Regulation of autophosphorylation controls PLK4 self-destruction and centriole number

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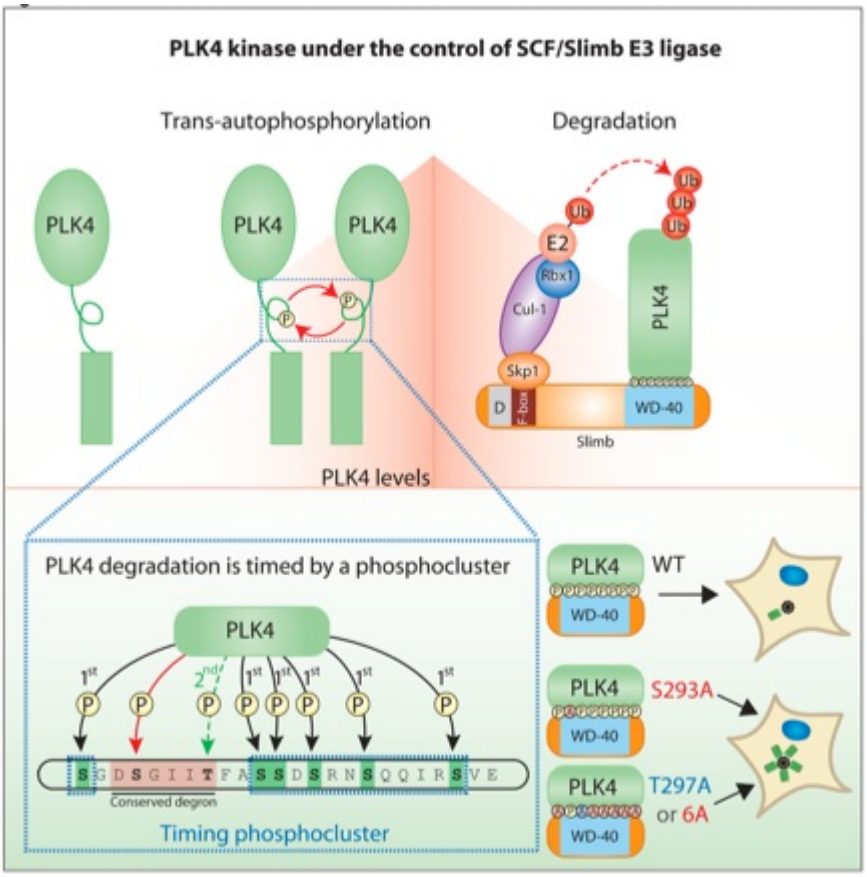
Summary

Centrosomes and their core structures, the centrioles, are the major microtubule organizing centers in animal cells. Polo like kinase 4 (PLK4) is a major player in centriole biogenesis: in its absence centrioles fail to form, while in excess leads to centriole amplification [1–5]. PLK4 levels are regulated by the SCF-Slimb/βTrCP E3-ubiquitin ligase through recognition of a conserved phosphodegron [6–13]. SCF-Slimb/βTrCP substrate binding is normally regulated by phosphorylation cascades, which integrate diverse signals and regulate timing of protein degradation in complex processes, such as circadian clocks and morphogenesis [14, 15]. Here, we show that PLK4 is a suicide kinase, autophosphorylating in residues that are critical for SCF-Slimb/βTrCP binding. We demonstrate a multisite *trans*-autophosphorylation mechanism, likely to ensure that both a threshold of PLK4 activity is attained and a sequence of events is observed before PLK4 can autodestruct. First, PLK4 *trans*-autophosphorylates other PLK4 molecules on both Ser293 and Thr297 within the degron, and these residues contribute differently for PLK4 degradation, the first being critical and the second maximising the auto destruction process. Second, PLK4 *trans*-autophosphorylates a phospho-cluster outside the degron, which regulates Thr297 phosphorylation, PLK4 degradation and centriole number. Finally, we study the physiological relevance of this process and show that PLK4-Slimb/βTrCP is a major mechanism controlling centriole number, operating in both soma and germline in *Drosophila*. As βTrCP, PLK4 and centriole number are deregulated in cancers of different tissues [14–18] our work provides novel links between centriole number control and tumorigenesis.

Graphical abstract



Highlights

1. PLK4 *trans*-autophosphorylates both degron residues Ser293 and Thr297 and a phosphocluster that surrounds them
2. Ser293 is critical whereas Thr297 maximises PLK4 degradation, both ensuring centriole number control
3. Thr297 phosphorylation is regulated by phosphocluster phosphorylation
4. The PLK4 degron controls kinase protein levels and centriole number in different tissues in the fly

Results

The levels of PLK4 in *Drosophila* and human cells, and of its homologue Zyg1 in *C.elegans*, are regulated by the SCF-Slimb/βTrCP E3 ubiquitin ligase complex [6–13, 19]. PLK4 contains a conserved phospho-degron (DpSGXXpT), whose sequence is often found in Slimb/βTrCP substrates ([6–13]; Figure 1A). Typically these substrates present one or more phospho-degrons within their sequence, which mediate binding to SCF-Slimb/βTrCP upon phosphorylation [14, 15]. Degron identity and regulation of phosphorylation are crucial aspects to control protein degradation and ultimately cellular and developmental processes [14, 15]. To fully understand how SCF/Slimb-βTrCP substrates are regulated it is particularly important to address how phosphorylation of each individual site in the degron is modulated and how the activity and localization of the kinase(s) that phosphorylate the degron(s) is controlled.

We and others found that phosphorylation of two residues (Ser293 and Thr297) within the PLK4 SCF-Slimb/βTrCP degron is critical for PLK4 degradation and depends on PLK4 activity. First, a PLK4 variant that displayed both residues mutated to alanine, hence not being targeted for phosphorylation, showed much weaker affinity for Slimb/βTrcp, resulting in high protein stability and centriole amplification [7, 9–11, 13, 20]. Second, inactive PLK4 kinase was shown to be stable when compared to the WT [6, 10, 20]. Moreover, PLK4 was shown to autophosphorylate a region that includes the degron [6, 10, 11], but the identity of the phosphorylated residues was not determined due to their close packing [6, 10]. Therefore it was suggested that autophosphorylation of degron residues and/or residues surrounding the degron contribute to PLK4 binding to Slimb/βTrCP and consequent PLK4 degradation [6, 10, 11].

Although progress was made towards elucidating PLK4-induced auto destruction, we lack mechanistic understanding on how that process is regulated, ensuring a window of time for PLK4 activity on centriole biogenesis. It is not currently known which specific phosphorylation residues are involved in PLK4 degradation, how their phosphorylation is regulated and how this impacts on centriole number control. Furthermore it is not known and is highly debated whether this is a direct mechanism (discussed in [11, 21, 22]). On one hand, PLK4 may autophosphorylate residues within and/or outside of the degron that regulate binding to Slimb/βTrCP [9, 10]. Alternatively, phosphorylation of PLK4 residues that promote

its degradation occurs through an indirect process mediated by a yet unknown kinase whose activity is dependent on PLK4 (discussed in [11, 21, 22]). Understanding this complex mechanism is critical to elucidate how PLK4 levels, hence centriole numbers, are regulated.

PLK4 autophosphorylates both the Ser293 and Thr297 within the degron

We first validated that *Drosophila* PLK4 kinase activity is required for its own degradation through the Slimb pathway. We used cell lines in which PLK4 WT (Wild Type) and PLK4 KD (Kinase Dead) mutant are fused with Protein A (ProtA) and could be expressed from the inducible methallothionein promoter (pMT). Endogenous *PLK4* (*PLK4*) was depleted using dsRNA against the 5' and 3' UTR (Figure S1 A, B and S2 A, B) to prevent phosphorylation of ectopic PLK4 by the endogenous kinase. We investigated the levels of PLK4 for a period of 6 hours after inhibition of protein synthesis by cycloheximide treatment. While ProtA PLK4 KD levels remained stable throughout the course of the experiment, ProtA PLK4 WT was degraded (Figure S1 A, B). As expected, PLK4 KD mutant expressed always much higher protein levels than its WT counterpart in the context of the GFP negative control RNAi (Figure S1 C). *Slimb* depletion led to an accumulation of ProtA PLK4 WT as compared to the GFP negative control, but had minimal impact on the levels of ProtA PLK4 KD (Figure S1 C), suggesting Slimb-mediated PLK4 degradation depends on PLK4 catalytic activity. Moreover, unlike the WT counterpart [7, 13], ProtA PLK4 KD did not interact with Slimb or SkpA, although other known interactors like Asterless [23–25] were still present in PLK4 KD immunoprecipitation assays (Figure S1 D). Together, these data show that *Drosophila* PLK4 protein kinase activity is required for PLK4 degradation via the Slimb pathway.

