Supplementary Information

Regulation of autophosphorylation controls PLK4 self-destruction and centriole number

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1. Inventory of Supplemental Information

Figure S1 supports Figure 1 by expanding on results presented on the main text.
Figure S2 supports Figures 1, 2, 3 and 4 by demonstrating the efficiency of endogenous PLK4 depletion.
Figure S3 supports Figure 3 showing that dimerization through the Polo boxes 1 and 2 is required for Ser293 phosphorylation
Figure S4 supports Figure 4 showing that residues outside the degron are autophosphorylated and required for proper Slimb binding
Figure S5 supports Figure 5 by demonstrating the PLK4 ND is more stable than PLK4 WT in *Drosophila* female and male germline.
Figure S6 supports the discussion with a model for PLK4 trans-autophosphorylation and degradation
Table S1 describes the list of primers used for site-directed mutagenesis of PLK4.
Table S2 describes the list of primers used for dsRNA synthesis.

2. Supplemental Figures and Legends, Tables, Experimental Procedures, and References

Figure S1

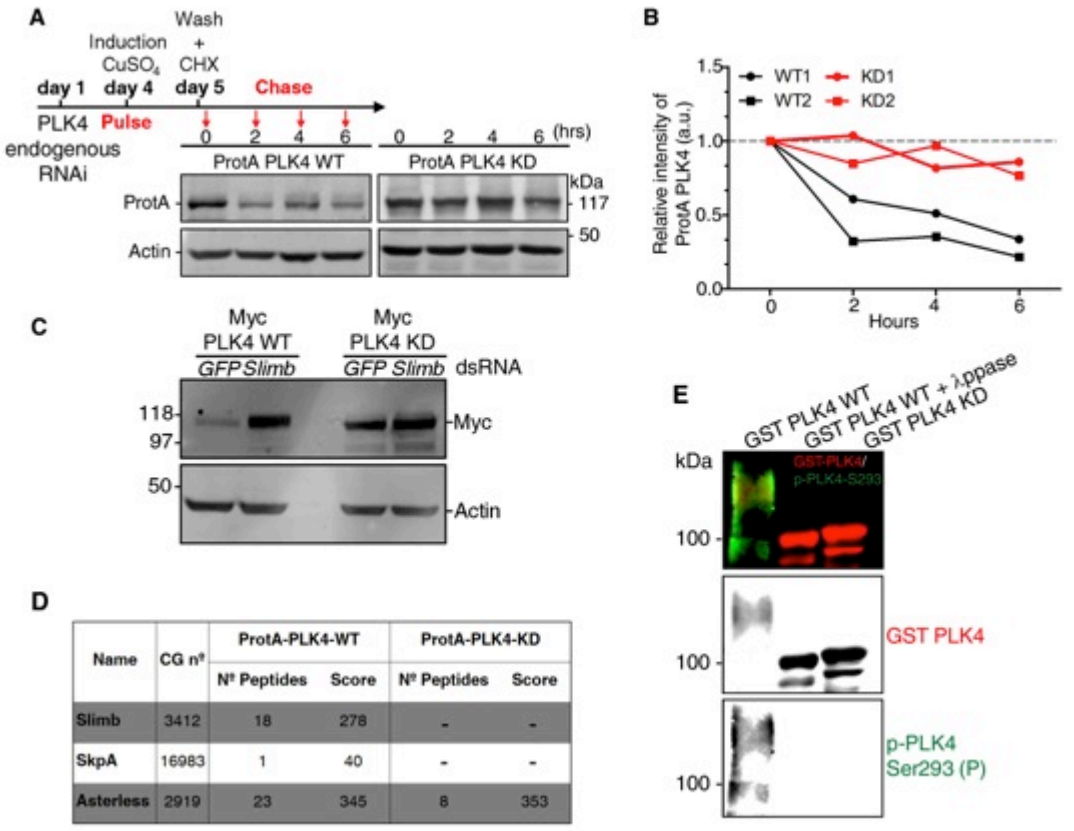

Figure S1, Related to Figure 1. PLK4 autophosphorylates and its kinase activity is required for its own degradation via the Slimb pathway. (A, B) **Kinase Dead PLK4 is a stable protein**. (A) pMT-ProtA PLK4 WT or pMT-ProtA PLK4 KD transfected DMEL cells were induced after endogenous depletion of *PLK4* (*PLK4*). Cells were washed 18 hours later, incubated in medium supplemented with cycloheximide, and harvested at the indicated time points. Cell extracts were then prepared and analyzed by western blotting for ProtA and actin (loading control). (B) Quantification of ProtA PLK4 relative intensities shown in panel (A). ProtA PLK4 intensity values were normalized to the actin loading control. Two experiments are shown. Note that while ProtA PLK4 WT levels decrease with time, ProtA PLK4 KD protein remains stable. (C) ***Slimb*** **depletion**

stabilizes Myc PLK4 WT but has minimal effect on Myc PLK4 KD. DMEL cells were transfected with dsRNA against *Slimb* or GFP (GFP dsRNA was used as a negative control) followed by transient transfection with the constructs act5-Myc PLK4 WT or act5-Myc PLK4 KD. Cell extracts were then prepared and analyzed by western blotting for Myc. Note that Myc PLK4 WT protein is stabilized upon *Slimb* depletion, whereas the KD version is already more stable in the presence of Slimb. (D) **PLK4 KD does not interact with Slimb.** Results of pulldowns of ProtA PLK4 WT and KD fusion proteins. The maximum MASCOT scores and the number of identified peptides for each interacting protein are indicated. Note that neither Slimb nor SkpA were identified in the ProtA PLK4 KD pulldown, but that Asl, a PLK4 interactor was [1–3]. (E) **PLK4 autophosphorylates the Ser293 degron residue**. A different antibody from Figure 1A is shown. pGEX2RBS-GST PLK4 WT, WT + λPPase and KD were expressed in bacteria at 25ºC. The soluble fractions were probed for p-PLK4-Ser293 (antibody P) and GST. Note that the p-PLK4-Ser293 (P) antibody only recognizes the WT kinase and not the WT co- expressed with phosphatase showing that it is a bona fide phosphospecific antibody. There is a higher mobility band that is likely to correspond to a degradation product of PLK4, which is more obvious in the kinase dead or phosphatase-treated WT kinase.

