotting
GFP-binding protein fused to Human IgG was coupled to Protein-A conjugated Dynabeads (Invitrogen) and cross-linked to the beads with dimethyl pimelimidate dihydrochloride (Sigma). Beads were blocked overnight in PBS-0.1% Triton X-100 + 1% BSA. S2 cells were lysed in IP buffer (50 mM Tris pH 7.2, 125 mM NaCl, 1 mM EGTA, 0.5% Triton X-100, and 0.4 mM NaN₃, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL soybean trypsin inhibitor (SBTI), and SIGMAFAST protease inhibitor cocktail (Sigma), then precleared by centrifugation at 10,000 rcf for 5 minutes at 4°C. A sample of cleared lysate was used for input blots. Beads were equilibrated in IP buffer, then 50 μL of beads were added to the remaining lysate and rocked at 4°C for 30 minutes. Beads were washed 4x with IP buffer by resuspension and then harvested with a magnet between washes. Beads were transferred to a new tube during the final wash. Samples were eluted by boiling in Laemmli buffer (40 μL of 2x). Inputs and IPs were resolved by SDS-PAGE and transferred to nitrocellulose membrane, then analyzed by Western blot using mouse anti-GFP (1:3000, clone JL-8; Clontech Laboratories, Catalogue #632381), mouse anti-α-Tubulin (1:3000, clone DM1a; Sigma, Catalogue #T9026), guinea pig anti-Spd2 (1:500, polyclonal), mouse anti-α-Tubulin (1:1500, Clone HA-7; Sigma, Catalogue #H3663), or mouse anti-V5 (1:1500; Invitrogen; Catalogue #46-0705) primary antibodies, followed by fluorescently-labeled goat anti-mouse (1:3000; Li-COR IRDye 800CW, Catalogue #926-32210) or donkey anti-guinea pig (1:3000; Li-COR IRDye 800 CW, Catalogue #926-32411) secondaries. Detection was performed on a LICOR odyssey cl-X fluorescent imaging system at medium quality and 84 nm resolution.

Synthesis of Spd2 dsRNA
dsRNA was synthesized by in vitro T7 transcription using PCR product amplified from Spd2 EST (CG17286, Clone ID LD24702, DGC EST Library 1.0) with the primers Spd2-UTR-For: 5' – TAATACGACTCACTATAGGGTTTTCGCGTTCGCACTGCAAACTGTAA CTGTTTAAAGGACAAAGCGGATTGTGTTTTATTTGTGCTG and Spd2-UTR-Rev: 5’ – TAATACGACTCACTATAGGGCTTTAGGAAA CAAAGCG.

Image Acquisition
Images of squashed preparations were collected with a x100 objective on a Leica DM6B epifluorescence microscope, this microscope was equipped with an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu) and controlled by the Metamorph software (Gustafsson et al., 2008). The system is equipped with an APO TIRF SR 100x 1.49NA oil immersion, a laser illumination (488nm 200mW, 561nm 100mW, 640nm 100mW) and an EMCCD DU-897 Andor camera. Images were acquired with the following protocol, a Z stack (0.12 micron steps) was acquired. Images were then reconstructed using Nikon elements software.

QUANTIFICATION AND STATISTICAL ANALYSIS
Characterization of Centriole Behavior
The characterization of centriole movement was performed on raw data taking into account the Z-stacks required to follow centriole behavior in x, y, and z. Z-stacks were used to generate projections to follow the centriole behavior over time. In Ctrl cells, after mitosis, the two centrioles display stereotypic movements as described previously (Rebollo et al., 2007; Rusan and Peifer, 2007) and this behavior was taken for the basis of characterization of centriole behavior in the different genotypes. Centrioles were considered “Apical” when behaving as Ctrl apical centrioles- maintaining a stable and fixed position closer to the apical cell cortex throughout
interphase. The apical cell cortex was established by the position of the large aster, at the end of telophase, typical of the asymmetric spindle in NBs. Centrioles were categorized as apical mobile when they were maintained within the apical hemisphere (top half of the cell), Centrioles that moved away from the apical cortex and were positioned towards the basal hemisphere (bottom half of the cell) were considered as “Basal-like” centrioles. Frequently, they displayed a random jumping movement. In certain cases, the NB moved and the neighboring cells were used as landmarks to position the apical cortex at different time points. For the characterization of centriole behavior in the different Plk4 transgenes, 20 NBs from 3 Ctrl brains, 26 NBs from 7 Plk4KD brains and 20 NBs from 7 Plk4WT brains were analyzed. In the same type of analysis in the Fzrmut background, 12 NBs from 3 Fzrmut brains and 18 NBs from 5 Plk4KD,Fzrmut brains were analyzed.