To test whether PLK4 phosphorylates its own degron, and which specific residues are involved, we developed polyclonal phosphospecific antibodies against the PLK4 degron residues, Ser293 and Thr297 (Figure 1 B and S1 E). While phosphorylation at both residues was detected with GST PLK4 WT, it was absent from GST PLK4 WT kinase co-expressed with Lambda Phosphatase (λppase [26]) (Figure 1 B, Figure S1 E), validating those as phospho antibodies. Additionally, these antibodies did not recognize proteins specifically mutated to alanine in those residues (S293A and T297A) (Figure 1 D and Figure 4 F, G). To address whether PLK4 is able to target directly the degron residues we expressed PLK4 WT in the presence or absence of λppase, and KD in bacteria, and probed with different antibodies (Figure 1 B and

S1 E). PLK4 WT is highly autophosphorylated in bacteria, which is first evidenced by its slower mobility on gel when compared to the condition where PLK4 is co-expressed with λppase or when expressing PLK4 KD mutant (Figure 1 B and S1 E middle panels). Whereas PLK4 WT is phosphorylated at both residues in the degron, phosphorylation is lacking in PLK4 KD mutant indicating that PLK4 autophosphorylates the degron (Figure 1 B and S1 E lower panels). As kinases can extensively autophosphorylate within bacteria due to high concentrations and long incubation periods, we performed an alternative assay. We purified His MBP PLK4 that had previously been dephosphorylated in bacteria and followed its gradual autophosphorylation *in vitro* after addition of ATP (Figure 1C). Autophosphorylation of both residues within the degron was observed very early upon addition of ATP (Figure 1 C), showing that PLK4 autophosphorylates both residues within the degron in a direct manner. These results indicate that PLK4 can autophosphorylate directly both residues within the typical Slimb degron, in the absence of other eukaryotic kinases.

To investigate whether other kinases can phosphorylate the degron *in vivo*, PLK4 autophosphorylation of the degron was further evaluated in *Drosophila* tissue culture cells expressing different GFP PLK4 constructs after *PLK4* endogenous depletion (Figure 1 A, D, S2). Both antibodies failed to detect the corresponding single alanine (S293A and T297A) mutants, or the ND (Non-degradable; both residues mutated (S293A/T297A)) mutant (Figure 1 A, D), validating the site-specificity of the antibodies. Whereas GFP PLK4 WT was phosphorylated at both Ser293 and Thr297, the KD mutant was not phosphorylated in the degron, showing that PLK4 kinase activity is required for phosphorylation of both degron residues *in vivo* (Figure 1 B, D). We also observed that PLK4 ND mutant showed a broader band with slower mobility than its WT counterpart (Figure 1 D). This mobility disappeared in the context of the KD mutation (PLK4 KDND; Figure 1 D), showing that the slower mobility of the ND mutant results from extensive PLK4-dependent phosphorylation of stable PLK4 variants in residues other than the degron. This suggests PLK4 is likely to autophosphorylate many residues outside of the degron not just *in vitro* but *in vivo* as well.

Differential requirement of Ser293 and Thr297 phosphorylation for SCF-Slimb-dependent degradation of PLK4

Since PLK4 autophosphorylates both residues within the degron we wondered what is the impact of each of those events on PLK4 stability. Many SCF-Slimb substrates have the typical DS/TG(X)2+nS/T degron sequence. Both β−catenin degron phosphoserines *DSGIHS* form specific hydrogen bonds and electrostatic interactions with residues in the βTrCP WD-40 domains, suggesting a requirement for both residues concerning βTrCP docking [27]. However, this is not always the case and the contribution of each phospho-residue for efficiency and timing of substrate degradation can be different, modulating protein degradation [14, 15]. To investigate how the individual Ser293 and Thr297 within the conserved Slimb degron contribute for PLK4 degradation we analyzed the kinetics of degradation of two single site degron mutants, ProtA PLK4 S293A and ProtA PLK4 T297A. While ProtA PLK4 ND levels remained stable throughout the course of the experiment, ProtA PLK4 WT was degraded (Figure 2 A, B). Importantly, ProtA PLK4 S293A remained stable throughout the course of the experiment (similarly to ProtA PLK4 ND), whereas ProtA PLK4 T297A showed slower degradation, indicating different requirements for each residue in PLK4 degradation (Figure 2 A, B). These results suggest that while autophosphorylation of S293 is critical for PLK4 degradation, autophosphorylation of Thr297 sets the speed at which PLK4 is degraded and thus determines the efficiency of this process.

If the phosphorylation of the residues within the degron imparts differently the kinetics of PLK4 degradation, we would expect ultimately to find differences in centriole number upon expression of the different mutants. To investigate how the relative stability of each mutant impinges on centriole biogenesis, we analyzed the formation of supernumerary centrosomes. We expressed different GFP-tagged PLK4 variants, at very low levels from the leakiness of the pMT promoter, in an RNAi background for endogenous *PLK4* (Figure 2 C, D and Figure S2 A, B), and used GFP PLK4 ND as a positive control [13]. As expected we observed a systematic increase in centriole amplification (> 4 centrioles) with GFP PLK4 S293A -expressing cells relative to GFP PLK4 WT (Figure 2 C, D). GFP PLK4 T297A elicited a modest, but significant increase in centriole number compared with the WT, showing that maximum efficiency in PLK4 degradation is necessary to prevent deregulation of centrosome number (Figure 2 C,

D). The regulation of autophosphorylation of both residues is thus important for controlling the levels of PLK4 and ultimately centriole number.

PLK4 phosphorylates both Ser293 and Thr297 in trans

Our results show that PLK4 is a suicide kinase, *i.e.* whose activity determines its own degradation through autophosphorylation of two residues. This is in contrast to many other processes regulated by Slimb/βTrCP, where a third molecule (sometimes also a fourth) is required to phosphorylate the Slimb/βTrCP substrate, hence determining the time of degradation [28, 29]. How could PLK4 self-destruction be regulated so that it is not immediately degraded and PLK4 can have the time to trigger centriole biogenesis? One possibility is that phosphorylation of at least one of the residues within the degron is regulated by one or more events dependent on PLK4, but independent of other eukaryotic proteins, as degron phosphorylation can also occur *in vitro* (Figure 1 B, C).

Interaction of PLK4 molecules in *trans* is important for PLK4 degradation, but it is not known which specific residues might be phosphorylated and how this is regulated [6, 8, 11]. We tested whether PLK4 can phosphorylate one or both degron residues in *trans,* i.e. one molecule of PLK4 phosphorylating another PLK4 molecule, hence providing some temporal and/or spatial control of degradation. We co-expressed Myc PLK4 WT and GFP PLK4 KD in *Drosophila* tissue culture cells (Figure 3 A-C). We chose those tags to ensure different protein size. In this experiment we expressed much lower levels of the WT kinase as compared to the KD to reduce the probability of encounter between two WT molecules and preferentially examine the behavior of the KD in the presence of the WT (Figure 3B, right panel, left and middle lanes). When GFP PLK4 KD was co-expressed with GFP empty control (not shown) it showed a strongly defined band as expected (Figure 3 B, middle lanes). Co-expression of the WT and KD led to a shift in the mobility of the latter, likely to be associated with KD phosphorylation by the WT kinase (Figure 3 B, middle and right lanes). This shift in mobility in PLK4 KD was accompanied by phosphorylation of the degron residues (Figure 3 C, lower panels in each blot). This data suggests that PLK4 *trans*-phosphorylates both the Ser293 and Thr297 residues within the degron *in vivo*.

