APC/C-dependent degradation of Spd2 regulates centrosome asymmetry in Drosophila neural stem cells

Francesco Meghini1,†, Torcato Martins1,2,†, Qian Zhang3,†, Nicolas Loyer4,‡, Michelle Trickey5, Yusanjiang Abula3,‡, Hiroyuki Yamano5,‡, Jens Januschke4,† & Yuu Kimata1,3,‡,*

Abstract

A functional centrosome is vital for the development and physiology of animals. Among numerous regulatory mechanisms of the centrosome, ubiquitin-mediated proteolysis is known to be critical for the precise regulation of centrosome duplication. However, its significance beyond centrosome copy number control remains unclear. Using an in vitro screen for centrosomal substrates of the APC/C ubiquitin ligase in Drosophila, we identify several conserved pericentriolar material (PCM) components, including the inner PCM protein Spd2. We show that Spd2 levels are controlled by the interphase-specific form of APC/C, APC/C\textsuperscript{Cdc20}, in cultured cells and developing brains. Increased Spd2 levels compromise neural stem cell–specific asymmetric PCM recruitment and microtubule nucleation at interphase centrosomes, resulting in partial randomisation of the division axis and segregation patterns of the daughter centrosome in the following mitosis. We further provide evidence that APC/C\textsuperscript{Cdc20}-dependent Spd2 degradation restricts the amount and mobility of Spd2 at the daughter centrosome, thereby facilitating the accumulation of Polo-dependent Spd2 phosphorylation for PCM recruitment. Our study underpins the critical role of cell cycle–dependent proteolytic regulation of the PCM in stem cells.

Keywords asymmetric cell division; cell cycle; centrosome; stem cell; ubiquitination

Subject Categories Cell Cycle; Post-translational Modifications & Proteolysis; Stem Cells & Regenerative Medicine

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Introduction

The centrosome is a non-membrane-bound organelle that acts as a major microtubule-organising centre in animal cells, orchestrating cytoskeletal network in interphase and promoting bipolar spindle assembly in mitosis (Bornens, 2021; Hoffmann, 2021). Each centrosome contains a pair of rod-shaped structures, centrioles, at its core and the surrounding proteinous matrix, pericentriolar material (PCM; Woodruff et al, 2014; Conduit et al, 2015; Fu et al, 2015; Bornens, 2021). PCM contains microtubule polymerisation factors such as β-Tubulin (β-Tub) and is responsible for the microtubule nucleation activity of the centrosome. Prior to mitosis, PCM rapidly increases its size and microtubule organising capacity (a process known as centrosome maturation), preparing for spindle assembly. Several evolutionarily conserved proteins are known to be required for this process, which include Polo kinase (Polo-like kinase 1, Plk1 in vertebrates) and PCM components Spd2 (Cep192 in vertebrates) and Centrosomin (Cnn, CKD5RAP2 in vertebrates; Sunkel & Glover, 1988; Golsteyn et al, 1995; Lane & Nigg, 1996; Megraw et al, 1999; Pelletier et al, 2004; Dix & Raff, 2007; Gomez-Ferreria et al, 2007; Giansanti et al, 2008; Zhu et al, 2008b; Lizarraga et al, 2010; Chinen et al, 2021). In the current model for the Drosophila mitotic centrosome assembly, Spd2 and Cnn cooperate to form a mesh-like structure around centrioles that serves as a scaffold for other mitotic PCM components; Polo-dependent phosphorylation of Spd2 and Cnn stimulates the assembly of this PCM scaffold (Conduit et al, 2014a, 2015; Feng et al, 2017; Alvarez-Rodrigo et al, 2019).

Although some details may differ, elements of this model are likely evolutionarily conserved (Woodruff et al, 2017; Lee et al, 2021).

In multicellular organisms including humans, the centrosome plays a critical role in the regulation of development and tissue homeostasis, often through its cell type–specific functions (Tang & Marshall, 2012; Araújo, 2019; Bornens, 2021; Qi & Zhou, 2021). As a prime example, in the Drosophila larval neural stem cell or...
neuroblast (NB), which undergoes stereotypical asymmetric cell division (Gallaud et al., 2017), the centrosome regulates spindle orientation and division axis maintenance through a unconventional behaviour specific to this cell type (Rebollo et al., 2007; Rusan & Peifer, 2007). Unlike most somatic cells, in which centrosomes lose microtubule nucleation activity during interphase, the NB retains microtubule nucleation capacity at one of the two centrosomes (Rebollo et al., 2007; Rusan & Peifer, 2007; Januschke & Gonzalez, 2010). The active centrosome is attached to the apical cortex via an astral microtubule network, which allows the NB to form mitotic spindle along the apico basal axis upon mitotic entry, by fixing one pole. Through this mechanism, the NB can maintain the division axis over consecutive divisions, delivering its differentiating daughters (ganglion mother cells, GMCs) to close basal positions (Rebollo et al., 2007; Rusan & Peifer, 2007; Januschke & Gonzalez, 2010). Several evolutionally conserved centrosome components and regulators, including Polo, Plk4, Centrinob, CEP135, DPLP (Pericentrin in mammals) and Wdr62, have been identified to regulate this NB-specific asymmetric centrosome behaviour (Januschke et al., 2013; Lerit & Rusan, 2013; Singh et al., 2014; Conduit et al., 2014a; Ramdas Nair et al., 2016; Gamarotto et al., 2019). However, how the activities of these regulators are spatiotemporally coordinated to regulate the differential centrosome behaviour remains largely elusive.

The behaviour and activity of the centrosome are tightly coordinated with the cell cycle. Various posttranslational modifications on centrosome components are implicated in this coordination of the centrosome cycle with the cell cycle (Nigg & Stearns, 2011; Blanco-Ameijeiras et al., 2022). Of these, ubiquitination, followed by proteasomal degradation of ubiquitinated proteins, is considered key to the regulation of centriole duplication (Darling et al., 2017; Badaru deen et al., 2021). However, the importance of ubiquitination-mediated proteolysis beyond centrosome number control remains to be established.

The APC/C (anaphase-promoting complex/cyclosome) is a ubiquitin ligase complex that plays a central role in cell cycle control (Darling et al., 2001). The APC/C is activated upon mitotic exit to trigger chromosome segregation and mitotic exit, while APC/C-Ctcm is activated upon mitotic exit and remains active during interphase (Pines, 2011; Chang & Barford, 2014). It has been shown that APC/C-Ctcm controls centrosome duplication by ubiquitinating various centriolar proteins, such as SAS6 and STIL (Strnad et al., 2007; Arquint & Nigg, 2014). APC/C-Ctcm also targets major regulators of PCM and centrosome maturation, Plk1 and Aurora A (AurA) for degradation (Lindon et al., 2017; Badar udeen et al., 2021). The active centrosome is attached to the apical cortex via an astral microtubule network, which allows the NB to form mitotic spindle along the apico basal axis upon mitotic entry, by fixing one pole. Through this mechanism, the NB can maintain the division axis over consecutive divisions, delivering its differentiating daughters (ganglion mother cells, GMCs) to close basal positions (Rebollo et al., 2007; Rusan & Peifer, 2007; Januschke & Gonzalez, 2010). Several evolutionally conserved centrosome components and regulators, including Polo, Plk4, Centrinob, CEP135, DPLP (Pericentrin in mammals) and Wdr62, have been identified to regulate this NB-specific asymmetric centrosome behaviour (Januschke et al., 2013; Lerit & Rusan, 2013; Singh et al., 2014; Conduit et al., 2014a; Ramdas Nair et al., 2016; Gamarotto et al., 2019). However, how the activities of these regulators are spatiotemporally coordinated to regulate the differential centrosome behaviour remains largely elusive.