**Stabilization and Tracking Centriole Dynamics in Time-lapse Movies**

To stabilize the full set of images that comprise each time-lapse movie, we used the “SetLandmark” and “StabilizeMovie” plugins from ImageJ. With the “SetLandmark” we defined a landmark on each frame to identify the apical and basal axis of each NB cell. Subsequently, translations and rotations were applied to align the center of the previously established landmarks at the center of the image. Centrosome/centriole tracking was performed with the “Tracking” plugins from ImageJ. In each time frame the centriole was identified and labelled manually in order to allow tracking of their movement. After this step, we used “PrintTracking” from imageJ to draw the tracking of each centrosome/centriole.

**Analysis of Spindle Orientation after Two Consecutive Mitosis**

Analysis of spindle orientation was performed on time-lapse movies where two consecutive mitosis of the same NB could be identified. In both mitosis spindle axis was determined at early anaphase. The angle between the two spindles was measured using the “Angle tool” from Fiji (NIH).

**Analysis of Spindle Orientation in Mitotic NBs**

Analysis of spindle orientation was performed on metaphase or anaphase NBs that contained a clear aPKC crescent. The angle between the spindle and the middle of the aPKC crescent was determined using the “Angle tool” from Fiji (NIH).

**Analysis of Centrosome Position in NBs**

We only analyzed NBs were a clear group of GMCs could be seen to identify the position of the apical cortex at the opposite side of the GMCs. First, a line was drawn from the apical cortex to the basal to measure the apical-basal axis length with the Fiji (NIH) “Straight line” tool. Then a second line, positioned at the same position than the first line (at the apical side), was drawn till it reached the centrosome, labelled with two centrosome markers (only one is shown in the Figure). The two values for each NB (apical-basal axis length and centrosome distance from the apical cortex) were plotted on an excel sheet. Centrosome position was then calculated by dividing the second measure by the first one.

**Characterization of Centrosomal Protein Levels in Interphase Centrioles**

Quantification of the levels of centrosome proteins in interphase were performed in at least ten NBs for each genotype from 3 independent experiments. For each set of immunostaining experiments, the same acquisition settings were kept for all conditions. Images were analyzed with Fiji (NIH) and quantifications done as follows. The fluorescence mean grey value (Fcy) was measured by drawing the area occupied by the protein of interest with the freehand tool in a single Z plane, representing the center of the centrosome. The cytoplasmic fluorescence (Fcy) was measured by drawing a round area as big as about one-fourth of the cell size and then subtracted from Fc to obtain the net fluorescence of the centrosome (Vcy=Fcy-Fcy). The signal of the background (Fbkg) outside the cell was also measured to obtain the net fluorescence of the cytoplasm (Vcy=Fcy-Fbkg). Finally, centrosome enrichment was measured as ratio between Vcy and Vcy (Vcy/Vcy).

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism 7. A two-tailed unpaired t-test was used to assess statistical differences. All details of statistical analyses, including n and p values, are found in the text and Figures. All results are presented as mean ± SD.
Supplemental Information

Plk4 Regulates Centriole Asymmetry and Spindle Orientation in Neural Stem Cells

Davide Gambarotto, Carole Pennetier, John M. Ryniawec, Daniel W. Buster, Delphine Gogendeau, Alix Goupil, Maddalena Nano, Anthony Simon, Damien Blanc, Victor Racine, Yuu Kimata, Gregory C. Rogers, and Renata Basto
Figure S2- Gambarotto et al

A

Centriolar GMC

Acentriolar Nb

Centriolar GMC

Acentriolar Nb

B

Acentriolar GMC

Acentriolar Nb

C

Percentage of NBs

0 centrosome

1 centrosome

2 centrosomes

>2 centrosomes

D

Sas-6 Tubulin

G2 Prometaphase Interphase

Sas4mut

06:00 06:30 12:00 33:30 46:00
Figure S3 - Gambarotto et al

A

Ctrl Plk4KD Plk4WT

Avg angle = 9.9° ± 0.7°
Var = 2.3° and 45.0°

Avg angle = 15.3° ± 1.2°
Var = 5.4° and 60.9°

Avg angle = 23.5° ± 2.1°
Var = 3.9° and 89.6°

B

p = 0.013

p = 0.007

p < 0.0001

C

Actin Plp Cnn DNA

D

Centrosome position along the apical-basal axis

< 0.0001
< 0.0062
< 0.0001

Colcemid:
Ctrl Plk4KD Plk4mut Plk4WT

E

F

Centrosome position along the apical-basal axis

< 0.0003
< 0.0003
< 0.0164

Colcemid:
Fzrmut Plk4KD Fzrmut
SFigure S4 - Gambarotto et al
Figure S5 - Gambarotto et al