Figure S2

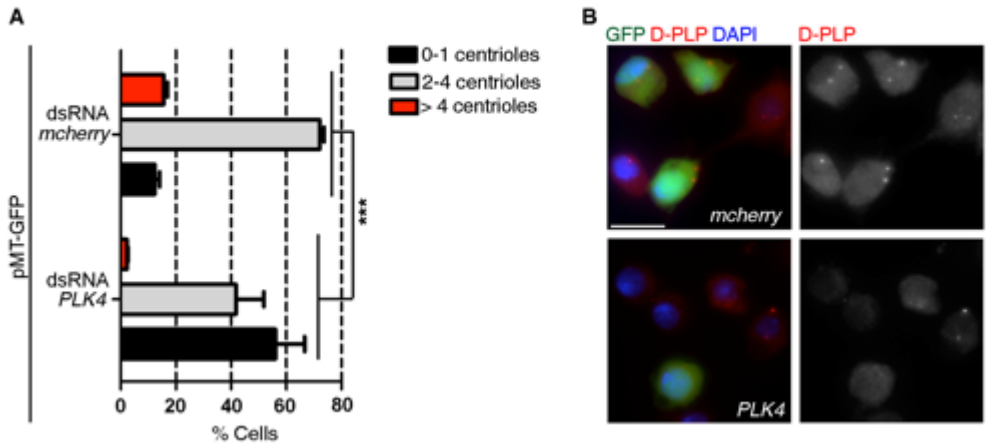

Figure S2, Related to Figure 1, 2, 3 and 4. Endogenous depletion of *PLK4* is efficient, resulting in centriole loss. (A-B) DMEL cells were transfected with dsRNA against the UTR region of endogenous *PLK4* (*PLK4*) (mcherry dsRNA was used as a negative control), followed by transient transfection with a pMT-GFP plasmid (as a negative control for PLK4 constructs used in main figures – 1, 2 3 and 4). To score centrioles, cells were fixed and stained against centrosomal D-PLP and DNA (counterstained with DAPI). Data are the average of 3 experiments (n=100 cells in each experiment). Note that depletion of *PLK4* is highly efficient and blocks centriole duplication, with a large increase in the population of cells with zero and one centrioles ($p<0{,}001$ Pearson's $\chi 2$ test). *Drosophila* cultured cells normally show some centriole amplification and some cells with zero centrioles in the negative control (mcherry) [4].

Figure S3

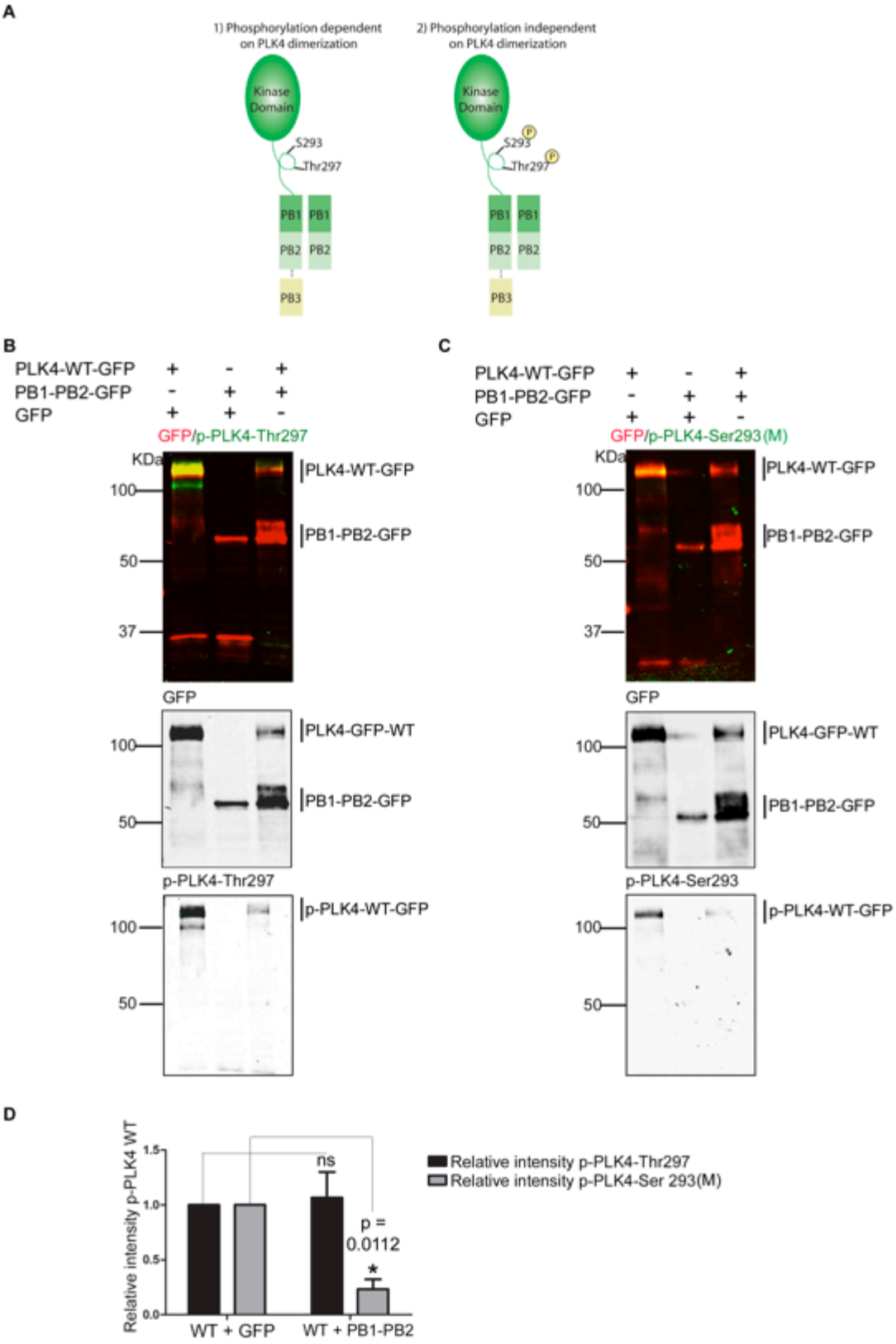
A
1) Phosphorylation dependent on PLK4 dimerization
2) Phosphorylation independent on PLK4 dimerization
Kinase Domain
S293
Thr297
PB1
PB2
PB3
B
PLK4-WT-GFP + - +
PB1-PB2-GFP - + +
GFP + + -
GFP/p-PLK4-Thr297
KDa
100
50
37
PLK4-WT-GFP
PB1-PB2-GFP
GFP
PLK4-GFP-WT
p-PLK4-Thr297
p-PLK4-WT-GFP
C
GFP/p-PLK4-Ser293(M)
p-PLK4-Ser293
D
Relative intensity p-PLK4 WT
1.5
1.0
0.5
0.0
ns
p = 0.0112
*
WT + GFP
WT + PB1-PB2
Relative intensity p-PLK4-Thr297
Relative intensity p-PLK4-Ser 293(M)