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The APC/C (anaphase-promoting complex/cyclosome) is a ubiquitin ligase complex that plays a central role in cell cycle control (Pines, 2011; Chang & Barford, 2014). The APC/C associates with one of two co-activators, Fizzy (Fzy) and Fizzy-related (Fzr) (also called CDC20 and CDH1 or Fzr1, respectively). APC/C-dependent proteolysis can be reconstituted in vitro (Yamano et al., 2009). Drosophila orthologues of mitotic cyclins, well-established canonical APC/C substrates (King et al., 1995; Sudak in et al., 1995), were degraded upon APC/C activation in this assay (Fig EV1A). We screened 55 Drosophila centrosomal proteins, including 51 proteins that were previously identified in RNAi and proteomic screens as critical regulators of the centrosome (Andersen et al., 2003; Dobbe laere et al., 2008; Müller et al., 2010; Dobbel laere, 2015), as well as Sg1, γTub37C, Pen and Cnot1, which we previously identified as putative in vivo APC/C-interacting proteins (Martins et al., 2009; Haider et al., 2015; Meghini et al., 2016; Fig EV1B). Ana1, Ana3, Plk4, Red1 and Red2 showed APC/C-dependent degradation in mitotic extracts while Asterless (Asl, CEP152 in vertebrates), A urA, CP110, Feo, Klp61F, Nek2, Polo, Red4 and Spd2 showed degradation in interphase extracts (Fig EV1C and D). The degradation of all the five mitotic substrates could be only partially inhibited by addition of an APC/C inhibitor S. pombe MeS1 (Kimata et al., 2008), indicating a certain degree of intrinsic instability of these proteins in this assay (Fig EV1C). Meanwhile, among the 9 interphase substrates, all but Polo showed a clear dependency on APC/C-Fzr activity (Fig EV1D). Nek2 and A urA showed rapid and nearly complete degradation, whereas the rest, including Spd2, showed slow and/or incomplete degradation (Fig EV1D).

We validated some of these candidates in cultured D.mel-2 cells. Inhibition of mitotic APC/C activity induces extensive apoptosis. We therefore focused our analysis on the candidates of APC/C-Fzr substrates. Similar to the control CyclA and CycB, the cellular levels of endogenous Spd2, Polo, A urA and Asl proteins, as well as GFP- CP110 and Feo-GFP, expressed by transient transfection, were increased upon addition of the proteasome inhibitor MG-132, while the levels of γTubulin (γTub23C) was unaffected (Fig EV2A). In alignment with this result, RNAi against Fzr or APC/C core subunit Apc4 caused accumulation of Spd2, Polo, A urA, Asl and Nek2-GFP (Fig EV2B–E). We also induced hyper-activation of APC/C-Ctcm by depleting endogenous Rca1 (Emi1 in vertebrates), an APC/C-Fzr inhibitor (Grosskortenhaus & Sprenger, 2002), by RNAi and found that the levels of Spd2, Polo, A urA, Asl and Nek2-GFP were decreased upon Rca1 depletion (Fig EV2F and G). Together, these results suggest that Spd2, A urA, Polo, Asl and Nek2 are centrosomal targets of APC/C-Ctcm-dependent proteolysis in Drosophila.
Spd2 protein levels are controlled by APC/C<sup>Fzr</sup>-dependent degradation in vivo

We previously found that Spd2 acts as a centrosomal loading factor for Fzr, by directly binding it and recruiting it to the interphase centrosome. Spd2 binds the WD40 repeat domain of Fzr via D-box and KEN-box sequences (Davey & Morgan, 2016) and is ubiquitinated and degraded by APC/C<sup>Fzr</sup> in vitro, in a manner similar to canonical APC/C substrates (Meghini et al., 2016). However, a potential function of APC/C-dependent Spd2 ubiquitination/degradation in vivo has not been determined yet.

To investigate the role of APC/C-dependent degradation of Spd2, we examined the effect of Spd2 stabilisation/degradation on the centrosome using the larval NB as a model. To this end, we first created a series of *Drosophila* lines in which increasing levels of Spd2 proteins are expressed in NBs (Fig 1A, also see Materials and Methods). We generated Spd2 overexpression lines (Spd2WT-OE and Spd2DK-OE), in which GFP-tagged wild-type Spd2 (Spd2WT) or a stable mutant form of Spd2 (Spd2DK), which is not recognised by APC/C<sup>Fzr</sup> due to mutations in all its degrons, four D-boxes and one KEN-box (Meghini et al., 2016), is overexpressed in a wild-type background by an NB-specific Gal4 driver, *worntu-Gal4* (wor-Gal4; Zhu et al., 2008a), using the Gal4/UAS system (Brand & Perrimon, 1993; Fig 1A). We also complemented the *spd2* null mutant (*spd<sup>2-25,5711</sup>*;*Df(3L)BSC561*) with RFP-tagged Spd2WT or Spd2DK mildly expressed under a constitutive polyubiquitin gene promoter (pUbq; Lee et al., 1988; hence, called “rescue”, Fig 1A). In addition, we generated an Fzr knockdown line (*fzrRNAi*) in which endogenous Fzr is depleted in NBs by inducing *fzrRNAi* by wor-Gal4 (Fig 1A).

Immunoblot analyses using the larval brain extracts from Spd2WT-OE and Spd2DK-OE lines showed that, despite being expressed under the same transcriptional control, GFP-Spd2DK proteins showed higher accumulation than GFP-Spd2WT proteins (2.07-fold difference in the mean normalised band intensities, Fig 1B). Similarly, higher accumulation of RFP-Spd2DK proteins, compared with RFP-Spd2WT proteins, were observed in brain extracts from Spd2WT-RES and Spd2DK-RES lines (2.08-fold difference, Fig 1C), confirming that the D-Boxes and KEN-box regulate Spd2 protein levels posttranslationally in *vivo*. In this analysis, we could not observe any significant change in endogenous Spd2 levels in *fzrRNAi* (Fig 1C); this is perhaps expected as *worntu* is expressed only in a small fraction of cells (i.e. NBs) in the larval brain.

We also analysed Spd2 protein levels in larval NBs in different conditions quantifying fluorescence intensities using an Spd2 antibody. We measured the local signal intensities of Spd2 immunofluorescence at the centrosomes and in the cytoplasm (Fig 1D, see Materials and Methods for more details). Spd2WT-RES NBs did not show any significant increase in Spd2 levels when compared with control both at the centrosomes and in the cytoplasm (Fig 1D). *fzrRNAi* NBs showed a subtle but statistically significant increase in Spd2 levels at the centrosome, compared with control (1.29 ± 0.50-fold increase in the normalised mean signal intensities, *P* = 0.0078, Student *t*-test, Fig 1D). Spd2WT-OE NBs showed substantial increases both in the centrosomal and cytoplasmic Spd2 levels, compared with control (1.93 ± 1.08-fold and 3.62 ± 1.46-fold, respectively, Fig. 1D). These data indicate that Spd2 levels in wild-type and Spd2WT-RES larval NBs are comparable, while Spd2 levels are slightly increased in *fzrRNAi* NBs and progressively increased in Spd2WT-OE and Spd2DK-OE NBs, supporting the interpretation that Spd2 protein levels are regulated by APC/C<sup>Fzr</sup>-dependent degradation in vivo, at least in larval NBs.