It is possible that PLK4 phosphorylation in *trans* involves oligomerization of the kinase [6, 8, 11]. PLK4 has a triple polo box architecture [8] that facilitates oligomerization and targeting [8, 11, 30]. It has

been suggested that homodimerization through PB1 and PB2 is important to promote PLK4 binding to Slimb and degradation [8, 11].The fact that co-expression of the dimerization domain PLK4 PB1-PB2- leads to stabilization of PLK4 WT and centriole amplification [8, 11] suggests that PLK4 targets the degron in *trans* within a dimer. Those experiments suggest that C-terminal constructs containing the polo boxes and lacking the kinase domain sequester full length PLK4 through dimerization but cannot phosphorylate it on the degron. In order to test whether dimerization through PB1 and PB2 is necessary for Ser293 and/or Thr297 phosphorylation, we co-expressed the dimerization domain of PLK4 (PLK4 PB1-PB2) with the full length in the context of RNAi for the endogenous PLK4, and accessed the impact on degron phosphorylation. If dimerization of PB1-PB2 is required for phosphorylation either at Ser293 or Thr297, then those residues should be less phosphorylated in PLK4 WT when co-expressed with PB1-PB2, as compared with GFP alone. While we observed phosphorylation at the two residues in the WT kinase, Ser293 phosphorylation was markedly reduced upon co-expression of PLK4 PB1-PB2, suggesting that dimerization through the PB1-PB2 domain is necessary for *trans*-phosphorylation at that specific residue (Figure S 3). The same was not true for Thr297, suggesting that dimerization through PB1-PB2 (cryptic polo box, CPB) is not required for Thr297 *trans*-autophosphorylation. We conclude that autophosphorylation of both residues within the degron is likely to be differently regulated, with the critical S293 residue requiring dimerization through CPB. A window of activity for PLK4 might thus be created by the fact that sufficient levels of PLK4 need to accumulate so that one molecule of PLK4 encounters another to dimerise and phosphorylate it at S293, the critical destruction residue.

A phosphocluster around the typical Slimb/βTrCP degron regulates degron phosphorylation and controls timely degradation of PLK4

Could additional mechanisms regulate the timing of PLK4 degradation? In other substrates of Slimb/βTrCP, phospho-residues outside the typical Slimb degron regulate the interaction with Slimb/βTrCP directly by raising the affinity of the substrate to the WD-40 domain in Slimb/βTrCP and/or indirectly regulating degron phosphorylation [29, 31–33]. Multisite phosphorylation-triggered degradation is a common strategy in signaling and cell cycle transitions, creating a temporal threshold [34]. *Drosophila* Slimb sequence is ~73% similar to that of human βTrCP (data not shown). The crystal structure of βTrCP

displays seven WD repeats, which help to form the conical frustum and the substrate binding groove. The positive charged amino acids in βTrCP, which play crucial role in binding to the phosphorylated, hence negatively charged, destruction motif of the substrate (e.g., β-catenin [27]), are conserved in *Drosophila* Slimb. A careful observation of the residues on the surface of the WD40 repeat region of *Drosophila* Slimb shows that positive charged residues are regularly distributed along the slant surfaces of the conical frustum (data not shown). *Drosophila* PLK4 has several putative phospho-residues around the *DSGIIT* degron (Figure 4 A), several of which might be autophosphorylated as they fit into the broad PLK4 phosphorylation consensus site (reviewed in [21]). This part of the protein is highly phosphorylated in murine PLK4 but the identity of these residues is unknown due to their close packing that hindered their identification [6, 10]. Six of those residues in *Drosophila* are conserved in murine PLK4, where they are likely to be autophosphorylated in *vitro* and known to participate in PLK4 degradation [10] (Figure 4 A). Mass spectrometry of *in vitro* reactivated PLK4 enabled us to identify individual phosphorylation in at least 5/6 of those residues, plus the Ser293 and Thr297 at the degron (Figure 4 A and S4 A). The phosphorylated residues present around the destruction motif of PLK4 might thus play a very important role in regulating the kinetics of PLK4 binding to Slimb, through electrostatic interactions.

To address whether and how multisite phosphorylation impacts in PLK4 degradation and centriole number control, we generated a mutant where we converted all of those conserved residues to alanine (PLK4 6A; 6 alanine mutant; Figure 4 A). One hypothesis is that phosphorylation of those residues regulates direct binding to Slimb. To evaluate whether phosphorylation at the 6 conserved phospho-residues around *Drosophila* Slimb degron is important for Slimb binding and PLK4 degradation we analyzed PLK4 6A affinity for Slimb and its degradation, using ProtA PLK4 ND as a positive control. Binding to Slimb and PLK4 degradation kinetics were both impaired with PLK4 6A in comparison to the negative control (ProtA PLK4 WT) (Figure S 4, 4 B). Impairing binding to Slimb and degradation with the PLK4 6A mutant should ultimately lead to centriole amplification *in vivo*. Indeed we observed that expression of PLK4 6A leads to centriole amplification to a similar extent to PLK4 Thr297A (Figure 4 D,E). As with other degron mutants PLK4 6A is localized at the centrioles (Figure 4 E). These data indicate that phosphorylation around the degron in *Drosophila* PLK4 is required for efficient binding to Slimb and hence for centriole number control.

In other Slimb/βTrCP substrates, phosphorylation of similar clusters primes the timed phosphorylation of the conserved degron [29, 31–33]. Phosphorylation at those clusters controls when the conserved degron is phosphorylated and consequently determines when the substrate is degraded [28]. We tested whether there are regulatory phosphorylations that modulate PLK4 levels, which could enforce a sequence of events. We found that neither phosphorylation of Ser293 or Thr297 depends on each other phosphorylation (Figure 4 F,G). However, while phosphorylation of Ser293 was not affected in a PLK4 6A mutant (Figure 4 F), phosphorylation of the Thr297 residue was highly impaired (Figure 4 G), suggesting that PLK4 phosphorylation around the degron primes PLK4 autophosphorylation at the Thr297 residue. The fact that Ser293 phosphorylation is well detected in the PLK4 6A mutant, together with its proper centriole localization and ability to induce centriole formation (Figures 4 D,E and F) strongly suggest that those mutations do not substantially alter the structure of the protein, and can be used to infer the behavior of non-phosphorylated PLK4 at the area surrounding the degron. Increasing the phospho-occupancy around the degron thus regulates the phosphorylated state of the Thr297 residue within the degron. This result suggests that phospho-occupancy around the degron is important to set PLK4 degradation speed.

Finally, we tested whether phosphocluster phosphorylation may also occur in *trans*. For this experiment we used priming of Thr297 phosphorylation as an indirect readout of phosphocluster phosphorylation. We expressed simultaneously Myc PLK4 ND with either GFP PLK4 KD 6A or GFP PLK4 KD and asked whether phosphorylation of PLK4 KD 6A was impaired at Thr297. Myc PLK4 ND was used to simplify the experiment as it is not detected by the Ser293 and Thr297 phosphospecific antibodies. If phosphorylation of the cluster were to occur in *cis* then neither GFP PLK4 KD 6A or GFP PLK4 KD should be phosphorylated in the cluster when co-expressed with Myc PLK4 ND; as such both should show reduced levels of phosphorylation at the Thr297 residue. However, if phosphorylation of the phosphocluster were to occur in *trans*, we would expect GFP PLK4 KD 6A to show decreased phosphorylation at the Thr297 when compared with GFP PLK4 KD control. Indeed, when PLK4 KD 6A is coexpressed with Myc PLK4 ND there is a marked decrease in phosphorylation at Thr297, indicative that phosphorylation of the phosphocluster occurs *in trans in vivo* (Figure 4 H,I).

Together these results show that *trans*-autophosphorylation of a phosphocluster regulates Thr297 phosphorylation, suggesting both the need for a PLK4 concentration threshold, plus the presence of sequential events in timing PLK4 phosphorylation and subsequent degradation.

PLK4 degradation in the organism

Total centriole number can vary between tissues [35] .While cycling cells keep their number of centrioles constant between 2 and 4, multiciliated cells assemble 200 to 300 centrioles/basal bodies [35]. In the male germline centriole duplication can be uncoupled from the cell cycle. In the female germline, centrioles disappear during oogenesis, after which centriole number is reconstituted during fertilization with the entry of the sperm centriole ([35], Figure 5A). PLK4 plays a universal role in centriole number control [2–5, 36, 37]. Its activity is essential for centriole formation in somatic cells [4, 5] and in the germline in *Drosophila* [2–4, 37], and the formation of multiple centrioles/basal bodies in multiciliated cells correlates with very high levels of PLK4 [38]. If SCF/Slimb-mediated degradation of PLK4 is a pathway that is always present to control PLK4 levels, and hence centriole number, we would expect it to operate in different tissues. Neuroblasts of *Slimb* mutants in *Drosophila* show extra centrosomes, suggesting that the SCF/Slimb complex is important for PLK4 degradation *in vivo* in somatic cells [39]. A similar phenotype was found in fibroblasts from mice that are mutant for βTrCP. However, direct control of PLK4 degradation and consequent centriole number by the SCF/Slimb complex has only been shown in somatic cells [6–11, 13]. To understand if this mechanism is a general pathway for centriole number control, we investigated whether the PLK4 degron is required for regulation of PLK4 levels in both the male and female germlines in *Drosophila*. We created transgenic lines expressing either PLK4 WT or PLK4 ND. The constructs were integrated by recombination within the same defined genomic loci to ensure the same mRNA expression levels between different lines, thus allowing for direct comparison between PLK4 WT and ND protein levels [40].