Figure S3, Related to Figure 3. Dimerization through the Polo boxes 1 and 2 is required for Ser293 phosphorylation. (A) Schematic representation of PLK4 *trans*-autophosphorylation and expected regulation of phosphorylation at Ser293 and Thr297. PLK4 has a triple polo box architecture that facilitates oligomerization and targeting as PB1-PB2 is known to dimerise with PLK4 WT [5, 6, 7]. *Trans*-autophosphorylation has been proposed to be dependent on dimerization through the polo-boxes, in particular PB1-PB2 [5, 6, 7]. (1) If phosphorylation of PLK4 is dependent on dimerization through PB1-PB2 then co-expression of a full length PLK4 kinase with the PB1-PB2 alone should result in impaired phosphorylation at the degron (Ser293 and Thr297 residues) as PB1-PB2 sequesters PLK4 WT but cannot phosphorylate it. (2) If PLK4 phosphorylation at the degron is not dependent on dimerization through PB1-PB2 then overexpression of PB1-PB2 with the full leght PLK4 kinase should not block phosphorylation at the degron residues. **(B-C) Combinations (as indicated) of pMT-PLK4 WT-GFP, pMT-PB1-PB2-GFP (PB1-PB2 spans residues 382-602[5]) and empty pMT-GFP constructs were transfected in DMEL cells.** The pMT constructs were induced with 500 μM $CuSO_4$ after endogenous depletion of *PLK4* (*PLK4)*. Cell extracts were then prepared, centrifuged and cell pellets (enriched for centrosomes[8], maximizing the signal for the phosphoantibodies) analyzed by western blotting for p-PLK4 Ser293 (P) or p-PLK4 Thr297 and GFP (to detect total PLK4). Note that it is possible that PB1-PB2 fragment is *trans*-phosphorylated when co-transfected with PLK4 WT as it shows a broader band in this condition. **(D) Quantification of relative phosphorylation at Ser293 and Thr297 in panel B, C**. The relative intensity of Ser293 and Thr297 for each sample was obtained by normalization with the respective total PLK4 levels. Data are the average of 3 experiments ± SEM (t-Test). Quantifications were performed using the *Odyssey* infrared image *system* (LI-COR). Note that Ser293 is less phosphorylated in PLK4 WT when cotransfected with PB1-PB2, which is indicative of phosphorylation within a dimer form, but Thr297 is not.

Figure S4

A

Mr(expt)	ppm	Score	Counts	Peptide	Positions
2073.70	-3.65	71	23	NVFSQ**pS**MoxESGD**pS**GII**pT**F	S287, S293, T297
1780.62	-5.84	39	2	FSQSMoxESGD**pS**GII**pT**F	S293, T297
1860.59	-3.14	50	15	F**pS**QSMoxESGD**pS**GII**pT**F	S285, S293, T297
1633.56	1.30	61	3	SQSMoxESGD**pS**GII**pT**F	S293, T297
1713.52	-4.68	72	9	SQ**pS**MoxESGD**pS**GII**pT**F	S287, S293, T297
1331.43	0.18	31	2	MoxESGD**pS**GII**pT**F	S293, T297
1184.40	-3.15	52	2	ESGD**pS**GII**pT**F	S293, T297
1507.58	4.06	40	3	AS**pS**DSRN**pS**QQIR	S301, S306
1587.55	3.98	29	1	A**pS**SD**pS**RN**pS**QQIR	S300, S303, S306
824.35	4.17	26	2	N**pS**QQIR	S306
1236.53	-0.30	43	1	**pS**VENSGPQQVL	S311
1316.50	0.68	31	4	**pS**VEN**pS**GPQQVL	S311, S315
1541.61	-3.39	27	1	**pS**VEN**pS**GPQQVLPQ	S311, S315
1810.79	-4.29	101	2	**pS**VEN**pS**GPQQVLPQIR	S311, S315

B

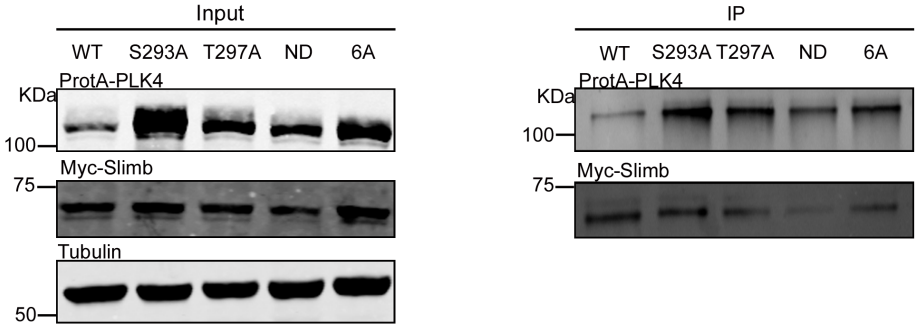

Figure S4, Related to Figure 4. Several residues within and ouside the PLK4 degron are autophosphorylated and regulate direct bindig to Slimb. (A) Identification of PLK4 phospho-peptides by mass spectrometry. List of phosphorylated peptides extracted from Mascot search results. Mr (expt) – mass of the measured peptide, ppm – mass difference between measured mass and theoretical mass based on a peptide sequence, Score – Mascot peptide score;

score value is given for a highest scored peptide only, Counts – number of ions corresponding to a given peptide. Phosphorylated residues are bold and marked with "p". Total protein coverage was 92% and multiple phosphorylation sites were identified (data not shown). Non-phosphorylated PLK4 was reactivated as shown in Figure 1C. Protein phosphorylation was determined by specific isolation of phosphorylated peptides by incubation with titanium dioxide. In order to improve protein coverage and phospho sites mapping, trypsin digested peptides were additionally digested with immobilized pepsin. Note that phosphorylated amino acids were present only in reactivated samples, i.e incubated with ATP (data not shown for non-phosphorylated peptides). **(B) Immunoprecipitation of Simb with PLK4.** Western blot of pull down of ProtA from cell lines expressing either pMT-ProtA PLK4 WT or PLK4 mutants (S293A, T297A, ND, 6A) and act5-Slimb Myc (*left*: input fractions; *right*: IP fractions) Western was first probed for Myc to detect Slimb-Myc and then for IgG rabbit to detect PLK4. α–tubulin was used as a loading control. One representative experiment is shown. Note that all samples express similar levels of Slimb-Myc (*left*: input). Note that a large difference in binding efficiency to Slimb is observed between the positive control (PLK4 WT) and mutants, including PLK4 6A.