Spd2 accumulation compromises the stem cell–specific control of interphase centrosome activity

To monitor the dynamic behaviour and microtubule nucleation activity of the centrosome, we adapted an *ex vivo* whole-mount brain culture technique to observe the asymmetric cell divisions of larval NBs (Pampalona et al., 2015; Januschke & Loyer, 2020). We confirmed that expression of RFP-tagged Spd2WT or Spd2DK in a *spd2* null background (i.e., Spd2WT/DK-RES) can restore PCM accumulation and microtubule nucleation at mitotic centrosomes as previously shown (Meghini et al., 2016). Indeed, in Spd2WT-RES and Spd2DK-RES larval brains, most NBs formed mitotic spindles with two clearly focused poles and underwent proper chromosome segregation and asymmetric cell division, similar to NBs in control brains (Movies EV1–EV3). This was also true for most NBs analysed in *fzrRNAi*, Spd2WT-OE and Spd2DK-OE brains (Movies EV4–EV6). A small fraction of NBs of these genotypes transiently exhibited abnormal spindle morphologies, such as multipolar, monopolar spindle or “bent” spindles (Fig EV3A and B; Movie EV6). High levels of Spd2 proteins did not seem to be accountable for these defects, as we did not observe such abnormalities in Spd2WT-OE NBs overexpressing GFP-Spd2WT (0.0%, *n* = 17, Fig EV3B; Movie EV5). The large majority of NBs from all lines, however, formed bipolar spindle with asters (Fig EV3A and B; Movies EV1–EV6). These data suggest that the mitotic function of the centrosome, i.e. centrosome maturation and microtubule nucleation at spindle poles, is robust to increased Spd2 levels, which is in line with our results that Spd2 did not appear to be a target of APC/C in *vivo* in mitotic extracts (Fig EV1; Meghini et al., 2016).

We next examined the effect of Spd2 accumulation on the interphase centrosome. Larval NB centrosomes exhibit an unconventional behaviour during interphase (Rebollo et al., 2007; Rusan & Peifer, 2007). The daughter centrosome retains PCM and microtubule nucleation activity after mitotic exit and is tethered to the apical cell cortex through astral microtubules throughout interphase (Fig 2A; Rebollo et al., 2007; Rusan & Peifer, 2007; Conduit & Raff, 2010; Januschke et al., 2011). As we found that Spd2 was a target of the interphase-specific APC/C<sup>Fzr</sup>, but not of the mitotic APC/C<sup>Fzr</sup>, we speculated that APC/C<sup>Fzr</sup>-dependent degradation of Spd2 may play a role in regulating interphase centrosomes in NBs.

Consistent with previous reports, in all the NBs examined in control (pUbq-Sas6-GFP or pUbq-GFP-Fzr, expressing Sas6-GFP or GFP-Fzr as a centrosome marker) and Spd2WT-RES brains (n = 29 or 45, respectively), one centrosome kept nucleating microtubules during interphase and was stably anchored at the proximity of the apical cell cortex (Fig 2C; Movies EV1, EV2, and EV7). However, in significant fractions of NBs in the lines with increased Spd2 levels (*fzrRNAi*, Spd2DK-RES and Spd2WT/DK-OE), both centrosomes lost microtubule nucleation capacity during interphase, and the apical centrosome became detached from the cortex to start wandering around the cytoplasm (Fig 2A–C; Movies EV3–EV5). Intriguingly, the frequencies of this phenotype (“apical centrosome detachment”)
Increased with increasing Spd2 levels observed in these conditions (Fig 1 and 2C): approximately 21.3% (n = 80) of NBs in fzrRNAi or Spd2DK-RES larval brains showed apical centrosome detachment in interphase which rose to 65.1% (n = 43) in Spd2WT-OE brains and even 90.0% in Spd2DK-OE (n = 40, Fig 2C). This observation shows that the microtubule nucleation ability and the anchoring of the daughter centriole to the apical cortex are sensitive to an increase in Spd2 levels and may suggest that the APC/C-dependent...
degradation of Spd2 is critical for regulating asymmetric centrosomes behaviour in larval NBs.

It was previously shown that various PCM proteins are differentially recruited to the two centrosomes in the NB, regulating their behaviour (Lerit & Rusan, 2013). We therefore examined the effect of altered Spd2 levels on the recruitment of major PCM proteins to centrosomes in interphase NBs. To this end, we measured the fluorescent signal intensities of the PCM proteins at the two centrosomes in individual NBs and determined the degree of their asymmetric distribution, as described previously (Lerit & Rusan, 2013; Fig 2D and E, see Materials and Methods for details). In interphase NBs in control and Spd2WT-RES brains, the PCM proteins Spd2, Cnn, AurA and γ-Tubulin (γ-Tub) showed an asymmetric distribution, being enriched on one centrosome, while the pan-centriolar component Asl (Fu & Glover, 2012; Mennella et al., 2012) was symmetrically distributed (Fig 2D and E). In contrast, in Spd2DK-RES NBs, Spd2 and γ-Tub showed significantly reduced asymmetric localisation patterns at the two centrosomes and become more symmetrically distributed, while Cnn appears to be unaffected (Fig 2D and E).

Combined together, these data suggest that increased Spd2 levels may alter the retention of PCM components on interphase centrosomes. This could potentially explain the loss of microtubule nucleation activity of the centrosome and its detachment from the apical cortex in interphase larval NBs.

**Altered Spd2 levels partially randomise the NB division axis**

Larval NBs keep their axis of division stably over multiple rounds of divisions, delivering their differentiating daughters (i.e. GMCs) always at their basal pole (Fig 3A). It has been shown that the active interphase centrosome acts as a positional cue to orient the axis of NB division (Januschke & Gonzalez, 2010). We therefore...
tested whether reduced PCM asymmetry and apical centrosome detachment in interphase, caused by altered Spd2 levels, affect the division axis in three dimensions and analysed the deviation between consecutive divisions (Loyer & Januschke, 2018; see Materials and Methods, Fig 3B and C). Consistent with a previous report (Januschke & Gonzalez, 2010), NBs in the control brains maintained their division axis stably over consecutive divisions (28.24 ± 20.43°, n = 36, Fig 3B and C; Movies EV1 and EV7). In some of the larval brains with higher Spd2 levels, NBs showed significantly larger axis deviations: fzrRNAi and Spd2DK-OE brains, and the brains in which GFP-Spd2WT and fzrRNAi were co-expressed by wor-Gal4 (Spd2WT-OE + fzrRNAi) showed mean deviations of 39.62 ± 20.43°, 60.14 ± 38.79° and 46.00 ± 31.05° (n = 66, 16, and 74, respectively. Fig 3B and C; Movie EV8).

To determine whether these division orientation deviations are attributable to apical centrosome detachment, we separately analysed the division axis deviations in consecutive mitoses in NBs in which the apical centrosome stayed attached to the cortex in interphase and compared them with those in NBs in which it did not (Fig 3D). We observed strong correlations between the occurrence of apical centrosome detachment in interphase and larger division axis deviations in the subsequent mitosis (Fig 3D). These data show that the detachment of the apical centrosome during interphase caused by increased Spd2 levels significantly alters the division axis maintenance of larval NBs.

All together, these results strongly suggest that in larval NBs, the correct regulation of Spd2 protein levels by APC/C is critical for maintaining PCM and microtubule nucleation activity at the apical centrosome, required for NB division axis maintenance.

Spd2 accumulation compromises the segregation pattern of the daughter centrosome

Each centrosome consists of a pair of daughter and mother centrioles: the daughter centriole is generated using the mother centriole as the template. Due to the semi-conservative manner of the centriole duplication, two centrosomes present in each cell are different molecular ages: one centrosome contains the mother centriole that is older than the mother centriole of the other. Hence, they are commonly referred to as mother and daughter centrosomes (Nigg & Stearns, 2011; Blanco-Ameijeiras et al., 2022).