A

Actin
Actin + Centrosomes
Actin

Actin Centrosomes DNA

Ctrl

Actin Centrosomes DNA

Plk4

B

Prophase
Interphase
Interphase
Metaphase

Sas-6 Tubulin

+ Cytochalasin

Plk4

KD Tubulin

00.00 67.00 121.00
Supplementary Figure 1 – Diagram of Plk4 constructs analyzed

(A, B) Transgenes were under the control of the UAS promoter and their expression induced through the UAS/GAL4 system. (A) The UAS sequence was fused to the GFP coding region, upstream of Plk4 WT cDNA containing the entire coding region from the initial starting ATG to the stop codon, while the Plk4KD contains a mutation in the kinase catalytic site of Aspartic Acid 156 to Asparagine (D156N) that prevents centriole duplication, (Brownlee et al., 2011, Habedanck et al., 2005, Holland et al., 2010). The GFP and Plk4 sequences are spaced by a linker of 12 bp encoding G-G-T-G-G-T. (B) The UAS sequence was fused to the RFP coding region upstream of Spd2WT or Spd2DE or Spd2AA coding regions. The RFP and Spd2 sequences are spaced by a linker of 12 bp encoding G-G-T-G-G-T). Related with Figure 1 and Figure 4

Supplementary Figure 2 – Analysis of centrosome numbers in NBs of centriole mutants

Related with Figures 1-3

(A, B) Schematic explaining the absence of centrioles in NBs of Sas-4 mutants (Sas-4mut). (A) At mitotic exit, the NB inherits a single unduplicated centriole. Through centriole maturation, the single centriole becomes a mother centriole and, consequently, loses apical anchoring and starts moving randomly into the basal side throughout interphase. In the following mitosis, it will most likely form the pole that will be segregated into the GMC giving rise to an acentriolar NB. (B) In the following mitosis, the acentriolar NB will form an acentrosomal spindle, mainly using the chromatin-mediated microtubule assembly pathway. (C) Graph of centriole numbers in NBs of the indicated genotypes. Error bars represent standard error of the mean.(D) Still images from time-lapse movies of a Sas-4mut NB. Tubulin is shown in red and RFP-Sas-6 in green. The Sas-4mut NB lost its centrioles in the previous cell cycles as judged by the absence of RFP-Sas-6 positive dots. Time is shown in minutes. Scale, 3 µm.

Supplementary Figure 3 – Analysis of spindle positioning in mitotic NBs

Related with Figures 2-3

(A) Immunostaining of Ctrl, Plk4KD and Plk4WT NBs using antibodies against aPKC (green), tubulin (blue) to label the mitotic spindle and centrosomes (red). The diagrams
below recapitulate spindle position relative to the aPKC crescent. Scale, 4 µm. (B) Quantification of mitotic spindle orientation during mitosis in Ctrl, Plk4\textsuperscript{KD} and Plk4\textsuperscript{WT} NBs. (C) On the left, picture of whole mount brain lobe projection to illustrate NB and GMC organization within the central brain revealed by actin labeling with phalloidin (shown in red) and labeled with antibodies against Plp and Cnn (green and white respectively) to label centrosomes. DNA in blue. The dashed blue lines surrounds the central brain region and the dashed square illustrates the NB-GMC progeny in the higher magnification on the right. (D) Dot plot of centrosome positioning along the apical basal axis with (+) and without (-) colcemid for the indicated genotypes (Ctrl-9.9±0.4 Ctrl+ 33.8±2.2; Plk4\textsuperscript{KD}-10.6±0.7 Plk4\textsuperscript{KD}+ 14.5±1.3; Plk4\textsuperscript{mut}+ 11.5±0.5 Plk4\textsuperscript{mut}+ 20.3±0.9; Plk4\textsuperscript{WT} 36.6±18.7). Error bars represent means ± SD from at least 3 independent experiments. Statistical significance was assessed by unpaired t-test. (E) Immunostaining of Ctrl ,Plk4\textsuperscript{KD}, Fzr\textsuperscript{mut} and Fzr\textsuperscript{mut}Plk4\textsuperscript{KD} NBs, treated with (right) or without (left) colcemid. Plp is shown in green, phalloidin-labeled actin in red and DNA (blue). Scale, 5µm. (F) Fzr\textsuperscript{mut} 22.7±2.2 Fzr\textsuperscript{mut} + 33.6±3.4; Plk4\textsuperscript{KD}, Fzr\textsuperscript{mut} - 39.2±3.9 Plk4\textsuperscript{KD}, Fzr\textsuperscript{mut} +52.1±3.4. Error bars represent means ± SD from at least 3 independent experiments. Statistical significance was assessed by unpaired t-test.