Figure S5

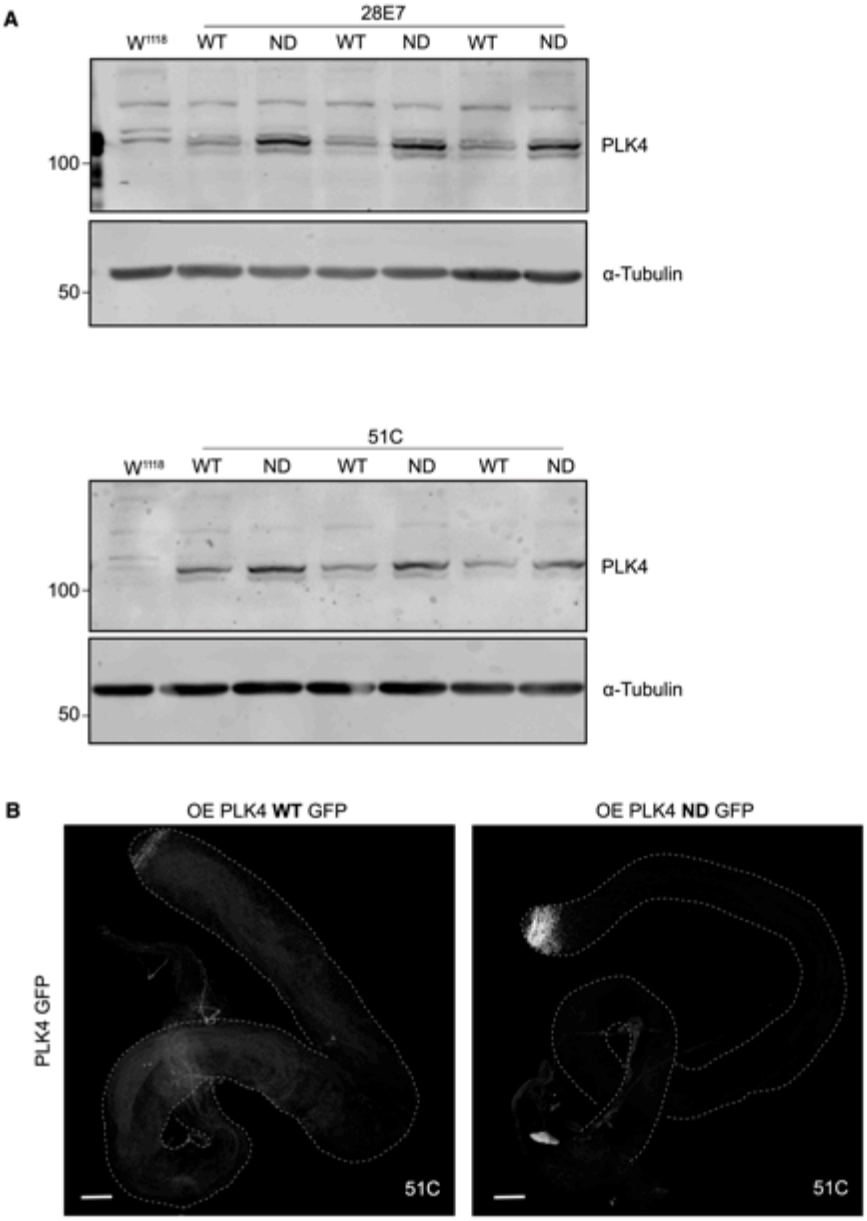

Figure S5, Related to Figure 5. PLK4 ND GFP is stabilized in both male and female *Drosophila* germlines. (A) **PLK4 ND GFP is stabilized in *Drosophila* female germline.** *pUASp-PLK4 WT GFP* and *pUASp-PLK4 ND GFP* integrated at the recombination loci 28E7 or

51C were expressed under the control of the ovariole specific driver *V32Gal4*. Ovary extracts from 3-day-old females were prepared and analysed by western blot with PLK4 antibody (α-Tubulin was used as a loading control). Note that as in other experimental systems, endogenous PLK4 is not seen by western blot. Three samples were loaded to show reproducibility. (B) **PLK4 ND is stabilized in *Drosophila* male germline.** *pUASp-PLK4 WT GFP and pUASp-PLK4 ND GFP* integrated at 51C, were expressed under the male germline specific driver *BamGal4.* Immunostaining of whole mount testis and acquisition of images using the same exposure shows that PLK4 ND GFP is stabilized in the male germline (line inserted in 28E7 is shown in main text).

