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Supplemental Information

Plk4 Regulates Centriole Asymmetry and Spindle Orientation in Neural Stem Cells

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Figure S1- Gambarotto et al

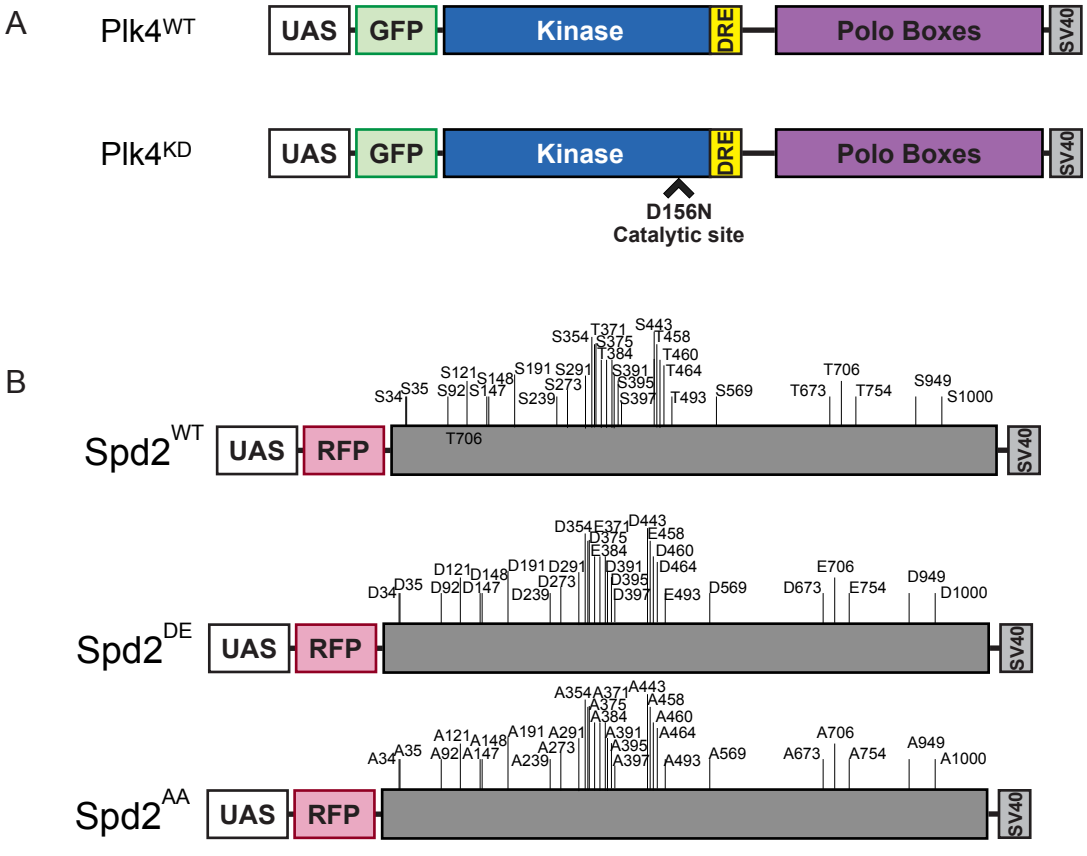
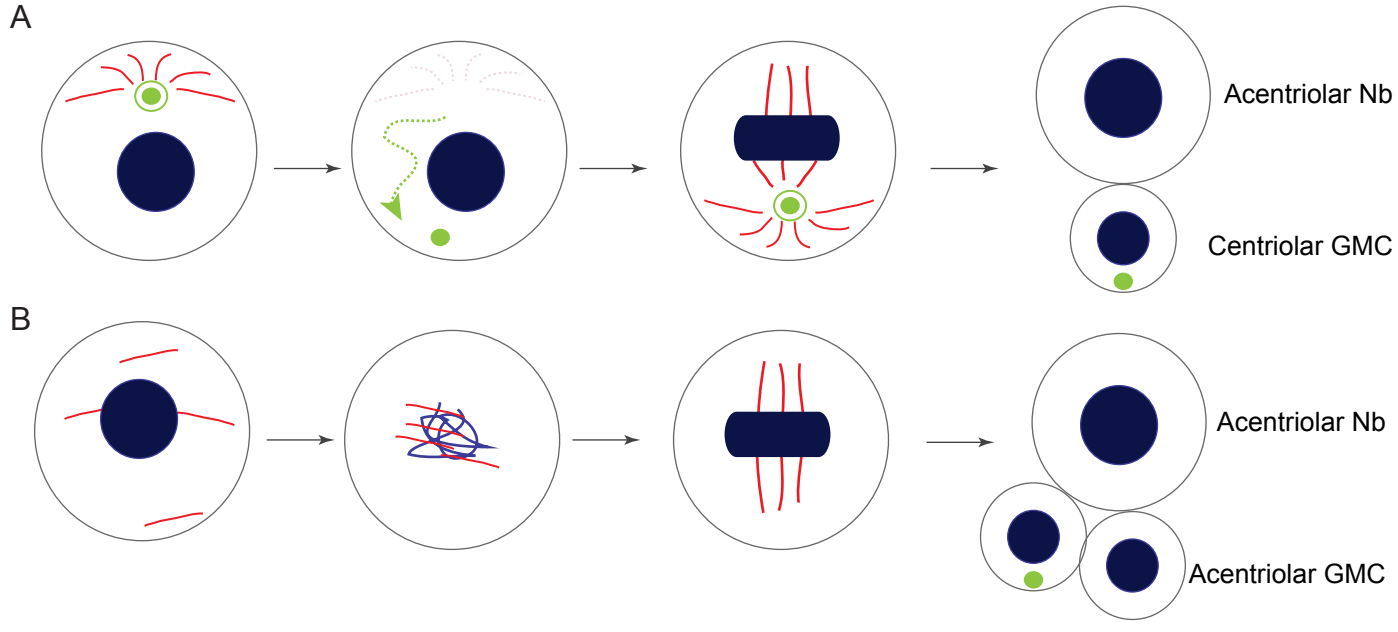
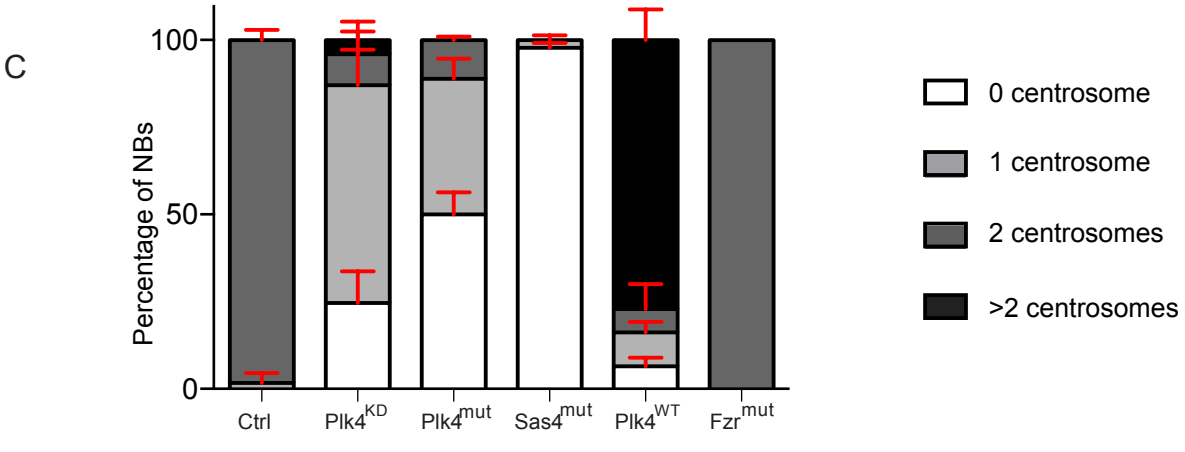