**Supplementary Figure 4 – Analysis of the role of Fzr in centriole apical anchoring**

Related with Figures 2-3

(A-C) Images from time-lapse movies of Ctrl, Plk4\textsuperscript{KD}, and Plk4\textsuperscript{WT} NBs expressing RFP-Fzr. For each genotype, the top panel shows Tubulin (red) and RFP-Fzr (green). The bottom panels show a higher magnification fire LUT representation of RFP-Fzr levels at the centriole (purple=low levels, white=high levels). The blue arrow points to the apical centrosome or centriole inherited by NB at the end of mitosis, while the white arrow points to the basal centriole. Time, minutes. Scale, 4µm. (D) Images from time-lapse movies of Fzr\textsuperscript{mut} NBs. Tubulin (grey). Blue arrows point to the apical centriole, which maintains a strong aster throughout interphase. Time, minutes. Scale, 4 µm. (E) Images from time-lapse movies of GFP-Cdc27 NBs. Tubulin (red) and GFP-Cdc27 (green). During interphase, GFP-Cdc27 does not localize to centrosomes. Time, minutes. Scale, 3 µm. (F) Confocal images show the maintenance of an apical aster in Ctrl NBs incubated either with DMSO (left) or the proteasome inhibitor BZ (middle). Brains were immunostained for Plp (green), tubulin (red in top panels) and Cnn (red in bottom panels). DNA(blue). Scale, 4 µm. (G) Graph shows the percentage of interphase Ctrl NBs...
that display an apical centriole and aster after incubation with DMSO (n=126 NBs) or BZ (n=98 NBs).

**Supplementary Figure 5 - Centrosomes in Drosophila NBs are not associated with an actin-based structure**

Related with Figure 3

(A) Immunostaining of Ctrl (top) and Plk4\textsuperscript{KD} (bottom) NBs stained with Phalloidin to label the actin cortex (red) and immunostained for centrosomes (green). DNA, blue. The insets on the right show higher magnification regions of the centrosome-cortex region Scales, 4 mm and 1mm. (B) Images from time-lapse movies of Ctrl (expressing Sas-6 GFP) and Plk4KD NBs expressing tubulin-RFP in the presence of cytochalasin D. The blue arrow points to the apical centrosome (in Ctrl) or centriole (in Plk4KD). The white arrow points to the basal centriole in the Ctrl NB. Time, minutes. Scale, 4 μm.

**Supplementary Figure 6 - Plk4 extensively phosphorylates Spd2, and Spd2 phospho-mutations do not effect Spd2 homodimerization or its association with Cnn**

