A COMPREHENSIVE ANALYSIS OF POLO-LIKE KINASE 4’S REGULATION AND ROLE IN CENTRIOLE BIOGENESIS

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Abstract

Plk4 has been termed a ‘suicide kinase’ because it promotes its own destruction to regulate protein levels. We identified numerous autophosphorylated residues within a region of Plk4 called the Downstream regulatory element (DRE). We find that phosphorylation of a single residue is sufficient for Slimb recruitment and phosphorylation of the surrounding residues builds a high affinity Slimb-binding site. These autophosphorylation events are dependent on Plk4 homodimerization, although the domains that mediate this dimerization are unknown. We show that Plk4 homodimerization is mediated by interactions between the PB1-PB2 cassette. We find that like all Polo kinases, Plk4 encodes a mechanism of kinase autoinhibition. Unlike other Polo kinases, which rely on external inputs for relief of inhibition, Plk4 is self-sufficient in relieving kinase inhibition. This relief of autoinhibition is regulated by PB3 of Plk4 and is dependent on homodimerization, thereby making homodimerization a necessary step in formation of the Slimb phosphodegron on Plk4.

Polo Boxes are known as multifunctional domains, and the Polo Boxes of Plk4 are no different. We identified numerous Slimb-mediated ubiquitination sites on PB1 as well as PB2. Furthermore, the PB1-PB2 cassette mediates the interaction between Plk4 and the N-terminus of Asterless. In Drosophila cells, Plk4 requires Asterless for centriolar localization and Asterless overexpression drives centriole amplification in a Plk4 dependent manner. This is a fascinating result as endogenous Plk4 protein levels are undetectable in S2 cells, making it hard to envision a scenario where overexpression of Asterless could shuttle a non-existent Plk4 population to the centriole to initiate duplication. We found that in addition to shuttling Plk4 to the centriole, Asterless stabilizes Plk4, likely protecting Plk4 at the centriole to allow it to ‘license’ the centriole for duplication. Moreover, we show that Asterless encodes two distinct Plk4 binding
sites: the previously described N-terminal binding site as well as a novel C-terminal binding site. We found that the interaction between the C-terminal of Asterless and Plk4 is necessary for centriole duplication while the interaction between the N-terminal of Asterless and Plk4 is expendable. Together these findings provide significant insight into Plk4 biology and the mechanisms which limit its activity.
CHAPTER 1: POLO-LIKE KINASE 4 AUTODESTROCKS BY GENERATING ITS SLIMB BINDING PHOSPHODEGRON

*All S2 cell culture work performed by J.Kleba, all in vitro work performed by D. Buster*

1.1 Summary

Polo-like Kinase 4 (Plk4) is a conserved master-regulator of centriole assembly [1]. Previously, we found that *Drosophila* Plk4 protein levels are actively suppressed during interphase [2]. Degradation of interphase Plk4 prevents centriole overduplication and is mediated by the ubiquitin-ligase complex, SCF^{Slimb/βTrCP} [3, 4]. Since Plk4 stability depends on its activity [5, 6], we studied the consequences of inactivating Plk4 or perturbing its phosphorylation-state within its Slimb-recognition motif (SRM). Mass spectrometry of in vitro phosphorylated Plk4 and Plk4 purified from cells reveals that it is directly responsible for extensively autophosphorylating and generating its Slimb-binding phosphodegron. Phosphorylatable residues within this regulatory region were systematically mutated to determine their impact on Plk4 protein levels and centriole duplication when expressed in S2 cells. Notably, autophosphorylation of a single residue (Ser293) within the SRM is critical for Slimb binding and ubiquitination. Our data also demonstrate that autophosphorylation of numerous residues flanking S293 collectively contribute to establishing a high-affinity binding site for SCF^{Slimb}. Taken together, our findings suggest that Plk4 directly generates its own phosphodegron and can do so without the assistance of an additional kinase(s).
1.2 Results and Discussion

Centriole over-duplication is prevented by limiting centriole duplication to one occurrence per cell cycle, and while numerous proteins affect centriole number, much remains to be discovered about their coordination and regulation [7]. A key component is Polo-like kinase 4 (Plk4)/Sak, a conserved master-regulator of centriole duplication [8, 9]. During interphase, Plk4 is degraded which prevents centriole over-duplication [3, 4]. Features of this degradation mechanism support the following model: Plk4 homodimerizes and phosphorylates several residues within a hydroxyl-rich region called the Downstream Regulatory Element (DRE) (Figure 1A), although precise sites of phosphorylation are unknown [5, 6, 10, 11]. Embedded within the DRE is a conserved Slimb-Recognition Motif (SRM: DpSGXXpT) whose serine/threonine residues are phosphorylated, generating a phosphodegron recognized by the F-box protein Slimb in *Drosophila* or β-TrCP in humans [3-6, 12]. F-box proteins are substrate-targeting subunits of the SCF ubiquitin-ligase which ubiquitinates Plk4, marking the kinase for proteasomal degradation [3-6, 12].

Several important questions concerning Plk4 regulation remain to be answered. First, although purified Plk4 extensively phosphorylates its DRE in vitro [5], the specific phosphorylation sites required for Slimb recognition have not been defined. Second, it is not known which specific residues are autophosphorylated. Third, it is not clear whether the Plk4 phosphodegron is generated directly by autophosphorylation or whether another unidentified kinase is required [6, 13, 14]. Thus, important features of Plk4 down-regulation remain to be discovered. To address these mechanistic unknowns, we examined which DRE residues are autophosphorylated, explored the consequences of inactivating Plk4, and determined what happens to Plk4 stability when the normal DRE phosphorylation pattern is perturbed.
Previous work has shown that kinase-dead (KD)-Plk4 is stable when expressed in cells, suggesting that kinase activity precedes its degradation [2, 5, 6, 15, 16]. KD-Plk4 expression also induces centriole over-duplication in tumor-derived cell lines when endogenous Plk4 is present [5, 6, 9]. The latter result is counterintuitive, but it was proposed that the SRM within KD-Plk4 is phosphorylated in trans after heterodimerizing with endogenous Plk4, and that phosphorylated KD-Plk4 then sequesters β-TrCP/Slimb, decreasing the available β-TrCP. Thus, endogenous Plk4 levels would increase and stimulate centriole amplification [6].

We examined whether centriole amplification is a universal consequence of KD-Plk4 expression or unique to transformed cells. We expressed metallothionein-inducible KD-Plk4 and other Plk4-EGFP constructs in S2 cells, an immortalized Drosophila cell line. After 3 days of expression, centrioles were visualized by immunostaining for PLP [17], a centriole protein that coats the surface of mature centrioles [18], and manually counted. Due to their small size, mother and daughter centrioles cannot be distinguished within an engaged pair using standard light microscopy in most fly cells. Nevertheless, the number of PLP spots is an accurate readout of centriole loss (<2 spots) and amplification (>2 spots) in these cells [2-4, 8, 10]. As expected, all Plk4 constructs localized to centrioles, though KD-Plk4 also formed small puncta in the cytoplasm that lacked PLP (Figure 1B). Under low expression conditions, only non-degradable Plk4 Slimb-binding mutant (SBM; a S293A/T297A double mutation within the SRM that blocks Slimb binding [3, 4]) significantly increased the percentage of cells with >2 centrioles (Figure 1C), as shown previously [3, 4]. However, under high expression conditions, wild-type (WT)-Plk4 induced significant centriole amplification, consistent with the model that increased Plk4 activity drives centriole over-duplication (Figure 1C). Surprisingly, KD-Plk4 high expression had the opposite effect: the percentage of cells with <2 centrioles significantly increased (Figure
Therefore, our results suggest that KD-Plk4 behaves as a dominant/negative in S2 cells, inhibiting centriole duplication. A similar result was also observed in KD-Plk4-overexpressing early Drosophila embryos [19].

Because KD-Plk4 formed puncta within the cytoplasm, we reasoned that KD-Plk4 overexpression might perturb localization of essential centriole components, such as the Plk4-binding protein Asterless (Asl) [20-22]. To test this, anti-Asl antibodies (Figure S1A) were used to examine the association of Asl with KD-Plk4 and centrioles. Endogenous Asl co-immunoprecipitated with WT- and KD-Plk4-EGFP but not control EGFP (Figure 1E). Unlike WT-Plk4, high expression of KD-Plk4 completely blocked Asl localization to centrioles (Figure 1D). This effect was directly due to KD-Plk4 binding because expression of a mutant form of KD-Plk4 lacking the Asl-binding domain (KD-Plk4-ΔPB1-PB2-EGFP) (Figure 1E) [10, 20-22] displayed normal centriole numbers and Asl localization (Figure 1C, D). These results suggest that KD-Plk4 blocks centriole duplication by sequestering Asl, thus preventing Asl from localizing endogenous Plk4 to centrioles (Figure S1B,C). The disparity in KD-Plk4 overexpressed in human cancer and Drosophila cells might reflect a fundamental difference in Plk4 regulation in these systems.

Previous work in transformed cells suggests that Plk4 homodimerizes and trans-phosphorylates to induce its degradation [5, 6]. To test whether trans-phosphorylation is a prerequisite for SCFSlimb recognition, we co-expressed EGFP- and myc-tagged KD- and WT-Plk4 in cells and evaluated their relative stabilities using quantitative immunoblotting. As expected, Plk4 was almost undetectable in cells co-expressing two WT constructs (Figure 2A, lane 1). (We verified WT-Plk4-Myc expression by depleting Slimb to stabilize this protein; Figure S2A.) In contrast, KD-Plk4 is strongly stabilized when co-expressed with another KD
protein (Figure 2A, lane 3). However, KD-Plk4 is clearly less stable when co-expressed with WT-Plk4 and migrates as a smear on SDS-PAGE (Figure 2A, lane 2), suggesting that KD-Plk4 has been phosphorylated. Therefore, the ability to trans-phosphorylate controls Plk4 stability.

We tested this further by assaying Slimb association with Plk4 and its ubiquitination state. To assess Plk4 ubiquitination, S2 cells were co-transfected with 3xFLAG-Ubiquitin (Ubi). Immunoprecipitation of EGFP-tagged WT-Plk4 retrieved the second Myc-tagged WT-Plk4 as well as endogenous Slimb, and both Plk4 proteins were robustly ubiquitinated (Figures 2B, lane 1; S2B) while negative control GFP was not (Figure S2C). In contrast, ubiquitination diminished when two KD-Plk4 constructs were co-expressed (Figures 2B, lane 2; S2B), and, as predicted from their increased stabilities, Slimb failed to associate (Figure 2B, lane 2).

If trans-phosphorylation stimulates Slimb binding, then dimerization of KD-Plk4 with WT-Plk4 should result in KD-Plk4’s trans-phosphorylation, ubiquitination, and destabilization (Figure S2B). In this case, Slimb associated with KD-Plk4-EGFP, displayed the smeary appearance of a phosphorylated species, and was robustly ubiquitinated (Figure 2B, lane 3).

The above observations support a conserved mechanism of degradation whereby trans-phosphorylation within a Plk4 homodimer induces its ubiquitination. We next used an in vitro system to ask whether dimerization affects Plk4 autophosphorylation. A construct of human Plk4 encoding the kinase domain and its SRM (amino acids 1-289) was tagged with GST to induce dimerization. Autophosphorylation of the dimeric Plk4 and the monomeric kinase (generated by cleaving GST) was compared. Whereas monomeric Plk4 displayed some capacity to trans-phosphorylate, dimeric Plk4 kinase strongly autophosphorylated under the same conditions (Figure 2C, lanes 1 and 2). Phosphorylation was specific as monomeric Plk4 did not phosphorylate GST added to the reaction (Figure 2C, lane 3). Thus, Plk4 dimerization is an
efficient mechanism for trans-phosphorylation and self-destruction.

Previous studies revealed that Slimb interacts with Plk4 through a phosphorylated motif (the SRM) near the kinase domain (Figure 2D) [3, 4, 23]. This region of 50 amino acids, the Downstream Regulatory Element (DRE), contains the SRM, is serine/threonine rich, and is extensively autophosphorylated in vitro [2, 5]. However, specific autophosphorylated DRE residues are unknown, and it is not clear whether Plk4 directly generates its own phosphodegron to recruit Slimb or whether another kinase is responsible [6, 13, 14]. First, we mapped autophosphorylated residues in vitro using tandem mass spectrometry (MS) of Drosophila Plk4 containing the kinase domain and DRE (amino acids 1-317) and identified 10 (of 13 possible) autophosphorylated DRE residues (Figures 2E and S2D; Table S1). Significantly, both SRM residues S293 and T297 were autophosphorylated as were 8 phosphorylation sites flanking the SRM. Tandem MS was also used to examine the in vivo phosphorylation state of the DRE of Plk4-EGFP immunoprecipitated from S2 cell lysates. Of the 10 residues identified in vitro, 7 were phosphorylated in vivo, including S293 (Figures 2E and S2E; Table S2). Although our results do not exclude the possibility that additional DRE residues are autophosphorylated in cells, our findings demonstrate that Plk4 can directly autophosphorylate its DRE, including the key SRM residues, S293 and T297.

To investigate what impact the phosphorylation states of DRE residues have on Plk4 stability, we mutated each hydroxyl residue to alanine within full-length Plk4. To avoid neglecting a phosphorylated residue not identified in our MS analysis, we individually mutated all 13 serine/threonine DRE residues (Figure 3A). Each Plk4-EGFP mutant was expressed in S2 cells and quantitative immunoblots were used to measure protein levels. Unexpectedly, mutation of only one residue, S293, extensively stabilized Plk4 (Figure 3B). Interestingly, the second
SRM residue, T297, had only a small impact on Plk4 stability (Figure 3B). Thus, S293 is the key residue for Slimb recognition whereas T297 makes a minor contribution. Mutation of other residues downstream of T297A also slightly increased Plk4 stability compared to WT-Plk4, suggesting that phosphorylation of these residues may also contribute to Slimb binding (Figure 3B).

To determine if mutation of S293 alone blocks Slimb binding, immunoprecipitations were performed from cells co-expressing 3xFLAG-Ubi. S293A failed to bind Slimb and ubiquitination decreased ~10-fold, whereas T297A had a moderate impact (Figure 3C-E), consistent with the observation that T297A is slightly more stable than WT-Plk4 (Figure 3B). Overexposure of the FLAG immunoblot revealed one persistent ubiquitinated species for S293A and SBM-Plk4 (Figure 3C), suggesting that an unidentified Slimb-independent ubiquitin-ligase may also regulate Plk4.

Mutations that increase Plk4 stability should induce centriole amplification [3, 4]. To test this, Plk4-EGFP constructs were expressed for 3 days and centriole numbers were measured. As expected, both S293A and T297A mutants localized to centrioles (Figure 3F). Whereas S293A significantly increased centriole number (>2 centrioles per cell) to an extent nearly identical to SBM-Plk4, T297A did not produce a significant increase (Figure 3G). Thus, S293 is the key residue for Slimb recognition, whereas T297 modestly increases the efficiency of Slimb binding but is nonessential.

We next examined whether phosphorylated DRE residues collectively regulate Plk4 by systematically mutating every hydroxyl residue, such that each new Plk4 construct contained a steadily accumulating number of alanine mutations (Figure 4A). Mutation of the 5 residues upstream of S293 had little effect on Plk4 stability, even when mutated together (Figure 4B, A1-
A5). However, once S293A was included, Plk4 stability increased significantly (Figure 4B, A6). Plk4 mutants with additional alanine substitutions downstream of S293A did not significantly elevate levels greater than A6 (Figure 4B, A7-A12) with the exception of Plk4 harboring all 13 alanine mutations (A13) which displayed a ~3-fold average increase in protein level (compared to A6; P=0.0001). These results suggest that numerous hydroxyl DRE residues, in combination with S293, function collectively to recruit Slimb.

Since S293A alone prevents Slimb binding, we predicted that Plk4 mutants harboring the S293A mutation (A6-A13) would not bind Slimb and exhibit diminished ubiquitination. Surprisingly, mutants that include the S276A mutation (A2-A5) displayed reduced Slimb binding and ubiquitination compared to WT-Plk4. As expected, mutants of Plk4 lacking S293 (A6-A13) fail to bind Slimb and are not extensively ubiquitinated (Figure 4C). Therefore, the phosphorylation state of hydroxyl DRE residues preceding the SRM can influence Slimb binding. All A1-A13 mutants also localized properly to centrioles and those constructs with increased stability (A6-A13) increased the percent of cells with >2 centrioles (Figure 4D, E, and data not shown). Thus, the trend in centriole amplification duplicates the trend in Plk4 stability.

The previous mutant series revealed that some hydroxyl residues flanking the SRM contribute to Slimb recognition and ubiquitination. To examine how these residues affect regulate Plk4, we generated a new mutant series which progressively accumulated alanine substitutions without mutating S293 (Figure S3A). Strikingly, though Plk4 stability is minimally affected by either the single T297A mutant or the aggregate mutation of the 5 hydroxyl residues upstream of the SRM (A5), the combination of these mutations (A14) stabilized Plk4 to a level almost 4-fold greater than T297A, and constructs containing additional mutations downstream of T297A are just as stable (Figure S3B). Elimination of upstream hydroxyl residues and T297
(A14) reduced Slimb binding and the extent of ubiquitination by ~50% (Figure S3C), confirming that upstream hydroxyl DRE residues contribute to Slimb binding. Additional mutations downstream of the SRM decreased Slimb binding by another 2-fold (Figure S3C, A15-A20), but did not further increase Plk4 stability consistently (Figure S3B, A15-A20). Thus, DRE residues besides S293 collectively contribute to Slimb binding and ubiquitination.

To completely isolate the contribution of non-SRM residues to Plk4 stability, we generated constructs possessing an unaltered SRM but with all hydroxyl residues flanking S293/T297 mutated to alanine (Figure S4A). This series showed a clear pattern: progressively accumulating mutations had little effect on Plk4 stability until residues downstream of the SRM were mutated (compare A1-A5 of Figure 4B with Figure S4B). Therefore, mutation of upstream hydroxyl residues has limited impact on Plk4 stability and only becomes appreciable when T297 (Figure S3B) and/or downstream residues are also mutated. As further downstream residues were mutated, protein levels displayed a near steady increase; this was accompanied by a ~5-fold reduction in the amount of associated Slimb (Figure S4B, C). All mutants localized to centrioles (Figures S3D, S4D and data not shown), and all stabilizing mutations increased centriole numbers (Figures S3E, S4E). Thus, our findings suggest that the SRM is flanked by phosphorylatable residues that positively regulate Slimb recognition. Their cumulative effect is substantial as Slimb binding is inhibited to almost as great an extent by loss of flanking hydroxyl residues (e.g., A26) as by KD or SBM (Figure S4C). Even though S293 is required for Slimb binding (Figure 3C), loss of the other hydroxyl residues can diminish ubiquitination as strongly as S293A (Figure S3C). Our data suggest that hydroxyl residues downstream of the SRM (Figure S4, A21-A26) exert a greater influence on Slimb binding, Plk4 stability, and centriole duplication than those upstream of the SRM (Figure S3, A1-A5), although the mechanistic basis
Our findings demonstrate that the Plk4 DRE influences Slimb binding, ubiquitination, and centriole duplication. Previous studies demonstrated that S293 and T297 are critical in regulating Plk4 stability [3-6], and our results support functional roles for other phosphorylatable DRE residues [5]. Furthermore, self-regulation by trans-autophosphorylation is conserved [5, 6], because co-expression of WT-Plk4 converts KD-Plk4 into a Slimb-binding species and promotes its ubiquitination and degradation in Drosophila. Moreover, dimerized Plk4 autophosphorylates in vitro more readily than monomeric Plk4.