Various stem cells preferentially inherit either the mother or daughter centrosome. The function of this preferential centriole inheritance is unclear but may act as a form of cellular memory (Chen & Yamashita, 2021; Gonzalez, 2021). The Drosophila larval NB preferentially inherits the daughter centrosome (Conduit & Raff, 2010; Januschke et al., 2011). The asymmetric activity of the interphase centrosomes is thought to play a critical role in regulating this centrosome segregation pattern: it is the daughter centrosome that retains PCM and is anchored to the apical cortex in interphase, thereby being segregated into the daughter NB (Rebollo et al., 2007; Rusan & Peifer, 2007; Conduit & Raff, 2010; Januschke et al., 2011; Fig 4A).

To examine whether altered Spd2 levels affects the pattern of centrosome inheritance, we used live NB imaging and followed the segregation of centrosomes. Control NBs correctly segregated centrosomes (96.7 ± 20.43°, n = 30, Fig 4A–C; Movies EV1 and EV7). Upon altered Spd2 levels, NBs largely segregated centrosomes correctly when the daughter centrosome kept nucleating microtubules in interphase (Fig 4C). An exception were NBs in Spd2WT-OE brains, in which the daughter centrosome was frequently mis-segregated into the GMC for unclear reasons (50.0%, n = 10, P = 0.0003 in comparison with control, Pearson’s chi-squared test. Fig 4C).

We further addressed whether centrosome detachment upon altered Spd2 levels affects centrosome segregation. Due to rapid free movement of the detached centrosomes, it was difficult to track them throughout interphase in an unbiased fashion. Interestingly, we noticed that, in control NBs, the daughter centrosome always matures slightly earlier than the mother centrosome upon mitotic entry, starting expanding PCM and increasing microtubule nucleation (100%, n = 36, Figs 4B and EV4A and C; Movies EV1 and EV7). We also observed asynchronous maturation of two centrosomes in...
NBs with detached centrosomes (Figs 4A and B, and EV4A and C; Movies EV4, EV5, and EV8), although the incidence of synchronous centrosome maturation was increased in the presence of higher Spd2 levels (Fig EV4A and B; Movie EV9). We therefore assumed that the centrosome that matures earlier upon mitotic entry is the daughter centrosome.

Using this assumption, we monitored the fate of the daughter centrosome in the larval NBs with detached interphase centrosomes. Surprisingly, in the large number of the NBs in \textit{fzrRNAi} and \textit{Spd2DK-RES} brains, despite its detachment from the cortex, the daughter centrosome was correctly segregated to the NB (87.5%, \(n = 8\), and 22.2%, \(n = 9\), respectively, Fig 4D; Movies EV4 and EV5), indicating that, despite the lack of the cortical interaction, the daughter centrosome can maintain its preferential segregation pattern into the NB. However, in NBs with higher Spd2 levels, the daughter centrosome was frequently mis-segregated into the GMC (Fig 4A, B, and D; Movies EV8 and EV10). In \textit{Spd2WT-OE} and \textit{Spd2DK-OE} NBs, for instance, the segregation of the daughter centrosome was randomised: (50.0%, \(n = 12\), and 65.2%, \(n = 23\), respectively, Fig 4D). These data indicate that altered Spd2 levels...
Figure 3. Altered Spd2 levels partially randomise the NB division axis.

A

A diagram summarising the division axis maintenance in wild-type larval NBs and NBs with altered levels of Spd2. Wild-type NBs maintain division axes over consecutive divisions while NBs with increased Spd2 levels partially randomise division axes.

B

Selected images from time-lapse microscopy of NBs in the whole-mount larval brains in the control (pUbg-GFP-Fzr) and Spd2WT-OE lines (see also Movies EV7 and EV8). Centrosomes were visualised by Sas6-GFP or GFP-Spd2 signals and microtubules by mCh–α-Tub signals. Multi-channel images showing GFP signals in green and mCh signals in red are shown in the upper panels. Single-channel images showing mCh–α-Tub signals in grey are shown in the lower panels, in which dotted white lines outline NBs and asterisks indicate forming GMCs. The blue and pink arrows indicate the NB division axes upon the first and second mitosis, respectively, and the deviations between the two axes (θ) were determined. Control NBs mostly maintain the division axes over consecutive divisions (top panels), whereas Spd2DK-OE NBs often deviate at significantly different angles, over 90° (lower panels). Scale bars: 10 μm.

C

In larval NBs in the indicated fly brains, division angles were determined in the 3D context, and deviations of the angles in consecutive divisions were quantified (see Loyer & Januschke, 2018 and Materials and Methods for details). The number of NB divisions (n) analysed from at least four brains and the average deviations (mean ± SD) were Control: 28.24° ± 20.43 (n = 36), Spd2WT-RES: 35.57° ± 17.10 (n = 31), fzrRNAi: 39.62° ± 27.24 (n = 66), Spd2DK-RES: 28.13° ± 47.7 (n = 81), Spd2DK-OE: 60.14° ± 38.79 (n = 16), Spd2WT-OE combined with fzrRNAi (Spd2WT-OE + fzrRNAi): 46.00° ± 31.03 (n = 74). Spd2WT-OE brains were not analysed due to the low sample number (n = 4). P-values were calculated using Mann–Whitney U tests comparing with control and are shown above the plots. fzrRNAi, Spd2DK-OE and Spd2WT-OE + fzrRNAi showed statistically significant differences in division angle deviations.

D

Division axis deviations were separately analysed in NBs with apically attached interphase centrosomes (“attached”) and those with centrosomes being detached from the cortex (“detached”) for each line, using the same data set as in Fig 3C. The same control data as in Fig 3C are shown for reference. fzrRNAi-detached (mean ± SD: 34.62° ± 23.39), Spd2DK-OE detached (64.01° ± 33.93), and Spd2WT-OE + fzrRNAi attached (39.55° ± 27.08) and detached (59.08° ± 34.88) brains showed statistically significant differences from control, while NBs with cortically attached centrosomes in any of the lines did not show significant differences. n: the number of NB divisions analysed from at least four brains for each line. P-values were calculated using Mann–Whitney U tests comparing with control and are shown above the dots.

Spd2 accumulation does not affect the centrosomal localisation of Fzr

We asked how the increase of Spd2 levels impedes PCM maintenance and microtubule nucleation at the daughter centrosome. We previously showed that Spd2 recruits Fzr to interphase centrosomes, which promotes APC/C-dependent degradation of AurA at the centrosomes (Meghini et al., 2016). Since AurA is a PCM regulator (Berdnik & Knoblich, 2002), increased Spd2 levels may affect PCM recruitment at interphase centrosomes through recruiting more Fzr to the centrosomes. We therefore examined whether increasing Spd2 levels can affect the centrosomal localisation of Fzr in larval NBs. As we detected Fzr by using pUbg-GFP-Fzr transgene, we generated fly lines in which HA-tagged wild-type Spd2 can be induced under the UAS promoter (Materials and Methods). HA-Spd2WT overexpression by wor-Gal4 (HA-Spd2WT-OE) caused centrosome detachment (26.7%, n = 30, P = 0.003, Pearson’s chi-squared test, compared with control, Fig EV5A and B) and reduced an asymmetry distribution of Spd2 (Fig EV5C and D), as expected. Nevertheless, GFP-Fzr levels at interphase centrosomes and its asymmetric distribution were unaffected (Fig EV5C–E). Similarly, the dynamics of GFP–Fzr at the centrosomes during mitosis was not affected by HA-Spd2 overexpression (Fig EV5F and G; Movies EV11 and EV12). Therefore, Spd2 accumulation likely affects PCM recruitment at the daughter centrosome independently of Fzr recruitment.