Related with Figure 4-5

(A) Spd2 was bacterially-expressed and purified as four fragments that collectively span the entire protein: MBP-Spd2-NT1 (amino acids 1-84), GST-Spd2-NT2 (amino acids 85-340), GST-Spd2-M (amino acids 341-662), and GST-Spd2-C (amino acids 663-1146). Spd2 fragments were incubated with Plk4 kinase domain (amino acids 1-317) and MgATP, then resolved by SDS-PAGE, and the excised Spd2 bands processed for analysis by tandem mass spectrometry (MS/MS) to identify phosphorylated serine and threonine residues. From 76% coverage of full-length Spd2, MS identified 28 in vitro Ser/Thr phosphorylated residues. The positions of the phospho-Ser/Thr residues are indicated in the Spd2 linear map. (B-D) In vitro kinase assays of purified His\textsubscript{6}-tagged Plk4 kinase domain (amino acids 1-317) mixed with various Maltose-Binding Protein (MBP) and Glutathione -S-transferase (GST) Spd2 fusion proteins. The Coomassie-stained SDS-PAGE protein gels and their corresponding autoradiographs are shown. As expected, active Plk4 autophosphorylates (green arrowheads) but does not phosphorylate purified MBP (B) or GST (C, D). Plk4 does phosphorylate MBP- and GST-tagged-Spd2 fragments NT1 (B), NT2 (C), M and C (D) (red arrowheads). Some
proteolytic fragments of GST-Spd2-NT2 are visible and were phosphorylated. (E) RFP-Spd2 and endogenous Spd2 protein levels were analysed by immunoblotting lysate of larval brain extracts probed with Spd2 antibodies. (F) GFP-Spd2 and endogenous Spd2 protein levels were analyzed by immunoblotting lysate of S2 cells in a 7 day RNAi and replacement experiment. Transgenic GFP-Spd2 expression was induced with 0.5 mM CuSO4. Immunobots were probed with anti-GFP and anti-Spd2 antibodies. *** represents a non-specific band recognized by the anti-GFP antibody. (G) Anti-GFP immunoprecipitates (IPs) were prepared from lysate of S2 cells transiently expressing the indicated combinations of GFP- and V5-Spd2 phosphomutants. Endogenous Spd2 was depleted using RNAi. Immunobots were probed with anti-GFP and anti-V5 antibodies. (H) Anti-GFP immunoprecipitates (IPs) were prepared from lysate of S2 cells transiently expressing the indicated combinations of GFP-Spd2 phosphomutants and V5-Cnn. Endogenous Spd2 was depleted using RNAi. Immunobots were probed with anti-GFP and anti-V5 antibodies.

SUPPLEMENTARY and MOVIE LEGENDS

Movie S1: Centriole behavior in Ctrl NBs
Related with Figure 1
Ctrl NB expressing Sas6 (in green) and α-tubulin (in red). Time is shown in minutes: seconds. Movie related to Fig. 1B. Centriole and centrosome behavior can also be followed with the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and on the tracking on the right panel, which overlaps with the movie shown on the left. Before the first mitosis, the apical centrosome (green full circle in the middle panel and empty blue circle on the right panel) and the basal centrosome (pink full circle in the middle panel and empty pink circle on the right panel) can be seen. After cytokinesis, only the apical centrosome is labeled as a full green circle and a blue empty circle on the middle and right panels respectively. At (T00.31min), the two centrioles disengage (pink full and empty circles in the middle and right panels label the basal centriole while a full green circle and a blue empty circle on the middle and right panels label the apical centriole).

Movie S2: Centriole behavior in Plk4KD and Plk4WT NBs
Related with Figure 1. This movie gathers three different movies from three
different NBs from the following genotypes: The first two are Plk4\textsuperscript{KD} expressing NB (in green) and the third is Plk4\textsuperscript{WT} expressing NB (in green). All NBs express α-tubulin (in red). Time is shown in minutes: seconds.

Movie S2A: from frame 2-192: Plk4\textsuperscript{KD} expressing NB- Centriole depicting apical behavior. Movie related to Fig. 1C. Centriole behavior can also be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and with the tracking on the right panel, which overlaps with the movie shown on the left. The apical centriole (blue full circle in the middle panel and empty blue circle on the right panel) can be seen oscillating in small trajectories always very close to the initial apical position throughout interphase. As mitosis starts (T114.00min), increased MT nucleation can be noticed and the amplitude of movements also increases. The spindle is initially nucleated from the centriole-containing pole, but a bipolar mitotic spindle is formed subsequently.

Movie S2B: from 193 to 326: Plk4\textsuperscript{KD} expressing NB- Centriole depicting apical mobile behavior. Movie related to Fig. 1D. Centriole behavior can also be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and with the tracking on the right panel, which overlaps with the movie shown on the left. The NB membrane is show as black empty circles in the middle and right panels. During interphase (from T6.00-57.00min), the apical centriole (blue full circle in the middle panel and empty blue circle on the right panel) is maintained closely associated with the apical cortex, even if displaying trajectories of larger amplitudes than the ones shown in Movie 2. Indeed the centriole moves laterally from one side of the cell to the other and it was thus named as apical mobile. Remarkably, even if the membrane of this NB is deformed, as the neighboring cell positioned above the apical cortex undergoes mitosis, the centriole is maintained associated with the apical hemisphere.