Although autophosphorylation is necessary for Plk4 degradation, it was not previously known whether Plk4 directly generates its own phosphodegron or whether autophosphorylation serves to prime and recruit an additional kinase(s) to phosphorylate the SRM. The latter is true for the Slimb/-TrCP substrates -catenin, Ci, and Wee1; multiple kinases sequentially phosphorylate and generate the phosphodegron in these Slimb/-TrCP substrates [24-26]. Significantly, a majority of the Plk4 hydroxyl DRE residues are autophosphorylated. Therefore, our data supports a model whereby Plk4 directly generates its phosphodegron unassisted. This is consistent with our previous RNAi screen of the Drosophila kinome which did not reveal the involvement of an additional kinase in regulating Plk4 levels [2]. Our systematic mutagenesis of the DRE indicates that S293 is the critical phospho-residue for Slimb binding, and that multiple residues flanking S293, particularly those downstream, collectively generate a higher-affinity Slimb-binding region. However, mutation of all hydroxyl DRE residues except for S293 significantly increases Plk4 stability, suggesting that phosphorylation of S293 alone does not promote efficient degradation. Notably, the Plk4 DRE is not the only autophosphorylated region [13]. The importance of these additional phospho-residues in regulating Plk4 activity and/or its
1.3. Figures

Figure 1.1. High Expression of Kinase-dead Plk4 Blocks Centriole Duplication by Preventing Asterless Targeting to Centrioles

(A) Linear map of *Drosophila* Plk4 showing functional and structural domains. The Downstream Regulatory Element (DRE) is a span of approximately 50 amino acids containing the phylogenetically conserved Slimb-recognition motif (SRM). Plk4 contains three Polo-boxes
(PB) [10]. PB1 and PB2 comprise the Asl-binding region [10, 20-22].

(B) S2 cells co-expressing the indicated Plk4-EGFP construct (green) and Nlp-EGFP (a nuclear protein used as a transfection marker; green nuclei) were immunostained for PLP (red) to mark centrioles. DNA (blue). Expression of Plk4 constructs was induced with 50\(\mu\)M CuSO\(_4\). KD-Plk4 targets centrioles (arrowhead) but also forms cytoplasmic punctate aggregates (arrows). Scale, 5\(\mu\)m.

(C) Transfected S2 cells were induced to express Plk4-EGFP constructs at low (50\(\mu\)M CuSO\(_4\)) or high (1mM CuSO\(_4\)) levels for three days, immunostained for PLP, and their centrioles counted. Centriole amplification (a significant, increased frequency of >2 centrioles/cell) occurs in cells expressing SBM-Plk4 (P=0.0001) or a high level of WT-Plk4 (P=0.0002). In contrast, centriole duplication is inhibited (a significant, increased frequency of <2 centrioles/cell) in cells expressing a high level of KD-Plk4 (P<0.0001) but not KD-Plk4-ΔPB1-PB2 (P=0.29). At least three experiments were performed per construct (n = 600 cells/construct). Error bars in all figures, S.E.M.

(D) S2 cells co-expressing the indicated Plk4-EGFP construct (green) and the transfection marker, Nlp-EGFP (green nuclei), were immunostained for PLP (red) and Asterless (blue; bottom row). DNA (blue; top row). Expression of Plk4 constructs was induced with 1mM CuSO\(_4\). Scale, 5\(\mu\)m.

(E) Anti-GFP immunoprecipitates from S2 cell lysates transiently-expressing the indicated Plk4-EGFP construct (or control GFP) were probed for GFP and endogenous Asterless. Note that co-precipitating Asl is absent in the control and KDΔPB1-PB2 samples.
Figure 1.2. Plk4 is Destabilized by Trans-Autophosphorylation and Directly Autophosphorylates its SRM In Vitro

(A) The relative protein stabilities of different combinations of Plk4 constructs were analyzed by immunoblotting lysates of S2 cells transiently co-expressing the indicated EGFP- and Myc-tagged Plk4 constructs and Nlp-EGFP (used as a loading control and expressed under its
endogenous promoter). Anti-Myc immunoblots are shown at short and long exposures.

(B) Anti-GFP immunoprecipitates (IPs) were prepared from lysates of S2 cells transiently expressing 3xFLAG-ubiquitin and the indicated combinations of EGFP- and Myc-tagged Plk4 constructs. Blots of the IPs were probed with anti-GFP, FLAG, and Slimb antibodies. Note that robust ubiquitination of Plk4 corresponds to the presence of endogenous Slimb, and that co-expression of KD-Plk4 (lane 2) prevents phosphorylation (as indicated by the lack of gel-shift), Slimb binding, and ubiquitination.

(C) Autophosphorylation of Plk4 kinase domain is more efficient as a dimer compared to a monomeric species. Lanes 1-3, both purified recombinant GST-tagged (dimeric) human Plk4 kinase domain + SRM and monomeric kinase (cleaved of its GST-tag) autophosphorylates in vitro. Plk4 does not phosphorylate purified GST (lane 3). Top panel, Coomassie-stained SDS-PAGE gel; bottom panel, corresponding autoradiograph. Equimolar amounts of dimeric and monomeric kinase were assayed.

(D) The DRE contains a conserved, high percentage (~20-40%) of hydroxyl amino acids (highlighted) that are potential sites of phosphorylation. These include the conserved Ser and Thr residues within the SRM (boxed).

(E) In vitro phosphorylation sites within the DRE were identified by tandem mass spectrometry (MS) analysis of purified fly His6-Plk4 (amino acid residues 1-317, comprising the kinase and DRE domains) incubated with MgATP. Above the DRE sequence, in vitro phosphorylation sites identified with high confidence are indicated with a ‘P’ encircled with a solid line; a low confidence site (S285) is indicated with a ‘P’ encircled with a dashed line. In vivo phosphorylation sites within the DRE were identified by MS analysis of full-length Plk4-EGFP immunoprecipitated from S2 cell lysates. Identified in vivo sites are marked below the DRE.
sequence. (A very low confidence site, S271, is not marked.) At least nine of the hydroxyl residues within the DRE are phosphorylated in vitro, and seven of these residues are also phosphorylated in vivo (bottom row).
Figure 1.3. S293 of the SRM is the Critical DRE Residue for Slimb Recognition

(A) 13 hydroxyl amino acids (red) within the DRE of full-length fly Plk4 were individually mutated to alanines and used to evaluate the impact of each residue on Plk4 stability and centriole duplication. S293 and T297 reside within the SRM (yellow highlight).

(B) (Top) Anti-GFP immunoblot of lysates prepared from S2 cells transiently co-expressing the indicated Plk4-EGFP construct and Nlp-EGFP (loading control). All Plk4 mutants are single alanine mutants except SBM (a double mutant of S293A/T297A). (Bottom) Plk4-EGFP intensities were measured by densitometry of the anti-GFP immunoblot and normalized with their respective Nlp-EGFP loading controls. The plotted values are the normalized Plk4 intensities relative to the WT-Plk4 treatment.

(C) Anti-GFP immunoprecipitates of lysates prepared from S2 cells transiently expressing 3xFLAG-ubiquitin and the indicated Plk4-EGFP construct were probed with anti-GFP, FLAG, and Slimb antibodies. Short and long exposures of the anti-FLAG immunoblot are shown.

(D) Amounts of associated endogenous Slimb were determined by densitometry of the anti-Slimb immunoblot and then normalizing the measurements with the amounts of Plk4-EGFP present in the IPs. The plotted values are relative to the WT-Plk4 treatment.

(E) The relative amounts of total Plk4 FLAG-Ubi were calculated using the densitometry method described in (D).

(F) S2 cells co-expressing the indicated Plk4-EGFP construct (green puncta) and Nlp-EGFP (green nuclei) were immunostained for PLP (red) to mark centrioles. DNA (blue). Insets show higher magnifications of the boxed regions.

(G) The centrioles of S2 cells treated and immunostained as in (F) were counted. Centrioles are amplified in cells expressing SBM (P<0.0001) or S293A (P<0.0001) but not T297A (P=0.06).
There is no significant difference in centriole loss (black bars) in these treatments. Four experiments were performed per construct (n = 600 cells/construct).

Figure 1.4. Mutation of All 13 Hydroxyl DRE Residues Display Only a Subtle Difference in Plk4 Stability Compared to the S293A Mutant

(A) Hydroxyl residues (blue) within the DRE were systematically mutated to non-phosphorylatable alanines (red) to generate a series of Plk4 constructs containing an increasing number of mutations.

(B) Plk4 is stabilized by mutation of residue S293, but this stability is slightly modulated by neighboring phosphorylatable residues within the DRE. (Top) Anti-GFP immunoblot of lysates
from S2 cells transiently co-expressing the indicated Plk4-EGFP construct and Nlp-EGFP (loading control). (Bottom) Relative amounts of Plk4 protein were determined by measurement of the integrated intensities of the Plk4 bands, normalized to their respective loading controls, and then plotted relative to the normalized intensity of WT-Plk4. Measurements were obtained from three experiments.

(C) Blots of anti-GFP immunoprecipitates from lysates of S2 cells transiently expressing 3xFLAG-Ubi and the indicated Plk4-EGFP construct were probed with anti-GFP, FLAG, and Slimb antibodies. Slimb binding is reduced by mutation of upstream DRE serines (A2-A5). As expected, Slimb binding is eliminated in mutants containing the S293A mutation (A6-A13).

(D) S2 cells co-expressing the indicated Plk4-EGFP plasmid (green puncta) and Nlp-EGFP (green nuclei) were immunostained for PLP to mark centrioles (red). DNA (blue).

(E) Expression of stabilized Plk4 mutants increases the percent of cells with excess centrioles (>2). Centrioles in 100 cells were measured per construct.
Figure S1.1. Kinase-dead Plk4 Could Exert its Dominant/Negative Effect on Centriole Duplication in *Drosophila* by Sequestering Asterless

(A) Anti-Asterless (Asl) antibodies used in this study are specific. Four different guinea pig polyclonal anti-Asl antibodies were raised against purified recombinant full-length GST-Asl and affinity-purified against MBP-Asl. The specificity of these antibodies (Asl-1-4) on immunoblots against S2 cell lysates is shown. All antibodies react with the identical ∼125 kDa polypeptide
and immunostain centrioles in S2 cells (see Figure 1D).

(B) High expression of wild-type (WT)-Plk4-EGFP in S2 cells drives centriole amplification. Plk4 (both endogenous and transgenic) (red) binds endogenous Asterless (Asl) (purple) through its Polo Boxes (PB) 1 and 2 (aka ‘the Cryptic Polo Box’). Asl then targets Plk4 to the centriole (blue) surface where it initiates centriole duplication (procentriole assembly).

(C) High expression of kinase-dead (KD)-Plk4-EGFP (red, marked with ‘X’) in S2 cells inhibits centriole duplication. KD-Plk4 binds endogenous Asl and forms cytoplasmic aggregates. By sequestering Asl and preventing it from localizing to centrioles, centriole duplication is blocked. Likely, this is due to the inability of endogenous Plk4 to target centrioles. However, Asl is a multi-functional scaffolding protein and may play additional unidentified roles in the duplication process. This dominant/negative effect on centriole duplication is specifically due to sequestering Asl because it is rescued by expression of KD-Plk4 lacking the Asl-binding domain (delta-PB1-PB2) (not shown in this illustration).
Figure S1.2. Plk4 Trans-Autophosphorylation is Probably Primarily Responsible for the Phosphorylation of the Downstream Regulatory Element (DRE) In Vitro and In Vivo

(A) Immunoblots of lysates from control or Slimb-depleted S2 cells transiently co-expressing Plk4-Myc and Nlp-EGFP (loading control). Slimb-depletion was used to stabilize the expressed Plk4-Myc, making the protein readily apparent and demonstrating that Plk4-Myc is expressed in transfected cells.

(B) Models depicting Plk4 dimerization scenarios. (1) Homodimerization of WT-Plk4 leads to trans-autophosphorylation, promoting Slimb-mediated ubiquitination of Plk4 and subsequent degradation of both molecules. (2) If WT-Plk4 heterodimerizes with KD-Plk4, then only KD-Plk4 is trans-phosphorylated leading to KD-Plk4 ubiquitination and degradation. (3) KD-Plk4 homodimerization is predicted to block phosphorylation, ubiquitination and degradation.
(C) 3xFLAG-tagged ubiquitin (Ubi) specifically labels Plk4-GFP but not GFP. Anti-GFP immunoprecipitates of lysates prepared from S2 cells transiently expressing 3xFLAG-ubiquitin and either EGFP or Plk4-EGFP were probed with anti-GFP and FLAG antibodies.

(D, E) Samples of Plk4 were prepared for MS analysis from either in vitro autophosphorylated Plk4 (D) or transgenic Plk4-EGFP immunoprecipitated from S2 cell lysates (E). To prepare the in vitro sample (D), bacterially expressed and purified Plk4-DRE-His\(_6\) containing only the kinase domain and DRE (amino acids 1-317) was incubated with MgATP and then resolved by SDS-PAGE. The band corresponding to Plk4 was cut from the gel, processed, and then analyzed by tandem mass spectrometry (MS). An example of a mass spectrum obtained from the analysis of in vitro autophosphorylated Plk4 is shown (D). The sequence of the peptide is shown in bold; the phosphorylated residues (S293 and T297) are indicated with asterisks. (We note that this spectrum only weakly demonstrates T297 phosphorylation; however, other spectra verify the phosphorylation of this residue.) To prepare the in vivo sample (E), S2 cells were transfected with Plk4-EGFP-encoding plasmid, and then the transgenic Plk4 was immunoprecipitated from S2 cell lysates, resolved by SDS-PAGE and similarly processed for tandem MS. An example of a mass spectrum obtained from the analysis of in vivo phosphorylated Plk4 is shown (E). The sequence of the peptide is shown in bold; the phosphorylated residue (S293) is indicated with an asterisk.
Figure S1.3. Hydroxyl Residues Flanking the S293 Contribute to Slimb Binding and Plk4 Stability

(A) A series of Plk4 constructs (A14-A20) was created by incrementally mutating all of the hydroxyl amino acids (blue) within the DRE, except S293, to alanines (red).

(B) Elimination of phosphorylatable residues within the DRE increases Plk4 levels even though the key residue, S293, is unaltered. (Top) Anti-GFP immunoblot of lysates prepared from S2 cells transiently co-expressing the indicated Plk4-EGFP mutant and Nlp-EGFP (loading control). (Bottom) The normalized Plk4 intensity values were obtained as described in the Figure 4B legend. Graphed values are relative to T297A. Data were acquired from three experiments.

(C) (Top) Anti-GFP immunoprecipitate of lysates from S2 cells transiently expressing 3xFLAG-Ubi and the indicated Plk4-EGFP mutant were probed with anti-GFP, FLAG, and Slimb
antibodies. (Bottom) Plk4 ubiquitination levels and associated Slimb levels measured from the quantitative FLAG and Slimb immunoblots, respectively. Graphed values are relative to the WT-Plk4 control.

(D) S2 cells co-expressing the indicated Plk4-EGFP construct (green puncta) and Nlp-EGFP (green nuclei) were immunostained for PLP-centrioles (red). DNA (blue).

(E) Cells expressing different Plk4-EGFP constructs were immunostained as in (D) and their centrioles were counted. Graph shows the percentage of cells with the indicated number of centrioles per cell. Note that loss of hydroxyl DRE residues (not including S293) can have as large an impact on centriole amplification as expression of SBM-Plk4. Centrioles in 100 cells were measured per construct.
Figure S1.4. Hydroxyl Residues Flanking the SRM Collectively Contribute to Slimb Binding and Plk4 Stability

(A) A series of Plk4 constructs (A21-A26) was created by mutating all of the hydroxyl amino acids (blue) within the DRE, except for S293 and T297, to alanines (red).

(B) Elimination of phosphorylatable residues within the DRE increases Plk4 stability even when the SRM is unaltered. (Top) Anti-GFP immunoblot of lysates prepared from S2 cells transiently co-expressing the indicated Plk4-EGFP construct and Nlp-EGFP (loading control). (Bottom) Graphed values of normalized Plk4 intensities are relative to the WT-Plk4 treatment from three separate experiments.

(C) Elimination of about half the hydroxyl residues within the DRE diminishes Slimb binding by over 5-fold, even though the SRM is intact (compare A21 and WT). (Top) Anti-GFP immunoprecipitate of lysates from S2 cells transiently expressing the indicated Plk4-EGFP construct were probed with anti-GFP and Slimb antibodies. (Bottom) Slimb binding was measured by densitometry of the Slimb immunoblot and normalized to WT-Plk4.

(D) S2 cells co-expressing the indicated Plk4-EGFP plasmid (green puncta) and Nlp-EGFP (green nuclei) were immunostained for PLP (red) to mark centrioles. DNA (blue).

(E) S2 cells expressing the indicated Plk4 constructs were immunostained as in (D) and their centrioles counted. Graph shows the percentages of cells with the indicated numbers of centrioles per cell. Centrioles in 100 cells were measured per construct.
Table S1. *In vitro* phosphorylation sites of *Drosophila* Plk4 DRE. Partial list of recovered MS/MS peptides covering the DRE of *Drosophila* Plk4. Two autophosphorylated Plk4-kinase-DRE samples were analyzed by different facilities. The # symbol follows residues that are
modified; all Ser/Thr modifications are phosphorylations. Note that not all recovered peptides are listed. Mascot parameters were obtained from Scaffold 4.0.7 (Proteome Software).

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Table S1.2. *In vivo* phosphorylation sites of the *Drosophila* Plk4 DRE. Partial list of recovered MS/MS peptides covering the DRE of *Drosophila* Plk4. The # symbol follows residues that are modified; all Ser/Thr modifications are phosphorylations. Note that not all recovered peptides are listed. Parameters were obtained from Scaffold 4.0.7.

### 1.5 Materials and Methods

**Cell culture and double-stranded RNAi (dsRNA Interference)**

*Drosophila* S2 cell culture, in vitro dsRNA synthesis, and RNAi treatments were performed as previously described [27]. In brief, cells were cultured in Sf900II SFM media (Life Technologies). RNAi was performed in 6-well plates containing cells at 50–90% confluency by applying 10μg dsRNA in 1ml media and replenishing with fresh media/dsRNA every day for 4-5d. Control dsRNA was synthesized from control DNA template amplified from a non-GFP sequence of the pEGFP-N1 vector (Clontech) using the primers 5’-

CGCTTTTCTGGATTTCATCGAC-3’ and 5’-TGAGTAACTGAGGCTATGG-3’ (all primers used for dsRNA synthesis in this study begin with the T7 promoter sequence 5’-

TAATACGACTCACTATAGGG-3’). DNA template for Slimb dsRNA was generated using the primers 5’-GGCCGCCACATGCTGCG and 5’-CGGTCTTGTCTCATCTTTGGG to amplify a region of coding sequence from a Slimb cDNA.
**Immunofluorescence microscopy**

For immunostaining, S2 cells were fixed and processed exactly as previously described [27] by spreading S2 cells on concanavalin A–coated, glass-bottom dishes and fixing with 10% formaldehyde. Primary antibodies were diluted to concentrations ranging from 1 to 20μg/ml. They included rabbit and guinea pig anti–PLP [2]. Secondary antibodies (conjugated with Cy2, Rhodamine red-X, or Cy5 [Jackson ImmunoResearch Laboratories, Inc.]) were used at manufacturer-recommended dilutions. Hoechst 33342 (Life Technologies) was used at a final dilution of 3.2μM. Cells were mounted in 0.1M n-propyl galate, 90% (by volume) glycerol, and 10% PBS solution. Specimens were imaged using a DeltaVision Core system (Applied Precision) equipped with an Olympus IX71 microscope, a 100× objective (NA 1.4), and a cooled charge-coupled device camera (CoolSNAP HQ2; Photometrics). Images were acquired with softWoRx v1.2 software (Applied Science).