Figure S6

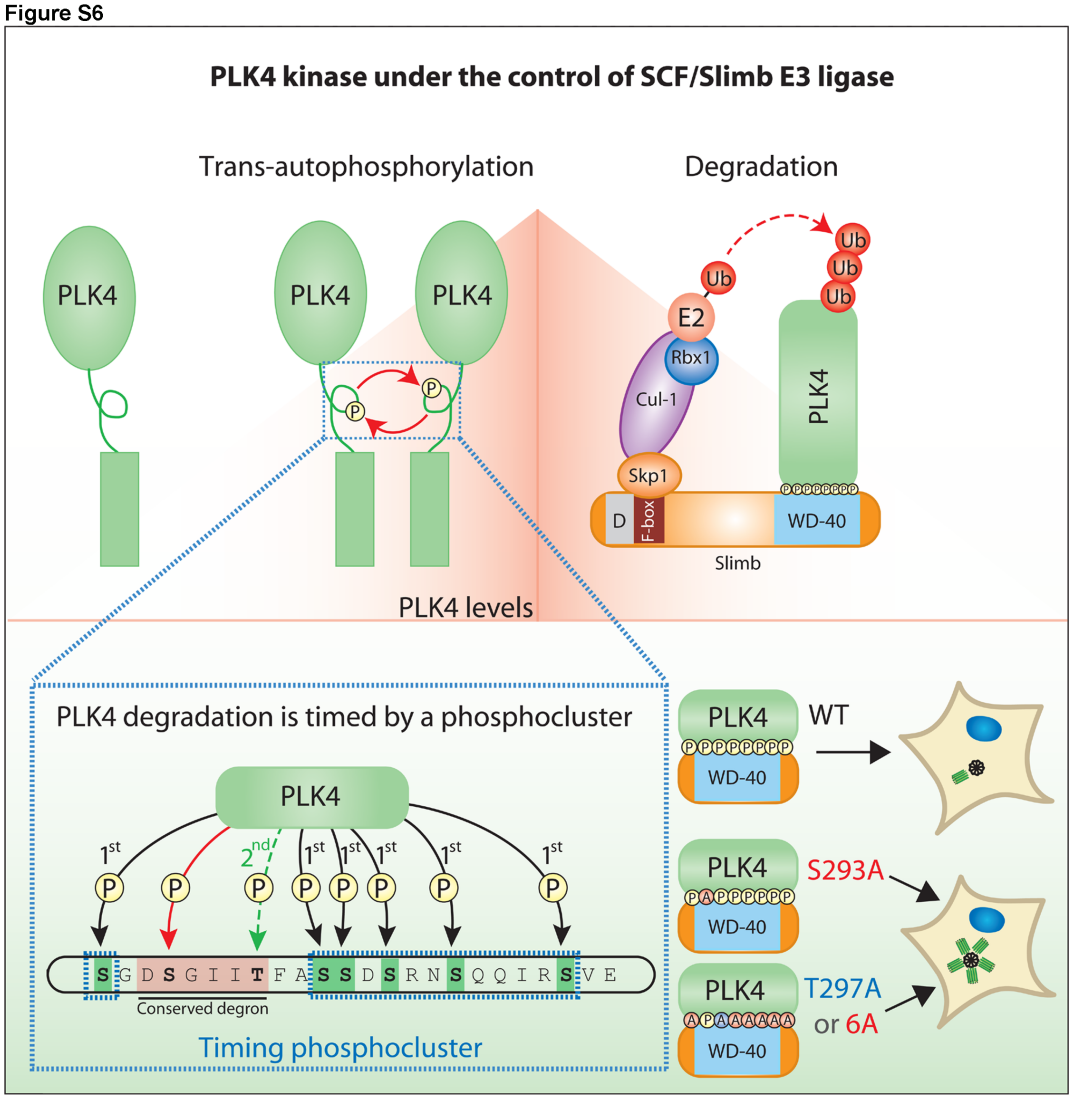

Figure S6, Related to the discussion. PLK4 levels are regulated by *trans*-autophosphorylation and a phosphocluster whose phosphorylation ensures a sequence of events. (A) PLK4 *trans*-autophosphorylation plays a very important role in its degradation. PLK4 *trans*-autophosphorylates a phosphocluster and its degron. As a consequence, degradation by the SCF/Slimb E3 ligase is likely to be dependent on a threshold of PLK4 protein levels: the increase in PLK4 concentration allows for one molecule of PLK4 to encounter another and engage in *trans*-autophosphorylation. This may require dimerization of the kinase (eg. for Ser293 phosphorylation) or not. Once PLK4 is phosphorylated within the degron the kinase binds Slimb

within the SCF/Slimb E3 ligase, which ubiquitinates it, leading to its degradation by the proteasome. Note that the represented increase/decrease in PLK4 levels is merely a scheme as we presently do not know the kinetics of PLK4 level regulation. (B**) PLK4 induced self-degradation is temporally controlled by a cascade of phosphorylation events.** Maximum efficiency for PLK4 phosphorylation at Thr297 within the typical degron is dependent on previous phosphorylation events executed by PLK4 on the surrounding six residues flanking the degron, which we called phosphocluster. A requirement for sequential phosphorylation events most likely creates a delay in PLK4 degradation, allowing it to play a role in centriole biogenesis. (C**) Ser293 is a critical residue in PLK4 degradation.** When PLK4 is phosphorylated at the residues within the degron, centriole numbers are properly controlled: PLK4 is degraded at the right time allowing for proper control of centriole duplication. Mutagenesis of Ser293 to a non-phosphorylatable residue fully blocked PLK4 degradation leading to centriole amplification. This suggests that most likely Ser293 is playing a critical role. Mutagenesis of Thr297 or of the flanking 6 residues delayed PLK4 degradation with concominant centriole amplification showing the delay imposed by the sequential phosphorylation of these residues is critical to properly control centriole duplication.

Supplemental Experimental Procedures

Plasmid constructs

All the vectors constructed in our lab were done using the Gateway system (Invitrogen, USA) according to the manufacturer instructions. The destination vector with a Myc tag and Actin 5C promoter were acquired at DRGC (Indiana, USA). The pcBLAST vector was obtained from Invitrogen. The pMT-ProtA destination vector (with N-terminal ProtA tag and metallothionein promoter) was constructed and kindly provided by Luisa Capalbo (David Glover, Cambridge UK). The PMT-GFP N-terminal plasmid was a kind gift of João Rocha (University of Cambridge, UK). The PMT-PLK4 GFP and PMT-PB1-PB2 GFP vectors were kindly provided by Greg Rogers[5]. The pACYC-RIL-λppase for expression in *E.Coli* was a kind gift from Dr. Jonathan M. Elkins (Oxford University, UK) [9]. The PLK4 entry vector has been described elsewhere [10]. The PLK4 Kinase Dead (KD) mutant includes three mutations in the PLK4 catalytic domain of PLK4: K43M in the nucleotide binding motif, K22M in the phosphate anchor, and D156A in the activation loop. This mutant has been described elsewhere [11]. The dicistronic vector pGEX-2RBS (created by Anna De Antoni) was a kind gift from Andrea Musacchio (Milan, Italy)[12]. For the creation of pGEX-2RBS-GST PLK4 WT, PLK4 was amplified by PCR from a pDONOR221-PLK4 entry vector (Gateway) and cloned into pGEX-2RBS using StuI and SpeI restriction sites. For the creation of pGEX-2RBS-GST PLK4 KD, PLK4 KD (K22M, K43M, D146A) was amplified by PCR from a pDONOR221-PLK4 KD entry vector (Gateway) and cloned into pGEX-2RBS using KpnI sites. The PLK4 Non-degradable (ND) mutant includes two mutations within the *DSGIIT* degron, S293A and T297A. This mutant has been described elsewhere [13]. PLK4 6 alanine mutant includes mutations at S290, S300, S301, S305, S309 and S311 to alanine and was synthesized from PLK4 entry clone (Gateway) at GenScrip, USA. To create the PLK4 KD 6A construct, a 1520 bp sequence of the pDONOR221-PLK4 6A (GenScrip, USA), which includes the