The analysis of total PLK4 levels in the female germline revealed a two-fold increase in PLK4 ND GFP as compared to the PLK4 WT GFP control (Figure 5 A, B and S5 A). We and others have observed that centrioles still disappear during oogenesis, even upon overexpression of high protein levels of PLK4 in the female germline [2, 3]. However, in virgin females overexpressing high levels of PLK4, upon exit of

meiosis, centrioles are formed *de novo* [3, 37]. We looked into the centriole phenotype of eggs laid by virgin females expressing PLK4 WT GFP or PLK4 ND GFP from the same loci and promoter, at a time where centriole *de novo* formation upon PLK4 overexpression was previously described to begin [2]. We observed a two fold increase in the number of centrioles formed *de novo* with expression of PLK4 ND GFP when compared with PLK4 WT GFP (Figure 5 C).

Expression of PLK4 ND in the male germline resulted in a very strong effect on PLK4 levels (Figure 5 D, E), using a promoter that becomes active before the meiotic divisions (Bam-Gal4). Using the same exposure for both PLK4 WT GFP and PLK4 ND GFP we could observe a much wider band of expression of the mutant, showing that ND PLK4 GFP is stabilized after induction of its expression as compared to PLK4 WT GFP. PLK4 stabilization was associated with centriole amplification (Figure 5 F, G), and male sterility (Figure 5H). Together, these data shows that the PLK4 degron is functional *in vivo* in different tissues and suggests the SCF/Slimb complex is part of a molecular module that generally controls centriole number.

Discussion

PLK4 levels are regulated by the SCF-Slimb/βTrCP E3-ubiquitin ligase complex; as a consequence, in the absence of this control several centrioles are generated around each mother [6–13, 20]. Slimb/βTrCP-containing regulatory circuits normally operate using built-in time delays that couple protein degradation to different biological processes [14, 15, 41]. Simultaneous mutation to alanine of the two phosphorylatable residues within the PLK4 Slimb/βTrCP phosphodegron was shown to impact on PLK4 degradation [7, 9–11, 13, 20]. Moreover, PLK4 degradation via Slimb/βTrCP was reported to be mediated by its own activity [6, 10, 20]. Here we show how PLK4 controls its protein levels (Summarised in Figure S6). *Drosophila* PLK4 autophosphorylates both Ser293 and Thr297 residues within the phosphodegron recognised by SCF-Slimb/βTrCP (Figure 1 and S1). Here we show that each residue within the degron is regulated differently in a PLK4 dependent manner. While phosphorylation of both Ser293 and Thr297 occurs in *trans* only the former depends on PLK4 dimerization (Figure S3). Moreover, while Ser293 is critical for PLK4 degradation, phosphorylation of Thr297 maximizes the efficiency of degradation (Figure 2). Finally, PLK4 trans-autophosphorylates a phosphocluster that regulates Thr297

phosphorylation and binding to Slimb. In summary, two broad mechanisms are likely to ensure a window of PLK4 activity before it self-destructs: i) multiple transphosphorylating events are likely to ensure that a minimal amount of PLK4 accumulates before it targets itself for degradation; ii) phosphocluster phosphorylation ensures an order of events. Finally, we show that PLK4 and the SCF/Slimb complex control centriole number in different tissues, being essential both in cultured somatic cells and in the germline in the fly (Figure 5 and S5).

PLK4 self destructs

A destruction motif, DS/TG$(X)_{2+n}$S/T, where the Ser/Thr residues are phosphorylated by specific kinases [14, 15, 41] is thought to determine the timed degradation of SCF-Slimb/βTrCP substrates [14, 15, 41]. Here we show for the first time that PLK4 phosphorylates both Ser293 and Thr297 residues within the degron (Figure 1 and 4 A). We could not detect phosphorylation in the PLK4 KD mutant *in vivo* (Figure 1 D), showing that no other kinase can account for phosphorylation of those sites in a PLK4-independent manner *in vivo*. Detection of phosphorylated PLK4 in bacteria in both residues (Figure 1 B, S1 E, 4 A and S 4A) shows that PLK4 can target these sites directly. Moreover, reactivation experiments using purified PLK4 show that autophosphorylation in the degron can occur within a very short time *in vitro*, supporting the specificity of this mechanism (Figure 1C). It is still formally possible that another kinase might target the degron in a PLK4 dependent manner. However, a kinome RNAi screen assaying PLK4 levels failed to produce a candidate [6], supporting our evidence that PLK4 regulates its own degradation in a direct manner.

Deciphering the PLK4 degron, a phosphocluster and the accuracy of PLK4 degradation

SCF-Slimb/βTrCP-mediated degradation normally requires phosphorylation of both phospho-residues within a typical degron [14, 15, 41], but each residue may have different roles in the regulation of degradation [42, 43]. We show for the first time that Ser293 and Thr297 within the PLK4 degron have different contributions for PLK4 degradation, the Ser293A mutation rendered PLK4 as stable as the ND or KD form, whereas PLK4 T297A showed slower degradation (Figure 2 and S1 A, B). It is thus likely that Ser293 is the docking residue to bind to Slimb as in other substrates of this complex [43], being critical but not sufficient to ensure accurate PLK4 destruction. Thr297 phosphorylation might increase PLK4-Slimb affinity and thus accelerate targeting and maximise timely destruction of PLK4. In accordance with this we

found that mutagenesis of Thr297A alone impairs binding to Slimb (Figure S4 B). Individual phosphorylation of both residues is thus critical as expression of GFP PLK4 Thr297A or GFP PLK4 Ser293A mutants led to significant centrosome amplification (Figure 2 B, C).

We investigated a phosphocluster that regulates the phosphorylation of Thr297 and maximizes the efficiency of PLK4 degradation. Most of the conserved Ser around the PLK4 degron fit into the broad PLK4 consensus (reviewed in [21]) and are phosphorylated *in vitro* by PLK4 (Figure 4 A and S 4A). Moreover mutation of those residues (6A mutant), leads to impaired phosphorylation at the Thr297 residue within the degron *in vivo*. This mutant shows less binding to Slimb, slower degradation of PLK4 and centriole amplification (Figure 4 and S 4). It is possible that those residues additionally regulate binding to Slimb/βTrCP in a Thr297 independent manner, perhaps by increasing the negative charges that interact with the positive charged amino acids in βTrCP/Slimb, which play crucial role in binding to the destruction motif of the substrate (e.g., β-catenin [27]).

Mechanisms coupling PLK4 activity and degradation

While degradation of other substrates of Slimb/βTrCP is determined by extrinsic factors, such as the activity of degron kinases, we have uncovered two aspects of PLK4 degron autophosphorylation that may provide a window of time for PLK4 to promote centriole biogenesis before it autodestructs. First, since phosphorylation occurs in *trans* in all phosphorylation events tested, a minimal concentration of PLK4 needs to be present before a PLK4 molecule encounters another and phosphorylates it. Thus, the requirement for Thr297 regulatory phosphorylations, and the fact that they occur in *trans*, contributes to further delay full degron phosphorylation, ensuing timely PLK4 degradation and centriole number control.

How is PLK4 autoregulation linked to timing centriole biogenesis in the cell cycle? Both PLK4 mRNA and protein levels increase from interphase to mitosis ([20, 44] and our own unpublished results). Higher PLK4 levels in mitosis suggest that PLK4 attains higher activity at this stage and therefore is likely to be more phosphorylated at the degron. However, it has been shown that PP2A/Twins phosphatase counteracts Slimb mediated degradation of PLK4 in mitosis [6]. One possibility is that an additional layer of regulation is conferred by the concerted action of this and/or other phosphatases at both the degron phospho-residues and phosphocluster that surrounds them. The combination of the factors discussed

here is likely to lead to the described maximum PLK4 levels found in mitosis, linking to the cell cycle and perhaps other aspects of cellular physiology, before PLK4 promotes its own degradation.

Implications of PLK4 degradation in vivo

Previous work showed that PLK4 and Zyg1 regulation by the SCF/Slimb complex is essential in Human and *Drosophila* somatic tissue culture cells and *C. elegans* embryos [6–13]. Neuroblasts from *Drosophila Slimb* mutants [39] and fibroblasts from βTrCP mutant mice [45] also show centrosome amplification, suggesting that regulation of PLK4 by this ubiquitin ligase is also important in somatic cells *in vivo* in *Drosophila*. However, those phenotypes in flies and mice could be due to misregulation of other Slimb/βTrCP substrates. We now show that the SCF/Slimb complex regulates PLK4 levels *in vivo*, being required for centriole number control in both the female and male germline and impacting on their fertility. The SCF/Slimb and PLK4 generally control centriole number in animals and are deregulated in many cancers. Centriole number is altered in the majority of cancers and its manipulation is widely discussed for diagnosis and therapeutics [14–17]. The knowledge that βTrCP controls centriole number in different tissues may offer new opportunities to interfere with centriole number in disease.