**Immunoblotting**

S2 cell extracts were produced by lysing cells in cold PBS and 0.1% Triton X-100. Laemmli sample buffer was then added and boiled for 5 min. Samples of equal total protein were resolved by SDS-PAGE, blotted, probed with primary and secondary antibodies, and scanned on an Odyssey imager (Li-Cor Biosciences). Care was taken to avoid saturating the scans of blots. The integrated densities of fluorescent bands (measured from the digitized scans using ImageJ software [National Institutes of Health]) were normalized relative to the integrated densities of the corresponding loading controls. Transfected Nlp-EGFP (a constitutively-expressed nuclear protein; [3]) was used as loading control and transfection marker. Antibodies used for Western blotting include anti-Slimb [2], anti-GFP monoclonal JL8 (Clontech), anti-Myc (Cell Signaling...
Technologies) and anti-FLAG (Sigma-Aldrich) used at 1:1,000 dilutions. IRDye 800CW secondary antibodies (Li-Cor Biosciences) were prepared according to the manufacturer’s instructions and used at 1:1,500 dilutions.

Constructs and transfection

Full-length cDNA of Drosophila Plk4 [3] was subcloned into a pMT vector containing in-frame coding sequence for EGFP (or Myc) and the inducible metallothionein promoter. QuikChange II (Agilent Technologies) was used according to manufacturer’s instructions to generate the series of Plk4 mutants. Transient transfections of S2 cells were performed using a Nucleofector II and nucleofector kit V (Lonza) according to manufacturer’s instructions. Expression of all Plk4 constructs (and GFP control) was induced by addition of 50 µM–2 mM copper sulfate to the culture medium. In Figure 1, addition of 50µM copper sulfate to cell medium was used to induce transgene expression to a low level, while 1mM copper sulfate was used to induce a high level of transgene expression.

Immunoprecipitation

Polyclonal and monoclonal antisera were bound to equilibrated protein A (or protein G) Sepharose (Sigma-Aldrich) by gently rocking overnight at 4°C in 0.2 M sodium borate. In some cases, the prebound antibody was cross-linked to the resin by incubating with 20mM dimethyl pimelimidate dihydrochloride in PBS, pH 8.3, 2 h at 22°C, and then quenching the coupling reaction by incubating with 0.2 M ethanolamine, pH 8.3, 1 h at 22°C. Antibody-coated beads were washed three times with 1.5 ml of cell lysis buffer (CLB; 50 mM Tris, pH 7.2, 125 mM NaCl, 2 mM DTT, 0.1% Triton X-100, and 0.1 mM PMSF). Transfected cells expressing
recombinant proteins were lysed in CLB, and the lysates clarified by centrifugation. Antibody-
coated beads were rocked with lysate for 1 h at 4°C, washed two times with 1 ml CLB, and then
boiled in Laemmli sample buffer. For GFP immunoprecipitations, GFP-binding protein (GBP)
[28] was fused to the Fc domain of human IgG (pIg-Tail) (R&D Systems), tagged with His₆ in
pET28a (EMD Biosciences), expressed in E. coli and purified on HisPur resin (ThermoFisher)
according to manufacturer’s instructions. Purified GBP was bound to Protein A-coupled
Sepharose, and then cross-linked to the resin using the method described above. In vivo
ubiquitination assays were performed by co-expressing Plk4-EGFP constructs with triple FLAG-
tagged Drosophila ubiquitin (CG32744) (also under the metallothionein promoter and Cu-
induced) and then probing the immunoblot of the cell lysate with anti-FLAG antibody (Sigma-
Aldrich).

In Vitro Autophosphorylation of Plk4

Drosophila (His)₆-tagged Plk4 kinase domain + DRE (amino acids 1-317) was cloned into the
pET28a vector, expressed in BL21DE3 bacteria, and purified on HisPur resin (ThermoFisher)
according to manufacturer’s instructions. Purified Plk4 was autophosphorylated by incubating
with 50μM ATP, 1-2 h, 25°C, in reaction buffer (40mM Na HEPES, pH 7.3, 150mM NaCl,
5mM MgCl₂, 0.5mM MnCl₂, 1mM DTT, 0.1mM PMSF, 10% glycerol). (To identify
phosphorylated residues that are not generated by autophosphorylation, a sample of the same
purified Plk4 is left untreated.) Samples were resolved by SDS-PAGE, Coomassie stained, and
then processed for mass spectrometry (below).

Mass Spectrometry
Mass spectrometry was performed at the Taplin Mass Spectrometry Facility (Harvard Medical School) and the NHLBI Proteomics Core Facility (NIH). Better coverage of Plk4 was obtained by the NHLBI facility using the following procedure. Samples were reduced (10µM dithiothreitol, 55°C, 1hr), alkylated (55mM iodoacetamide, room temperature, 45min), and trypsin digested (~1µg trypsin, 37°C, 12hrs) in-gel, and then extracted. Peptide samples were loaded onto a Zorbax C18 trap column (Agilent Tech., Santa Clara, CA) to desalt the peptide mixture using an on-line Eksigent (Dublin, CA) nano-LC ultra HPLC system. The peptides were then separated on a 10 cm Picofrit Biobasic C18 analytical column (New Objective, Woburn, MA). Peptides were eluted over a 90 min linear gradient of 5-35% acetonitrile/water containing 0.1% formic acid at a flow rate of 250 nL/min, ionized by electrospray ionization (ESI) in positive mode, and analyzed on a LTQ Orbitrap Velos (Thermo Electron Corp., San Jose, CA) mass spectrometer. All LC MS analyses were carried out in “data-dependent” mode in which the top 6 most intense precursor ions detected in the MS1 precursor scan (m/z 300-2000) were selected for fragmentation via collision induced dissociation (CID). Precursor ions were measured in the orbitrap at a resolution of 60,000 (m/z 400) and all fragment ions were measured in the ion trap.

LC MS/MS data acquired from tryptic digests were searched independently using the MASCOT algorithm. All data were searched against the Drosophila NCBInr protein database for peptide and protein identifications. Trypsin or chymotrypsin was specified as the digestion enzyme, allowing for up to 2 missed cleavage sites. Carbamidomethylation (C) was set as a static modification and Oxidation (M) and Phosphorylation (S,T,Y) were selected as variable modifications. Precursor and fragment ion mass tolerances were set to 20 ppm and ± 0.8 Da, respectively. Following MASCOT searches, database search results were combined to obtain a
comprehensive map of all peptides identified from Plk4.

Statistical Analysis

The statistical significance of differences in average measurements was evaluated using one-way ANOVA and Dunnett’s multiple comparisons post-test (GraphPad Prism 6.0). Reported P values are adjusted for multiplicity. Differences in averages are deemed significant if P < 0.05.

1.6 References


CHAPTER TWO: PLK4 ENCODES A MECHANISM OF AUTOINHIBITION AND RELIEF

*All S2 cell culture work performed by J. Klebba, all in vitro work performed by D. Buster*

2.1 ABSTRACT

Polo-like kinase 4 (Plk4) is a master-regulator of centriole duplication and its hyperactivity induces centriole amplification. Unlike monomeric Plk1 whose activity is autoinhibited, homodimeric Plk4 has been shown to autodestruct by promoting its ubiquitination, thus preventing centriole amplification. Unlike other Plks, Plk4 contains three rather than two Polo-Boxes, and the function of its third Polo-Box (PB3) is unclear. Here, we performed a functional analysis of Plk4’s structural domains. Surprisingly, Plk4 does possess a previously unidentified autoinhibitory mechanism mediated by a linker (L1) near the kinase domain. However, autoinhibition is relieved by either L1 phosphorylation or PB3. Thus, autoinhibition is a conserved feature of Plks and, in the case of Plk4, is important for homodimerization because kinase activity promotes dimer separation. We propose a model for Plk4 activation and degradation and discuss the implications for Plk4 function and regulation.

2.2 INTRODUCTION

The Polo-like kinase (Plk) family of proteins function as master-regulators of cell cycle progression, cell division, centrosome maturation and centriole duplication\textsuperscript{1-3}. All four members (Plk1-4) of this serine/threonine protein kinase family share sequence similarity and domain structure with the founding member, \textit{Drosophila} Polo kinase (Plk1)\textsuperscript{4}. Plks are highly expressed in proliferating cells and overexpressed in a variety of cancers where they have the potential to promote chromosomal instability and tumorigenesis\textsuperscript{5-9}. Previous studies have shown that Plk
kinase activity can be limited to brief periods within the cell cycle through mechanisms involving the localization, degradation, and autoinhibition of the kinase\textsuperscript{10-13}. New regulatory mechanisms of Plks continue to be identified\textsuperscript{14-16}, making it clear that our understanding of Plk regulation is incomplete.

All Plks contain an N-terminal kinase domain followed by one or more Polo Box (PB) motifs and interrupted by linkers of varying length\textsuperscript{4}. PBs are \textasciitilde 100 amino acid multifunctional domains that serve as hubs of protein interaction and are important for dimerization, substrate binding, intracellular targeting and autoinhibition of kinase activity\textsuperscript{1,4,10,11,17}. Whereas Plk1-3 contain two PBs, Plk4 contains three distinct PBs\textsuperscript{18}. Of all Plk members, Plk1 regulation is the most well understood, due in part to the recent crystallization of the kinase domain in complex with its PB-linker elements\textsuperscript{16}, revealing new insights into its mechanism of autoinhibition. The two PBs of Plk1 form an intramolecular dimer joined by two linkers\textsuperscript{19} and, together, make extensive contact with the kinase domain\textsuperscript{16,20,21}. This interaction rigidifies the hinge region of the kinase domain, thereby decreasing flexibility of the ATP cleft and likely crippling nucleotide hydrolysis\textsuperscript{16}. Inhibition is relieved either by phosphopeptide binding to the PB dimer or by phosphorylation within the kinase domain\textsuperscript{22-26} which disrupts the kinase domain-PB/linker interaction\textsuperscript{16}. In addition, full Plk1 activity requires phosphorylation of its activation loop by Aurora A\textsuperscript{27,28}, but this is hindered by the interdomain linker that connects the kinase domain to the PB dimer\textsuperscript{16}. Thus, Plk1 is normally inactive due to autoinhibition and requires multiple cell cycle-dependent inputs to achieve full mitotic activation.

Plk4 is the master-regulator of centriole duplication and its hyperactivation drives centriole amplification\textsuperscript{29-35}, a phenomenon observed in cancer\textsuperscript{36}. Plk4 is distinct from its monomeric relatives because it forms a homodimer and contains an additional PB\textsuperscript{18,37} (PB3; \textbf{Fig. 1a}).
Crystal structure analysis suggests that Plk4 homodimerization is mediated by protein-protein interactions between the PB domains\(^{18,38}\), and its stability is regulated primarily through ubiquitin-mediated proteolysis\(^{12,13,39}\). Plk4 generates its own phosphodegron that ultimately results in degradation\(^{37,40-42}\). Following assembly into a homodimer, Plk4 extensively trans-autophosphorylates its Downstream Regulatory Element (DRE) containing the phylogenetically conserved Slimb-Recognition Motif\(^{37,40-42}\). This phosphorylation recruits the SCFSlimb/β-TrCP ubiquitin-ligase (Fig. 1b) to ubiquitinate Plk4, although the specific ubiquitinated residues are not known.

To understand how the PBs and linker regions regulate Plk4, we studied their impact on Plk4 activity and stability in cultured *Drosophila* S2 cells. Our analyses reveal that PBs are crucial not only for Plk4 homodimerization and ubiquitination, but that they also relieve autoinhibition caused by Linker 1. Relief of autoinhibition is mediated by the downstream Linker 2-PB3 regions, demonstrating a new role for this third PB and supporting a multistep model for Plk4 activation. Thus, autoinhibition is a conserved regulatory mechanism of the Plk family. Furthermore, the presence of a third PB in Plk4 allows self-activation, whereas Plk1, which does not encode a third PB, is reliant on external factors to relieve its autoinhibition.

### 2.3 RESULTS

*Polo Boxes (PB) involved in Plk4 dimerization*

Structures of purified fly PB1-PB2 and mouse PB3 have been solved, and although each PB is unique, they all adopt a classic PB-fold and form stable homodimers *in vitro*\(^{18,38}\). In the case of the purified PB1-PB2 cassette, homodimerization is mediated by contacts at both the PB1-PB1 and PB2-PB2 interfaces. These findings have led to a model where all three PBs mediate Plk4 homodimerization (Fig. 1b), but this has not been tested in cells.
To determine which PBs actually mediate homodimerization, we performed dimerization assays using S2 cells co-expressing different EGFP- and myc-tagged Plk4 constructs. Plk4-EGFP protein was immunoprecipitated, and quantitative immunoblotting was used to measure the amounts of associated Plk4-myc. As expected, wild-type (WT)-Plk4-myc binds WT-Plk4-EGFP (Fig. 1c, lane 1). We next evaluated the importance of the PB1-PB2 tandem as a single unit because these PBs are adjacent and separated by only a short Thr-Pro linker. Plk4 lacking PB1-PB2 was unable to bind full-length Plk4 (Fig. 1c, lane 2; Supplementary Fig. 1a, lane 2). Failure to bind was not due to a difference in the size of these proteins (which might cause the domains of a potential heterodimer to be out of register) because deletion of PB1-PB2 from both co-expressed Plk4 constructs dramatically reduced their association (Fig. 1c, lane 4). Thus, PB1-PB2 is critical for Plk4 dimerization.

In contrast, PB3 is not required for dimerization. Plk4 lacking the PB3 domain could still associate with full-length (WT) Plk4 (Fig. 1c, lane 3) and Plk4 constructs dimerize even if both lack PB3 (Fig. 1c, lane 5). However, binding was reduced by approximately 25% suggesting that, although not required for dimerization, PB3 may stabilize the dimer. In addition, binding was nearly abolished between Plk4-ΔPB1-PB2-EGFP and Plk4-ΔPB3-myc (Fig. 1c, lane 6), demonstrating that PB dimerization occurs through equivalent domains; i.e., PB1-PB2 binds PB1-PB2 but not PB3.

The individual contributions of PB1 and PB2 to dimerization were examined by deleting either domain from Plk4-EGFP and then measuring association with full-length (WT) Plk4-myc. Deletion of either PB1 or PB2 dramatically reduced binding but neither had as strong an effect as deletion of the PB1-PB2 tandem, underscoring the importance of the PB1-PB2 cassette in dimerization (Supplementary Fig. 1a). We note that the PB1 and PB2 deletions are not
equivalent because deletion of PB2 resulted in the greater decrease of association with WT-Plk4-myc. Using a similar assay, we also examined the sufficiency of EGFP- and myc-tagged PB fragments for dimerization. As expected, PB1-PB2-myc associated with PB1-PB2-EGFP but not with PB3-EGFP (Supplementary Fig. 1b, lanes 1-4). Surprisingly, PB3-myc did not associate with PB3-EGFP (Supplementary Figs. 1b, lanes 10-12 and 1c). This latter result is unexpected given the previous finding that mouse PB3 is capable of dimerizing in vitro and in cells\textsuperscript{38}, but is consistent with our results that PB3 plays a minor role in dimerization in the context of the full-length protein. Thus, Plk4 homodimerization is primarily mediated through contacts between PB1-PB2 cassettes.

Ubiquitination sites in Plk4

Specific sites of Plk4 ubiquitination are unknown. To identify ubiquitinated residues, we immunoprecipitated Plk4-EGFP expressed in S2 cells and mapped di-glycine (Gly-Gly) modified residues using tandem mass spectrometry (MS). Trypsin-treatment cleaves poly-ubiquitin (Ubi) chains, leaving behind diagnostic di-glycines of Ubi linked to their target lysines\textsuperscript{43}. MS analysis of full-length Plk4 (total coverage was 97% and included all Lys residues) identified several Lys-linked Gly-Gly modifications (Supplementary Fig 2. a, b; Supplementary Table 1). Seven of the modified residues reside in PB1, a region in close proximity to the Slimb-binding DRE, and five of these sites are conserved in vertebrates (Fig. 2a). Examination of the Drosophila PB1 structure revealed that the modified residues cluster in two regions (Fig. 2b). K496 and K498 are found in the C-terminus of the single 1α1-helix, and K392 is positioned in a nearby loop. The remaining four residues orient their side chains toward the opposite face of PB1 and are located near the N-terminus of 1α1 (K484), within β-strands 1
(K400) and 3 (K420), and within the 1β1-1β2-linker (K402).

To test which PBs are important for ubiquitination, we expressed various PB deletion-EGFP mutants together with 3xFLAG-Ubi in S2 cells and then assessed their ubiquitination states. As expected, WT-Plk4 co-precipitated with endogenous Slimb and was robustly ubiquitinated, whereas non-degradable Plk4-SBM did not bind Slimb, nor was it ubiquitinated (Fig. 2c, lanes 1,2). As previously reported\textsuperscript{18}, PB1-PB2 deletion reduces Slimb binding and abolishes incorporation of FLAG-Ubi, probably due to the inability of this mutant to dimerize and \textit{trans}-autophosphorylate (Fig. 2c, lane 3). Note that Plk4-ΔPB1-PB2 migrates as a sharp band on SDS-PAGE, indicating less phosphorylation compared to WT-Plk4 which migrates as a diffuse phosphorylated species. Likewise, deletion of PB2, which severely impairs dimerization, also reduces Slimb binding and ubiquitination (Fig. 2c, lane 5). Deletion of PB1, which is less important for dimerization than PB2, does not appear to block autophosphorylation. This mutant displayed the diffuse appearance of a phosphorylated species on SDS-PAGE and bound a similar amount of Slimb as WT-Plk4 (Fig. 2c, lane 4). Strikingly, although Plk4-ΔPB1 binds Slimb, it was not ubiquitinated (Fig. 2c, lane 4), suggesting that PB1 is a major site of Plk4 ubiquitination. Moreover, Plk4-ΔPB1 total protein levels were dramatically elevated in cells, similar to non-degradable Plk4-SBM (Fig. 2d).

Next, we tested whether the seven Gly-Gly modified lysines in PB1 contributed to Plk4 ubiquitination. To block their ubiquitination, all seven residues were mutated to alanine (K7A) in an otherwise WT Plk4-EGFP background, and then co-expressed with 3xFLAG-Ubi in cells. Immunoprecipitation of K7A revealed that it efficiently recruits Slimb, suggesting that it is capable of dimerizing and autophosphorylating, but ubiquitination was reduced almost to the same extent as Plk4-ΔPB1 (Fig. 2e). The reduced ubiquitination of K7A was accompanied by an
increase of its protein levels in cells, although Plk4-ΔPB1 was noticeably more stable (Fig. 2f). These findings indicate that at least some of the lysines in PB1 are physiologically important targets of Plk4 ubiquitination. The fact that K7A did not completely eliminate Ubi-labeling suggests that other ubiquitinated residues remain to be identified.

Polo Box 3 (PB3) regulates Plk4 kinase activity

Aside from the minor role of PB3 in dimerization and its weak targeting to centrioles when expressed as a GFP fusion, the function of this third PB is unknown. Therefore, we performed a functional analysis of PB3 by first expressing a PB3 deletion (ΔPB3) mutant of Plk4-EGFP and examining its protein level in S2 lysates. Surprisingly, deletion of PB3 dramatically stabilized the mutant protein compared to WT-Plk4 (Fig. 3a, lanes 1,3). Levels of Plk4-ΔPB3 were comparable to kinase-dead (KD)-Plk4 which is unable to autodestruct (Fig. 3a, lane 2). When co-expressed with 3xFLAG-Ubi, immunoprecipitated Plk4-ΔPB3-EGFP displayed reduced ubiquitination and Slimb binding (Fig. 2c, lane 6). This suggests that kinase activity is impaired in Plk4-ΔPB3, reducing its ability to trans-autophosphorylate and recruit Slimb and thereby increasing its stability.