Figure S2- Gambarotto et al





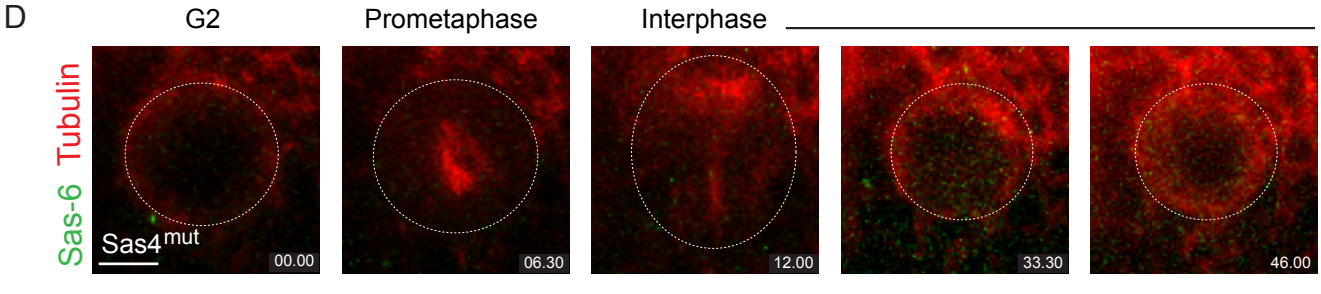
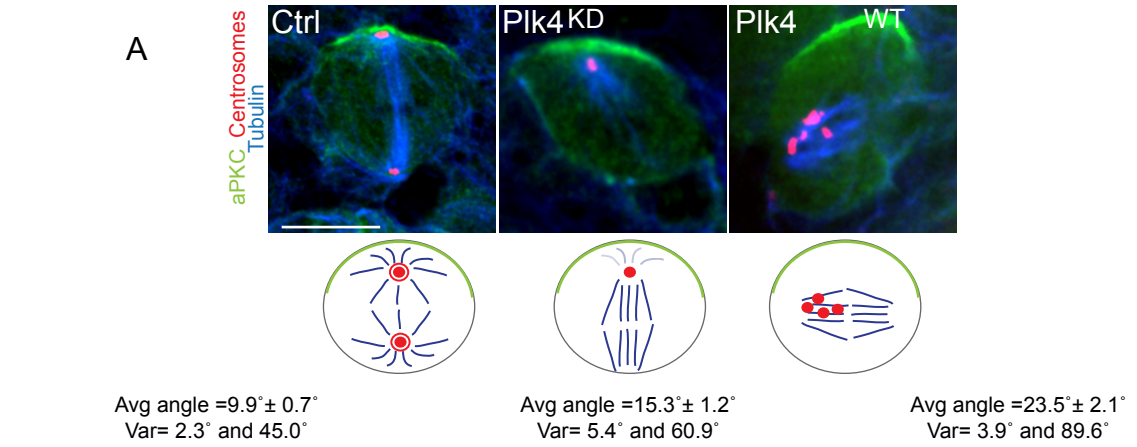
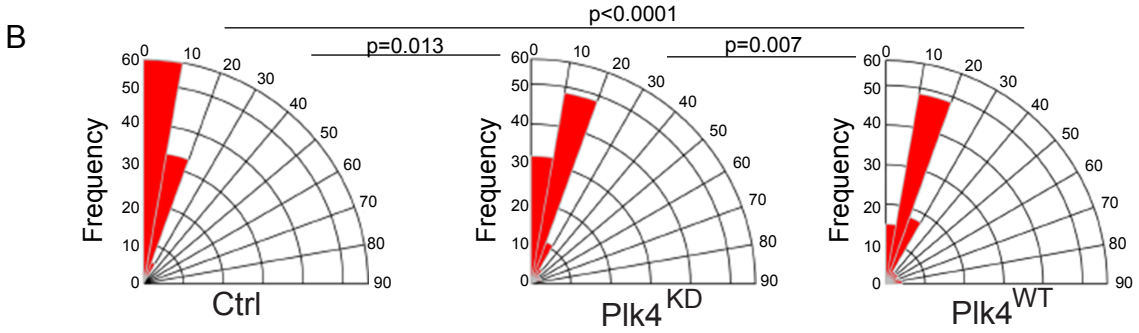
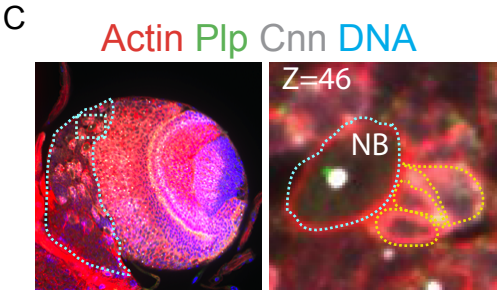
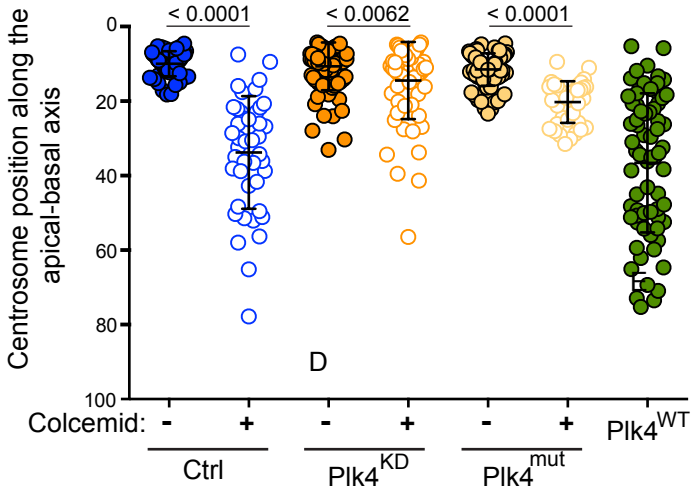