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Figures and Legends

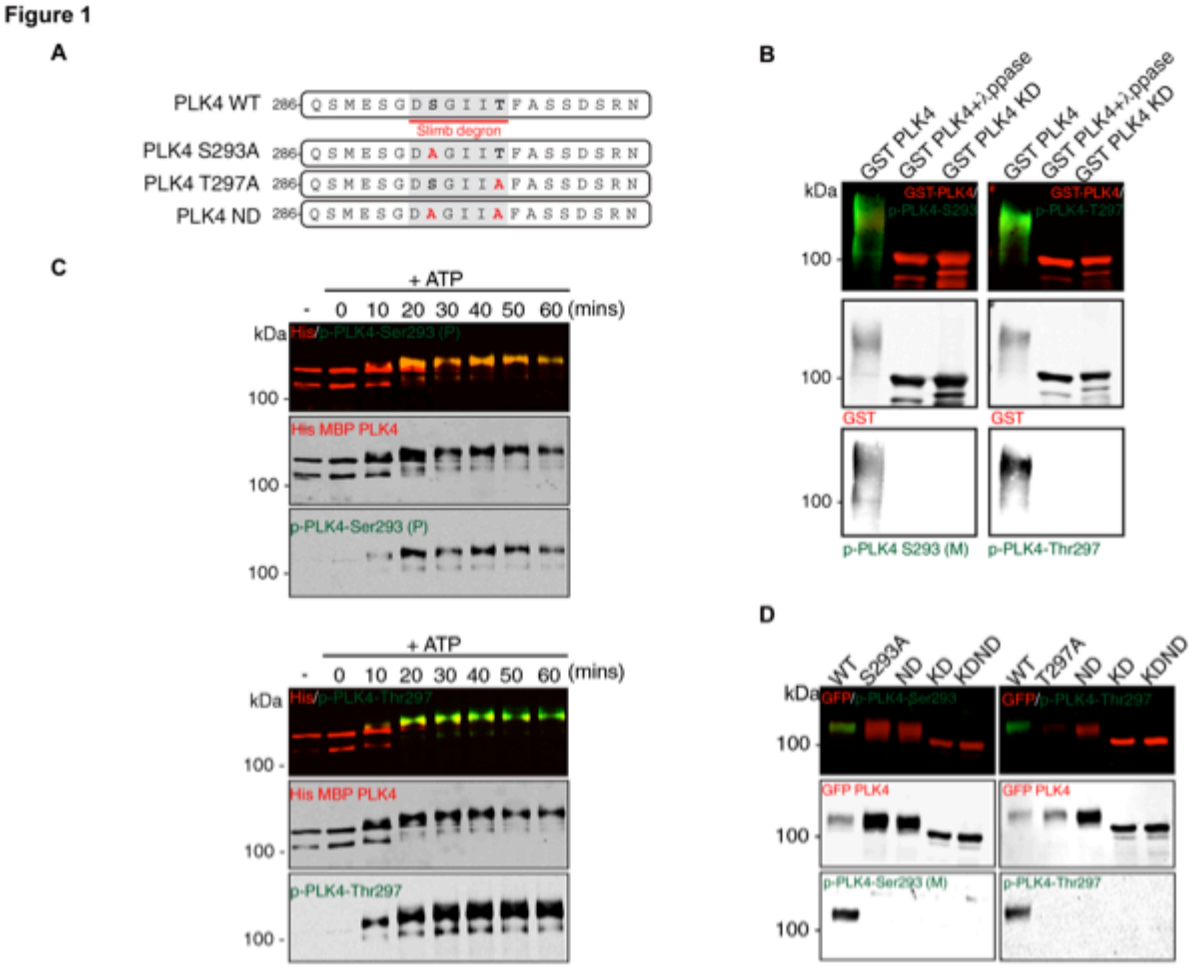

Figure 1. PLK4 targets itself for degradation by autophosphorylation of both Ser293 and Thr297 residues within the degron. (A) **Consensus degron in PLK4 sequence for Slimb binding**. Position of the first aminoacid residue is shown. Degron is highlighted in gray and mutated Ser293 and/or Thr297 to alanine are shown in red. PLK4 WT, wild type; PLK4 S293A, S293 was mutated to alanine; PLK4 T297A, T297 was mutated to alanine; PLK4 ND, non-degradable, both residues were mutated to alanine. (B) **PLK4 autophosphorylates the degron directly in bacteria**. pGEX-2RBS-GST PLK4 WT, WT + λppase and KD were expressed in bacteria at 25ºC. The soluble fractions were probed for p-PLK4-Ser293 (M) or p-Thr297 and GST. Note that with PLK4 KD or WT co-expressed with λppase there is no autophosphorylation of either Ser293 or Thr297 residues. PLK4 is highly autophosphorylated in bacteria,

leading to the appearance of a smear, which disappears upon expression of the phosphatase. We always observed the appearance of a lower mobility form, which might be the product of degradation. (C) **PLK4 autophosphorylates the Ser293 and Thr297 *in vitro.*** Autophosphorylation of purified dephosphorylated His MBP PLK4 was followed for 60 minutes after reactivation of the kinase, following addition of ATP. Samples were taken at the indicated time points and tested by western blot for phosphorylation at the Ser293 and Thr297 residues. Note that phosphorylation on the two residues is absent from the sample where ATP was not added, while a very faint signal can be seen as soon as ATP is added (0 mins) and clearly after 10 min, reinforcing the specificity of the phosphorylation. A likely product of degradation is always seen below the PLK4 band (D) **PLK4 autophosphorylates the degron *in vivo***. DMEL cells transiently transfected with pMT-GFP PLK4 WT, S293A, T297A, ND, KD, KDND (both KD and ND mutations) constructs after endogenous *PLK4* depletion were induced with 500 µM $CuSO_4$. Cell extracts were then prepared and analyzed by western blotting for p-PLK4 Ser293 (M) or p-PLK4 Thr297 and GFP (to detect total PLK4). Note that contrary to PLK4 WT, the PLK4 KD mutant is not phosphorylated at both degron sites. The ND mutant is highly stabilized and autophosphorylated; combination of the ND, KD (NDKD) mutations abolishes electrophoretic mobility (See also Figure S1 and S2).