6 mutations referred above, was cloned into pGEX-2RBS-GST PLK4 KD using NdeI and NcoI. For the creation of an entry vector with PLK4 KD 6A sequence, PLK4 KD 6A was amplified by PCR from the pGEX-2RBS-GST PLK4 KD 6A and cloned into a pDONOR221 entry vector (Gateway). For the construction of PLK4 S293A, PLK4 T297A and PLK4 KD ND mutants the mutations were introduced in the *DSGIIT* degron in the PLK4 WT (PLK4 S293A and PLK4 T297A) and KD (PLK4 KD ND) entry vectors (Gateway). We used the Quick-Change XL Site-Directed mutagenesis kit from Stratagene. The mutations were as follows: the serine in position 293 and threonine in position 297 were each mutated to alanine in the S293A and T297A mutants. For the construction of PLK4 KD ND mutant the mutations were as follows: the serine in the position 293 and threonine in position 297 were both mutated to alanine. Primers used for Site-Directed Mutagenesis are listed in **Supplementary Information Table S1.** All the constructs were sequenced and confirmed prior to recombination into destination vectors. pMT-PLK4 GFP and pMT-PB1-PB2-GFP were kindly provided by Greg Rogers [5].

Protein depletion, transient plasmid transfection and stable cell lines

Drosophila cells (DMEL) were cultured in Express5 SFM (Gibco,USA) supplemented with 1x L-Glutamine-Penicilin-Streptomycin according to standard tissue culture techniques. dsRNA synthesis against GFP, mcherry, Slimb and PLK4 was performed as previously described [13]. dsRNA for endogenous PLK4 was synthesized from genomic DNA with an adjustment of the PCR annealing temperature to 45ºC. For transient transfection of dsRNA, we used 2 million cells and 40 μg of dsRNA. In the case of endogenous PLK4, we used a combination of 30 μg of PLK4 *3'* dsRNA, with 30 μg of PLK4 *5'* dsRNA. For the respective control experiments, we used 60 μg of mcherry dsRNA. The primers used for the production of dsRNA are listed in **Table 2**.

Transient plasmid transfections were performed with Effectene reagent (Qiagen, USA) according to the manual recommendations. Briefly, 3 million DMEL cells were plated per well (6-

well plate) in 1 ml antibiotic-free medium; 400 ng of total plasmid DNA were mixed with 3,5 μl Enhancer reagent and incubated for 5 min at RT; 10 μl Effectene transfection reagent were added to the previous solution, mixed and incubated at RT for 10 min; 2 ml antibiotic-free medium were added to the final mix and the solution was added to the cells in a drop wise manner. Transfections proceeded for 4 days at 25ºC. In experiments where the cells had previously been treated with dsRNA, plasmid transfection was performed on the second day of the depletion. In transient transfections with pMT-derived constructs, $CuSO_4$ induction was initiated 18 hours before harvesting.

Construction of stable cell lines was performed with Effectene reagent (Qiagen, USA) according to manufacturer recommendations. The protocol is similar to the one used for transient transfections, except that 120 ng of picoblast selection vector carrying a blasticydin resistance gene (Invitrogen, USA) were added to the DNA-enhancer mix, and this solution was incubated for 10 min at RT. On the second day of transfection, cells were washed 3 times in antibiotic-free medium and incubated for 24h in 4 ml complete medium. On the following day (day 3 of transfection), the medium was replaced with complete medium supplemented with 20 mg/ml blasticydin (Invitrogen, USA). The blasticydin selection was maintained for seven days after which the cell line was tested. Stable lines were routinely grown in complete medium supplemented with 20 mg/ml blasticydin.

Preparation of whole cell protein extracts

Whole cell lysates were prepared by ressupending cell pellets in lysis buffer containing 75 mM HEPES, 150 mM NaCl, 2mM $MgCl_2$, 0,1% NP-40, 5 mM DTT, 5% glycerol, 2 mM EGTA (all chemicals from Sigma, USA) and supplemented with 1x EDTA-free protease inhibitors (Roche). Laemmli buffer was added to the samples to 1x and then boiled at 99ºC for 5 min and centrifuged at 14.000 RPM for 1 min before being analyzed on polyacrylamide gels. For the preparation of whole cell extracts for pulse chase experiments the samples were screened for total protein concentration

using Bradford reagent and then normalized prior being analyzed on polyacrylamide gels. For detection of phosphorylated PLK4 on western blots the whole cell lysates were prepared by ressuspending cell pellets in lysis buffer containing 75 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0,1% NP-40, 5 mM DTT, 5% glycerol, 2 mM EGTA, 200 mM NaF, 150 mM, β-Glycerophosphate, 1 mM Na_3VO_4 (all chemicals from Sigma, USA) supplemented with 1x EDTA-free protease inhibitors (Roche). The lysate soluble and insoluble fractions were separated by centrifugation at 14.000 RPM for 10 min. The pellet was ressuspended in 0,8 M Urea and 1x Laemmli buffer. The extract was then boiled at 99ºC for 5 min and centrifuged at 14.000 RPM for 1 min before being analyzed on polyacrylamide gels.

Preparation of ovaries protein extracts

Ovaries from 3-day-old females expressing either PLK4 WT GFP or PLK4 ND GFP, were dissected in PBS containing protease inhibitors (Roche). For each sample, ovaries were squashed in SDS-PAGE sample buffer and boiled for 5 minutes before loading.

Recombinant PLK4 expression

Recombinant pGEX-2RBS-GST PLK4 WT, pGEX2RBS-GST PLK4 WT and pACYC-RIL λppase and pGEX2-RBS-GST PLK4 KD were each transformed into *Escherichia coli* BL21 cells. PLK4 expression was induced with 1 mM IPTG for 6 hr at 25ºC. For detection of phosphorylated PLK4, the soluble fractions were prepared according to the procedures described above.