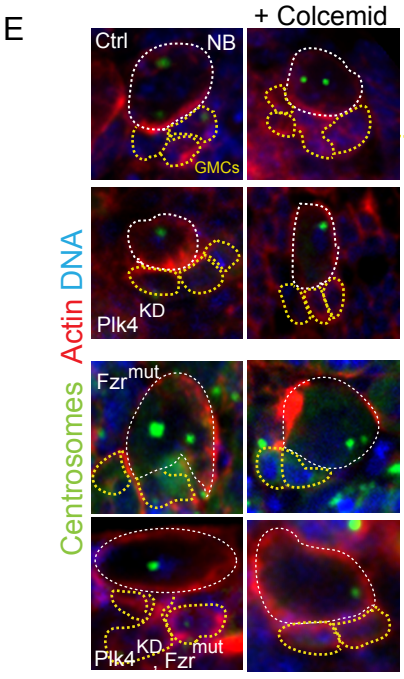
Figure S3- Gambarotto et al

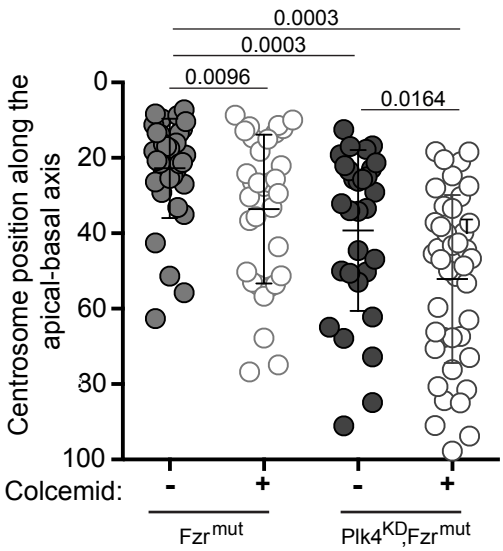












SFigure S4 - Gambarotto et al