To further test the impact of PB3 on kinase activity, we co-expressed Plk4-ΔPB3-EGFP with WT- or KD-Plk4-myc and examined their ability to trans-autophosphorylate within the heterodimers that form. As previously described, WT-Plk4-myc binds and trans-autophosphorylates KD-Plk4-EGFP, efficiently promoting degradation of this otherwise stable KD mutant (Fig. 3b, lane 4). Likewise, WT-Plk4-myc binds and converts Plk4-ΔPB3-EGFP into a slower migrating phosphorylated species and promoted its degradation, compared to cells expressing Plk4-ΔPB3-EGFP alone (Fig. 3b, lanes 1,2). In contrast, Plk4-ΔPB3 was not able to
promote KD-Plk4 degradation (Fig. 3b, lane 3) even though they heterodimerize and co-immunoprecipitate (Supplementary Fig. 3a), suggesting that Plk4-ΔPB3 lacks the ability to trans-autophosphorylate.

As an additional test of ΔPB3 mutant kinase activity, we examined its localization and ability to induce centriole overduplication in S2 cells. Whereas WT-Plk4-EGFP co-localized with PLP and Asterless (Asl)-labeled centrioles, centriole targeting was disrupted by deletion of either PB1-PB2, PB1 or PB2 (Fig. 3c; Supplementary Fig. 3b). This is consistent with the critical role PB1-PB2 plays in binding Asl which then targets Plk4 to centrioles. Plk4-ΔPB3-EGFP co-localized with centrioles and formed numerous small aggregates in the cytoplasm reminiscent of KD-Plk4 overexpression (Fig. 3c). Asl staining was also dramatically diminished on PLP-labeled centrioles in these cells, similar to the effects observed with KD-Plk4 overexpression (Fig. 3c).

Previously, we found that KD-Plk4 expression causes centriole loss by sequestering Asl and preventing its centriole targeting. Therefore, we tested the ability of Asl to co-immunoprecipitate with Plk4-EGFP and PB deletion mutants. Consistent with previous work, Asl associated with WT-Plk4 but not with PB1-PB2 or PB1 deletion mutants (Fig. 3d). In contrast, deletion of PB3 had no effect on Asl binding (Fig. 3d), suggesting that, like KD-Plk4, Plk4-ΔPB3 may sequester Asl and prevent its centriole localization. Therefore, we expressed Plk4-EGFP constructs in S2 cells for 3 days, immunostained them for PLP, and counted centrioles. As expected, WT-Plk4 expression significantly increased the percentage of cells with centriole amplification (>2 centrioles) (P=0.01), whereas Plk4-ΔPB1-PB2 had no effect (Fig. 3e). In contrast, Plk4-ΔPB3 expression significantly increased the percentage of cells with <2 centrioles (Fig. 3e; P<0.0001), similar to the effects of KD-Plk4 expression (Fig. 5d; control vs
KD, <2 centrioles, P=0.02). All of our functional assays lead to the conclusion that PB3 deletion severely impairs kinase activity, thus revealing a surprising new role for PB3 in promoting kinase activation.

The Plk4 phosphorylation pattern

The finding that PB3 deletion impairs kinase activity raises the possibility that Plk4 possesses a previously unidentified autoinhibitory mechanism that is relieved by PB3 and, thus, is normally not detected. In the case of Plk1, autoinhibition is relieved by phosphorylation of its kinase domain\textsuperscript{16,23-28}. Since Plk4 extensively autophosphorylates its DRE to recruit Slimb\textsuperscript{40-42}, it is plausible that similar autophosphorylation of other domains regulates a putative autoinhibitory mechanism. Therefore, it was necessary to better characterize the Plk4 phosphorylation pattern.

First, we isolated full-length Plk4-EGFP from S2 cells and mapped phosphorylated Ser/Thr residues using tandem MS. From 97% coverage, several clusters of phosphorylated residues were identified throughout the protein (Fig. 4a; Supplementary Fig. 4a,b and Table S2). Many of these residues were confirmed as \textit{in vitro} autophosphorylation targets by performing MS on purified fly Plk4 kinase domain (amino acids 1-317) incubated with GST-tagged proteins containing different downstream regions of Plk4 (Supplementary Fig. 4a,c and Table S2). Notably, autophosphorylation was not detected within any of the PBs but instead was primarily restricted to its linker and loop regions. Aside from the 10 previously described residues in the DRE\textsuperscript{42}, the phosphorylation sites include 3 residues in the activation loop (AL) of the kinase domain, one residue in the C-terminal lobe of the kinase domain, 2 residues near the end of Linker 1, 11 residues in Linker 2, and one residue in the C-terminal segment (Fig. 4a).
A mechanism for Plk4 autoinhibition

Phosphorylation of the Plk1 activation loop by Aurora A stimulates its activity and governs a critical step in Plk1 regulation\textsuperscript{20,24,26-28}. Our observation of autophosphorylated residues in the Plk4 activation loop (AL) suggested that Plk4 may be regulated in a similar manner and that autoinhibition might function to block autophosphorylation of this loop. We tested the importance of these residues by first mutating all three of the phosphorylated residues of the Plk4 AL to alanine (T172A/T176A/S228A), and then comparing the activity of the triple mutant (AL-Ala) to purified WT kinase domain protein in an \textit{in vitro} assay. Whereas WT protein robustly autophosphorylated, kinase activity was significantly reduced in the AL-Ala protein, nearly to the same extent as a kinase-dead (KD) mutant (\textbf{Fig. 4b}). Thus, phosphorylation of the activation loop is critical for \textit{in vitro} Plk4 kinase activity.

To test the effects of mutating the activation loop of Plk4 expressed in cells, we generated inducible forms of both the AL-Ala and triple phosphomimetic (T172E/T176E/S228D) (AL-PM) mutants within full-length Plk4-EGFP. Similar to KD-Plk4, the protein level of AL-Ala-Plk4 was dramatically stabilized compared to WT-Plk4 and migrated on SDS-PAGE mostly as a sharp, high-mobility band (as would be expected for a non- or low-phosphorylated protein) (\textbf{Fig. 4c, lanes 1-3}; \textbf{Fig. 4d, lane 2}). Moreover, when co-expressed with 3xFLAG-Ubi, AL-Ala is clearly less ubiquitinated and binds less Slimb compared to WT (\textbf{Fig 4d, lanes 1,2}), further demonstrating its inability to autophosphorylate and recruit Slimb. AL-Ala-Plk4 localized to centrioles (\textbf{Fig. 5c}), and, interestingly, induced both significant centriole amplification (P=0.03) and centriole loss (P=0.004) (\textbf{Fig. 5d}). This suggests that, although AL-Ala is kinase-impaired, high levels of protein in some cells may provide sufficient activity to induce centriole amplification. AL-PM-Plk4 phenocopied AL-Ala-Plk4; it was highly stable, displayed reduced
3xFLAG-Ubi labelling and Slimb binding, and its expression significantly decreased centriole numbers (control vs AL-PM, <2 centrioles, P=0.001) (Fig. 4e,f; Fig. 5d). Though our model predicts that the AL-PM mutant should be fully active, it is possible that Asp/Glu substitutions in the Plk4 activation loop do not adequately mimic phosphorylation and therefore disrupt kinase activity. Taken together, these results suggest that phosphorylation of the Plk4 activation loop is crucial for its kinase activity in cells, but some activity (~5 fold less) is present if the activation loop is not phosphorylated.

The previous findings reveal a potential autoinhibitory mechanism within Plk4. Previous studies have shown that Plk1 autoinhibits phosphorylation of its activation loop. In this case, the interdomain linker (IDL), a region immediately downstream of the kinase domain, masks the activation loop from Aurora A phosphorylation\textsuperscript{16,27,28}. During mitotic entry, the protein Bora relieves this autoinhibition by binding Plk1 and exposing the activation loop\textsuperscript{27,28}, allowing its phosphorylation and preventing further inhibition by the IDL. We hypothesized that a similar mechanism might operate within Plk4, specifically that Linker 1 prevents phosphorylation of the activation loop. As a result, Plk4 activity is significantly reduced. Furthermore, PB3 may act in a manner analogous to Bora and relieve Linker 1 autoinhibition of the kinase domain, thereby allowing autophosphorylation of the activation loop. Finally, Linker 1 may also be phosphorylated with the consequence that further autoinhibition is prevented.

MS analysis of Plk4 identified two phosphorylated residues within Linker 1 (S374/S378) (Fig. 4a). If Linker 1 (L1) is responsible for autoinhibition, then constructs containing L1 (but lacking PB3) should display reduced catalytic activity. To test this hypothesis, we first generated a series of Plk4 deletion constructs lacking C-terminal Linker 2-PB3, and examined their ability to autophosphorylate \textit{in vitro} (Fig. 5a). Purified Plk4 kinase domain (amino acids 1-317)
phosphorylates itself by trans-autophosphorylation and was stimulated by fusing it to GST to artificially induce dimerization (317-GST) (Fig. 5b), similar to human Plk4\(^{42}\). Strikingly, GST-602 autophosphorylates significantly less than 317-GST (Fig. 5b), supporting the hypothesis that the presence of L1 (without PB3) results in partial autoinhibition. Moreover, if phosphorylation of L1 blocks autoinhibition, then substituting L1 residues Ser374 and Ser378 with non-phosphorylatable alanines should severely impair kinase activity. Indeed, the double alanine mutation S374A/S378A within L1 (GST-L1-Ala-602) (Fig. 5a) decreased catalytic activity further, nearly to the level observed with a kinase-dead construct (GST-KD-602) (Fig. 5b). PB1-PB2 is unlikely to contribute to autoinhibition in the GST-602 constructs because first, PB1-PB2 does not bind the kinase domain when both are co-expressed in cells [Supplementary Fig. 1b, lanes 6, 8], and second, because purified PB1-PB2 does not inhibit kinase activity when mixed with 1-317-His\(_6\) protein\(^{18}\). These results suggest that L1 impairs autophosphorylation in vitro and that, when not phosphorylated, suppresses kinase activity.

To test the effects of mutating L1 on Plk4 activity in cells, we generated expression constructs of double Ala and phosphomimetic (S374D/S378D) mutants within full-length Plk4-EGFP. Identical to KD-Plk4, L1-Ala-Plk4 protein was dramatically stabilized and appeared to undergo little or no phosphorylation because it migrated as a sharp, high-mobility band on SDS-PAGE (Fig. 4c, lane 4; Fig. 4d, lane 3). As expected for a kinase-impaired mutant, L1-Ala also displayed reduced 3xFLAG-Ubi labeling and Slimb binding (Fig. 4d, lane 3). Although L1-Ala localized to centrioles (Fig. 5c), its expression had the same dominant-negative effect on centriole duplication as KD-Plk4 and significantly increased the percentage of cells with <2 centrioles (control vs L1-Ala, <2 centrioles, P=0.0007) (Fig. 5d). In contrast, the expressed phosphomimetic L1 mutant (L1-PM) was similar to WT because both proteins are present at low
levels, migrate on SDS-PAGE as a diffuse, highly-phosphorylated species, bind Slimb and are heavily ubiquitinated (Figs. 4e, lanes 1,4; 4f, lanes 1,3). Furthermore, L1-PM-Plk4 expression significantly induced centriole amplification (control vs L1-PM, >2 centrioles, P=0.008) (Fig. 5d). Taken together, these findings suggest that L1 is responsible for Plk4 autoinhibition and that inhibition can be relieved by phosphorylation of L1.

Relief of autoinhibition

Because PB3 deletion severely impairs kinase activity, we propose that an important function of PB3 is to contribute to autoinhibition relief. Linker 2 (L2) is adjacent to PB3 and a target of extensive phosphorylation (Fig. 4a), so we hypothesized that L2 might be required to position PB3 in a way that it can interact with L1 and the kinase domain. In this scenario, a change in L2 conformation (perhaps due to a change in its phosphorylation state) could reposition PB3 and alter kinase activity. For example, non-phosphorylated L2 could position PB3 in a conformation favorable to relieve autoinhibition and, subsequently, autophosphorylated L2 then repositions PB3 in a conformation that facilitates PB3-PB3 homodimerization.

To test the effects of L2 phospho-mutants on Plk4 activity in cells, we generated expression constructs harboring 11 alanine (L2-Ala) or phosphomimetic (Asp/Glu) (L2-PM) substitution mutations within full-length Plk4-EGFP. Similar to WT, L2-Ala protein was capable of efficient autodestruction (Fig. 4c, lane 5), was robustly labeled with 3xFLAG-Ubi, and associated with Slimb (Fig. 4d, lane 4), demonstrating that it retained kinase activity. Moreover, L2-Ala localized to centrioles (Fig. 5c) and induced significant centriole amplification (control vs L2-Ala, >2 centrioles, P=0.005) (Fig. 5d), demonstrating normal functionality. Therefore, non-phosphorylated L2 is compatible with Plk4 activity. Surprisingly,
our assays performed with L2-PM yielded similar results, demonstrating that this mutant was also able to efficiently autodestruct and increased the percentage of cells with >2 centrioles (control vs L2-PM, >2 centrioles, P=0.02), phenocopying L2-Ala in all respects (Figs. 4e, lane 5; 4f, lane 4; 5d). Thus, although the high level of phosphorylation of L2 is suggestive of a regulatory role, our attempts to use mutants to mimic the different phospho-states of L2 were inconclusive. Possibly, Asp/Glu mutations in L2 do not mimic phospho-modifications.

We devised a second approach to test whether L2 contributes to autoinhibition relief by combining the L2-Ala mutant with the nearly kinase-dead L1-Ala mutation. If non-phosphorylated L2 relieves L1-mediated autoinhibition, then non-phosphorylatable L2 should rescue the L1-Ala mutant phenotypes, allowing the protein to autodestruct and induce centriole amplification. Therefore, we generated a Plk4-EGFP mutant containing alanine substitutions in both L1 and L2 (L1-L2-Ala) and examined its expression in cells. Whereas L1-Ala protein levels were high compared to WT, addition of L2-Ala converted it to a less abundant protein and shifted most of it to a slower-migrating phosphorylated species on SDS-PAGE (Fig. 4g), indicating a partial restoration of kinase activity. L1-L2-Ala also induced significant centriole amplification. Therefore, converting the L2 domain to a non-phosphorylatable state rescued the loss-of-centrioles phenotype observed with the L1-Ala mutant (control vs L1-L2-Ala, >2 centrioles, P=0.01) (Fig. 5d). Thus, non-phosphorylated L2 helps relieve the autoinhibition generated by the L1-Ala mutation.

So far, our results indicate that PB3 is required for full kinase activity, that non-phosphorylated L1 autoinhibits Plk4, and that non-phosphorylated L2 can relieve L1-mediated inhibition. We hypothesize that PB3 specifically prevents L1-mediated inhibition, which predicts that L1 is responsible for inhibiting kinase activity in the PB3 deletion mutant. To test
this, we generated a new inducible Plk4-EGFP construct containing the phosphomimetic L1 mutation but lacking PB3 (L1-PM-ΔPB3). If our hypothesis is correct, then the inhibition caused by the ΔPB3 mutation should be rescued by the second L1-PM mutation. Strikingly, expression of the combined L1-PM and ΔPB3 mutant in cells resulted in a slower-migrating phosphorylated species with a dramatically decreased protein level compared to ΔPB3 (Fig. 4h). Moreover, expression of L1-PM-ΔPB3 rescued the loss of centrioles observed in cells expressing ΔPB3 (Fig. 3e), and significantly decreased the percentage cells containing <2 centrioles compared to ΔPB3 (ΔPB3 vs L1-PM-ΔPB3, <2 centrioles, P<0.001) (Fig. 5d). Therefore, our data support the remarkable conclusion that Plk4 itself contains domains that are responsible for both the suppression (L1) and rescue (PB3) of its activity.

We attempted to reconstitute PB3-mediated relief of autoinhibition in vitro using bacterially-expressed proteins, but were unable to express long Plk4 constructs containing L2 and PB3. Using purified proteins, we did find that PB3 mixed with GST-602 failed to rescue its full kinase activity in vitro (data not shown), suggesting that PB3 must be tethered to L2 in cis in order to release autoinhibition within the full-length polypeptide. Consistent with this idea, little kinase domain-myc protein co-immunoprecipitated with PB3-EGFP from cell lysates (Supplementary Fig. 1b, lane 9) indicating that the intermolecular interaction between these polypeptides is weak. Taken together, our results suggest that the primary function of PB3 is to relieve autoinhibition: L2-PB3 relieves L1-mediated autoinhibition, but PB3 is not necessary for Plk4-induced centriole assembly because expressed L1-PM-ΔPB3 drives centriole amplification.

A function for Plk4 autoinhibition

Plk4 is a suicide kinase that is active as synthesized and then robustly autodestructs. Our results
suggest that newly translated Plk4 is briefly inactivated by L1-mediated autoinhibition. Subsequently, Plk4 gains activity as autoinhibition is relieved. An obvious question is: what is accomplished by Plk4 autoinhibition? One possibility is that autoinhibition would favor Plk4 homodimer formation, while kinase function induces dimer separation. Such an activity has not previously been described for Plk4.

Recent studies have shown that kinase-dead (KD)-Plk4 expression induces centriole over-duplication in tumor-derived human cell lines when endogenous Plk4 is present. To explain this counterintuitive result, it was proposed that the DRE within KD-Plk4 is phosphorylated in trans after heterodimerizing with endogenous Plk4, and that phosphorylated KD-Plk4 then sequesters the Slimb/β-TrCP ubiquitin-ligase component, decreasing the available Slimb\textsuperscript{37}. The resulting increase in endogenous Plk4 levels would stimulate centriole amplification. However, this explanation is only valid if the Plk4 heterodimer can separate, allowing endogenous Plk4 to temporarily avoid proteasome-mediated destruction suffered by its phosphorylated KD partner and thus drive centriole amplification (Fig. 6a). Therefore, we examined whether kinase activity influenced dimerization in cells co-expressing different EGFP and myc-tagged Plk4 constructs. GFP immunoprecipitation and quantitative immunoblots were used to measure Plk4-myc binding to Plk4-EGFP. Although WT-myc binds WT-EGFP, a surprisingly small amount of WT-myc was recovered in the immunoprecipitate relative to WT-EGFP (Fig. 6b, lane 1). This difference was not due to the rapid turnover of the proteins because similar results were obtained using non-degradable Slimb-binding mutants (SBM) in both proteins (Fig. 6b, lane 2). Identical results were also observed by first depleting Slimb using RNAi to stabilize the WT-Plk4 proteins (Supplementary Fig. 5). Strikingly, we observed a ~4-fold increase in the amount of KD-Plk4-myc that associated with KD-Plk4-EGFP as
compared to the kinase active forms used in this assay (Fig. 6b, lane 3). However, dimerization was decreased when one subunit in the heterodimer was kinase active, which efficiently trans-phosphorylated KD-Plk4 and promoted its degradation (Fig. 6b, lanes 3,4). These results indicate that Plk4 kinase activity promotes dimer separation and that one active kinase domain in a heterodimer is sufficient to induce separation.

2.4 DISCUSSION

Multiple mechanisms constrain Plk activity to limited durations within the cell cycle and for good reason, since Plk overexpression is observed in a variety of cancers\(^5\text{-}^9\). Therefore, Plk members are attractive drug targets for developing anti-cancer therapies and the focus of several small molecule screens\(^47\text{-}^49\). Unlike monomeric Plk1 which autoinhibits and requires multiple external inputs for its activity, Plk4 was not known to autoinhibit but, instead, was thought to rely on degradation (stimulated by trans-autophosphorylation) as its sole means of regulation. Thus, inhibition of degradation activates Plk4 by allowing its protein levels to rise. During mitosis, Protein Phosphatase 2A\(^{\text{Twins}}\) fulfills this role by counteracting Plk4 autophosphorylation\(^50\).