Movie S2C: from frame 327 to 474: Plk4\textsuperscript{WT} expressing NB. Movie related to Fig. 1E. Centriole behavior can also be followed with the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and with the tracking on the right panel, which overlaps with the movie shown on the left. In Plk4\textsuperscript{WT}NBs, several centrosomes can be noticed. To facilitate comprehension, only one centrosome is shown in the tracking (full red circle in the middle panel and empty red circle on the right panel), which corresponds to the centrosome associated with the mitotic spindle pole.
closer to the apical cortex at the end of mitosis (T00:21 min). This centrosome displays an erratic movement throughout interphase, while other centrosomes are also present and moving throughout the cytoplasm. On the following mitosis, two other centrosomes start to nucleate at an apical position, while the tracked centrosome does not nucleate MTs even if back to the apical hemisphere. Eventually, a bipolar spindle is assembled, without the participation of this centrosome, showing the loss of apical identity from the tracked centrosome.

**Movie S3: Centriole behavior in Ctrl and Plk4KD NBs after colcemid treatment**

Related to Figures 2D-E. This movie gathers two different movies from two different NBs from the following genotypes after incubation with the MT depolymerizing drug colcemid. Ctrl NBs expressing Sas-6 GFP (in green) and Plk4KD expressing NB (in green). All NBs express α-tubulin (in red). Time is shown in minutes: seconds.

Movie S3A: from frame 2-153- Ctrl NB after colcemid incubation-Movie related to Fig. 2D. Centriole behavior can be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and on the tracking on the right panel, which overlaps with the movie shown on the left. To facilitate comprehension, only the apical centriole is shown in the tracking (full red circle in the middle panel and empty red circle on the right panel). After disengagement (T-0.5min), the apical centriole moves initially towards the apical hemisphere and then to the basal hemisphere, where it remains till the following mitosis (T71.30min), where tubulin can be seen filling in the nuclear space. We noticed that in the presence of colcemid, the fluorescence intensity of Sas-6 signal decreases substantially when compared to controls.

Movie S3B: from frame 154-234- Plk4KD expressing NBs after colcemid treatment. Movie related to Fig. 2E. Centriole behavior can be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and on the tracking on the right panel, which overlaps with the movie shown on the left. The centriole, which is shown in the tracking (full blue circle in the middle panel and empty blue circle on the right panel), remains initially associated with the apical hemisphere (until 16.00min), moves slightly towards the basal side, but remains more centrally located, never reaching the basal hemisphere as in the Ctrl with colcemid. We noticed that in the presence of colcemid, the GFP-Plk4KD signal decreases substantially
when compared to controls. We confirmed that the centriole is still present and followed its behavior by increasing fluorescence intensity levels in order to generate the tracks.

**Movie S4: Centriole behavior in Spd2\(^{WT}\), Spd2\(^{DE}\) and Spd2\(^{AA}\) NBs**

Related with Figure 4. This movie gathers three different movies from three different NBs from the following genotypes: Spd2\(^{WT}\), Spd2\(^{DE}\) and Spd2\(^{AA}\) (in green). All NBs express α-tubulin (in red). Time is shown in minutes: seconds.

Movie S4A: from frame 2 to 247 Spd2\(^{WT}\) NB. Movie related to Fig. 4C. Centrosome and centriole behavior can be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and on the tracking on the right panel, which overlaps with the movie shown on the left. The apical centrosome, which is shown in the tracking (full blue circle in the middle panel and empty blue circle on the right panel), disengages at 42.00 min. The apical centriole remains associated with the apical cortex, while the basal centriole is not detected throughout interphase, since it does not contain Spd2. At time 114 min, the basal centrosome is detected at the basal hemisphere (full orange circle in the middle panel and empty blue circle on the right panel). In the following interphase, apical centriole disengagement occurs at 168 min. The apical centriole is labeled as a full blue circle in the middle panel and an empty blue circle on the right panel, while the basal centrosome is labeled as a full green circle in the middle panel and an empty green circle on the right panel.