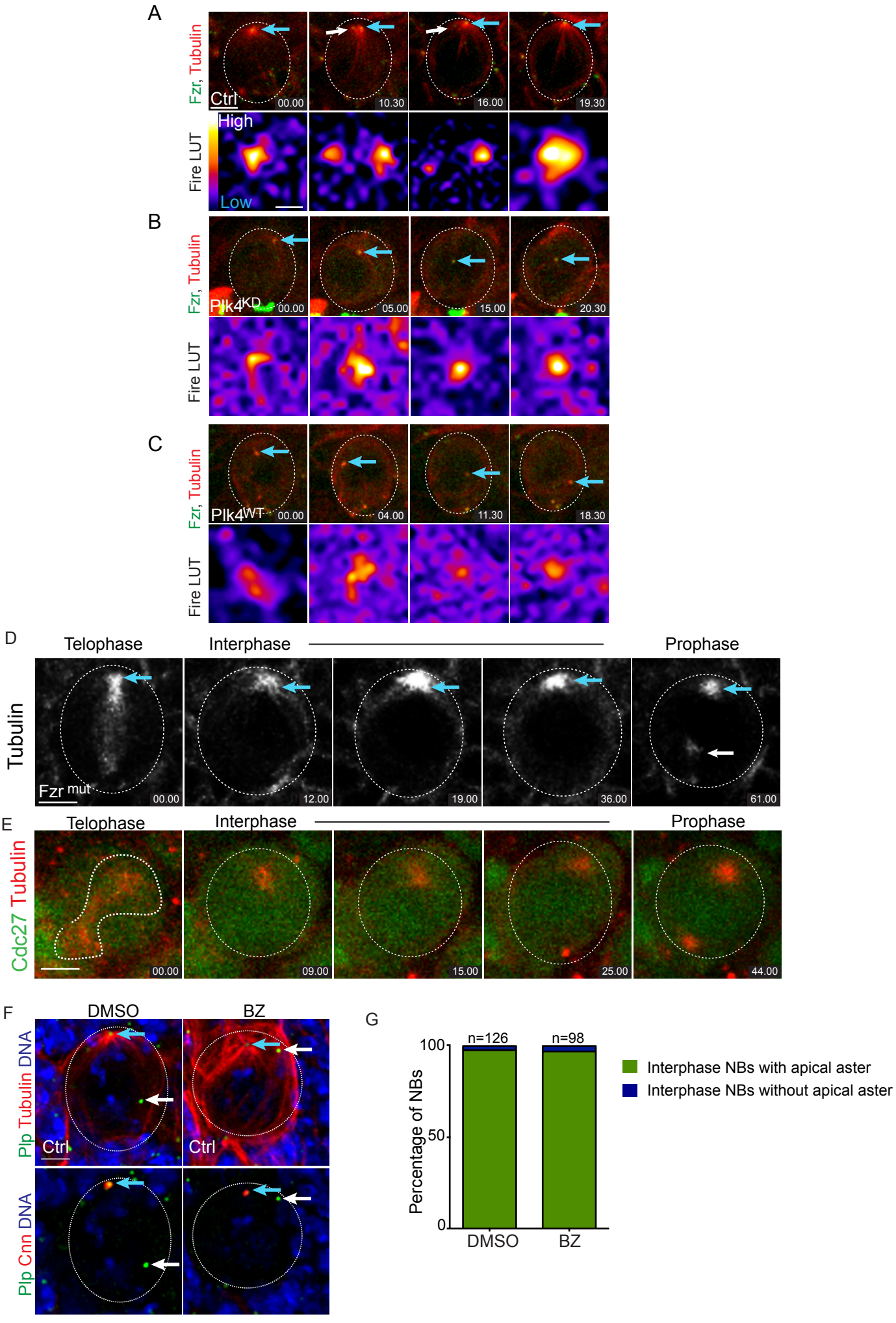


Figure S5 - Gambarotto et al

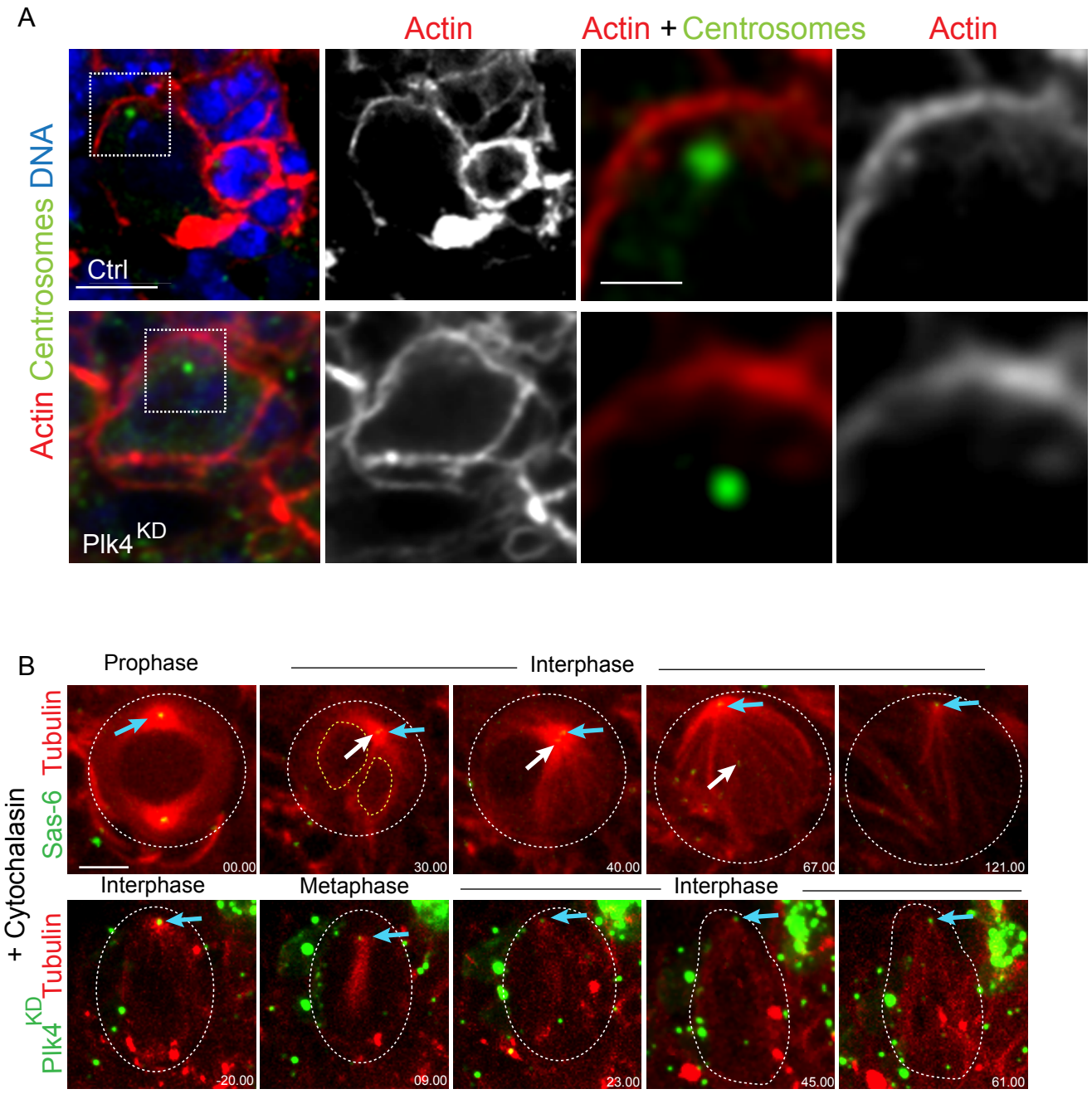
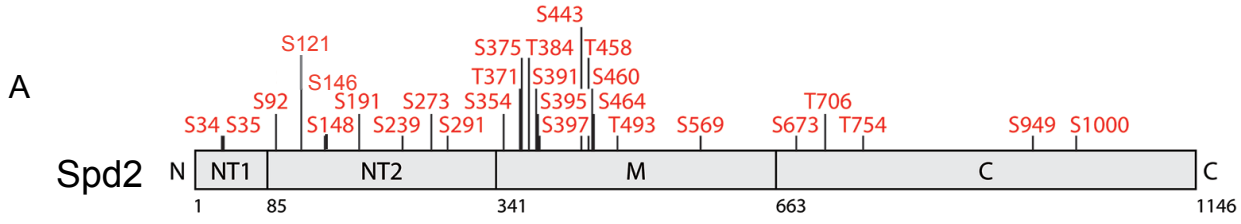