Immunoprecipitation

Immunoprecipitations of pMT-ProtA-PLK4-WT, S293A, T297A, 6A and ND fusion proteins was performed to test interaction between PLK4 and Slimb. DMEL cells were transiently transfected with each PLK4 construct and act5-Slimb-Myc constructs using Effectene reagent as

described above. Transiently transfected cells expressing both pMT-ProtA PLK4 (WT, S293A, T297A, ND or 6A) and act5-Slimb-Myc were used as input to immunoprecipitate PLK4. Because each mutant impairs on PLK4 stability in a different manner different concentrations of $CuSO_4$ were used in order to have similar amounts of expressed PLK4 in all samples at the beginning of the experiment. A constitutive promoter (Actin5c) was used for Slimb-Myc expression in both cell lines and similar levels of that fusion protein were expressed. Cells lysis and binding to IgG coated beads was performed with EB buffer (50 mM Hepes pH 7.5, 100 mM NaAc, 50mM KCl, 2mM $MgCl_2$, 0,1% NP-40, 5mM DTT, 2mM EGTA, 5% Glycerol, Complete Protease Inhibitors EDTA-free). Antibody coated beads were incubated with lysates for 4 hours, washed 5 times with 1 ml EB buffer. Beads were eluted with Laemlli buffer 1x concentrated and protein complexes were resolved by SDS-Page Polyacrilamide Gels followed by western blot analysis with mouse Myc antibody (Sigma) at 1:2000 dilution and mouse Horseradish Peroxidase antibody (Jackson Immunoreseach) at 1:10.000 dilution. After development of this western, detection for PLK4 was performed by incubation with HRP ChromPure rabbit IgG (Jackson Immunoresearch) at 1:5000 dilution. The protocol for pMT-ProtA PLK4 immunoprecipitation has been already described elsewhere [13].

Western blotting and pulse-chase experiments

Standard western blotting procedures involved blocking in TBS-T (0.1% Triton X-100 in TBS) supplemented with 5% milk powder, and 1% milk powder in TBS-T for antibody incubations and washes. For detection with phosphospecific antibodies, blocking was performed in TBS supplemented with 5% BSA and phosphatase inhibitors (NaF 50 mM, and 5 mM Na_3VO_4), primary antibody incubations were performed in TBS-T supplemented with 5% BSA and phosphatase inhibitors (NaF 50 mM, and 5 mM Na_3VO_4) and secondary antibody incubations were performed in 5% BSA and phosphatase inhibitors (NaF 50 mM, and 5 mM Na_3VO_4) supplemented with 0.01% SDS, while washes were performed in TBS-T.

PLK4 expression in pMT-ProtA PLK4 WT, S293A, T297A or ND stable cell lines was characterized by pulse-chase. For this, cells were induced with $CuSO_4$ for 18 hours. Different concentrations of $CuSO_4$ were used in order to have similar amounts of expressed PLK4 in all cell lines at the beginning of the experiment. Cells were then harvested by centrifugation, washed once, resuspended in medium supplemented with cycloheximide (1μg/ml), equally divided by four wells, and collected at two-hour intervals over a period of six hours. The same conditions were used to characterize the degradation kinetics of PLK4 KD (PLK4 WT was used as control). For these experiments endogenous PLK4 was depleted during a period of 4 days by RNAi. At the end of this period each PLK4 construct was induced with $CuSO_4$ and pulse chases followed as previously described above.

***In vitro* auto-phosphorylation of PLK4**

Recombinant dephosphorylated His-MBP PLK4 was expressed and purified by the Protein Expression and Purification Core Facility, EMBL, Heidelberg, Germany. His-MBP PLK4 was co-expressed with pACYC-RIL-λppase for dephosphorylation[9]. For *in vitro* autophosphorylation assays protein samples were incubated for 60 mins at room temperature in 50 mM Tris-HCl ph 7.5 buffer containing 1 mM ATP, 2 mM MgCl2 and 1 mM Na_3VO_4. Time points were taken every ten minutes for testing auto-phosphorylation over the course of the experiment.

Analysis of Plk4 Phosphorylation by Mass Spectrometry.

Recombinant dephosphorylated His-MBP PLK4 (EMBL, Germany) was autophosphorylated *in vitro*. PLK4 was incubated in 50 mM Tris-HCl ph 7.5 buffer containing 2 mM MgCl2, 1 mM Na_3VO_4 and 1 mM ATP. A sample without ATP was used as negative control. PLK4 was then digested by addition of trypsin (sequencing grade modified trypsin; Promega V5111) directly to the

reaction mixture and incubated overnight at 37 °C. Half of the digested sample was acidified and additionally digested by pepsin.

Isolation of phospho-peptides was performed as described by[14]. Briefly, peptides obtained after trypsin digestion were mixed with loading buffer (80% acetonitrile, 5% TFA, 1M phthalic acid) and incubated with titanium dioxide slurry (GL Sciences, Japan) for 10 min and centrifuged. Titanium beads were washed with 80% acetonitrile, 1% TFA and with 20% acetonitrile, 0.05% TFA. Phosphopeptides were eluted with water alkalized by ammonia to pH 10.5 and immediately after elution acidified with 100% TFA.

Obtained peptides were applied to RP-18 precolumn (nanoACQUITY Symmetry C18; Waters no. 186003514) using water containing 0.1% TFA as mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18; Waters no. 186003545) using an acetonitrile gradient (0–60% AcN in 120 min) in the presence of 0.05% formic acid with the flow rate of 150 nL/min. Column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis. Acquired raw data were processed by Mascot distiller followed by database search with the Mascot program (Matrix Science, 8-processor on-site license) against *D. melanogaster* (Flybase) database. Search parameters for precursor and product ions mass tolerance were 20 ppm and 0.6 Da, respectively, with none enzyme specificity, fixed modifications through cysteine carbamidomehtylation and the following allowed variable modifications: lysine carboxymethylation, methionine oxidation, serine, threonine, and tyrosine phosphorylation. Peptides with Mascot score exceeding the threshold value corresponding to <5% false positive rate, calculated by Mascot procedure, were considered to be positively identified. Peptides identified in the Mascot search runs as phosphorylated were subjected to the confirmation procedure on the basis of visual inspection of the fragmentation spectra corresponding to the modified (and unmodified, when detected) peptide and identification of a significant fraction of expected product ions.

Immunostaining

DMEL cells were plated onto glass coverslips, allowed to adhere for 1 hour and post-fixed in 4% formaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4mM $MgCl_2$). Cells were permeabilized and washed in PBS-T-B (PBS containing 0.1% Triton X-100 and 1% BSA). Immunostainings were performed as previously described [10]. Cells were mounted with a mounting medium containing DAPI to counterstain DNA (Vectashield - Vector Laboratories, USA). Cell imaging and centriole scoring were performed on a Leica DMRA2 microscope. Images were acquired as a z-series (300 nm apart) with a Cool SNAP HQ camera (Photometrics). Images are presented as maximal intensity projections. All images were prepared with Adobe Photoshop (Adobe Systems, USA) and ImageJ (NIH, USA).