Figure 2

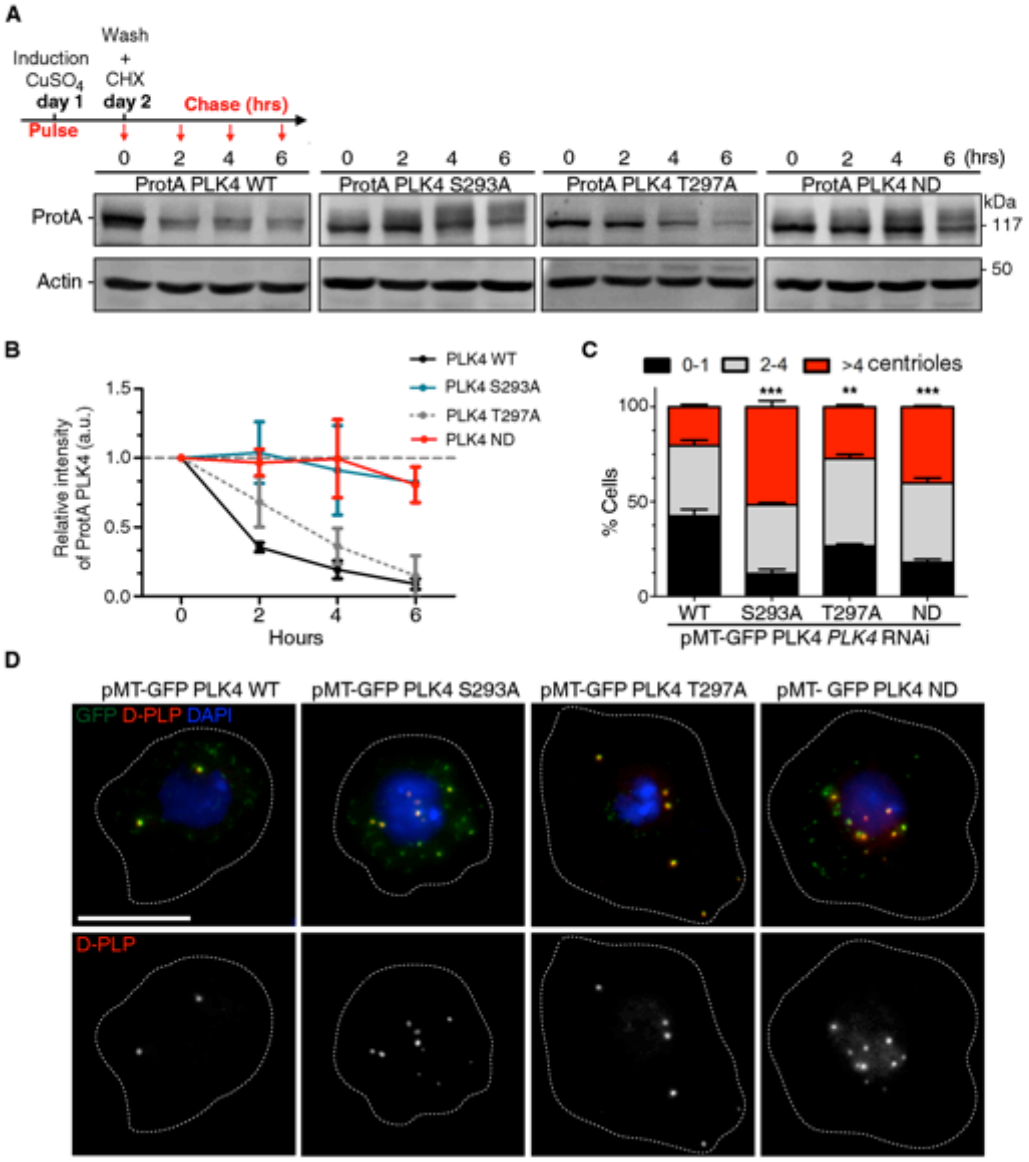

Figure 2. Phosphorylation of the Ser293 residue within PLK4 degron is critical, while phosphorylation of Thr297 maximises the efficiency of PLK4 degradation and centriole number control. (A, B) **Mutation of Ser293 to alanine blocks PLK4 degradation.** (A) pMT-ProtA PLK4 WT, S293A, T297A or ND transfected DMEL cells were induced with $CuSO_4$ so that similar levels of protein where present at the beginning of the pulse-chase. 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 intensity values shown in panel (A). These values were normalized to the actin loading control. Three independent experiments were used to generate the average for each time point. Error bars represent ± SEM. Note that while both ProtA PLK4 S293A and PLK4 ND remain stable, ProtA PLK4 WT and T297A levels decrease with time. (C, D) **Phosphorylation of both Ser293 and Thr297 residues is required to control centriole number.** (C) DMEL cells were transiently transfected with pMT-GFP PLK4 WT, S293A, T297A or ND constructs in an endogenous PLK4 RNAi background (*PLK4*). Due to the leaky nature of the metallothionein promoter (pMT), the experiments were conducted in a non-induced context. To score centrioles, cells were fixed and stained against D-PLP and DNA (counterstained with DAPI). Data are the average of 3 independent experiments ± SEM (n=100 cells in each experiment). Expression of PLK4 S293A, T297 and ND constructs led to a statistically significant increase in centrosome amplification (more than 4 centrioles) when compared to PLK4 WT (*** p<0,001, ** p<0,01, using Pearson's $\chi 2$ test). RNAi for PLK4 results in an increase in cells with 0 and 1 centrioles that is not fully rescued with expression of the WT construct, and is partially rescued with the other more stable constructs (for phenotype in the absence of depletion of endogenous PLK4 please see Supplemental Figure 2). (D) Representative images of cells analyzed in panel (C) GFP PLK4 proteins are represented in green, D-PLP in red, and DNA in blue. Individual cells are outlined by dashed lines that represent the cell outline as judged by the D-PLP background signal. Scale bar=10μm (See also Figure S2).

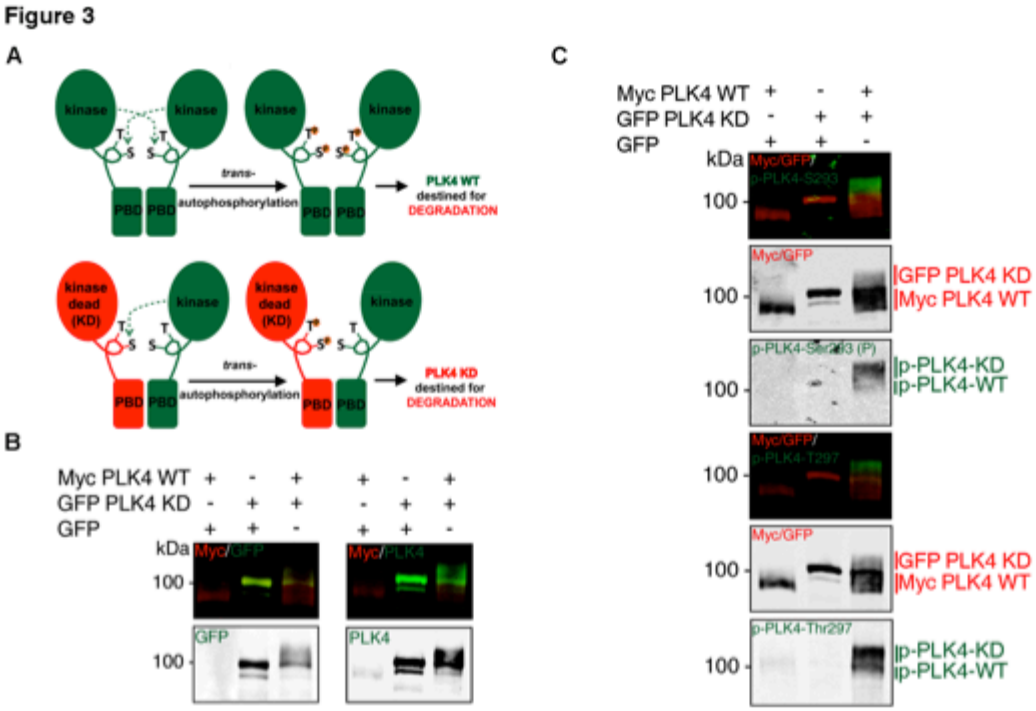

Figure 3. PLK4 trans-autophosphorylates the Slimb degron. (A) **Schematic representation of PLK4 *trans* auto-phosphorylation and expected regulation of protein levels.** PLK4 has a triple polo box architecture that facilitates oligomerization and targeting ([8, 30], here generally called PBD). Trans-phosphorylation might be dependent or not on dimerization through the polo-boxes, in particular PB1-PB2 [8, 11]. (B, C) **PLK4 *trans*-autophosphorylates the Slimb degron**. (B) ***Trans*-autophosphorylation destabilizes PLK4 KD.** *left,* Cell extracts were prepared and analyzed by western blotting for GFP (to detect GFP PLK4 KD) and Myc (to detect Myc PLK4 WT). Note that in the presence of Myc PLK4 WT, GFP PLK4 KD becomes phosphorylated (lower mobility bands). *Right,* Cell extracts were analyzed by western blotting for Myc and PLK4 antibodies (for comparison of PLK4 expression under the act5 (PLK4 WT) and pMT promoter (PLK4 KD – expressed 52 fold more)). (C) **PLK4 *trans*-auto phosphorylates both the Ser293 and Thr297 residues.** Combinations (as indicated) of act5-Myc PLK4 WT, pMT-GFP PLK4 KD and pMT-GFP constructs were transfected in DMEL cells after endogenous depletion of *PLK4* (*PLK4)*. The pMT constructs were induced with 500 μM $CuSO_4$, to ensure higher protein expression as compared to the act5 promoter. Note that co-expression of Myc PLK4 WT leads to phosphorylation of

both Ser293 and Thr297 in a PLK4 KD mutant. Note that since Myc PLK4 WT is expressed at much lower levels (panel B, *right*) it is impossible to detect its phosphorylation in the left lane (See also Figure S2 and S3).