Our findings demonstrate that Plk4 does autoinhibit, in a process that is as complex as it is for Plk1, and suggest a pathway for its regulation and activation (Fig. 7). One key feature of this pathway is that kinase activity facilitates homodimer separation. Without a mechanism of temporary autoinhibition, Plk4 dimer formation may be too transient to efficiently generate its phosphodegron, which occurs via trans-autophosphorylation within the homodimer. Consequently, without autoinhibition, Plk4 levels would accumulate and induce centriole amplification. According to our model, newly synthesized Plk4, like Plk1, is autoinhibited by
L1, preventing autophosphorylation of its activation loop (Fig. 7a). In turn, autoinhibition permits homodimerization through PB1-PB2 (Fig. 7b) and also prevents trans-autophosphorylation of the DRE. After homodimerization, L2 positions PB3 in a conformation to relieve L1-mediated autoinhibition, which then triggers extensive autophosphorylation throughout the molecule (Fig. 7c). On SDS-PAGE, Plk4 migrates as a series of phosphorylated polypeptides, appearing as a diffuse band or even a short ladder (e.g., Fig. 3b) and potentially revealing several distinct phosphorylation steps. Although the order in which the multiple phosphorylations occur is unknown, we propose that autophosphorylation of the activation loop likely occurs first.

L2 contains the largest number of autophosphorylated residues, but the role of phospho-L2 is not clear. Since overexpression of the L2-Ala mutant induces centriole amplification, L2 phosphorylation is not required for centriole assembly. Possibly, electrostatic repulsion due to numerous phosphorylations within L2 straightens the element, thereby repositioning PB3 in a conformation favoring PB3-PB3 binding and, consequently, stabilizing the homodimeric state (Fig. 7c). Phosphorylation of L1 subsequently blocks further autoinhibition, and the fully active Plk4 homodimer would rapidly trans-autophosphorylate the DREs to generate the Slimb-binding phosphodegrons which recruit the SCF^Slimb ubiquitin-ligase to tag nearby PB1 with multiple polyubiquitin chains (Fig. 7d). Finally, kinase activity stimulates dimer separation.

This study raises several intriguing questions. It is not understood how L1 autoinhibits Plk4 kinase activity, although it is possible that L1 could bind and “sequester” the activation loop, similar to models for Plk1 autoinhibition16. It is also unclear how L2-PB3 relieves autoinhibition; do they interact with the kinase domain, L1, or both? Obviously, several aspects of this model will not be clarified until the atomic structures of these elements are solved for
both the autoinhibited and uninhibited states of Plk4.

Importantly, Plk4 kinase activity induces dimer separation, but how this occurs mechanistically is not known. Our findings demonstrate that the PB1-PB2 cassette is the critical Plk4 dimerization domain and autophosphorylation could potentially disrupt dimerization. However, none of the PBs are targets of autophosphorylation, suggesting that phosphorylation of a Plk4 binding partner may facilitate dimer separation. Notably, the N-terminus of Asterless binds PB1-PB2\textsuperscript{44-46}, and this region in Cep152 (the human homolog of Asterless) is also a Plk4 substrate\textsuperscript{45}. Although Asterless targets Plk4 to centrioles\textsuperscript{44}, future studies of the Asterless-Plk4 interaction will be necessary to determine if the phosphorylation state of Asterless regulates Plk4 dimerization. Furthermore, what is the function of Plk4-induced dimer separation? One possibility is that dimer separation facilitates Plk4 degradation. It is not known whether the proteasome can process Plk4 as dimers or whether they must first separate. During mitosis, Plk4 also forms an aggregate on the surface of parent centrioles which is thought to serve as a platform for a newly emerging daughter centriole\textsuperscript{13,33,34}. Likely, Plk4 forms a higher order structure within this aggregate which may require disassembly before degradation of the individual subunits\textsuperscript{17}. In this regard, induced dimer separation could regulate disassembly of such a structure.

The Plk4 autophosphorylation pattern is complex and may select either down-regulation or activation, depending on which residues are modified. During mitosis when the Plk4 protein level peaks, its kinase activity somehow stimulates centriole assembly. Some of the \textit{in vivo} phosphorylated sites were not confirmed as \textit{in vitro} autophosphorylated residues and, potentially, additional kinases may regulate Plk4 activity. However, our previous RNAi screen of the \textit{Drosophila} kinome did not reveal additional kinases involved in regulating Plk4 levels\textsuperscript{50}. A
major future challenge will be to understand how Plk4’s phosphorylation pattern, and those of its centriolar targets, are spatially and temporally regulated as cells navigate through mitosis, so that Plk4 can induce centriole duplication without being inactivated.

2.5 Figures

Figure 2.1 Tandem Polo Boxes (PB1 and PB2) are critical domains for Plk4 homodimerization.
(a) Linear map of the *Drosophila* Plk4 polypeptide showing functional and structural domains including Polo boxes (PB1-3), the Downstream Regulatory Element (DRE) containing the Slimb-Recognition Motif (SRM), Linker 1 (L1), and Linker 2 (L2).

(b) Model of Plk4 autodestruction. Homodimerization facilitates *trans*-autophosphorylation, generating an extensive phosphodegron within each DRE. The SCF\(^{\text{Slimb/} \beta-\text{TrCP}}\) ubiquitin-ligase binds the phosphodegron and ubiquitinates Plk4, targeting it for proteasomal degradation.

(c) Anti-GFP immunoprecipitates (IPs) were prepared from lysates of S2 cells transiently co-overexpressing the indicated inducible EGFP and myc-tagged Plk4 constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP and myc. Amounts of Plk4-EGFP and associated Plk4-myc in the IPs were determined by densitometry of the anti-GFP and myc immunoblots and then normalizing the measurements with the amounts of WT-Plk4 present in the IPs. The plotted values are relative to the WT/WT co-IP in lane 1.
Figure 2.2 Polo Box 1 (PB1) contains multiple ubiquitination sites.

(a) Plk4 PB1 sequence alignment across four species. Fly PB1 contains nine lysine residues, seven of which were di-glycine modified (yellow highlight), indicating that these residues were ubiquitinated in vivo. Five of the ubiquitinated residues are conserved in vertebrate PB1.

(b) Quaternary structure of homodimerized PB1. Ubiquitinated lysine residues are shown in red.

(c) Anti-GFP immunoprecipitates from lysates of S2 cells transiently expressing 3xFLAG-Ubiquitin (Ubi) and the indicated Plk4-EGFP construct. Blots were probed with anti-GFP, anti-FLAG and anti-Slimb antibodies. Note that ubiquitination is greatly diminished in Plk4 lacking...
PB1 (lane 4) even though it binds endogenous Slimb.

(d) The relative protein stabilities of the indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cells transiently expressing the indicated inducible Plk4-EGFP constructs. (Co-transfected Nlp-EGFP was expressed under its endogenous promoter and served as a loading control in gels.)

(e) Anti-GFP immunoprecipitates from S2 cell lysates transiently expressing 3xFLAG-Ubi and the indicated inducible Plk4-EGFP construct. Blots were probed with anti-GFP, anti-FLAG and anti-Slimb antibodies. Note that although the K7A PB1 mutant binds Slimb, its ubiquitination is dramatically decreased.

(f) The relative protein stabilities of the indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cells transiently expressing the indicated inducible Plk4-EGFP constructs. (Co-transfected Nlp-EGFP was used as a loading control.)
Figure 2.3 Polo Box 3 (PB3) is required for full kinase activity.

(a) The relative protein stabilities of the indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cells transiently expressing the indicated inducible Plk4-EGFP constructs. Note the similar stabilities of KD and ΔPB3.

(b) The relative protein stabilities of the indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cells transiently co-expressing the indicated inducible Plk4-EGFP and myc constructs. Anti-GFP and myc immunoblots are shown at short and long exposures. Although WT phosphorylates Plk4-ΔPB3 and KD in trans, Plk4-ΔPB3 cannot phosphorylate KD.

(c) S2 cells co-expressing the indicated Plk4-EGFP construct (green) and Nlp-EGFP (a nuclear protein used as a transfection marker; green nuclei) were immunostained for PLP (to mark
centrioles; red) and Asterless (blue). DNA (blue). Plk4-ΔPB3 targets centrioles (white arrowhead) but also forms cytoplasmic punctate aggregates (yellow arrowheads).

(d) Anti-GFP immunoprecipitates from lysates of S2 cells transiently expressing the indicated inducible Plk4-EGFP construct (or control GFP) were probed for GFP and endogenous Asterless.

(e) Transfected S2 cells were induced to express Plk4-EGFP constructs for 3 days, then immunostained for PLP (a centriole marker), and their centrioles were counted. Nlp-EGFP transfected cells were used as control. Each bar shows the average percent of cells containing the indicated number of centrioles (n = 3 experiments; 300 cells counted per treatment, per experiment). Asterisks mark significant differences (relative to control) for comparisons mentioned in the text. Error bars indicate SEM. Centriole amplification (an increase in the percentage of cells with > 2 centrioles) occurs in cells expressing WT-Plk4 (P=0.01). Even though Plk4-ΔPB3 localizes to centrioles, it does not induce centriole amplification but instead significantly increases the percentage of cells with <2 centrioles (P<0.0001).
Figure 2.4 Plk4 autoinhibits, but inhibition can be relieved by Linker 1 phosphorylation or the presence of Linker 2-PB3.

(a) Linear map of Plk4 showing phosphorylated residues identified by tandem mass spectrometry. Note the high concentration of phosphorylated residues within the Downstream Regulatory Element (DRE) and Linker 2 (L2).
(b) *In vitro* autophosphorylation of purified His₆-tagged Plk4 kinase domain + DRE (amino acids 1-317) is significantly reduced in the triple alanine activation loop (AL-Ala) mutant, similar to kinase-dead (KD) Plk4. (Top) Coomassie-stained SDS-PAGE gel; (middle) corresponding autoradiograph. (Bottom) Quantitation of autophosphorylation activity (normalized by protein load and displayed relative to WT kinase activity). The average activity levels are significantly different (WT vs KD, P<0.0001; WT vs AL-Ala, P<0.0001; KD vs AL-Ala, P=0.009). Error bars indicate SEM.

(c) The indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cell co-expressing the indicated inducible Plk4-EGFP construct and Nlp-EGFP. Anti-GFP immunoblot is shown at short and long exposures.

(d) Anti-GFP IPs from lysates of S2 cells transiently co-expressing inducible 3xFLAG-Ubi and the indicated Plk4-EGFP construct. Blots were probed with anti-GFP, anti-FLAG and anti-Slimb antibodies.

(e) The indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cell co-expressing the indicated inducible Plk4-EGFP construct and Nlp-EGFP. Anti-GFP immunoblot is shown at short and long exposures.

(f) Anti-GFP IPs from S2 cell lysates transiently co-expressing inducible 3xFLAG-Ubi and the indicated Plk4-EGFP construct. Blots were probed with anti-GFP, anti-FLAG and anti-Slimb antibodies.

(g) The indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cells co-expressing the indicated inducible Plk4-EGFP construct and Nlp-EGFP. Note the shift in electrophoretic mobility of Plk4-L1-L2-Ala compared to the Plk4-L1-Ala, indicating greater phosphorylation of the kinase active Plk4-L1-L2-Ala mutant.
The indicated Plk4 constructs were analyzed by immunoblotting lysates of S2 cell co-expressing the indicated inducible Plk4-EGFP construct and Nlp-EGFP. Note the mobility shift of Plk4-L1-PM-ΔPB3 compared to Plk4-ΔPB3 (dashed lines), indicating that addition of L1-PM restored kinase activity to Plk4-ΔPB3.

Figure 2.5 Linker 1 autoinhibits Plk4 kinase activity in vitro, and expression of Plk4 phospho-
mutants influences centriole numbers.

(a-b) *In vitro* autophosphorylation of purified Plk4 deletion constructs reveal an autoinhibitory mechanism. (a) Schematic of Plk4 constructs used in this assay. All constructs were fused to GST to induce stable dimer formation. (b, top) Coomassie-stained SDS-PAGE gel; (middle) corresponding autoradiograph. (Bottom) Quantitation of autophosphorylation activity (normalized by protein load and displayed relative to 317-GST kinase activity). Asterisks indicate significant differences from GST-602 (vs 317-GST, P=0.01; vs GST-KD-602, P>0.0001; vs GST-L1-Ala-602, P<0.0001). Error bars indicate SEM.

(c) S2 cells co-expressing the indicated Plk4-EGFP construct (green) and Nlp-EGFP (green nuclei) were immunostained for PLP (red) to mark centrioles. DNA (blue).

(d) Transfected S2 cells were induced to express Plk4-EGFP constructs for 3 days, then immunostained for PLP, and their centrioles were counted. Nlp-EGFP transfected cells were used as control. Average percent of cells containing the indicated number of centrioles are shown (300 cells/treatment; three experiments). Asterisks mark significant differences (relative to control) for comparisons mentioned in the text. Error bars indicate SEM.
Figure 2.6 Plk4 kinase activity promotes homodimer separation.

(a) Model depicting how kinase-dead (KD)-Plk4 expression could stabilize endogenous Plk4 and amplify centrioles in cells. This mechanism explains published observations only if Plk4 dimers separate, sparing endogenous Plk4 from proteasomal degradation. Otherwise, both subunits would be degraded and centriole numbers would not change.
(b) Anti-GFP immunoprecipitations were prepared from lysates of S2 cells transiently co-overexpressing the indicated inducible EGFP and myc-tagged Plk4 constructs. (Top) Blots of the input lysates and IPs were probed for α-tubulin, GFP, and myc. (Bottom) Average amounts of Plk4-myc bound to Plk4-EGFP. For each treatment, a ratio of the measured amount of co-precipitated Plk4-myc to the measured amount of precipitated Plk4-EGFP was calculated. The graphed values are the calculated ratios relative to the WT-EGFP/WT-myc ratio in lane 1. Averages for WT/WT and KD/KD are significantly different (P=0.0002). Error bars indicate SEM.

![Speculative multistep model for Plk4 activation and regulation.](image)

Figure 2.7 Speculative multistep model for Plk4 activation and regulation. (a) Newly synthesized Plk4 is initially autoinhibited, allowing homodimer formation via PB1-PB2 interaction. (b) After dimerization, L2 appropriately positions PB3 to relieve autoinhibition. (c)
Plk4 autophosphorylates several domains with assorted consequences; our data do not mandate an order of phosphorylation. 1) Activation loop phosphorylation activates the kinase; 2) L2 autophosphorylation repositions PB3 to allow PB3-PB3 dimerization, stabilizing the homodimer; 3) \textit{trans}-autophosphorylation of the DRE generates the Slimb-binding phosphodegron; 4) L1 phosphorylation further relieves autoinhibition. (d) SCF$^{\text{Slimb}}$ is recruited and polyubiquitinates PB1 (green hexagons). (e) Kinase activity promotes dimer separation by an unknown mechanism. We propose that separation facilitates degradation during interphase.

2.6 Supplementary Data

Figure S2.1 PB1-PB2 is necessary and sufficient for homodimerization, whereas
PB3 does not homodimerize.

(a) PB1 and PB2 are critical for Plk4 homodimerization. Anti-GFP immunoprecipitates (IPs) were prepared from lysates of S2 cells transiently co-expressing the indicated inducible EGFP and WT-myc-tagged Plk4 constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP and myc. Amounts of Plk4-myc recovered in the GFP-IP were determined by densitometry of the immunoblots and then normalized to the amount of WT-Plk4-myc recovered in the WT-Plk4-EGFP IP in lane 1.

(b) PB1-PB2 is sufficient for dimerization. Anti-GFP IPs were prepared from lysates of S2 cells transiently co-expressing the indicated inducible EGFP and myc-tagged Plk4 constructs containing either EGFP alone, kinase domain, PB1-PB2, or PB3. Blots of the input lysates and IPs were probed for α-tubulin, GFP and myc. PB1-PB2 interacts strongly with itself, but PB3 does not. We note that Plk4 kinase domain associates with itself and also binds the GFP BPcoated-beads, explaining its presence in the PB1-PB2 and PB3 immunoprecipitates.

(c) PB3 does not homodimerize. PB3 expression was relatively low in the previous experiment, thus we induced greater expression of the PB3 constructs by addition of 2 mM CuSO4 and then assessed its ability to dimerize. Anti-GFP IPs were prepared from lysates of S2 cells transiently co-expressing the indicated inducible EGFP and myc-tagged PB3. Blots of the input lysates and IPs were probed for α-tubulin, GFP and myc.
Figure S2.2 Plk4 is labeled with numerous Gly-Gly modifications (ubiquitin signatures), several of which are found in PB1.

(a) Immunoprecipitation of WT-Plk4-EGFP from S2 cell lysates was subjected to tandem mass spectrometry (MS) to identify di-glycine modified residues. From 97% coverage of full-length Plk4 (which included all lysine residues), tandem MS identified 19 Gly-Gly modified lysines, and their positions are indicated in the linear map depicting Plk4 domain structure. Seven of the Gly-Gly modified lysines reside in PB1.

(b) Samples of Plk4 were prepared for MS analysis from in vivo ubiquitinated Plk4-EGFP immunoprecipitated from S2 cell lysates. Immunoprecipitated proteins were resolved by SDS-PAGE, and the band corresponding to Plk4-EGFP was cut from the gel, processed, and then analyzed by tandem MS. An example of a mass spectrum is shown. The sequence of the PB1
peptide is shown in bold; the Gly-Gly labeled Lys residue (K392) is indicated with an asterisk.

Figure S2.3. Unlike Plk4-ΔPB1-PB2, Plk4-ΔPB3 binds kinase-dead (KD)-Plk4 and localizes to centrioles. 

(a) Anti-GFP IPs were prepared from lysates of S2 cells transiently co-overexpressing the indicated inducible EGFP and myc-tagged Plk4 constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP and myc.
(b) Graph shows the percent of centrioles (labelled with the centriolar marker, PLP) that colocalize with the indicated Plk4-GFP protein. Co-localization was scored in cells that were clearly GFP positive (100 cells/construct and over 245 total centrioles were examined per construct).

Figure S2.4. Plk4 extensively autophosphorylates numerous residues within the linker and loop regions but not its Polo Boxes.

(a) Immunoprecipitation of WT-Plk4-EGFP from S2 cell lysates was subjected to tandem mass spectrometry (MS) to identify phosphorylated serine and threonine residues. From 97% coverage of full-length Plk4, MS identified 16 in vivo Ser/Thr phosphorylated residues, not including residues within the Downstream Regulatory Element (DRE) that were previously
described. *In vitro* samples of various GST- or His6-tagged downstream regions incubated with purified Plk4 kinase domain and ATP were also analyzed by MS. To prepare the *in vitro* samples, bacterially expressed and purified Plk4-DRE-His6 containing only the kinase domain and DRE (amino acids 1-317) was incubated with MgATP and GST, GST-PB1-PB2, GST. Proteins were then resolved by SDS-PAGE, phosphorylated bands were cut from the gel, processed, and then analyzed by tandem MS. The positions of the phospho-Ser/Thr residues are indicated in the linear map depicting Plk4 domain structure. Phosphorylated residues only observed in *in vivo* samples are highlighted in gray, those only observed *in vitro* are highlighted in purple, and those identified in both samples are highlighted in black.

(b) An example of a mass spectrum obtained from the analysis of *in vivo* phosphorylated Plk4 is shown. The sequence of the peptide is shown; the phosphorylated residue (S626) is indicated with an asterisk.

(c-e) *In vitro* kinase assays of purified His6-tagged Plk4 kinase domain + DRE (amino acids 1-317; denoted as 317-His) mixed with various Plk4 fusion proteins. The Coomassie-stained SDS-PAGE protein gels and their corresponding autoradiographs are shown. (c) 317-His autophosphorylates (lane 1) but does not phosphorylate purified His6-tagged PB1-PB2 (lane 2, red circle) or PB3 (lane 3, arrowhead) proteins. (d) 317-His does not phosphorylate purified GST protein alone (arrowhead) but does phosphorylate GST-Plk4 containing L1-PB3 (amino acids 318-741) (red circle). (e) 317-His also phosphorylates GST-PB1-PB3 protein (amino acids 382-741) (red circle). Both GST-L1-PB3 and GST-PB1-PB3 displayed some proteolysis and were phosphorylated.
Figure S2.5. Plk4 kinase activity promotes dimer separation.