Movie S4B: from frame 248 to 406 Spd2\(^{DE}\) NB. Movie related to Fig. 4D. Centrosome and centriole behavior can be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and on the tracking on the right panel, which overlaps with the movie shown on the left. The apical centrosome, which is shown in the tracking (full red circle in the middle panel and empty red circle on the right panel), can be noticed during the initial phases of interphase and it disengages at (T19.00 min) (the basal centriole is labeled as a full green circle in the middle panel and empty green circle on the right panel). Spd2\(^{DE}\) signal is rapidly lost from the basal (T21.00 min) and the apical centrioles (T23.00 min). Throughout interphase, Spd2\(^{DE}\) is not detected associated with the centrosomes, which most likely are mobile since centrosome association is noticed at the center and at the
basal side of the cell at (T11.00min) and (T12.00min) (full yellow circle in the middle panel and empty yellow circle on the right panel and full pink circle in the middle panel and empty pink circle on the right panel). The last centrosome to be detected and positioned at the basal side (pink in the tracking panels) moves towards the apical hemisphere as the mitotic spindle assembles. During interphase, certain regions with decreased fluorescence can be occasionally noticed like at (T56.00min), near the basal side of the hemisphere.

Movie S4C: from frame 407 to 533 Spd2AA NB expressing Spd2AA (in red) and α-tubulin (in green). Spd2AA signal is maintained at the centrioles throughout part of interphase. Time is shown in minutes: seconds. Movie related to Fig. 4C. Centrosome and centriole behavior can be followed on the tracking in the middle panel, which overlaps with a diagram where the apical position is on the top and on the tracking on the right panel, which overlaps with the movie shown on the left. The apical centrosome, which is shown in the tracking (full red circle in the middle panel and empty red circle on the right panel), can be noticed during the initial phases of interphase. Centriole disengagement is detected at 11.00min (one centriole is labeled in red while the other is labeled in orange) and both centrioles remain closer to the apical cortex until 44.00min. Only one centrosome (the one labeled in red) can be noticed until the following mitosis, where the second centrosome is again noticed on the basal side of the cell (time 94min).
Supplemental Table 1- In vitro phosphorylated residues of Spd2 (related with Figures 4-5 and Figure S6).