Figure 4

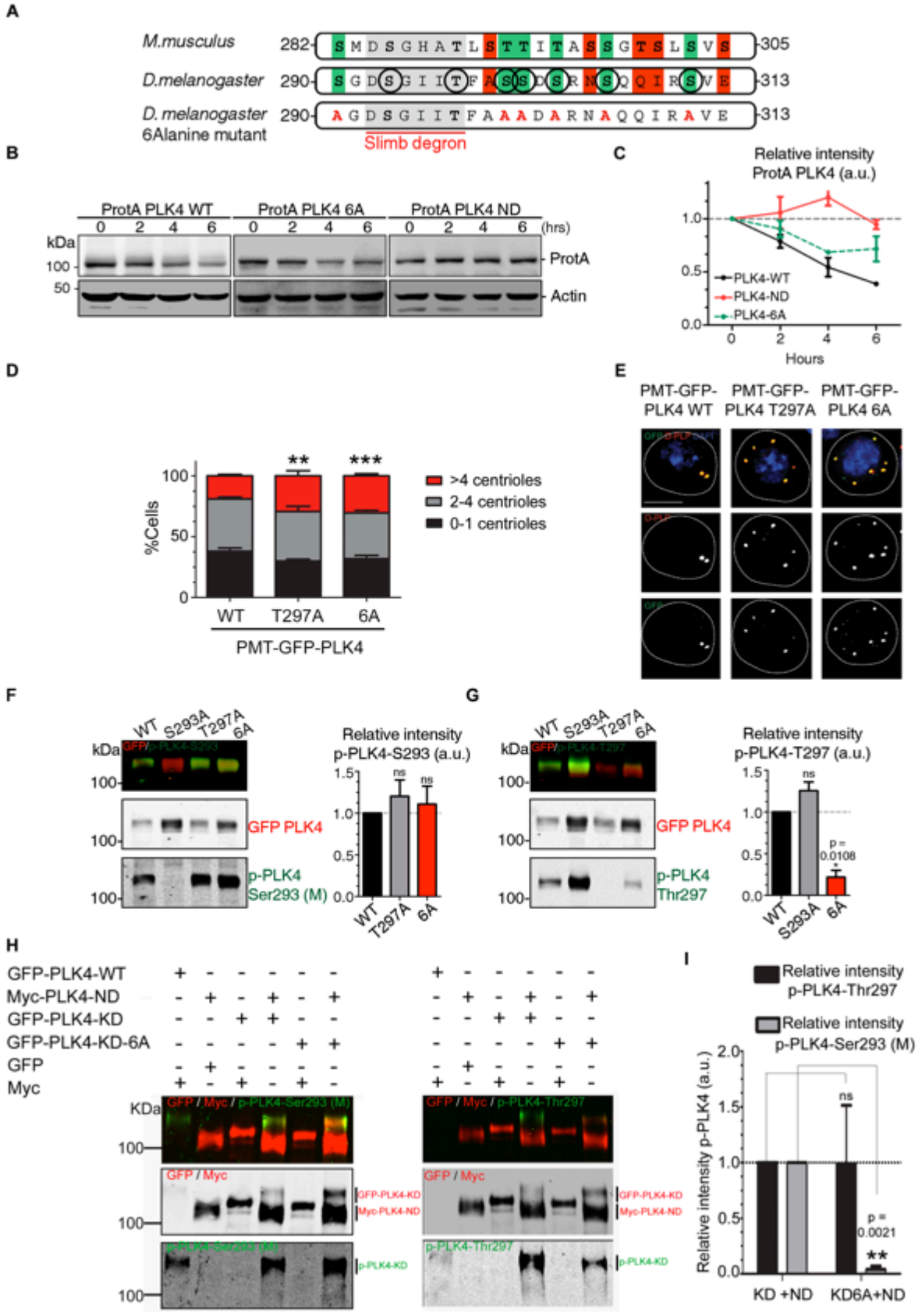
A
M.musculus
D.melanogaster
D. melanogaster
6Alanine mutant
Slimb degron
B
ProtA PLK4 WT
ProtA PLK4 6A
ProtA PLK4 ND
(hrs)
kDa
ProtA
Actin
C
Relative intensity
ProtA PLK4 (a.u.)
PLK4-WT
PLK4-ND
PLK4-6A
Hours
D
%Cells
>4 centrioles
2-4 centrioles
0-1 centrioles
WT
T297A
6A
PMT-GFP-PLK4
E
PMT-GFP-PLK4 WT
PMT-GFP-PLK4 T297A
PMT-GFP-PLK4 6A
F
GFP PLK4
p-PLK4 Ser293 (M)
Relative intensity
p-PLK4-S293 (a.u.)
G
GFP PLK4
p-PLK4 Thr297
Relative intensity
p-PLK4-T297 (a.u.)
H
GFP-PLK4-WT
Myc-PLK4-ND
GFP-PLK4-KD
GFP-PLK4-KD-6A
GFP
Myc
I
Relative intensity
p-PLK4-Thr297
Relative intensity
p-PLK4-Ser293 (M)
Relative intensity p-PLK4 (a.u.)
KD +ND
KD6A+ND

Figure 4. A phosphocluster outside the PLK4 degron controls phosphorylation within the degron, PLK4 degradation and centriole numbers. (A) Alignment of *D. melanogaster* and *M. musculus* PLK4 24-amino acid domain [10] shows that 6 phospho-residues outside the degron are conserved. From these 6 residues, we identified 5, plus Ser293 and Thr297, as being autophosphorylated *in vitro* by *D. melanogaster* PLK4 (residues highlighted with a black circle (see Figure S4 A for more details on experiment and peptides identified by mass spectrometry)). The sequence of the *Drosophila* PLK4 6 alanine mutant is also represented. The positions of the first and last amino acid residues are shown. Color code: green highlight – conserved residues; red highlight – non-conserved residues. (B, C) **Mutagenesis to alanine of the six conserved phospho-residues impairs PLK4 degradation.** (B) pMT-ProtA PLK4 WT, 6A, or ND transfected DMEL cells were induced with $CuSO_4$. 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 (the same experimental setup as indicated in Figure 2A). (C) Quantification of ProtA PLK4 relative intensity values shown in panel (B). These values were normalized to the actin loading control. Three independent experiments were used to generate the average for each time point. Error bars represent ± SEM. (D, E) **Phosphocluster phosphorylation is required for centriole number control.** Cells were transiently transfected with pMT-GFP PLK4 WT, T297A or 6A constructs in an endogenous *PLK4* RNAi background (*PLK4*). Due to the leaky nature of the metallothionein promoter (pMT), the experiments were conducted in a non-induced context. To score centrioles, cells were fixed and stained against D-PLP and DNA (counterstained with DAPI). Data are the average of 3 experiments ± SEM (n=100 cells in each experiment). Expression of PLK4 T297A and 6A constructs led to a statistically significant increase in centrosome amplification (more than 4 centrioles) when compared to PLK4 WT (*** p<0,001, ** p<0,01, using Pearson's $\chi 2$ test). RNAi for PLK4 results in an increase in cells with 0 and 1 centrioles that is not fully rescued with expression of the WT construct, and is partially rescued with the other more stable constructs. (E) Representative images of cells analyzed in panel (D) GFP PLK4 proteins are represented in green, D-PLP in red, and DNA in blue. Individual cells are outlined by dashed lines that represent the cell outline as judged by the D-PLP background signal. Note that the 6A mutant localizes to centrioles. Scale bar=10μm (F, G) **Phosphocluster autophosphorylation regulates Thr297 phosphorylation.**

DMEL cells transiently transfected with pMT-GFP PLK4 WT, S293A, T297A, and 6A constructs after endogenous *PLK4* (*PLK4*) depletion were induced with 500 µM $CuSO_4$. Cell extracts were then prepared and analyzed by western blotting for p-PLK4 Ser293 (F) or p-PLK4 Thr297 (G) and GFP (to detect total PLK4). The relative intensities of p-PLK4 Ser293 (F) or Thr297 (G) residues for each sample were obtained by normalization with the respective total PLK4 levels. Quantifications were performed using the Odyssey infrared image system (LI-COR). Note that the 6A mutant has impaired phosphorylation at Thr297 but not at Ser293 residue. Data are the average of 3 experiments ± SEM. (p=0,0106, t-Test). H) **Phosphorylation of the phosphocluster occurs in *trans in vivo*.** Combinations (as indicated) of pMT-GFP PLK4 WT (as a positive control for phosphorylation; low amounts are expressed), pMT-GFP PLK4 KD, pMT-GFP PLK4 KD 6A, act5-Myc PLK4 ND and GFP and Myc empty constructs were transfected in DMEL cells after endogenous depletion of *PLK4* (*PLK4)*. The pMT constructs were induced with 500 µM $CuSO_4$. Note that individual antibody blots were used to annotate which protein is which in the figure. I) Quantification of relative phosphorylation at Thr297 in panel H. The relative intensity of Thr297 for each sample was obtained by normalization with the respective total PLK4 levels. Quantifications were performed using the Odyssey infrared image system (LI-COR). Note that co-expression of Myc PLK4 ND with GFP PLK4 KD 6A leads to a marked reduction in Thr297 phosphorylation when compared with GFP PLK4 KD (See also Figure S2 and S4).