Anti-GFP IPs were prepared from lysates of S2 cells transiently co-overexpressing the indicated inducible EGFP- and myc-tagged Plk4 constructs and RNAi-treated with control or Slimb dsRNA for 5 days. Blots of the input lysates and IPs were probed for α-tubulin, Slimb, GFP and myc. The greatest recovery of co-precipitated Plk4-myc is observed when two kinase-dead (KD)-Plk4 proteins are co-expressed (lane 3), indicating that dimer association is most stable in absence of kinase activity. Dimerization was not influenced by turnover of the constructs because Slimb depletion dramatically stabilized Plk4 levels and, yet, had no qualitative effect on the amount of Plk4-myc recovered in the IP (lanes 3 and 4).
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Table S2.1. *In vivo* ubiquitination sites of *Drosophila* Plk4. Transgenic full-length Plk4-EGFP was affinity-purified from lysates of transfected S2 cells, resolved by SDS-PAGE, processed (e.g., trypsinized) for mass spectrometry, and then analyzed by MS/MS (see Methods). Multiple
experiments were analyzed and their combined results cover 97% of the Plk4 sequence. GlyGly modifications are indicated by a +114 mass increase and are the remnants of trypsinized ubiquitin modifications. Only lysines were observed to be GlyGly modified. In the table, the modified residues of recovered peptides are underlined and bold font; all lysine modifications are GlyGly. Highlighted rows refer to residues within PB1 of Plk4. The spectrum of the fragment marked with an asterisk (K392) is shown in Supplementary Fig. 2b. Note that not all recovered peptides are listed. Mascot parameters were obtained from Scaffold 4.2.1 (Proteome Software).

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<th>Modified Residue</th>
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<th>Mascot Identity Score</th>
<th>Mascot Delta Ion Score</th>
<th>Peptide Identification Probability (%)</th>
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Table S2.2. Phosphorylation sites of the Kinase, Linker 1, Linker 2 and C-terminal domains of *Drosophila* Plk4. To identify *in vivo* phosphorylated residues, transgenic full-length Plk4-EGFP was affinity-purified from lysates of transfected S2 cells, resolved by SDS-PAGE, processed (e.g., trypsinized) for mass spectrometry, and then analyzed by MS/MS (see Methods). Four *in vivo* experiments were analyzed and their combined results cover 97% of the Plk4 sequence. To identify *in vitro* phosphorylated residues, bacterially-expressed and purified Plk4 domains were incubated with purified and active Plk4 kinase domain, and then similarly processed and analyzed by MS/MS. In the table, the phosphorylated Ser or Thr residues of recovered peptides are underlined and bold font. The spectrum of the fragment marked with an asterisk (S626) is shown in Supplementary Figure 4. Note that not all recovered peptides are listed. Mascot parameters were obtained from Scaffold 4.2.1 (Proteome Software).
2.7 Materials and Methods

Cell culture and double-stranded RNAi. Drosophila S2 cell culture, in vitro dsRNA synthesis, and RNAi treatments were performed as previously described\(^5\). Briefly, cells were cultured in Sf900II SFM media (Life Technologies). RNAi was performed in 6 well plates. Cells (50-90% confluency) were treated with 5μg of dsRNA in 1ml of media and replenished with fresh media/dsRNA every day for 4-7 days. Control dsRNA was synthesized from control DNA template amplified from a non-GFP sequence of the pEGFP-N1 vector (Clontech) using the primers 5’-CGCTTTTCTGGATTCATCGAC and 5’-TGAGTAACCTGAGGCTATGG (all primers used for dsRNA synthesis begin with the T7 promote sequence 5’-TAATACGACTCACTATAGGG). Slimb dsRNA was synthesized from cDNA using the primers 5’-GGCCGCCACATGCTGCG and 5’-CGGTCTTGTTCTCATTGGG.

Immunofluorescence microscopy. For immunostaining, S2 cells were fixed and processed exactly as previously described\(^5\) by spreading S2 cells on concanavalin A–coated, glass-bottom dishes and fixing with 10% formaldehyde. Primary antibodies were diluted to concentrations ranging from 1 to 20 μg/ml. They included rabbit anti–PLP and guinea pig anti-Asl. Secondary antibodies (conjugated with Cy2, Rhodamine red-X, or Cy5 [Jackson ImmunoResearch Laboratories, Inc.]) were used at manufacturer-recommended dilutions. Hoechst 33342 (Life Technologies) was used at a final dilution of 3.2 μM. Cells were mounted in 0.1 M n-propyl galate, 90% (by volume) glycerol, and 10% PBS solution. Specimens were imaged using a DeltaVision Core system (Applied Precision) equipped with an Olympus IX71 microscope, a 100× objective (NA 1.4), and a cooled charge-coupled device camera (CoolSNAP HQ2; Photometrics). Images were acquired with softWoRx v1.2 software (Applied Science).
**Immunoblotting.** S2 cell extracts were produced by lysing cells in cold PBS and 0.1% Triton X-100. Laemmli sample buffer was then added and boiled for 5 min. Samples of equal total protein were resolved by SDS-PAGE, blotted, probed with primary and secondary antibodies, and scanned on an Odyssey imager (Li-Cor Biosciences). Care was taken to avoid saturating the scans of blots. Transfected Nlp-EGFP (a constitutively-expressed nuclear protein\textsuperscript{13}) was used as loading control and transfection marker. Antibodies used for Western blotting include anti-Slimb\textsuperscript{50}, anti-Asl\textsuperscript{42}, anti-GFP monoclonal JL8 (Clontech), anti-myc (Cell Signaling Technologies) and anti-FLAG (Sigma-Aldrich) used at 1:1,000 dilutions. IRDye 800CW secondary antibodies (Li-Cor Biosciences) were prepared according to the manufacturer’s instructions and used at 1:1,500 dilutions.

**Constructs and transfection.** Full-length cDNA of *Drosophila* Plk4 was subcloned into a pMT vector containing in-frame coding sequence for EGFP or myc and the inducible metallothionein promoter. Phusion polymerase (ThermoFisher) was used according to manufacturer’s instructions to generate the series of Plk4 deletion and point mutants. Transient transfections of S2 cells were performed using a Nucleofector II and nucleofector kit V (Lonza) according to manufacturer’s instructions. Expression of all Plk4 constructs (and GFP control) was induced by addition of 50 µM–2 mM copper sulfate to the culture medium.

**GFP immunoprecipitation assays.** GFP-binding protein (GBP)\textsuperscript{52} was fused to the Fc domain of human IgG (pIg-Tail) (R&D Systems), tagged with His\textsubscript{6} in pET28a (EMD Biosciences), expressed in *E. coli* and purified on HisPur resin (ThermoFisher) according to manufacturer’s
instructions. Purified GBP was bound to Protein A-coupled Sepharose, and then cross-linked to the resin by incubating with 20mM dimethyl pimelimidate dihydrochloride in PBS, pH 8.3, 2 h at 22°C, and then quenching the coupling reaction by incubating with 0.2 M ethanolamine, pH 8.3, 1 h at 22°C. Antibody-coated beads were washed three times with 1.5 ml of cell lysis buffer (CLB; 50 mM Tris, pH 7.2, 125 mM NaCl, 2 mM DTT, 0.1% Triton X-100, and 0.1 mM PMSF). Transfected cells expressing recombinant proteins were lysed in CLB, and the lysates clarified by centrifugation. GBP-coated beads were rocked with lysate for 1 h at 4°C, washed two times with 1 ml CLB, and then boiled in Laemmli sample buffer. In vivo ubiquitination assays were performed by co-expressing Plk4-EGFP constructs with triple FLAG-tagged *Drosophila* ubiquitin (CG32744) (also under the metallothionein promoter and Cu-induced) and then probing the immunoblot of the cell lysate with anti-FLAG antibody (Sigma-Aldrich).

**In vitro autophosphorylation assays.** *Drosophila* Plk4 kinase domain + DRE (amino acids 1-317) C-terminally tagged with FLAG-His₆ (also called 317-His) was cloned into the pET28a vector, expressed in BL21 (DE3) bacteria, and purified on HisPur resin (ThermoFisher) according to manufacturer’s instructions. Purified Plk4 was autophosphorylated by incubating with 50 µM total ATP and, in cases, spiked with \([\gamma^{32}P]\) ATP, 1-2 h, 25°C, in reaction buffer (40 mM Na HEPES, pH 7.3, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM MnCl₂, 1 mM DTT, 10% glycerol). (To identify phosphorylated residues that are not generated by autophosphorylation, a sample of the same purified Plk4 is left untreated.) Samples were resolved on SDS-PAGE, Coomassie stained, and then processed for mass spectrometry. To identify autophosphorylated regions downstream of the kinase domain and DRE, various Plk4 domains were cloned into pGEX-6p2, pGEX-JDK, or pET28a vectors, expressed in BL21 (DE3), purified and mixed with
317-His + ATP. These include PB1-PB2-His₆, PB3-His₆, GST-L1-PB3 (amino acids 318-741), and GST-PB1-PB3 (amino acids 382-741). Single GST-tagged constructs that contained the kinase domain plus downstream regions (amino acids 1-602) were also examined for autophosphorylation (Fig. 5a).

Mass Spectrometry. Tandem mass spectrometry was performed at the NHLBI Proteomics Core Facility (NIH). Following resolution of protein samples by SDS-PAGE, selected Coomassie-stained bands were cut from the gel, then destained, reduced, alkylated, trypsin digested, and then the peptides extracted. Peptide samples were loaded onto a Zorbax C₁₈ trap column (Agilent Tech., Santa Clara, CA) to desalt the peptide mixture using an on-line Eksigent (Dublin, CA) nano-LC ultra HPLC system. The peptides were then separated on a 10 cm Picofrit Biobasic C₁₈ analytical column (New Objective, Woburn, MA). Peptides were eluted over a 90 min linear gradient of 5-35% acetonitrile/water containing 0.1% formic acid at a flow rate of 250 nL/min, ionized by electrospray ionization (ESI) in positive mode, and analyzed on a LTQ Orbitrap Velos (Thermo Electron Corp., San Jose, CA) mass spectrometer. All LC MS analyses were carried out in “data-dependent” mode in which the top 6 most intense precursor ions detected in the MS1 precursor scan (m/z 300-2000) were selected for fragmentation via collision induced dissociation (CID). Precursor ions were measured in the orbitrap at a resolution of 60,000 (m/z 400) and all fragment ions were measured in the ion trap.

LC MS/MS data acquired from tryptic digests were searched independently using the MASCOT algorithm. All data were searched against the Drosophila NCBInr protein database for peptide and protein identifications. Trypsin or chymotrypsin was specified as the digestion enzyme, allowing for up to 2 missed cleavage sites. Carbamidomethylation (C) was set as a
static modification and Oxidation (M) and Phosphorylation (S,T,Y) were selected as variable modifications. Precursor and fragment ion mass tolerances were set to 20 ppm and ± 0.8 Da, respectively. Following MASCOT searches, database search results were combined to obtain a comprehensive map of all peptides identified from Plk4.

Statistical Analysis. Means of measurements were analyzed for significant differences by two-tailed t-test or one-way ANOVA (followed by the Holm-Sidak post-test to evaluate differences between treatment pairs) using Prism 6 (GraphPad) software. Means are assumed to be significantly different if P<0.05. P values shown for pairwise comparisons of the Holm-Sidak post-test are adjusted for multiplicity. In figures, “*” indicates 0.05>P≥0.01, “**” indicates 0.01>P≥0.001, and “***” indicates 0.001>P for the indicated pairwise comparison. Error bars in all figures indicate standard error of the mean (SEM).

2.8. References


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15. Rivers, D.M., Moreno, S., Abraham, M. & Ahringer, J. PAR proteins direct asymmetry of


834-843 (2007).


Chapter 3: Asterless crosslinks and stabilizes Polo-like kinase 4 through two distinct binding domains.

*All S2 cell culture work performed by J.Klebba, all yeast 2 hybrid work performed by B.Galleta*

3.1. INTRODUCTION

Centrosomes are the primary microtubule organizing centers (MTOC) of cells and so are involved in multiple microtubule-dependent processes, including nucleation of spindle microtubules to guide proper chromosome segregation during mitosis and for correctly orienting the spindle relative to cortical cell-fate determinants during asymmetric divisions. Additionally, centrosomes are the precursors to the basal bodies of cilia and flagella and are the catalytic hubs for a number of important pathways regulating processes like cell cycle transitions and responses to cell stress and DNA damage [1,2]. One factor affecting centrosome function is their number within a cell, with two being the maximum number of centrosomes typically observed in a healthy cell. This aspect of centrosome function emerges from its components: centrioles are sub-micron, cylindrical structures that lie within a centrosome and recruit a dense structurally-amorphous but functionally-important cloud of proteins (known as Pericentriolar Material [PCM]) and also serve as the duplicating elements of the centrosome [3].

Centriole duplication is tightly controlled and normally occurs only once during S-phase when a single nascent procentriole begins growing orthogonally from each mother centriole. Mysregulation of duplication results in abnormal centrosome numbers and thus may perturb spindle orientation and chromosome segregation during mitosis. Centriole amplification -- the
over-duplication and subsequent over-abundance of centrioles within cells -- can drive genomic instability (a tumorigenic factor) and is often observed in cancer cells [4-7]. On the other hand, too few centrioles can lead to various ciliopathies [2,8-10]. For example, the neurodevelopmental disorder, microcephaly, can arise due to abnormal centrosome number during neuronal stem cell asymmetric division, leading to aneuploidy and apoptosis, thus depleting the neuronal progenitor cell population [11]. Impaired function of the centriolar protein CEP152 has been linked to Seckel Syndrome, an inherited variant of microcephaly, due to an accumulation of genomic defects [10].

Polo-like Kinase 4 (Plk4) is a phylogenetically conserved regulator of centriole duplication, and the over-expression of Plk4 drives centriole amplification [12-15]. Studies with cultured Drosophila and human cells have demonstrated that the SCF\textsuperscript{Slimb/TcRP} E3 ubiquitin ligase complex is required to suppress Plk4 protein level during interphase to prevent centriole overduplication [15-17]. However, the Plk4 protein level peaks during mitosis and, initially, mitotic centrioles are coated with Plk4. As cells progress through mitosis, Plk4 is “trimmed” to a single spot on each centriole, and Plk4 is believed then to modify this site on the centriole somehow, making the centriole competent to spawn a single daughter centriole from this spot during S-phase [15]. Plk4 differs from other members of the Polo kinase family in part because of its ability to homodimerize through an intermolecular interaction between its central tandem Polo Boxes (formerly known as the Cryptic Polo Box)[18-20]. Upon dimerization, Plk4 autophosphorylates \textit{in trans}, especially within a region termed the Downstream Regulatory Element (DRE) which houses the Slimb Recognition Motif (SRM). Phosphorylation of the SRM recruits the SCF\textsuperscript{Slimb} E3 ubiquitin ligase, resulting in the ubiquitination and proteosomal degradation of Plk4 [17].
The *Drosophila* protein, Asterless, is also required for centriole duplication and, notably, is a very early resident at the spot on the mother centriole that marks the future assembly site of the procentriole[21]. In humans, disruptions in the function of CEP152 (the vertebrate analog of Asterless) have been linked to both microcephaly (MCPH9) and Seckel syndrome (SCKL5)[22,10]. Asterless is a large protein containing extensive predicted coiled-coil regions. Previous studies identified three distinct functional domains: Asterless-A, B and C (Figure 1A), and their results support the model that Asterless acts as a scaffold for procentriole assembly by binding centriolar proteins [21,23-26]. Additionally, Asterless is involved in the recruitment of PCM to the centriole [26]. The C-terminus of Asterless, Asl-C, has been reported to associate with the centriolar protein SAS-4 while the N-terminus, Asl-A, has been shown to associate with the central tandem Polo Boxes of Plk4 [21,23]. In *Drosophila* S2 cells, Plk4 must associate with Asterless to localize to centrioles [21]. In nematodes, the protein SPD-2 functionally replaces Asterless and is responsible for Plk4 localization to centrioles. Similarly, CEP152 (the human ortholog of Asterless) is required for normal recruitment of cytoplasmic Plk4 to centrosomes in human cells [23]. In fly cells, overexpression of Asterless (or CEP152) drives centrosome amplification and can even induce *de novo* centriole biogenesis, both in a Plk4-dependent manner [21]. The mechanistic basis for these phenomena remains unclear.

We combined *in vivo* studies utilizing *Drosophila* S2 cells with *in vitro* and yeast 2 hybrid work to perform a comprehensive analysis of the interaction between Plk4 and Asterless. We find that, in S2 cells, deletion of the N-terminal third of Asterless (Asl-A) does not compromise Asterless’ ability to facilitate centriole duplication. Surprisingly, we find that only the C-terminus of Asterless (Asl-C) is necessary for centriole duplication and that this region of Asterless also associates with the tandem Polo Boxes of Plk4 independently of the N-terminus.
Thus, Asterless encodes two distinct Plk4 binding domains. Furthermore, we define the function of both Plk4 binding sites: Asl-A promotes Plk4 dimerization while Asl-C crosslinks and stabilizes Plk4. Together, these two domains of Asterless shuttle Plk4 to the centriole and stabilize Plk4 by forming a complex that prevents Plk4 degradation.
3.2. RESULTS

Asl-C rescues the elimination of endogenous Asterless

Asterless is an essential centriolar scaffold protein that contains binding sites for two different centriolar proteins. The N-terminus of the protein, Asterless-A (Asl-A, aa 1-374), interacts with the tandem Polo Boxes 1 and 2 (PB1-2) of Plk4 while the centriolar protein SAS-4 binds to the C-terminus of Asterless (Asterless-C, Asl-C, aa 631-994) (Figure 1A) [21,23]. Even though several studies describe these interactions, no study has reported the functional competency of each Asterless fragment to promote centriole duplication. Therefore, we examined Asterless function in greater detail, specifically by identifying the roles of the N- and C-terminal regions in governing Plk4 activity.

First, is any fragment sufficient to rescue centriole duplication following depletion of endogenous Asterless? To assess this, endogenous Asterless protein in Drosophila S2 cells was depleted by RNAi and different portions of GFP-tagged Asterless ectopically expressed. Depletion of the endogenous Asterless was necessary to minimize its interaction with the expressed fragments, which otherwise might have skewed our results. Because we were not expressing full-length Asterless, we used dsRNA sequences complementary to exons in the non-expressed regions of Asterless; this strategy achieved significantly better knockdown than dsRNA targeting Asterless’ UTRs. Cells were treated with the indicated dsRNA for 3 days, after which the indicated GFP-tagged Asterless fragment was transfected and its expression was initiated. After an additional 3 days of dsRNA treatment, cells were immunostained and their centrioles were counted. Three days of Asterless RNAi are not sufficient to remove centrioles from a cycling S2 population (data not shown), thus our scheme assessed each fragment’s ability to promote procentriole growth from an existing “mother” centriole rather than de novo centriole
assembly. Centrioles were identified by immunostaining cells for PLP; though PLP is present in centrioles and PCM, interphase S2 cells shed their PCM and so PLP immunostaining serves as a bona fide marker for interphase centrioles (Figure 1D). Endogenous Asterless knockdown and transgene expression were confirmed with Western blots of cell lysates (Figure 1C).