Spd2 accumulation increases the mobility of Spd2 and may prevent Polo-dependent phosphorylation at the interphase centrosome

Polo/Pik1 is the conserved kinase whose activity is universally required for centrosome maturation and spindle formation in eukaryotic cells (Llamazares et al., 1991; Conduit et al., 2014a; Ramani et al., 2018). In interphase NBs, Polo is preferentially accumulated at the daughter centrosome, which is required and sufficient for PCM retention and microtubule nucleation activity at the centrosome (Rebollo et al., 2007; Rusan & Peifer, 2007; Januschke et al., 2013; Ramdas Nair et al., 2016; Gallaud et al., 2020). In cnb or wdr62 mutant NBs, Polo cannot localise at the daughter centrosome, and both centrosomes become inactive during interphase (Ramdas Nair et al., 2016; Gallaud et al., 2020), a phenotype similar to what we observed in the NBs with high levels of Spd2 (Fig 3). We therefore examined the centrosomal localisation of Polo in interphase NBs with excess Spd2 by expressing GFP-Polo under the native polo promoter (Moutinho-Santos et al., 1999). Surprisingly, the centrosomal levels of Polo-GFP and its asymmetric centrosomal distributions were unaffected in both Spd2DK-RES and HA-Spd2WT-OE NBs (Fig 5A–D), in which the daughter centrosome was still inactivated and detached (Figs 2C and EV5B). These data suggest that Spd2 accumulation blocks PCM recruitment and microtubule nucleation at the daughter centrosome downstream of Polo.

It was recently shown that Polo directly phosphorylates Spd2 at multiple sites, which become additional binding sites for Polo and target it for degradation (Devi et al., 2021). It is proposed that Polo and Spd2 may form a positive feedback loop that enables rapid PCM expansion through an autocatalytic mechanism upon centrosome maturation (Alvarez-Rodrigo et al., 2019; Cabral et al., 2019). We speculated that a similar feedback mechanism may operate at the daughter centrosome in interphase NBs. To test this, we used Spd2CONS, the unphosphorylatable mutant form of Spd2 in which all the conserved putative Polo phosphorylation sites are mutated (Alvarez-Rodrigo et al., 2019). We generated fly lines in which mCherry-tagged Spd2WT or Spd2CONS is expressed in an spd2 mutant (spd2Df(3L)BSC561 and mCh-Spd2CONS-RES; Alvarez-Rodrigo et al., 2019). We analysed interphase NBs in mCh-Spd2CONS-RES third instar larval brains and found that NBs showed a significant reduction in asymmetric distribution of Spd2 and γ-Tub (Fig 5E and F), similar to the NBs with altered Spd2 levels (Fig 2D and E). This data suggests that Polo-dependent phosphorylation of Spd2 is critical for PCM retention at beyond a certain threshold affects centrosome segregation in NBs independently of its detachment from the cortex.
the daughter centrosome, enabling microtubule nucleation activity and cortical association in interphase NBs.

We therefore hypothesised that excess Spd2 levels interfere with Polo-dependent phosphorylation at the daughter centrosome, thereby blocking PCM retention. We analysed the dynamics of the centrosomal association of Spd2 with the daughter centrosome by performing fluorescence recovery after photobleaching (FRAP) experiments in interphase NBs in Spd2WT-RES and Spd2DK-OE brains. We found that in Spd2DK-OE NBs, the Spd2 fluorescence recovered much faster and more completely than in Spd2WT-RES brains (Fig 5G and H; Movies EV12 and EV13). This suggests that in Spd2DK-OE NBs, a larger mobile Spd2 pool exists at the daughter centrosome, which may prevent accumulation of Polo-phosphorylated Spd2 on the daughter centrosome.

Discussion

In this study, we set out to identify centrosomal APC/C substrates in Drosophila and conducted an in vitro destruction screen to identify 14 candidate substrates. Although whether all the candidates are bona fide APC/C substrates remains to be tested, we demonstrated that the protein levels of Spd2, AurA, Polo, Nek2 and Asl are indeed regulated in a proteasome- and APC/C\(^{\text{CPR}}\) dependent manner in \(D\).mel-2 cells (Fig EV2). The vertebrate orthologues of Ana1, Ana4, CP110, Rcd1 and Feo contain multiple APC/C recognition motifs, some of which are evolutionarily conserved, pointing to the conserved role of the APC/C-dependent proteolysis in centrosome regulation. Although we failed to obtain any evidence that human CEP192 is targeted by APC/C\(^{\text{CPR}}\) (Meghini et al., 2016), it is
noteworthy that CEP192 has been shown to be targeted by another cell cycle–related E3 ligase, the SCF (Moser et al., 2013; Fung et al., 2018; Meitinger et al., 2020). Thus, although the APC/C may not take part, the cell cycle–dependent regulation of Spd2/CEP192 protein stability through the ubiquitin-proteasome pathway may be conserved through evolution.

The critical role of APC/C-dependent proteolysis in coupling the centriole duplication process to the cell cycle is widely acknowledged (Strnad et al., 2007; Arquint et al., 2012; Badarudeen et al., 2021). Yet, its importance beyond the duplication control is unclear. Key PCM regulators, such as Plk1 and AurA, have been known to be APC/C substrates. However, the stabilization/accumulation of these regulators appears to have little impact on the general functions of the mitotic centrosome, i.e. PCM accumulation and bipolar spindle formation (Lindon & Pines, 2004; Floyd et al., 2008). In alignment with this, we also showed that overexpression of Spd2, even the non-degradable mutant form Spd2DK, does not strongly affect centrosome maturation and bipolar spindle formation (Figs EV2 and EV4). However, we found in the Drosophila larval NB that Spd2 protein levels strongly impact the cell type–specific interphase function of the centrosomes, which serves as a positional cue for orienting the spindle in the following mitosis. Upon Spd2 stabilisation or accumulation, both centrosomes lose PCM and become inactive during interphase, and the daughter centrosome is detached from the cortex (Fig 2). This will result in partial randomisation of the division axis and the centriole segregation pattern (Figs 3 and 4). These results strongly suggest that APC/C-dependent degradation of Spd2 is critical for the NB-specific mode of regulation of the centrosomes, more specifically, the daughter centrosome.
It is surprising that in the NBs with excess Spd2, the division axis is still largely maintained, despite the detachment of the daughter centrosome during interphase (Figs 2 and 3). However, this result is line with previous reports that the complete loss of the centrosome (by sos4 or asl mutations) only partially affects the division orientation (Januschke & Gonzalez, 2010; Loyer & Januschke, 2018). We recently showed that the interface between the NBs and its last-born GMC provides an additional positional cue for the division axis with previous reports that the complete loss of the centrosome (Loyer & Januschke, 2018). Interestingly, we also observed that in many Spd2DK-RES and fzrRNAi NBs, despite the loss of its apical anchorage prior to mitosis, the daughter centrosome was still able to be segregated into the NB (Fig 4), which appears to be hampered by excess Spd2, suggesting an additional daughter centrosome-intrinsic mechanism that allows its preferential segregation into the NB, which involves Spd2.