Figure 5

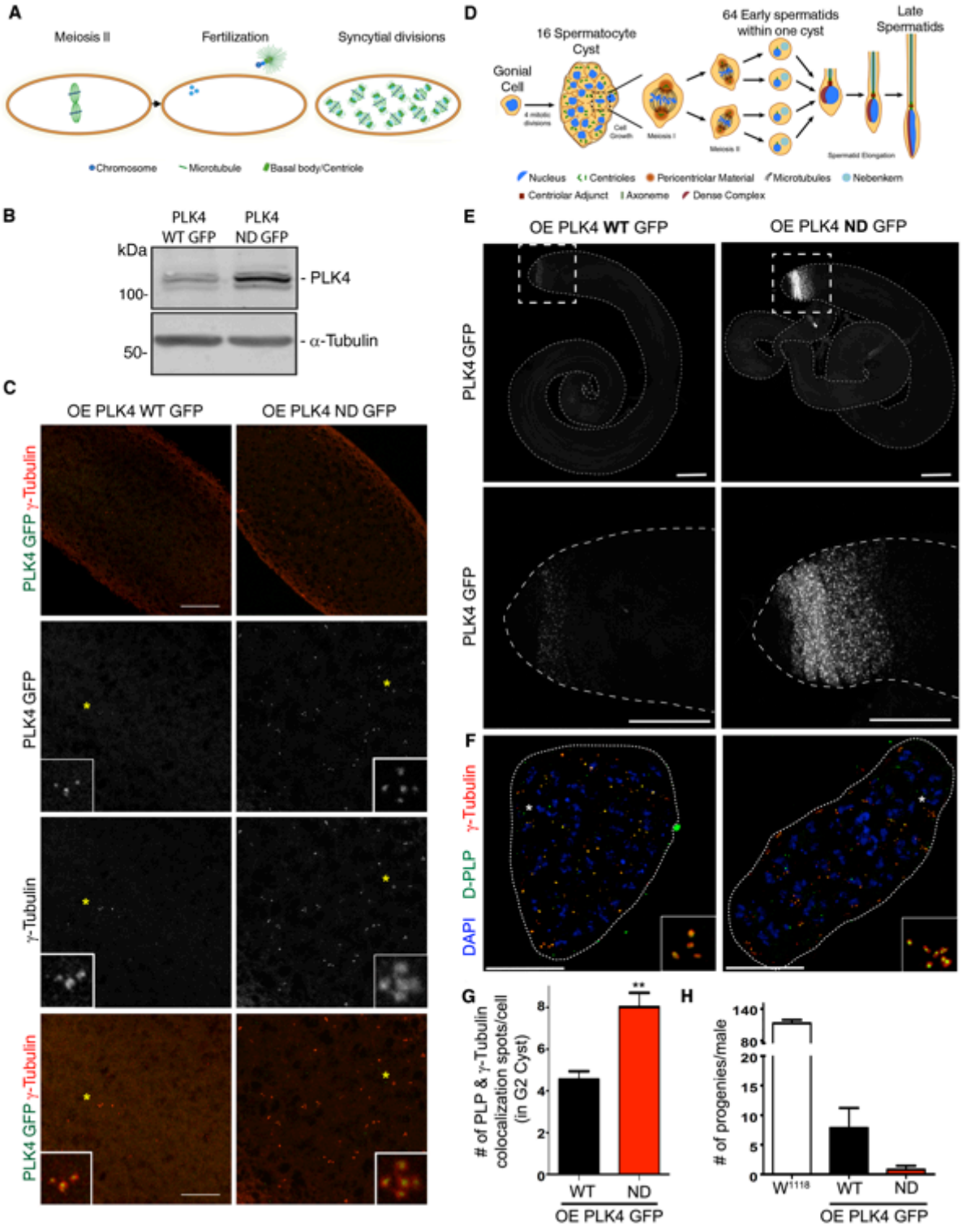
A
Meiosis II
Fertilization
Syncytial divisions
Chromosome
Microtubule
Basal body/Centriole
D
16 Spermatocyte Cyst
64 Early spermatids within one cyst
Late Spermatids
Gonial Cell
Nucleus
Centrioles
Pericentriolar Material
Microtubules
Nebenkern
Centriolar Adjunct
Axoneme
Dense Complex
B
PLK4 WT GFP
PLK4 ND GFP
kDa
100-
50-
- PLK4
- α-Tubulin
C
OE PLK4 WT GFP
OE PLK4 ND GFP
PLK4 GFP γ-Tubulin
PLK4 GFP
γ-Tubulin
PLK4 GFP γ-Tubulin
E
OE PLK4 WT GFP
OE PLK4 ND GFP
PLK4 GFP
PLK4 GFP
F
γ-Tubulin
D-PLP
DAPI
G
# of PLP & γ-Tubulin colocalization spots/cell (in G2 Cyst)
**
WT
ND
OE PLK4 GFP
H
# of progenies/male
W1118
WT
ND
OE PLK4 GFP

Figure 5. Phosphorylation at the Slimb degron is critical to control PLK4 stability and centriole number in the *Drosophila* germline. (A) ***Drosophila* early embryogenesis**. Centrioles are lost during oogenesis. Embryogenesis starts at fertilization when the sperm brings the first centriole of the egg and the two pronuclei meet. After the first mitotic division, syncytial divisions begin (Adapted from [46]) (B) **PLK4 ND GFP protein is stabilized in *Drosophila* female germline when compared with PLK4 WT GFP protein expressed from the same promoter and genomic loci.** *pUASp-PLK4 WT* GFP and *pUASp-PLK4 ND* GFP inserted by recombination in the same genomic loci 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). See Figure S3 3 for results using another insertion line. (C) **Expression of pUASp-PLK4 ND GFP leads to higher levels of centriole *de novo* formation as compared to pUASp-PLK4 WT GFP.** Note that at thirty minutes after egg laying, eggs overexpressing *pUASp-PLK4 ND GFP* show more centrioles as compared with the WT kinase. A magnified view of an area where centrioles are seen is shown. γ-Tubulin in red. PLK4 GFP in green. Scale bars are (top) 25 μm and (lower) 50 μm. Inset is 5X magnification. (D) ***Drosophila* spermatogenesis**. Cysts of 16 primary spermatocytes are formed upon the four mitotic divisions of the gonial cell. Each of these cells has four centrioles and undertakes a prolonged G2-phase, where both cells and centrioles grow. In meiosis I each centrosome is built up of two centrioles in a V-shape, whereas in meiosis II each centrosome contains only one centriole. Meiotic divisions produce a cyst of 64 interconnected spermatids, each with one centriole (Adapted from [47]). (E) **PLK4 ND GFP protein is highly stabilized in *Drosophila* male germline when compared with PLK4 WT GFP protein expressed from the same promoter and genomic loci.** *pUASp-PLK4 WT GFP and pUASp-PLK4 ND GFP* integrated at recombination site 28E7, were expressed under the male germline specific driver *BamGal4.* Immunostaining of whole mount testes shows that PLK4 ND GFP is stabilized in the male germline, being visible in many more cells. See Figure S3 3 for results using another insertion line. (F) Expression **of PLK4 ND in the male germline leads to centriole amplification.** Representative images of G2 cysts expressing pUASp-PLK4 WT GFP and pUASp-PLK4 ND GFP from the same promoter and genomic loci. Centrioles were immunostained with an antibody against the centrosome and centrioles, *Drosophila* pericentrin like protein (D-PLP) and γ-tubulin and DAPI (nucleus). Note the higher centriole

amplification observed upon expression of pUASp- PLK4 ND GFP as compared with pUASp- PLK4 WT GFP. (G) **Quantification of centriole number in pUASp-PLK4 WT and pUASp-PLK4 ND G2 cysts**. The average number of centrioles (labelled for D-PLP and γ-tubulin) per each cell in G2 cysts is represented. Data are the average of ≥8 cysts (≥128 G2 cells) from each genotype ± S.E.M. Expression of PLK4 ND, led to a statistically significant increase in centrosome amplification when compared to PLK4 WT (***,p<0,001, using Unpaired T test). (H) **Males expressing PLK4 ND GFP are sterile**. The average number of progeny produced by male of depicted genotypes is represented. Note that while males expressing pUASp-PLK4 ND GFP are fully sterile, expression *of* pUASp-PLK4 WT GFP from same promoter only leads to partial sterility. W118- Wild type strain with no insertion (See also Figure S5).