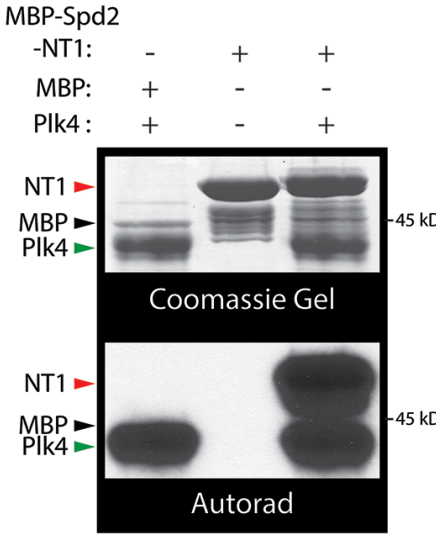
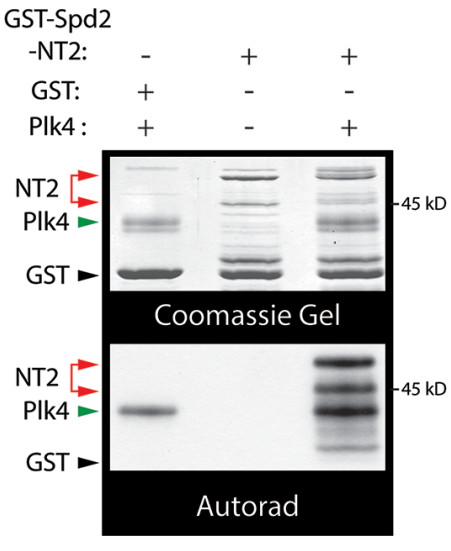
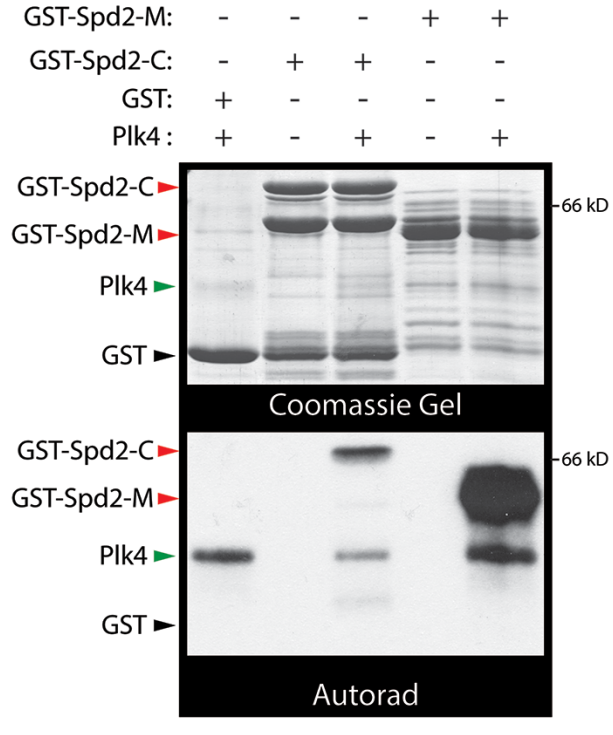