Spd2 was bacterially-expressed and purified as four fragments that, collectively, spanned the entire protein: MBP-Spd2-NT1 (amino acids 1-84), GST-Spd2-NT2 (amino acids 85-340), GST-Spd2-M (amino acids 341-662), and GST-Spd2-C (amino acids 663-1146). The Spd2 proteins were incubated with purified Plk4 kinase domain (amino acids 1-317) and MgATP, then resolved by SDS-PAGE, and the excised Spd2 bands processed for analysis by tandem mass spectrometry. Peptide sequences were identified with Sequest software. Confidence of the identification of the tryptic peptides was based primarily on Sequest Xcorr scores; a positive identification required an Xcorr score >1.5, >2.5 and >3.4 for singly, doubly and triply charged peptides, respectively. (This scheme was relaxed for the N-terminus [amino acids 1-340] because most candidate residues in this region did not display high probabilities [>95%] for both phosphate localization and peptide sequence.) Confidence of the peptide identification was further increased if the deltaCn score was >0.1. Probable phosphorylated residues are indicated with a box and were identified with Ascores and Phosphate Localization Probabilities (ScaffoldPTM, Proteome Software), using a phosphate localization probability of 95% as a threshold. (This scheme was relaxed for N-terminus residues.) No phosphorylated residues were observed in control samples of Spd2 (i.e., GST-Spd2 domains incubated with only MgATP prior to analysis). Coverage of Spd2 obtained from control GST-Spd2 samples: total = 77%, N-terminus (NT1+NT2) = 86%, M region = 83%, C-terminus = 68%. Coverage of Spd2 obtained from Plk4-treated GST-Spd2 samples: total = 78%, N-terminus = 83%, M region = 88%, C-terminus = 68%. Because of the proximity between S146 and S148 in the peptide sequence EKPSLSVAEIL from MBP-Spd2-NT1 with a Phosphate Localization Probability of 68%, we decided to mutagenize the two S encoding residues.
| Residue | Peptide Sequence | Phosphate Localization Probability | Ascore | Peptide Score | Scaffold: Peptide Probability | Sequest: Xcorr | Sequest: DeltaCN | Sequest: Peptide RankSp | Sequest: PeptideSp | Charge |
|---------|-----------------|-----------------------------------|--------|---------------|-------------------------------|----------------|-----------------|------------------------|-----------------|-------|
| S34     | GDL^FSK         | 73%                               | 7.38   | 51.74         | 69%                           | 1.795          | 0.445           | 1                      | 585.6           | 2     |
| S35     | VFGDL^ESF       | 81%                               | 6.20   | 26.57         | 46%                           | 1.994          | 0.322           | 1                      | 325             | 1     |
| S92     | LSTN^EVLYTDDTIDL | 100%                             | 22.93  | 69.11         | 80%                           | 2.128          | 0.307           | 1                      | 466             | 2     |
| S121    | GTNL^FEPAEITGR  | 100%                             | 45.93  | 120.86        | 100%                          | 3.88           | 0.492           | 1                      | 731             | 2     |
| S148    | EKPSL^VAEIL     | 68%                               | 3.34   | 29.68         | 75%                           | 1.693          | 0.231           | 1                      | 522.9           | 2     |
| S191    | GSSSLSD^FNCNR   | 100%                             | 41.42  | 120.89        | 100%                          | 3.371          | 0.589           | 1                      | 988.8           | 2     |
| S239    | AAEGQDESFAPAELMK | 100%                          | 132.14 | 106.82        | 100%                          | 3.05           | 0.425           | 1                      | 915.8           | 2     |
| S273    | IAAP^TEITSVNSVL | 90%                               | 10.28  | 77.08         | 93%                           | 2.462          | 0.553           | 1                      | 378.2           | 2     |
| S291    | IAAP^TEITSVNSVLAGDPDF^S | 100%                          | 16.91  | 55.40         | 100%                          | 2.705          | 0.43            | 1                      | 783.8           | 2     |
| S354    | TDAITG^NLGR     | 100%                             | 30.01  | 112.89        | 100%                          | 2.85           | 0.478           | 1                      | 344             | 2     |
| T371    | MQQDRIE^ALK     | 100%                             | 100.00 | 71.53         | 97%                           | 2.768          | 0.399           | 1                      | 117.1           | 2     |
| S375    | MQQDRIE^ALK     | 100%                             | 53.61  | 56.24         | 93%                           | 2.234          | 0.422           | 1                      | 313.8           | 2     |
| T384    | NGLAAK^KRPSSSEI^LSAIDK | 100%                          | 24.19  | 45.65         | 100%                          | 3.669          | 0.557           | 1                      | 228.7           | 3     |
| S391    | RPPSSSEI^LSAIDK | 98%                               | 19.02  | 132.11        | 100%                          | 3.178          | 0.471           | 1                      | 674             | 2     |
| S395    | RPPSSSEI^LSAIDK | 100%                             | 23.64  | 129.18        | 100%                          | 4.204          | 0.632           | 1                      | 276             | 2     |
| S397    | RPPSSSEI^LSAIDK | 100%                             | 51.96  | 97.15         | 100%                          | 3.233          | 0.427           | 2                      | 57              | 3     |
| S443    | GNYYDGENKENQSSN^HAE | 96%                               | 12.63  | 70.12         | 100%                          | 4.267          | 0.482           | 1                      | 645.6           | 3     |
| T458    | LTDF^MSFTD^VLNSTDFR | 99%                           | 22.30  | 162.62        | 100%                          | 4.267          | 0.588           | 1                      | 329             | 2     |
| ID | Sequence | Coverage | Mismatch | Deletion | Match | Quality | Matched Length |
|----|----------|----------|----------|----------|-------|----------|----------------|
| S460 | LTDTMFDTSVLSNDTSFR | 100% | 28.23 | 100% | 4.19 | 0.636 | 1 | 673.9 | 2 |
| S464 | LTDTMSFTDSVLSNDTSFR | 100% | 44.44 | 115.08 | 100% | 4.872 | 0.637 | 1 | 48 | 2 |
| T493 | KPLSPLADHPQIISR | 100% | 38.03 | 149.81 | 100% | 4.014 | 0.539 | 1 | 479 | 3 |
| S569 | RVATMGLIPR | 100% | 47.36 | 101.72 | 100% | 2.526 | 0.582 | 1 | 487.7 | 2 |
| S673 | GLGTSVAVPR | 96% | 16.83 | 86.93 | 100% | 3.042 | 0.476 | 1 | 442 | 2 |
| T706 | VTHTLCWGSTK | 100% | 35.92 | 108.10 | 97% | 2.627 | 0.499 | 1 | 527.5 | 2 |
| T754 | LQAMECR | 95% | 10.21 | 15.97 | 98% | 3.626 | 0.323 | 1 | 612.8 | 3 |
| S949 | EKLTSPMLDSWGEFPDEQPV | 99% | 17.35 | 115.47 | 99% | 3.947 | 0.287 | 1 | 945.1 | 3 |
| S1000 | DFDESSESMLPLEADTVLFR | 99% | 19.02 | 91.50 | 100% | 3.65 | 0.504 | 1 | 597.7 | 2 |