How does APC/C<sup>C<sub>Fzr</sub></sup>-dependent degradation of Spd2 regulate the asymmetric behaviour of the centrosomes in the larval NB? It has been shown that a daughter centriole-specific protein, Cnb, and a microtubule-associated protein, Wdr62, recruit Polo to the daughter centrosome where Polo-dependent Cnb phosphorylation is required for the retention of PCM and microtubule nucleation activity (Januschke et al., 2013; Ramdas Nair et al., 2016; Gallaud et al., 2020). In contrast, DPLP and CEP135 (also known as BLD10) are enriched at the mother centrosome to seed Polo and PCM (Lerit & Rusan, 2013; Singh et al., 2014). We observed that, in the presence of high levels of Spd2, the daughter centrosomes lose PCM and become inactive in interphase, similar to cnb and wdr62 mutants (Fig 5; Januschke et al., 2013; Ramdas Nair et al., 2016; Gallaud et al., 2020). However, unlike in these mutants, the asymmetric localisation of Polo and Cnn appears not to be affected by the presence of excess Spd2 (Figs 2D and E, and 5A–D), indicating that Spd2 levels control the PCM retention by the daughter centrosome downstream or independently of Polo. Recently, it was shown that Spd2 is directly phosphorylated by Polo at multiple sites, which create additional binding Polo sites (Alvarez-Rodrigo et al., 2019). We hypothesise that, in the interphase NB, Polo-dependent Spd2 phosphorylation may allow retention of a small fraction of active PCM at the daughter centrosome. In support of this, in the NBs of mCh-<sup>2CONS-RES</sup> brains, in which Spd2 cannot be phosphorylated by Polo, the asymmetric localisation of γ-Tubulin, but not Cnn, at the interphase centrosomes is significantly reduced (Fig 5E and F). We therefore propose a model in which APC/C<sup>C<sub>Fzr</sub></sup>-dependent Spd2 proteolysis functions to facilitate the accumulation of Polo-dependent Spd2 phosphorylation at the daughter centrosome to allow its retention of microtubule nucleating capacity (Fig 5G). Importantly, it was shown that a fraction of Spd2 is dynamically localised at the centrosome (Conduit et al., 2014b), which would rapidly dissociate and exchange with unphosphorylated Spd2 in the cytoplasm. Our FRAP analysis showed that, in the presence of excess nondegradable Spd2 proteins, the mobile fraction of the centrosomal Spd2 pool was substantially increased (Fig 5G and H). Thus, Spd2 degradation may promote accumulation of Polo-phosphorylated Spd2 by limiting not only the total volume of Spd2 but also the mobility of the centrosomal population of Spd2 (Fig 5I). Through this dual mechanism, APC/C<sup>C<sub>Fzr</sub></sup>-dependent Spd2 proteolysis can increase the sensitivity of the daughter centrosome PCM to the limited amount of Polo activity to ensure the differential activity of the two centrosomes in interphase NBs (Fig 5I). In the mitotic NB, although APC/C<sup>C<sub>Fzr</sub></sup> is turned off, excess Spd2 does not interfere with PCM loading, as more abundant and more active Polo is present at the mitotic centrosome (Macurek et al., 2008; Seki et al., 2008; Joukov et al., 2014).

In our previous study, we reported that Spd2 is the centrosomal loading factor of Fzr (Meghini et al., 2016). In this new study, we demonstrated that Spd2 is also a bona fide substrate of APC/C<sup>C<sub>Fzr</sub></sup>.
These results suggest an Spd2-Fzr negative feedback loop which can operate to maintain the centrosomal Spd2 levels within a specific range. This mechanism can prevent excessive Spd2 accumulation at the centrosome, which may overwhelm Polo activity, while preserving a fraction of Spd2 at the inner PCM core, which is required for maintaining the structural integrity of centrioles (Loncarek et al., 2008; Zhu et al., 2008b; Seo et al., 2015). Interestingly, some consensus Polo sites (S-T/P) are in the vicinity of the APC/C degrons of Spd2 (Alvarez-Rodrigo et al., 2019). Phosphorylation of these sites may prevent the recognition of Spd2 by Fzr, thereby turning off the Spd2-Fzr negative feedback loop. Such mechanism may contribute to allow the rapid PCM expansion and the dissociation of Fzr from the centrosome upon mitotic entry.

Dysfunctions and misregulations of the centrosome are tightly linked to various human disorders, including cancer, microcephaly, and ciliopathy (Nigg & Raff, 2009; Goundiam & Basto, 2021). Our study provides evidence that defects in the ubiquitin-dependent degradation of centrosomal components can affect cell-type-specific functions of centrosomes and abrogate the behaviour of stem cells, pointing to the potential involvement of the ubiquitin-dependent regulation of the centrosome in these pathologies. It was reported that CDH1 knockout mice show a microcephaly-like phenotype and increased susceptibility to sporadic tumours (Garci-Higuera et al., 2008; Delgado-Esteban et al., 2013; Eguren et al., 2013). Although these phenotypes are mainly attributed to increases genome instability due to premature S phase initiation, careful inspection of centrosomes in these mice might provide new insights on how the misregulation of centrosome components underlies some human diseases.

Materials and Methods

DNA constructs

All the sequences of Drosophila genes were obtained from the Drosophila Genomics Resource Center (DGRC) or amplified by PCR using cDNA libraries generated from fly embryos. Entry clones with the coding sequences encoding full length or fragments of these genes were generated using Gateway System (Thermo Fisher Scientific). Expression constructs were made by recombination between entry clones and the following destination vectors: pAGW (for actin5C promoter-driven N-terminal GFP fusion in D.mel-2 cells, DGRC), pMT-N-GFP, FLAG or HA (for inducible metallothionein promoter-driven N-terminal GFP, 3xFLAG or 3xAHA fusion in D.mel-2 cells), pURW (for pUbq-driven N-terminal RFP fusion in flies) and pPHW for ga4-driven expression of N-terminal HA fusion of Spd2 (for the HA-Spd2WT-OE line).

The generation of the spd2DK mutant genes was described previously (Meghini et al., 2016). The over-expression plasmids for generating Spd2WT-OE or Spd2DK-OE fly lines were constructed by classic ligation as follows. Spd2WT or Spd2DK genes were amplified and N-terminally fused to eGFp gene by PCR and inserted into not1 and Xba1 sites of pUAST-attB vector (Bischof et al., 2007). The resulting pUAST-eGFp-Spd2WT/OK::attB plasmids were then injected into embryos of y, w, M(eGFp, vas-int, dmRFP)ZH-2A; P{CaryP}attP40 lines for phiC31 integrase-mediated site-directed recombination (Groth et al., 2004). The candidate recombinant lines were screened by the eye colours in the progeny. The y, w, M(eGFp, vas-int, dmRFP)ZH-2A X chromosomes were replaced by the X chromosome carrying w^{1118} and the Gal4-mediated induction of GFP-Spd2WT/DK was confirmed by immunofluorescence and Western blotting. For generation of the fly line overexpressing HA-Spd2WT, the expression plasmid was generated by recombination between the Spd2WT entry clone and pPHW destination vector and was then injected into w^{1118} embryos for P-element-mediated recombination. The candidate recombinants were screened by the eye colours and the induction of HA-Spd2WT were confirmed by immunofluorescence and Western blotting.

His-Fzr constructs used for in vitro transcription and translation were generated by inserting full-length Fzr gene, N-terminally fused to 10 histidine sequences, into the PHY22 vector linearised with Ncol and BamHI restriction enzymes. The DNA sequences of all the constructs generated were confirmed by Sanger DNA sequencing (Source Bioscience).