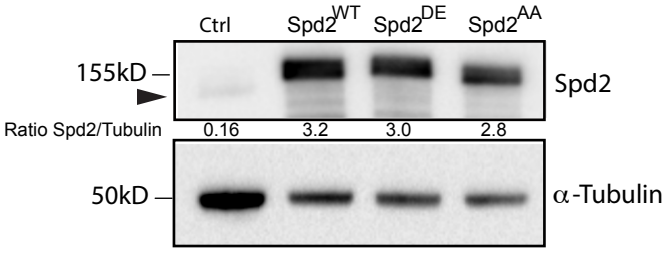
Figure S6- Gambarotto et al

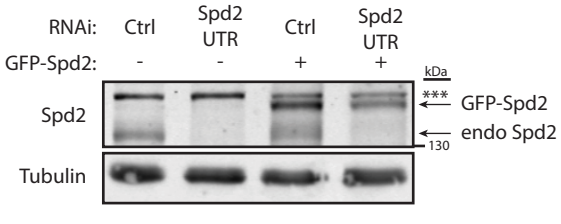


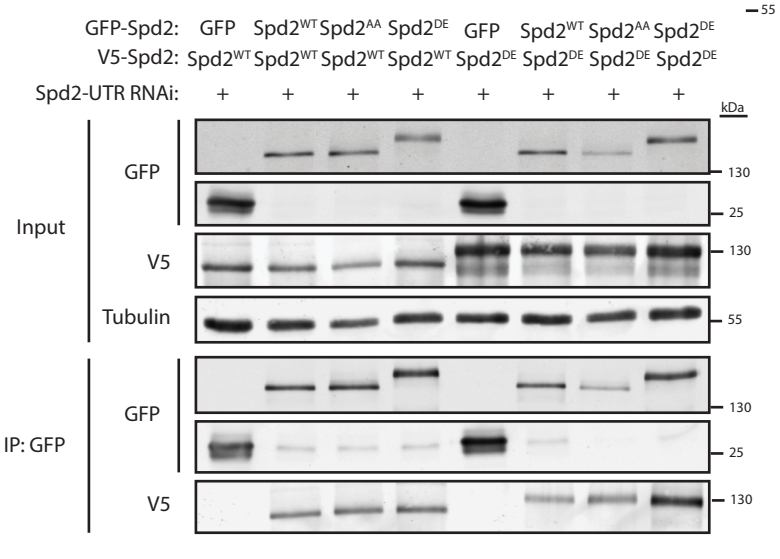


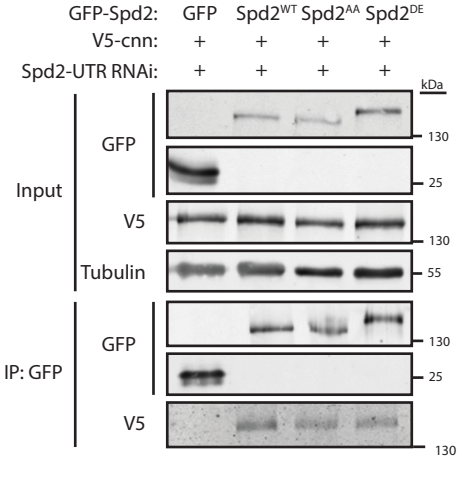












SUPPLEMENTARY FIGURE and MOVIE LEGENDS

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 $Plk4^{KD}$ 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 $Spd2^{WT}$ or $Spd2^{DE}$ or $Spd2^{AA}$ 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 ($Sas\text{-}4^{mut}$). (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 $Sas\text{-}4^{mut}$ NB. Tubulin is shown in red and RFP-Sas-6 in green. The $Sas\text{-}4^{mut}$ 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, $Plk4^{KD}$ and $Plk4^{WT}$ 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, $Plk4^{KD}$ and $Plk4^{WT}$ 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; $Plk4^{KD}$-10.6±0.7 $Plk4^{KD}$+ 14.5±1.3; $Plk4^{mut}$- 11.5±0.5 $Plk4^{mut}$+ 20.3±0.9; $Plk4^{WT}$ 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 ,$Plk4^{KD}$, Fzr^{mut} and $Fzr^{mut}Plk4^{KD}$ NBs, treated with (right) or without (left) colcemid. Plp is shown in green, phalloidin-labed actin in red and DNA (blue). Scale, 5μm. (F) Fzr^{mut}- 22.7±2.2 Fzr^{mut} + 33.6±3.4; $Plk4^{KD}$, Fzr^{mut} - 39.2±3.9 $Plk4^{KD}$, Fzr^{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, $Plk4^{KD}$, and $Plk4^{WT}$ 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^{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^{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 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_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. Immunoblots 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. Immunoblots 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. Immunoblots 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 Plk4^{KD} and Plk4^{WT} NBs