As expected, transfection of cells with only GFP fails to rescue centriole duplication following endogenous Asterless knockdown: the percent of treated cells with <2 centrioles is significantly greater than control RNAi cells (Figure 1B). Surprisingly, expression of GFP-tagged Asl-A moderately rescues centriole duplication because the percent of cells with 2 centrioles is increased (Figure 1B). This result differs from previous studies which found that Asl-A expressed in human cells acts as a dominant negative and inhibits centriole duplication [24]. Prior studies have shown that in S2 cells, Plk4 is dependent on Asterless for centriolar localization. Thus, it is possible that Asl-A, with Plk4 bound, is able to interact with residual endogenous Asterless to reach the centriole and begin the process of centriole biogenesis. To support this hypothesis, we note that Asl-A was able to weakly localize to centrioles in control RNA treated cells, presumably because of an interaction with endogenous Asterless (data not shown).

The central region of Asterless, Asl-B, is not known to interact with any other centriolar proteins in S2 cells and it did not rescue centriole duplication. Unexpectedly, Asl-C was an effective rescuer of centriole duplication as evidenced by an increase in the percentage of cells with >2 centrioles (Figure 1B). Nearly identical results were seen with Asl-B+C, where Asl-A has been deleted from the full length molecule. Previous studies have identified an Asterless mutant, Asl-M1, which was rendered incapable of binding Plk4 through deletion of residues 24-55 within the N-terminus of Asterless. Expression of this mutant had a dominant negative effect on centriole duplication in Drosophila S2 cells, while in Drosophila embryos and unfertilized
eggs expression of Asl-M1 produced free MTOCs that were devoid of centrioles [21]. This result suggests that Plk4’s interaction with Asl-A is necessary for centriole duplication, however it is difficult to understand how Asl-M1 could act as a dominant negative. This mutant form of Asl does not interact with Plk4, thus the cellular Plk4 population should be unperturbed and available to be localized to the centriole by endogenous Asl. Given the relatively quick half-turnover rate of Asterless at the centrosome, around 5 minutes [27], it seems unlikely that expression of Asl-M1 would monopolize all centriolar Asterless binding sites. Our data show that expression of Asl-C alone is able to rescue the loss of endogenous Asterless, and that Asl-A is expendable in regards to the centriole duplication process. As centriole duplication cannot occur without Plk4 activity at the centriole, these results raise the intriguing possibility that Asterless encodes a second Plk4 binding site within the C-terminus of the protein.

_Asterless stabilizes Plk4 through a direct interaction on its C-terminus_

Endogenous Plk4 protein levels in _Drosophila_ S2 cells are nearly undetectable by standard Western blotting, yet overexpression of Asterless is sufficient to drive centrosome amplification in a Plk4-dependent manner [21]. Given the relative protein levels of Plk4 (undetectable) and Asterless (easily detectable), it seems likely that Plk4 is the limiting factor in the initial steps of centriole biogenesis. In that case, it is surprising that further increasing the non-limiting factor by overexpressing ectopic Asterless would increase centriole duplication. A previous study proposed the explanation that Asterless may stabilize Plk4 at centrosomes and thus increases centriole duplication [21], but this hypothesis has yet to be tested. To assess what effect Asterless has on Plk4 protein level, we ectopically expressed Plk4-GFP and Asterless-V5 in S2 cells and analyzed Plk4 levels by immunoblotting cell lysates (Figure 2A).
protein nucleophosmin(Nlp-GFP) was co-transfected and used as a loading control.

Plk4 homodimerizes and autophosphorylates in trans, generating a phosphodegron targeted by SCF$^{\text{Slimb}}$, an E3 ubiquitin ligase, and ultimately directing Plk4’s destruction. Accordingly, protein levels of Plk4-GFP are not easily detectable (Figure 2A, lane 1). However, co-transfection of Asterless-V5 with Plk4-GFP resulted in a marked rise in Plk4 protein level as well as a shift in the electrophoretic mobility of Plk4 (Figure 2A, lane 2). The stabilizing effect of Asterless was not limited to the centriolar environment because centriole depletion by RNAi of SAS-6 did not disrupt Asterless’ stabilization of Plk4 (Supplementary Figure 1B). These data reveal a new aspect of Asterless’ control of Plk4 activity; in addition to localizing Plk4 to centrioles, Asterless stabilizes Plk4. Also, the change in electrophoretic mobility of Plk4 raises the interesting possibility that Asterless stimulates Plk4 kinase activity, increasing Plk4 autophosphorylation (thereby shifting its mobility) and potentially increasing phosphorylation of other Plk4 substrates. (We were unable to bacterially express full-length Drosophila Plk4, however, and so further in vitro experiments to study this possibility were not feasible.) To determine if Plk4 stabilization requires a direct interaction between Plk4 and Asterless, we expressed a Plk4 mutant lacking the critical Asterless-binding domains, Polo Boxes 1+2 (Plk4-ΔPB-1+2-GFP) (Figure 2B, lane 1). Expression of Asl-V5 had no effect on the protein level of the ΔPB1+2 mutant (Figure 2B, lane 2), indicating that Asterless must directly interact with Plk4 to stabilize the protein. Taken together, we show that the direct interaction between Asterless and Polo Box 1+2 stabilizes Plk4.

To identify the domain of Asterless stabilizing Plk4, we co-expressed V5-tagged Asterless fragments (A, B and C) (Figure 1A) with Plk4-GFP in S2 cells and then used immunoblotting to measure Plk4 protein levels in cell lysates (Figure 2A). Expression of Asl-A
or Asl-B did not alter Plk4 protein level (Figure 2A, lanes 3,4). (Interestingly, Asl-A expression appears to decrease the electrophoretic mobility of Plk4.) In contrast, expression of Asl-C clearly stabilizes Plk4 (Figure 2A, lane 5). To eliminate the possibility that endogenous Asterless is involved in generating this result, cells RNAi-treated with control or Asterless dsRNA were transiently transfected with Plk4-GFP alone or co-transfected with Asl-C-V5 (Figure 2C). Asl-C stabilized Plk4 to an approximately equal extent in the presence or absence of endogenous Asterless (Figure 2C, lanes 3,4). In both cases, Asl-C expression increases Plk4 protein levels relative to Plk4 expression alone (Figure 2C, lane 1). Notably, Asl-C expression does not visibly alter the electrophoretic properties of Plk4 (Figure 2C, lane 4), indicating that some Asterless domain other than Asl-C induces Plk4’s shift to slower mobility (suggesting increased Plk4 autophosphorylation); we propose that Asl-A has this function.

Our results are consistent with the idea that Asl-C directly interacts with Polo Boxes 1+2 of Plk4. To further test this possibility, we used multiple experimental approaches: *in vivo* immunoprecipitations, *in vitro* pull-downs, and a yeast 2 hybrid screen. S2 cells treated with Asl dsRNA were transiently transfected with Asl-C-V5 alone or with the indicated Plk4-GFP construct or GFP as a control. Western blots of the immunoprecipitated GFP-tagged proteins were used to determine Asl-C-V5 association with the different Plk4 constructs. Very little Asl-C-V5 bound the beads-only or GFP controls (Figure 2D). Conversely, Asl-C readily bound full-length Plk4, while deletion of Polo Boxes 1+2 (ΔPB1+2-GFP) abolishes the interaction (Figure 2D). Therefore, in S2 cells, Asl-C can bind Plk4 through Polo Boxes 1+2, and these Polo Boxes are necessary for binding. Furthermore, these findings were corroborated in a yeast two hybrid screen that revealed an interaction between Plk4 382-602 and Asl 625-993 (Supplementary Figure 3A). Additionally, we identified Asl-C mutants that abolish the interaction with PB1+2
through yeast 2 hybrid analysis (Supplementary Figure 3B). We were unable to identify a
specific portion of Asl-C necessary to bind PB1+2, suggesting that similar to Asl-A, the majority
of Asl-C is required to interact with PB1+2 [21].

We next tested whether Polo Boxes 1+2 were sufficient to bind Asl-C in vitro. GST-Polo
Box 1+2 and Asl-C-GST were bacterially expressed and purified. The GST tag was
proteolytically cleaved from Asl-C-GST (Supplementary Figure 2B) to prevent any GST/GST
dimerization between PB1+2 and Asl-C. 4 µM GST or 1 µM GST-PB1+2 were cross-linked to
glutathione beads, and then incubated with 1 µM Asl-C. While some Asl-C bound to the GST
control in these conditions, we see a marked increase in the amount of Asl-C that is pulled down
with GST-PB1+2 relative to GST alone. We note that increasing the amount of PB1+2 cross-
linked to the beads only stimulated a minor increase in the amount of Asl-C recovered,
suggesting that the stoichiometry of the Asl-C/PB1+2 interaction may be quite complex (data not
shown). Qualitatively, the N-terminus of Asterless displayed more robust binding to PB1+2 than
the C-terminus Asl-A pulled-down more readily with PB1+2 (Supplementary Figure 2A).

Asterless and Plk4 form aggregates in cells

Ubiquitination of multiple residues within Polo Box 1 of Plk4 is necessary for proper
degradation of the protein [20]. Asterless may stabilize Plk4 by binding this region of Plk4 and
interfering with ubiquitination of Plk4. If true, Asterless overexpression should decrease Plk4
ubiquitination. We ectopically expressed Plk4-GFP alone or with Asterless-V5 in S2 cells co-
transfected with 3xFLAG-Ubiquitin (Figure 3A), then immunoprecipitated Plk4-EGFP from cell
lysates and Western blotted to determine Plk4’s ubiquitination state and its association with
Slimb and Asterless. As shown previously [17], expressed Plk4 readily retrieves endogenous
Slimb and is robustly ubiquitinated (Figure 3A, lane 1). Co-expression of Asterless with Plk4 dramatically increased Plk4 protein levels (Figure 3A, lane 2, Input), but the increase in associated Asterless (Figure 3A, lane 2, IP) did not affect Slimb binding or ubiquitination. We conclude that Asterless does not inhibit Slimb-mediated ubiquitination of Plk4.

Typically, transiently expressed Plk4-GFP is observed only at centrioles (Figure 3B). When Asterless is co-expressed with Plk4, however, large aggregates containing Plk4, Asterless, and PLP appear in cells (Figure 3B). Interestingly, these aggregates are not simply a result of Plk4 over-abundance, because expression of a highly stable form of Plk4 (the Slimb-binding mutant of Plk4 [Plk4-SBM]) does not generate such aggregates [15]. Therefore, over-expressed Asterless promotes the formation of complexes containing centriolar proteins, and, at least in the case of Plk4, stabilizes even ubiquitinated protein.

Expression of just Asl-C phenocopies full-length Asterless expression and promotes the formation of Plk4/Asl complexes. Many of these complexes contain numerous centrioles (Figure 3C), indicating that proteins within these complexes retain their ability to drive centriole duplication. To gain a clearer understanding of the properties of these Plk4/Asterless complexes we performed structured illumination microscopy on cells ectopically expressing Plk4-GFP and Asterless-V5 (Figure 3E). We see that these complexes have a distinct structure. Plk4 fills the inside of the Plk4/Asterless complex, and appears to form a repeating pattern. Notably, Asterless forms a shell around Plk4 and is almost totally excluded from the interior of the Plk4/Asterless complex.
Asterless prevents turnover of Plk4

To our knowledge, this is the first demonstration that Asterless stabilizes Plk4, but it was surprising to us to find that Asterless does not interfere with the initial steps of Plk4 autoregulation: Asterless stabilizes Plk4 even though Slimb-binding and ubiquitination are normal. We hypothesized that formation of a Plk4/Asterless complex prevents proteasomal mediated destruction of Plk4. To test this, Plk4-GFP expression was induced overnight in S2 cells, and the ribosomal inhibitor cycloheximide (CHX) was introduced in fresh medium. Samples were taken every 2 hours and lysates of samples were Western blotted to measure Plk4 protein levels (Figure 4A). In S2 cells, Plk4 is relatively short-lived: 50% of expressed Plk4 was eliminated by 2 hrs after CHX addition and only ~20% remained by 6 hrs post-CHX addition (Figure 4B). As a control, Plk4-SBM-GFP -- a mutant not targeted by Slimb -- was subjected to the same assay and, as expected, the level of Plk4-SBM was constant throughout the time course. Finally, co-expression of Asterless-V5 significantly stabilized Plk4-GFP; by 6 hrs post-CHX addition, ~80% of the Plk4 protein remained. (Interestingly, Asterless protein turnover is not affected by Plk4 expression (Supplementary Figure2D). These results show that formation of Plk4/Asterless complex stabilizes Plk4 by protecting it from proteasomal degradation. Asterless’ incorporation into this complex is much more transient and normal turnover of the protein still occurs. It is possible that the centrosomal and cytoplasmic populations of Asterless are constantly exchanging, allowing Asterless to quickly respond to Plk4 stabilization and shuttle it to the centriole to begin the process of centriole biogenesis.

To assess which region of Asterless inhibits Plk4 turnover, we repeated the turnover assay, this time co-transfecting Plk4-GFP with V5-tagged Asterless fragments (Figure 4C). Expression of Asl-B does not affect the kinetics of Plk4 degradation, an expected result as this
fragment of Asterless does not interact with Plk4. Asl-C expression slows the turnover rate of Plk4, but the effect is not nearly as dramatic as what is seen with full length Asterless, suggesting that Asl-C is not as efficient in maintaining the Plk4/Asterless complex. Somewhat unexpectedly, overexpression of Asl-A results in a subtle inhibition of Plk4 turnover. Neither Asl-A nor Asl-C were nearly as efficient as full length Asterless is terms of stabilizing Plk4, suggesting that Asl-C is responsible for formation of the Plk4/Asterless complex but a secondary Asterless-Plk4 binding site found on Asl-A is required to maximally prevent protein turnover.

_Asterless promotes Plk4 autophosphorylation_

Our findings reveal that overexpression of Asterless stabilizes Plk4 through an interaction between Polo Boxes 1+2 of Plk4 and the “C” fragment of Asterless. Overexpression studies shed little light onto the nature of Asl-A’s interaction with Polo Box 1+2 of Plk4, leading us to deplete Asterless from cells expressing Plk4-GFP in an attempt to understand the significance of this association. S2 cells treated with control or Asl dsRNA were transiently transfected with Plk4-GFP and protein levels were assessed through immunoblotting (Figure 5A). Asterless depletion doubled the Plk4 protein level, but also of interest was the change in mobility of the Plk4 protein band. When resolved by SDS-PAGE, WT-Plk4 normally runs as a multi-banded smear, indicating that Plk4 has multiple phosphorylation states within cells (Figure 5A, lane 1). Depletion of Asterless altered the typical electrophoretic mobility of Plk4, increasing the prominence of the focused, fast-migrating band which probably corresponds to low- or non-phosphorylated Plk4 (Figure 5A, lane 2). We hypothesize that Asterless promotes Plk4 autophosphorylation.

To investigate this possibility, Plk4-GFP was expressed in cells that had been treated with
control or Asterless dsRNA and then Plk4 protein turnover was assayed. Plk4 turnover in control cells is efficient; by 6 hrs only a small portion of Plk4 protein remains (Figure 5B). In contrast, Asterless depletion significantly increases Plk4 stability. But not all Plk4 isoforms are equally spared in this sample. The focused, high-mobility (presumably non-phosphorylated) band of Plk4 persists longer through the time course than the diffuse, lower-mobility (presumably phosphorylated) Plk4. This observation is consistent with our knowledge of Plk4 autoregulation: Plk4 autophosphorylation in trans promotes its destruction. These findings support the hypothesis that Asterless increases Plk4 autophosphorylation, promoting its destruction.

*In vitro* and *in vivo* studies have shown previously that Asterless is not necessary for Plk4 dimerization and autophosphorylation, which seemingly disagrees with our current results [17]. However, the previous studies were performed in conditions that caused exogenous Plk4 to be expressed at levels greatly exceeding the physiological level of Plk4, which might obscure the impact of Asterless on Plk4 dimerization at its lower, physiological concentration. We propose that at low cellular Plk4 levels, Asterless binds Plk4 and promotes its autophosphorylation. To test this prediction, we transiently transfected control or Asterless dsRNA treated cells with Plk4-EGFP. Ectopic Plk4-EGFP expression was under control of the metallothionein promoter, allowing us to manipulate Plk4 expression levels by varying the concentration of CuSO4 added to the cell’s media (Figure 5C). Expressed Plk4-EGFP protein was immunoprecipitated, and quantitative immunoblotting was used to measure the amount of associated endogenous Slimb. Our hypothesis predicts that at low protein levels, Plk4 requires Asterless to properly *trans*-autophosphorylate, which is a prerequisite for Slimb binding [17,20]. As Plk4 expression is induced to higher non-physiological levels, the requirement for Asterless should diminish and
autophosphorylation (and thus Slimb recruitment) should become independent of Asterless. Our results confirm this prediction. When expressed at a low level (by induction with 0.5 mM CuSO₄), Plk4 protein was undetectable in control dsRNA cell lysate (Figure 5C, Lane 1, Input), and immunoprecipitated Plk4-EGFP readily pulled down Slimb (Figure 5C, Lane 1, IP), indicating that Plk4 had autophosphorylated and been down-regulated by SCF^{Slimb}. Asterless regulates low concentrations of Plk4 because Slimb binding sharply decreased (and Plk4 protein in the lysate increased) in Asterless depleted cells (Figure 5C, Lane 2). Increasing the expressed Plk4 protein levels partially (Figure 5C, Lanes 3,4) or even completely (Figure 5C, Lanes 5,6) relieved the need for Asterless to facilitate Plk4 autophosphorylation (as indicated by the increasing independence of Slimb binding from Asterless depletion). These data indicate that in physiological conditions, Asterless is necessary for Slimb-mediated degradation of Plk4 and add a new layer of complexity to Plk4 regulation and Asterless function in cells.

Newly synthesized Plk4 is monomeric and relatively stable due to its limited ability to autophosphorylate in cis. An early step in Plk4 autoregulation is homodimerization, which increases Plk4’s kinase activity, which in turn results in the autophosphorylation that precedes ubiquitination [15]. Plk4 dimerization is mediated by interactions between PB1+2 [20], the Asterless binding domain of Plk4. One simple explanation for our finding that Asterless facilitates Plk4 phosphorylation is that Asterless may interact with multiple Plk4 monomers, thus increasing the local Plk4 concentration and probability of dimerization. Furthermore, if the PB1+2 interactions within a dimer are tenuous, Asterless binding to each Plk4 protein could cross-link and stabilize the dimer. By doing so, Asterless binding would promote Plk4 trans-autophosphorylation and down-regulation. The effect of Asterless on Plk4 dimerization was assayed in S2 cells co-expressing Plk4-EGFP and Plk4-Myc, and dimerization was evaluated by
measuring the amount of Plk4-myc associated with immunoprecipitated Plk4-EGFP. As expected, Plk4-myc binds Plk4-EGFP and is readily recovered (Figure 6A, Lane 1). Overexpression of Asterless in these cells further increased the amount of Plk4-Myc bound to Plk4-EGFP by ~1.5 fold. To eliminate the possibility that the increased dimerization was simply a result of increased Plk4 protein levels due to Asterless expression, we performed the same assay using the hyper-stable Kinase Dead forms of Plk4-EGFP (KD-GFP) and Plk4-Myc (KD-myc), thereby allowing us to assess Asterless’ effect on Plk4 dimerization independently of the stability Asterless imparts to wt-Plk4. Nearly identical results were obtained with these stable Plk4 mutants (Figure 6A, Lanes 3,4). We conclude that Asterless interacts with Plk4 to increase or prolong its dimerized state, allowing for the necessary trans autophosphorylation to recruit Slimb and regulate Plk4 protein levels.

Asterless-A promotes Plk4 dimerization

To identify which fragment of Asterless increases Plk4 dimerization, we performed the dimerization assay using fragments of Asterless or full-length Asterless (as control). Expression of the N-terminal portion of Asterless (Asl-A) increased dimerization by ~1.5 fold and nearly matched the dimerization enhancement seen with full-length Asterless expression (Figure 6B, Lanes 2,3). Overexpression of either Asl-B, which does not bind Plk4, or Asl-C, which does bind PB1+2 of Plk4 (Figure 2D), did not affect Plk4 dimerization (Figure 6B, Lanes 4,5). Therefore, the two separate Plk4-binding domains of Asterless have distinctly different influences on Plk4: whereas Asl-C decreases Plk4 turnover, Asl-A increases the dimerized population of Plk4 in cells.