Antibodies

The following primary antibodies were used for Western blotting and immunofluorescence: rabbit anti-Spd2 (Rodrigues-Martins et al., 2007), rabbit anti-Fzr (Raff et al., 2002), mouse anti-GFP (Sigma-Aldrich, 1181446001), mouse anti-HA (Covance HA11), mouse anti-FLAG (M2, Sigma, F3165), mouse anti-γ-Tubulin (DM1A, Sigma-Aldrich), mouse anti-PSTAIRE (Sigma P7962), rabbit anti-Cnn (Bettencourt-Dias et al., 2005; a kind gift from Jinyang Fu), mouse anti-γ-Tubulin (GTU-88, Sigma T6557), rabbit anti-Asl (Dzhindzhiev et al., 2010) and guinea pig anti-Asl (1:40,000 for IF, a gift from Nasser Rusan; Lerit & Rusan, 2013). The following secondary antibodies were used (all 1:1,000): Goat α-Mouse, Rabbit, Rat or Guinea Pig Alexa 488, Alexa 568, Alexa 647 or HRP (all from Life Technologies).

Drosophila strains

All flies were raised at 25°C under standard conditions unless stated otherwise. The following stocks (described in FlyBase, unless otherwise stated) were used: Oregon R (as the wild-type), spd2^{255-5711} (Giansanti et al., 2008), Df(3R)BSC561 (as spd2 deficiency), pUbq-GFP-fzr (Raff et al., 2002), w;or-gal4 (Zhu et al., 2008a), UAS-mCherry-y6 tubulin (a gift from Chris Doe), UAS-fzrRNAi (v25550, VDRC), pUbq-AurA-GFP (Sabino et al., 2011), pUbi::polo-GFP (Logarinho & Sunkel, 1998) and pUbq-mCherry-Spd2WT and pUbq-mCherry-Spd2CONS (Alvarez-Rodrigo et al., 2019). The transgenic Spd2WT/OK::DK-RES fly lines were generated as described previously (Meghini et al., 2016). Spd2WT-OE, Spd2WT-OE and HA-Spd2WT-overexpression fly lines were generated as described above.

Drosophila cell culture and RNAi

Drosophila D.mel-2 cells (Thermo Fisher Scientific) were cultured in Express Five SFM medium (Thermo Fisher Scientific) supplemented with 2 mM l-glutamine and Pen Strep (ThermoFisher Scientific). DNA transfection was performed using FuGene HD transfection reagent (Promega) following the manufacturer’s instruction. RNAi experiments were performed as described previously (Bettencourt-Dias & Goshima, 2009). Briefly, dsRNAs were prepared using the T7
RiboMAX Express Large Scale RNA Production System (Promega) with cDNA templates and the following oligonucleotide primers:

T7-kanRRNAi-F: TAAATCAGACTCTATATAGGGAGAGCAGAAATTCTATCGCTTGATG.

T7-kanRRNAi-R: TAAATCAGACTCTATATAGGGAGAGCAGAAATTCTATCGCTTGATG.

T7-apc4RNAi-F: TAAATCAGACTCTATATAGGGAGAGCAGAAATTCAACAGCAAGCTC.

T7-apc4RNAi-R: TAAATCAGACTCTATATAGGGAGAGCAGAAATTCAACAGCAAGCTC.

T7-fzrRNAi-F: TAAATCAGACTCTATATAGGGAGAGCTCTTATGAA.

T7-fzrRNAi-R: TAAATCAGACTCTATATAGGGAGAGCTCTTATGAA.

In vitro APC/C-dependent destruction assay

To identify potential centrosomal destruction APC/C substrates, in vitro APC/C-dependent destruction assays were performed using mitotic or interphase Xenopus laevis egg extracts containing endogenous APC/C, as described previously (Yamano et al., 2009). Candidate centrosomal proteins were expressed and radioactively labelled in vitro coupled transcription and translation with 35 S-methionines and the plasmids or PCR products in reticulocyte lysates (Promega). The 35 S-labelled candidate proteins were added into destruction assays reconstituted in Xenopus egg extracts, and their degradation was monitored at different time points. To test for APC/C<sup>CDC20</sup>, dependent degradation, 0.4 mM calcium was added to Xenopus cytostatic factor-arrested egg extracts (CSF extracts) to release CSF inhibition and trigger rapid activation of APC/C<sup>CDC20</sup>. To confirm APC/C<sup>CDC20</sup> dependency, purified recombinant Mes1, the competitive APC/C inhibitor in fission yeast (Kimata et al., 2008) was added. To test for APC/C<sup>CDC11</sup>-dependent degradation (interphase assay), purified recombinant Xenopus CDH1 or Drosophila Fzr was added into Xenopus interphase egg together with 35 S-labelled candidate proteins, initiating CDH1-dependent destruction. Aliquots were collected into 2× Laemmli buffer at the indicated time points, boiled for 2 min and resolved by SDS-PAGE. The run-on gel was detected by autoradiograph by X-ray films.

Western blots

For the Western blots of Drosophila brain extracts, 10 third instar larval brains were dissected in 60 µl of PBS containing Pro tease and Phosphatase inhibitor (Roche). The samples were lysed using a homogenising pestle (Sigma-Aldrich), and the lysates were clarified by centrifugation. 2× Laemmli buffer was added to the cleared lysates, and the samples were boiled 2 min. For D.mel-2 cell extracts, 1 ml of confluent culture was harvested. After centrifugation, supernatants were removed, and cell pellets were washed once with 1× PBS and 200 µl of 2× Laemmli buffer was added to lyse the cells. The proteins were then resolved by SDS-PAGE. The electrophoretic run was performed using a Mini- PROTEAN Tetra Cell System (BioRad), in a Running Buffer solution (25 mM Tris, 192 mM glycine and 0.1% SDS, pH approx. 8.6, Sigma), at 200 Volts (V). The gel was assembled in a “transfer sandwich” (cushion pad-filter paper-gel-membrane-filter paper-cushion pad) and blotted on a nitrocellulose membrane (GE Healthcare) for 2 h at 60 V in Transfer Buffer solution (25 mM Tris, 190 mM Glycine, 20% Methanol). Protein transfer was verified by Ponceau S staining, and the membrane was incubated for 4 min at room temperature in a blocking solution containing 5% milk (Marvel) and 0.1% Tween (Sigma) in PBS. The membrane was then incubated in a primary antibody solution prepared in blocking solution for 1 h at room temperature. The membrane was washed three times for 10 min at room temperature with a solution of 0.1% Tween 20 in PBS (PBST) and then incubated in a secondary antibody solution prepared in blocking solution for 1 h at room temperature. The membrane was washed three times with PBST for 10 min at room temperature, incubated with a peroxidase ECL substrate (Pierce), and the proteins were detected by exposing an X-ray film (Fuji).

Band intensity measurements

The band intensity was calculated using Gel Analyser in Image J (Schneider et al., 2012). A box was drawn over the band and adjusted to only include the area of interest. Boxes in the same size and shape were used for all the bands of the same protein on a film. The intensity of each band was measured after the background subtraction and normalised to the loading control (CDK1 or α-tubulin). The relative intensity was calculated by dividing it by the control value.

Immunostaining of Drosophila larval brains

For fixation of larval NSCs, developing adult brains were dissected from climbing third instar larvae in PBS and then transferred to the solution of 4% formaldehyde in PBS supplemented with MgCl2 and EGTA for 25 min. The brains were then washed with PBS-Triton 0.3% and pre-incubated with PBSTB (PBS containing 0.3% Triton, 3% BSA). The fixed tissues were then incubated with the primary antibodies in PBSTB overnight at 4°C. After three washes in PBSTB, the samples were incubated in PBSTB with the secondary antibodies (1:1,000) and DAPI (1:1,000) for 2 h at room temperature. After three washes in PBSTB, tissue samples were mounted in Vectashield. Samples were analysed on the Nikon C2 confocal microscope.