Phosphospecific antibodies

Short phospho-peptides spanning each residue were used for the production of each antibody. The p-PLK4-Ser293 (P) was raised with a phosphoSer293-peptide that includes the Thr297 residue, whereas p-PLK4-Ser293 (M) lacks this residue in the peptide. The p-PLK4-Thr297 antibody does not include the Ser293 within the phosphoThr297-peptide. For priming experiments both the p-PLK4-Ser293 (M) and p-PLK4-Thr297 were tested by dot blot against the corresponding phospho-peptide and the non-phosphorylated peptide. The phospho-peptide used to raise the p-PLK4-Ser293 (M) includes additionally the Ser290 residue that is mutated to alanine in the PLK4 6A mutant. Dot blots were performed against a phospho-Ser293 peptide with Ser290 mutated to Alanine, validating this antibody for priming experiments (data not shown). Likewise the phosphoThr297-peptide includes the Ser300 residue that is also mutated to alanine in the PLK4 6A mutant. Dot blots were performed against a phospho-Thr297 – S300A peptide, validating this antibody for priming experiments (data not shown). For western blot of whole cell extracts, p-PLK4-Ser293 (M), p-

PLK4-Ser293 (P) and p-PLK4 Thr297 were used at 1:100, 1:500 and 1:1000 respectively. To detect bacterially-expressed PLK4, p-PLK4 Ser293 (M) was used at 1:250, p-PLK4-Ser293(P) at 1:1000 and p-PLK4-Thr297 at 1:5000. For detection of phosphorylated PLK4 upon reactivation p-PLK4-Ser293 (P) was used at 1:500 and p-PLK4-Thr297 at 1:5000. All antibodies were supplemented with the corresponding non-phosphorylated peptide at 1000 ng/ml in TBST supplemented with 5% BSA and phosphatase inhibitors for western blot.

Other Antibodies

The antibodies used for western blotting were the following: mouse α-tubulin (1:500000 dilution, clone B512, Sigma, USA), rabbit actin (1:10000 dilution, Sigma, USA), mouse GFP (1:100 dilution, Roche, USA), and mouse cMyc (1:10000 dilution, clone 9E10, Santa Cruz Biotechnology, USA), mouse GST (1:10000, clone 26H1, Cell Signalling, USA), mouse Histidine (1:2500, Novagen, USA) and rabbit PLK4 antibody (1:100 dilution; [11]) HRP ChromPure rabbit IgG, HRP secondary antibodies (Jackson Immunoresearch Laboratories, USA) and IRDye secondary antibodies (Odyssey, LI-COR Biosciences) were used at 1:10000 dilution. Chicken anti-*Drosophila*-PLP (1:1000 dilution; [10]) was used as a primary antibody in immunostaining. Rhodamine Redex secondary antibody with minimal cross reactivity against other species was used at 1:100 dilution (Jackson Immunoresearch, USA).

Centrosome scoring

Number codes were assigned to both control and sample slides in order to score centrioles as a blind assay. A total of 100 cells were scored per sample per slide. Cells were categorized according to the number of centrioles (0, 1, 2, 3, 4 or more than 4).

Flies and husbandry

To generate transgenes, PLK4 WT GFP and PLK4 ND GFP were cloned into XbaI / KpnI sites of a pUASp attB vector (kind gift fom Jiongming Lu, University of Bern). These transgenes were generated using PhiC31 integrase-mediated transgenesis system, which is based on the site-specific bacteriophage PhiC31 integrase [15]. For insertions of both transgenes (p*UASp-PLK4 WT GFP* and p*UASp-PLK4 ND GFP*), two different integration sites (28E7 and 51C) in the fly genome were chosen. All flies were reared according to standard procedures and maintained at 25ºC.

Egg Immunostaining

Eggs from 4- to 5-day-old virgin female flies were collected at 25^{o}C on small agar plates. Short time collections of 30 minutes were performed. Eggs were dechorionated in a 50% freshly made bleach solution and washed with distilled water. Vitteline membrane was removed in a 50% solution of methanol and heptane for 3 min with strong agitation. Eggs were fixed and stained as described [16].

Testes Immunostaining

Testes from adult flies were dissected in a buffer containing 183 mM KCl, 47 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl pH 6.8] transferred to poly-L-lysine glass slides and snap frozen in liquid nitrogen as previously described [17]. Samples were mounted in Vectashield mounting media (Vector Laboratories). Testes samples were imaged as a z-series (0.4 µm apart) on a point scanning confocal (Leica SP5). Images are presented as maximum-intensity projections and processed using ImageJ and Adobe Photoshop (Adobe Systems).

Fertility Tests

Fertility tests of males expressing p*UASp-PLK4 WT GFP* (28E7) and p*UASp-PLK4 ND GFP* (28E7) under the *BamGal4 driver* were performed by crossing a transgenic male with three wild type females during ten days. The progeny was scored and averaged for 10-15 males.

Statistical analysis

All the statistical analysis was performed with Graphpad Prism® version 5.0 software.

Table S1. List of primers used for PLK4 site-directed mutagenesis

Name	Forward (5'-3')	Reverse (5'-3')
PLK4 S293A	GGAAAGTGGCGATGCTGGAATCATA ACATTTGCTAGCAG	CTGCTAGCAAATGTTATGATTCCAGCATC GCCACTTTCC
PLK4 T297A	GGAAAGTGGCGATAGTGGAATCATA GCTTTTGCTAGCAG	CTGCTAGCAAAAGCTATGATTCCACTATCGC CACTTTCC
PLK4 ND	CCTGATGAGCGCCATATGGCCATG TGTGGAACTCCGAAC	GTTCGGAGTTCCACACATGGCCATATGGCGC TCATCAGG

TableS2. List of primers used for dsRNA synthesis

Name	CG number	Forward (5'-3')	Reverse (5'-3')
GFP	-	TAATACGACTCACTATAGGGAG ACTTCAGCCGCTACCCC	TAATACGACTCACTATAGGGAGA TGTCGGGCAGCACG
mcherry	-	TAATACGACTCACTATAGGGAT GGTGAGCAAGGG	TAATACGACTCACTATAGGGGTTG ACGTTGTAGG
PLK4 5'	7186	TAATACGACTCACTATAGGGAG AATTAATCCCAGGGCTGCATTA	TAATACGACTCACTATAGGGAGA AGCTAGCCTTTTTTCTGTAGAC
PLK4 3'	7186	TAATACGACTCACTATAGGGAG ATAATTGAATCAAAACTTAATT C	TAATACGACTCACTATAGGGAGA AACCTCACACTTATACAAAAAG
Slimb	3412	TAATACGACTCACTATAGGGAG AGCACAGGCCTTCACAACCACT ATG	TAATACGACTCACTATAGGGAGA TTGCAGACCAGCTCGGATGATTT

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