These data suggest that Asl-A is sufficient to promote Plk4 dimerization and trans-
autophosphorylation. To assess this, we again used Slimb-binding as a reporter for Plk4 autophosphorylation in cells. Expressed Plk4-EGFP was immunoprecipitated from S2 cells manipulated with four different combinations of Asterless RNAi and Asl-A co-transfection treatments (Figure 6C). The amounts of Plk4-associated endogenous Slimb were measured with Western blotting. Expression of Asl-A-V5 did not alter the level of endogenous Slimb bound to immunoprecipitated Plk4-EGFP observed in control cells (Figure 6C, Lanes 1,2), indicating that the level of Plk4 autophosphorylation was unchanged. As noted above, Slimb-binding was clearly diminished in Asterless RNAi treated cells (Figure 6C, Lane 3), indicating that endogenous Asterless promotes Plk4 autophosphorylation. Slimb binding was restored by expression of Asl-A-V5 in Asterless RNAi treated cells (Figure 6C, lane 4). Our results are consistent with the model that Asterless plays a key role in limiting cellular Plk4 levels by enhancing Plk4 dimerization, thereby promoting Plk4 autophosphorylation and initiating its autodestruct mechanism.

3.3. DISCUSSION

Our study reveals several important properties of Asterless, some of which are novel and some of which are contrary to the conclusions of previous studies or the implications of current Asterless models. It is possible that our contrary results arise from real differences between Drosophila S2 cells and the experimental systems used in other studies, but could simply arise from differences in experimental conditions. For instance, we find that the C-terminus of Asterless, Asl-C, is a region of particular importance in S2 cells; Asl-C can bind the Polo Boxes 1+2 of Plk4 and is sufficient to support centriole duplication. Then what is the function of the N-terminus of Asterless, Asl-A, if it is not required for centriole duplication? Our results show that
Asl-A can bind Plk4 (which agrees with other studies), promote dimerization of Plk4, and facilitate Plk4 autophosphorylation. Therefore, both termini of Asterless can interact with Plk4 but exert distinctly different effects. Asl-A ultimately down-regulates Plk4 by expediting the modification of Plk4 to become an SCF-Slimb substrate. In contrast, Asl-C assists Plk4 to duplicate centrioles. Our data do not demonstrate how Asl-C assists Plk4-mediated centriole duplication, but our observation that expressed Asterless and Asl-C both trigger formation of centriole-containing Plk4 patches within cells suggests that the Asl-C domain assembles Plk4-dense structures capable of fostering new centrioles.

We believe the following model of Asterless function is consistent with our current results (Figure 8). Within the cell are two distinct Asterless populations: a concentrated population at the centrosome and a diffuse cytoplasmic pool. Our evidence suggests that at the centrosome, Asterless oligomerizes to form a lattice like network via intermolecular interactions on its C-terminus. Centrosomal Asterless is not static however, quite the contrary as this population of Asterless has a half turnover rate of around 5 minutes [27]. We believe the relatively quick exchange between the centrosomal and cytosplasmic Asterless populations allows Asterless to quickly respond to changes in cellular Plk4 levels. Throughout most of the cell cycle, Plk4 levels are kept quite low, a result of Plk4’s self-regulating nature. However, in order for this autodestruct mechanism to be effective, Plk4 must dimerize. We propose that as Asterless binds cytoplasmic Plk4 to localize it to the centriole, it also increases the efficiency of Plk4 dimerization by serving as a scaffold for Plk4 monomers to associate. While the possibility that Asterless binds already dimerized Plk4 cannot be ruled out, it seems unlikely as Plk4 dimers are quite transient[20]. Instead, Asterless likely binds Plk4 monomers and serves as a platform for dimerization, initiating Plk4’s autodestruct mechanism (Figure 8A). Therefore, throughout
the majority of the cell cycle, Asterless instigates destruction of Plk4. As the cell nears mitosis, the protein phosphatase PP2A counteracts Plk4 autophosphorylation to stabilize Plk4 [28], allowing Asterless to successfully shuttle Plk4 to the centriole. Plk4 is held at the centriole by direct interactions with both Asl-A and Asl-C (Figure 8B), preventing Plk4 from being degraded by the proteasome. This allows Asterless to build a population of stable, dimerized Plk4 on the centriole (Figure 8C). The deposition of dimerized, and therefore maximally active Plk4 [20] on the centriole is critical, as this likely allows Plk4 to phosphorylate the substrates necessary to begin centriole duplication. Thus, Asterless delivers fully activated Plk4 to the centriole, stabilizes it, and allows for centriole duplication to occur.

A longstanding question in the field of centriole biology is what restrains a single daughter to assemble on a mother centriole. We previously showed that during mitosis Plk4 asymmetrically localizes on the centriole for a brief period and then is degraded [15]. Our study describes a mechanism by which Asterless can quickly respond to PP2A mediated stabilization of Plk4 and localize Plk4 to the centriole. We show that Asterless likely stabilizes Plk4 on the centriole, providing a mechanistic explanation for the asymmetric Plk4 spot seen on centrioles during mitosis. As the cell progresses through mitosis, Plk4 is completely removed from the centriole but how this occurs remains a mystery. Plk4 heavily phosphorylates the N-terminus of Asterless but no physiological consequence has been identified with this phosphorylation. It is conceivable that phosphorylation of Asterless may function to separate Plk4 from Asterless, and in turn dislodge Plk4 from the centriole surface, preventing aberrant Plk4 activity during interphase. Our analyses show that Asterless plays a very dynamic role in centriole duplication and open new lines of investigation into the initial steps of centriole biogenesis.
3.4. Figures

Figure 3.1. Asterless-A is expendable in centriole duplication

(a) Asterless is separated into 3 distinct functional domains. Schematic of interaction sites between Asl and its binding partners.

(b) Transfected S2 cells were induced to express the indicated Asl-EGFP fragments for 3 days, then immunostained for PLP, and their centrioles were counted. EGFP transfected cells were used as control. Average percent of cells containing the indicated number of centrioles are shown (300 cells/treatment; three experiments). Cell lysates were immunoblotted to verify expression of the Asl-EGFP fragments and knockdown of endogenous Asterless(c).
Figure 3.2. Asterless stabilizes Plk4 through a direct interaction with Plk4’s tandem Polo Boxes.

(a) Immunoblots of S2 cells transiently expressing Plk4-GFP and the indicated Asl-V5 construct. Note that expression of full length Asterless-V5 and Asl-C-V5 stabilize Plk4. Nlp-GFP was co-transfected and used as a loading control.
(b) Immunoblots of S2 cells expressing Plk4-ΔPB1+2-GFP or co-expressing Plk4-ΔPB1+2-GFP and Asl-V5. Deletion of Plk4’s tandem Polo Boxes prevents the interaction between Plk4 and Asterless.

(c) S2 cells treated with control or Asterless dsRNA were transfected with Plk4-EGFP alone or co-transfected with Plk4-EGFP and Asl-C-V5. Cell lysates were analyzed through immunoblotting to determine Plk4 protein levels.

(d) Anti-GFP IPs were prepared from lysates of S2 cells treated with Asterless dsRNA and transiently co-expressing Asl-C-V5 with the indicated Plk4-EGFP construct. Blots were probed with anti-GFP, anti-Asl and anti-V5 antibodies.
Figure 3.3. Plk4 and Asterless form large cytoplasmic complexes

(a) Anti-GFP IPs from lysates of S2 cells transiently co-expressing inducible 3xFLAG-Ubi and Plk4-EGFP alone or with Asl-V5. Blots were probed with anti-GFP, anti-tubulin, anti-FLAG and anti-Slimb antibodies.

(b) S2 cells co-expressing Plk4-EGFP (green) and Asl-V5 constructs were immunostained for PLP (red) to mark centrioles and V5 to identify transfected V5-tagged constructs. DNA (blue). Notably, Asl-V5 and Plk4-EGFP co-localize into cytoplasmic complexes, or “clouds”, which recruit PLP.

(c) S2 cells co-expressing Plk4-EGFP (green) and Asl-V5 were immunostained for PLP (red) to mark centrioles. The nuclear protein nucleophosmin (Nlp-GFP) was co-transfected and used as a transfection marker(green). DNA (blue).

(d) Quantitation of PLP/Plk4 “clouds” when Plk4-EGFP is expressed alone or with Asl-V5.

(e) S2 cells co-expressing Plk4-EGFP (green) and Asl-V5 were analyzed using Structured Illumination Microscopy (SIM). Cells were immunostained for V5 (white) to mark Asl-V5. DNA (blue).
Figure 3.4 Plk4 protein turnover is prevented by Asterless

(a) S2 cells co-expressing the indicated constructs were treated with cycloheximide (CHX) to inhibit protein translation. Plk4-EGFP protein turnover was tracked over a 6 hour time course through quantitative immunoblotting and values are plotted (Panel B&C). Expression of Asl-V5 inhibits degradation of Plk4-EGFP (b) as does expression of Asl-C-V5 (c).
Figure 3.5. Asterless promotes Slimb recruitment to Plk4

(a) S2 cells were treated with control or Asterless dsRNA and transiently transfected with Plk4-EGFP. Immunoblots were performed on cell lysates to determine Plk4 protein levels. Nlp-GFP was co-transfected and used as a loading control.

(b) S2 cells treated with control or Asterless dsRNA were transiently transfected with Plk4-EGFP. Plk4-EGFP was induced to express overnight and cells were treated the following morning with cycloheximide to inhibit protein translation. Plk4-EGFP protein turnover was tracked over a 6 hour time course through quantitative immunoblotting and values are plotted below.
(c) S2 cells treated with control or Asterless RNA were transiently transfected with inducible Plk4-GFP. Expression of Plk4-GFP was induced with varying amounts of Copper Sulfate (CuSO4) and anti-GFP IPs were performed. Blots of the input lysates and IPs were probed for α-tubulin, anti-GFP, anti-Asl and anti-Slimb antibodies. Notably, at low Plk4 expression levels, Asterless is required for maximal recruitment of Slimb to Plk4.

Figure 3.6. Plk4 dimerization is prolonged by Asterless binding

(a) Anti-GFP IPs were prepared from lysates of S2 cells transiently expressing the
indicated constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP, V5, Asl and myc. (Bottom) Amounts of Plk4-myc bound to Plk4-EGFP. For each treatment, a ratio of the measured amount of co-precipitated Plk4-myc to the measured amount of precipitated Plk4-EGFP was calculated. The plotted values are relative to WT-GFP/WT-myc(Lane 1) or KD-GFP/KD-myc(Lane 3).

(b) To identify which portion of Asterless was increasing Plk4 dimerization, the identical procedure described in panel A was performed, this time using V5-tagged fragments of Asterless(Lanes 3-5) as well as the full length molecule as a control(Lane 2). Blots of the input lysates and IPs were probed for α-tubulin, GFP, V5, Asl and myc. (Bottom) Amounts of Plk4-myc bound to Plk4-EGFP. For each treatment, a ratio of the measured amount of co-precipitated Plk4-myc to the measured amount of precipitated Plk4-EGFP was calculated. The plotted values are relative to WT-GFP/WT-myc(Lane 1).
Figure 3.7. Expression of Asl-A increases Slimb recruitment to Plk4 in the absence of endogenous Asterless.

(a) S2 cells were treated with control or Asterless dsRNA and then transiently transfected with the indicated constructs. Anti-GFP IPs were performed and immunoblots of the input lysates and IPs were probed for α-tubulin, GFP, V5, and Asl. As previously noted, loss of endogenous Asterless prevents proper Slimb recruitment to Plk4 (Lane 3) but this is rescued by expression of Asl-A (Lane 4).
Figure 3.8. Asterless asymmetrically stabilizes Plk4 on the centriole.

(a) Schematic of Asterless’ interaction with Plk4. Note that Asterless houses two distinct Plk4 binding domains.

(b) Zoomed image of Plk4 and Asterless on the centriole. Both Asl-A and Asl-C bind Plk4, but the interaction between Plk4 and Asl-C is critical for stabilizing Plk4 at the centriole.
(c) Asterless asymmetrically stabilizes Plk4 on the centriole, allowing for a single spot to be ‘licensed’ for centriole duplication. PP2A provides an initial stabilization of Plk4, allowing Asterless to build the Plk4 spot.

3.5 Supplementary Data

Figure S3.1. Asterless stabilizes Plk4 independent of the centriolar environment.

(a) S2 cells treated with control or SAS-6 dsRNA were transfected with the indicated constructs and then immunostained for PLP to mark centrioles. Lysates were collected and immunoblots
were performed to analyze Plk4-EGFP protein levels and to verify SAS-6 knockdown(b). As anticipated, depletion of SAS-6 prevents centriole duplication and results in far fewer centrioles in the S2 cell population treated with SAS-6 dsRNA. However, loss of SAS-6 does not alter the stabilization of Plk4 through Asterless overexpression.

Figure S3.2. Both Asterless-A and C interact with Polo Boxes 1&2 of Plk4.

(a) Bacterially expressed and purified GST-PB1+2 and Asl-A-His<sub>6</sub> were mixed <em>in vitro</em> and a GST pulldown was performed. Protein are visualized by Coomassie stain.

(b) Asl-C-GST was purified and then incubated with Precission Protease to specifically cleave off the GST tag.
(c) Asl-C from panel B was mixed with purified GST-PB1-2 \textit{in vitro} and a GST pulldown was performed. Protein are visualized by Coomassie stain.

(d) S2 cells were transiently transfected with the indicated constructs. The transfected proteins were induced to express overnight and cells were treated the following morning with cycloheximide to inhibit protein translation. Asl-V5 protein turnover was tracked over a 6 hour time course through quantitative immunoblotting and values are plotted.

Figure S3.3. A yeast two hybrid screen reveals an interaction between Asl-C and Polo Boxes 1&2 of Plk4.
(a) Yeast two hybrid analyses show a strong interaction between Asl-C(Asl-F3) and Polo Boxes 1&2 of Plk4(Plk4-F2).

(b) Low fidelity PCR was performed to generate Asl-C mutants. A yeast two hybrid screen was performed to identify mutants that were no longer able to interact with Polo Box 1&2 of Plk4.

(c) Description of the mutations found in the mutant forms of Asl-C analyzed in panel B.
3.5. REFERENCES


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4.1 Conclusions and Future Directions

My thesis has focused on understanding the mechanisms by which Polo-like kinase 4 (Plk4) activity is regulated within the cell. My work, as well as numerous other high profile studies, has shown Plk4 to be an extremely potent nucleator of centriole duplication; even very small increases in cellular Plk4 levels can trigger centriole amplification. In Chapter 1 of my thesis, I show that Plk4 is a ‘suicide kinase’, meaning that the protein self-regulates its protein levels by promoting its own destruction. Plk4 is able to do this by homodimerizing and creating a phosphodegron through trans-autophosphorylation of numerous residues within a region of the protein known as the Downstream Regulatory Element (DRE). Phosphorylation of a single residue, Serine 293, is sufficient to recruit the E3 ubiquitin ligase SCF \( \text{Slimb} \) to Plk4 while phosphorylation of flanking hydroxyl residues creates a high affinity binding site for Slimb. This ensures that Slimb is able to effectively ubiquitinate Plk4 and mark it for destruction. We find that Slimb mainly ubiquitinates Polo Box 1 of Plk4 and using in vivo mass spectrometry we identified and validated 7 residues that are Slimb ubiquitination targets. Taken together, my work describes how Slimb is efficiently recruited to Plk4 and where Slimb ubiquitinates Plk4 to mark the protein for destruction by the 26S proteasome.

After initial synthesis, Plk4 exists as stable monomer as monomeric Plk4 is unable to cis-autophosphorylate its DRE to recruit Slimb. We find that although stabilized, monomeric Plk4 is unable to drive centriole duplication because Plk4 encodes a mechanism of autoinhibition that keeps the protein inactive in this state. Therefore, we now know that in addition to promoting its
own destruction, Plk4 limits its activity by self-regulating kinase activity. Autoinhibition is a characteristic found in the other members of the Polo kinase family, and my work now establishes that kinase autoinhibition is a conserved feature throughout the Polo kinase family. Unlike other members of the Polo kinase family, we show that Plk4 is able relieve its own autoinhibition and that Polo Box 3 plays a crucial role in this process. The first step in the relief of autoinhibition is dimerization, which I have identified as being mediated through interactions between Polo Boxes 1&2 of Plk4. Dimerization only leads to a partial activation of Plk4 however, and using in vivo mass spectrometry and mutational analyses we have identified residues on Plk4’s activation loop and two linker regions that must be phosphorylated for Plk4 to become maximally active, which then initiates the autodestruct mechanism described in Chapter 1 of my thesis. Importantly, the linker regions of Polo kinase have also been shown to play a role in autoinhibition, suggesting that the mechanism of autoinhibition is also conserved throughout the Polo kinase family.

Chapter 3 of my thesis provides an in-depth characterization of the interaction between Plk4 and the centriolar protein Asterless. Numerous prior studies have provided a cursory description of this interaction and all report similar findings: Asterless, specifically the N-terminal region of the protein, interacts with Polo Boxes 1&2 of Plk4 and localizes Plk4 to the centriole. In this chapter, I show that in addition to localizing Plk4 to the centriole, Asterless also stabilizes Plk4, and that this stabilization occurs during mitosis. Surprisingly, this stabilization is due to an interaction between Polo Boxes 1&2 of Plk4 and the C-terminus of Asterless. Therefore, we now know that Asterless encodes two distinct Plk4 binding domains: one on the C-terminus and another on the N-terminus. I further go on to describe the interaction between the N-terminus of Asterless and Plk4 and show that this portion of Asterless serves as a scaffold for
Plk4 monomers to dimerize, thereby helping to regulate Plk4 protein levels. We find that this interaction predominates throughout interphase of the cell cycle, providing another mechanism by which Plk4 activity is regulated. Altogether, my work provides insight into the initial steps of centriole biogenesis and describes novel interactions between Plk4 and Asterless.

The work I have performed during my graduate career has furthered our knowledge of how Plk4 functions and how the protein is regulated to prevent aberrant centriole formation, but many questions still remain. Although I have shown that Polo Box 3 of Plk4 is necessary for relief of Plk4 autoinhibition, the actual mechanism for how Polo Box 3 performs this function has remained elusive. Further studies will be necessary to determine how Polo Box 3 is relieving autoinhibition and if another kinase may also play a role in this process. Additionally, crystallization of Plk4 will be necessary to fully understand Plk4 autoinhibition and how the residues we identified work to suppress kinase activity. Further characterization of the interaction between Plk4 and Asterless will also strengthen our understanding of their roles in the process of centriole biogenesis. My data suggest that Asterless binding stimulates Plk4 kinase activity and this is an attractive hypothesis as Plk4 kinase activity is required to form a centriole. Therefore, it is possible that in addition to localizing Plk4 to the centriole and stabilizing the protein, Asterless also ensures that Plk4 is maximally active so it can begin the process for centriole formation. In vitro kinase assays will be required to test this hypothesis. I have shown that Asterless increases Plk4 dimerization, but the mechanism by how this happens is still unclear. One possibility is that Asterless binds dimerized Plk4 and holds the protein in the state while another possibility is that Asterless binds Plk4 monomers and brings them together, allowing for dimerization. Distinguishing between these two possibilities will be difficult and will require identification of mutations on Polo Boxes 1&2 that inhibit Plk4 dimerization but do
not interfere with the interaction between Asterless and Plk4. Answering these questions will help our understanding of the fundamental processes involved in centriole biogenesis.

5.1 References


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