Live imaging of Drosophila NBs in whole-mount larval brains

For long-term imaging of whole-mount larval brains, the samples were prepared essentially adapting the clot method previously described (Januschke & Loyer, 2020). Briefly, third instar larvae were dissected into complete Express Five SFM medium (Thermo Fisher Scientific; 2 mM l-glutamine, Pen Stre p, 1 g/l glucose). The brains were included in a 10 µl droplet of complete medium pre-
warmed at 25°C and supplied with 10 mg/ml of fibrinogen and placed on a 35 mm glass bottom dish (MatTek). The brains were oriented with the dorso-anterior parts of the optic lobes facing down. The droplet was spread until the brain was slightly squashed, then 0.5 µl of 0.5 U/ml thrombin was dropped on the brain to stimulate the formation of a fibrin clot. The brains were incubated in the dark for 5–10 min to allow the formation of the clot, then they were washed three times with 200 µl of complete medium. Images were acquired on a Zeiss Axiosvert200 microscope fitted with a PerkinElmer RSIII spinning disc confocal unit (PerkinElmer Life Sciences) and running the Volocity v6.3. During the acquisition, the temperature was maintained at 25°C by a stage incubator. Single optical sections were captured at 90 s intervals with a 40× lens. Data sets were imported into ImageJ and Photoshop for movie export and figure generation, respectively.

**Measurement of centrosomal and cytoplasmic signal intensities and asymmetric indexes**

For the signal intensity measurements, the third instar larval brains were dissected, prepared in parallel and treated the same. The control was analysed first to establish the confocal settings to be used, which remained unaltered for all images acquired. The brains were mounted with the ventral side facing at the cover slips, and confocal images of maximal 15 µm-thick z-stack images of the dorso-ventral regions of the brains were acquired with 0.5 µm intervals using a Nikon C2 confocal microscope. Signal measurement was performed using ImageJ (Schneider et al., 2012). In the acquired confocal images, interphase NBs were selected based on the uncondensed round morphology of DNA and maximum intensity projections of the z-stacks covering the two centrosomes were generated for individual NBs. In the projection images, two equal-sized and -shaped ROIs were selected for each of the two centrosomes (cen1 and cen2) and two in their proximity in the cytoplasm (cyto1 and cyto2) in the same NB. The mean values were measured in each of the regions, and they were defined as $S_{cen1}$, $S_{cen2}$, $S_{cyto1}$ and $S_{cyto2}$. The average of $S_{cyto1}$ and $S_{cyto2}$ was used as cytoplasmic signals ($S_{cyto}$). Relative centrosome enrichment (corr.$S_{cen}$) of each centrosomal protein at each centrosome, the following equation was applied: corr.$S_{cen} = (S_{cen} - S_{cyto})/S_{cyto}$ (Fig EV5D). Asymmetric indexes were used to assess the degrees of asymmetric distributions of centrosomal proteins between the two centrosomes in NBs (Figs 2E and, 5B and D, and EV5E), following Lerit & Rusan (2013) with a slight modification. In the selected interphase NBs, corr. $S_{cen}$ of each centrosomal protein at each of the two centrosomes was determined as described above. The asymmetric index (AI) in each NB was then calculated as $AI = |S_{cen1} - S_{cen2}|/(S_{cen1} + S_{cen2})$. AI indicates that the protein is equally distributed between the two centrosomes while $AI = 1$ indicates that the protein is specifically localised at only one of the two centrosomes.

For quantifications of the centrosomal and cytoplasmic signal intensities of Spd2 (Fig 1D), projection images of interphase NBs were obtained, and $S_{cen1}$, $S_{cen2}$, $S_{cyto1}$ and $S_{cyto2}$ were measured as above. However, instead of $S_{cyto1}$ and $S_{cyto2}$, their averages were used as centrosomal signals ($S_{cen}$). In addition, two additional ROIs were selected outside of the NBs, and the averages of their mean values were defined as the background signal (Sback) and used to determine cytoplasmic signals (i.e. $S_{cyto} - S_{back}$) and for normalisation. Normalised centrosomal and cytoplasmic signal intensities of individual NBs were defined as follows: norm.$S_{cen} = (S_{cen} - S_{cyto})/S_{back}$ and norm.$S_{cyto} = (S_{cyto} - S_{back})/S_{back}$. The relative values to control (as 1.00) were plotted on Fig 1D.

For the measurement of the centrosome enrichment of GFP-Fzr signals using the time-lapse images of NBs during mitosis (Fig EV5E and F), corr.$S_{cen}$ for the apical centrosome (one segregated into a daughter NSC after division) was determined at each time point over the time course as described above. In Fig EV5F, the values were then normalised using the highest corr.$S_{cen}$ values in each time course as the reference.

**Estimation of NB Spd2 levels in the series of fly lines**

Using the normalised centrosomal and cytoplasmic signal intensities (norm.$S_{cen}$ and norm.$S_{cyto}$) measured above (Fig 1D), the approximate ratios of average Spd2 protein levels in individual NBs were estimated as control: Spd2WT-RES: fzrRNAi: Spd2WT-OE = 1: 1.00–1.18: 1.06–1.29: 1.93–3.62. Meanwhile, based on the Western blot analysis (Fig 1B and C), the ratios of the Spd2 protein levels between Spd2WT-OE NBs and Spd2DK-RES NBs and between Spd2WT-OE NBs and Spd2DK-RES NBs areSpd2WT-OE: Spd2DK-RES = 1: 2.04, and Spd2WT-RES: Spd2DK-RES = 1: 2.08, respectively. Collectively, we estimated the ratios of NB Spd2 levels between these fly lines as wild-type (or control): Spd2WT-RES: fzrRNAi: Spd2DK-RES: Spd2WT-OE: Spd2DK-RES = 1: 1.00–1.18: 1.06–1.29: 2.08–2.45 (≈1.00–1.18 × 2.08): 1.93–3.62: 3.94–7.38 (≈1.93–3.62 × 2.04). To note, the Spd2 levels between Spd2DK-RES and Spd2WT-OE are not directly comparable.

**Measurements of NB division angles and division axis deviations**

The angles of the division axes of individual NBs were determined in three dimensions as previously described (Loyer & Januschke, 2018). Briefly, the 3D coordinates of each spindle pole (centrosomes) at telophase were used to define a 3D vector of the mitotic spindle, i.e. the division axis. The angle between two 3D vectors defined for two successive divisions (the division axis deviation) was calculated using these coordinates.

**Fluorescence recovery after photobleaching (FRAP) analysis of Spd2 in larval NB**

For the analysis of the dynamics of centrosome signals of Spd2, third instar larvae brains were dissected in PBS and were mounted on a microscope slide with 50 µl of PBS, then slightly squashed under a coverslip to allow NBs to be physically dissociated from the brain tissue. Photobleaching and image acquisition were performed using the Nikon C2 confocal microscope at 25°C by a stage incubator. The centrosomes were identified as clearly defined spots with the mitotic spindle, i.e. the division axis. The angle between two 3D vectors defined for two successive divisions (the division axis deviation) was calculated using these coordinates.
Image processing

The images were processed using the NIS-Elements software or ImageJ (Schneider et al., 2012). For better visualisation, a 0.5 Gaussian blur filter was applied to every image shown.

Statistical analyses

Statistical analysis was performed with GraphPad Prism or Microsoft Office Excel. Throughout this study, unpaired nonparametric Mann-Whitney U tests were used for data containing individual values to assess statistical significance. For results with categorical variables (e.g., presence or absence of phenotypes), P-values were calculated by performing Pearson’s chi-squared tests and were used to assess significant differences between the data set and the control.

Data availability

This study includes no data deposited in external repositories. Original data and materials will be available from the corresponding author upon request.

Expanded View for this article is available online.

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Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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