Related with Figure 1. This movie gathers three different movies from three

different NBs from the following genotypes: The first two are Plk4^{KD} expressing NB (in green) and the third is Plk4^{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^{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^{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^{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^{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 Plk4^{KD} 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 Plk4^{KD} 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- Plk4^{KD} 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-Plk4^{KD} 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 42.00min. 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 114min, 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 168min. 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 (T19.00min) (the basal centriole is labeled as a full green circle in the middle panel and empty green circle on the right panel). Spd2DE signal is rapidly lost from the basal (T21.00min) and the apical centrioles (T23.00min). Throughout interphase, Spd2DE 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 (T121.00min) (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 $Spd2^{AA}$ NB expressing $Spd2^{AA}$ (in red) and α-tubulin (in green). $Spd2^{AA}$ 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[S]SFSK	73%	7.38	51.74	69%	1.795	0.445	1	585.6	2
S35	VFGDLS[S]F	81%	6.20	26.57	46%	1.994	0.322	1	325	1
S92	LSTNI[S]ELVTDDTIDL	100%	22.93	69.11	80%	2.128	0.307	1	466	2
S121	GTNI[S]FEPAEITGR	100%	45.93	120.86	100%	3.88	0.492	1	731	2
S148	EKPSL[S]VAEIL	68%	3.34	29.68	75%	1.693	0.231	1	522.9	2
S191	GSSSSLSD[S]FNCSR	100%	41.42	120.89	100%	3.371	0.589	1	988.8	2
S239	AAEGQDESFAPAELMQ[S]K	100%	132.14	106.82	100%	3.05	0.425	1	915.8	2
S273	IAAPT[S]EIETSVSNSVL	90%	10.28	77.08	93%	2.462	0.553	1	378.2	2
S291	IAAPTSEIETSVSNSVLAGDPDF[S]L	100%	16.91	55.40	100%	2.705	0.43	1	783.8	2
S354	TDAITG[S]NLGR	100%	30.01	112.89	100%	2.85	0.478	1	344	2
T371	MQQDRIE[T]ALK	100%	1000.00	71.53	97%	2.768	0.399	1	117.1	2
S375	MQQDRIETALK[S]R	100%	53.61	56.24	93%	2.234	0.422	1	313.8	2
T384	NGLAAKE[T]KRPPSSSEILSLSAIDK	100%	24.19	45.65	100%	3.669	0.557	1	228.7	3
S391	RPPSS[S]EILSLSAIDK	98%	19.02	132.11	100%	3.178	0.471	1	674	2
S395	RPPSSSEIL[S]LSAIDK	100%	23.64	129.18	100%	4.204	0.632	1	276	2
S397	RPPSSSEILSL[S]AIDK	100%	51.96	97.15	100%	3.233	0.427	2	57	3
S443	GNNYDDGENKENQSSN[S]HAER	96%	12.63	70.12	100%	4.267	0.482	1	645.6	3
T458	LTD[T]MSFTDSVLNSTDFR	99%	22.30	162.62	100%	4.267	0.588	1	329	2

S460	LTDTM[S]FTDSVLNSTDFR	100%	28.23	117.63	100%	4.19	0.636	1	673.9	2
S464	LTDTMSFTD[S]VLNSTDFR	100%	44.44	115.08	100%	4.872	0.637	1	48	2
T493	KPLSPLADHPQI[T]ISR	100%	38.03	149.81	100%	4.014	0.539	1	479	3
S569	RV[S]IATMGLIPR	100%	47.36	101.72	100%	2.526	0.582	1	487.7	2
S673	GLGT[S]SVAVPR	96%	16.83	86.93	100%	3.042	0.476	1	442	2
T706	VTHT[T]LCWGSTK	100%	35.92	108.10	97%	2.627	0.499	1	527.5	2
T754	LGIQGPGFQLVGTDSSTI[T]LQAMECR	95%	10.21	15.97	98%	3.626	0.323	1	612.8	3
S949	EKLTSPMLD[S]IWGEFPDEQPVR	99%	17.35	115.47	99%	3.947	0.287	1	945.1	3
S1000	DFDESSES[S]LMFLPEADETVLFR	99%	19.02	91.50	100%	3.65	0.504	1	597.7	2