# A STRUCTURAL STUDY OF CONSERVED CENTRIOLE DUPLICATION MACHINERY 

Lauren K. Slevin

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Approved by:
Kevin Slep
Kerry Bloom
Robert Duronio
Matthew Redinbo

Marcey Waters
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#### Abstract

Lauren K. Slevin: A structural study of conserved centriole duplication machinery (Under the direction of Kevin C. Slep)

Centrioles are microtubule-based cylindrical structures that act within organelles responsible for nucleating polarized microtubule networks. Centrioles have an inherent ninefold radial symmetry with species-dependent dimensions. Despite the fact that centrioles have been described by biologists for over a century, the process by which cells license and assemble nascent centrioles has only recently started to come to light. It is now known that centrioles, like DNA, duplicate via a cell cycle-regulated mechanism, and that a core set of five conserved proteins is necessary for centriole duplication. Polo-like Kinase 4 (Plk4) is a highly conserved serine/threonine kinase required for centriole duplication licensing. Plk4, along with all other Plk family members, contains arrayed Polo Box (PB) domains, a motif that undergoes hetero- or homo-dimerization to bind targets, localize to subcellular structures, and regulate kinase activity; however, the mechanism by which Plk4 employs its PBs to license centriole duplication has been unclear. Here, we harness x-ray crystallography, biochemistry, and cell biology to show that Drosophila melanogaster Plk4 contains three complete PB domains with distinct functions. The first two, PB1-PB2, form a homodimer in trans in both crystallographic form and in solution. We use in vitro pulldowns to demonstrate that PB1-PB2 are collectively needed to bind Asterless, a centriole scaffolding protein that localizes FL Plk4 to centrioles. Additionally, the PB1-PB2


homodimer is required for downregulation of the FL molecule, limiting centriole duplication. Finally, we use D.m. S2 cells to show that PB1-PB2 localizes to centrioles via conserved electrostatic interactions. The C-terminal PB domain, PB3, also forms a canonical PB fold, yet it shows species-dependent architecture and oligomerization states, demonstrating that PB3 is a structurally variable domain with species-dependent functions. Further work examines the role of a dynein light chain (LC8) in oligomerizing Anastral-2 (Ana2), a downstream centriole component that is a candidate for Plk4 phosphorylation. LC8-based Ana2 tetramerization has further implications for the role of Ana2 during centriole duplication. Collectively, our work delineates novel domains and interactions in the fundamental centriole licensing and assembly proteins Plk4 and Ana2, and implicates conserved mechanisms in centriole biogenesis.

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## PREFACE

Chapter 2 is a published manuscript describing work done in collaboration with the lab of Gregory Rogers (University of Arizona). This work was published in Structure in November 2012. Jonathan Nye carried out centriole count assays while Dan Buster completed all pull-down experiments and Western Blots (both from the Rogers lab). Derek Pinkerton (an undergraduate in the Slep lab) assisted with centriole localization experiments. I purified Plk4 PB1-PB2, obtained crystals, and assisted Kevin Slep with structure refinement, as well as designed, cloned, and performed the centriole localization assay (as well as the basic patch mutant assay, unpublished; described in detail in Chapter 6). Kevin Slep, Gregory Rogers and I designed experiments and drafted the manuscript.

Slevin, L.K., Nye, J., Pinkerton, D.C., Buster, D.W., Rogers, G.C., and Slep, K.C. (2012). The structure of the Plk4 cryptic polo box reveals two tandem polo boxes required for centriole duplication. Structure. 20, 1905-1917.

Chapter 3 describes an ongoing project that is a collaborative effort between Kevin Slep and me to define the role of Plk4's C-terminal PB3 in centriole duplication. I designed the constructs, collected diffraction data on the D.m. PB3 crystals, processed the data, built, and refined the structure, while Kevin Slep crystallized the protein and solved the structure. I designed, cloned, and performed all microscopy work to determine the localization and centriole phenotype of PB3 in both D.m. S2 cells and H.s. RPEI cells. A manuscript detailing this work is currently being drafted for publication.

Chapter 5 is a published manuscript co-authored by Erin Romes, a former Slep lab graduate student, and me. This work was published in The Journal of Biological Chemistry in August 2014. Erin Romes cloned LC8, designed Ana2 peptides, purified proteins for crystallization trials, crystallized LC8-Ana2 pep1, and assisted with ITC. I performed ITC, solved both structures as presented, analyzed comparative LC8 binding targets, and designed and carried out the SEC-MALS assays. Mary Dandulakis (a Slep lab undergraduate student) crystallized the LC8-Ana2 pep2 complex. I drafted all figures and wrote the manuscript. Kevin Slep designated the proposed LC8 binding sites within Ana2 and oversaw experimental design.

Slevin, L.K.,* Romes, E.M.,* Dandulakis, M.G., and Slep, K.C. (2014). The mechanism of dynein light chain LC8-mediated oligomerization of the Ana2 centriole duplication factor. Journal of Biological Chemistry. 289, 20727-20739. *indicates equal contributions

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## LIST OF ABBREVIATIONS AND SYMBOLS

| Å | Ångstrom |
| :---: | :---: |
| Asl | Asterless |
| ACD | asymmetric cell division |
| ASU | asymmetric unit |
| $\beta-\mathrm{ME}$ | $\beta$-mercaptoethanol |
| BSA | bovine serum albumin |
| C.e. | Caenorhabditis elegans (nematode) |
| CMV | cytomegalovirus |
| $\mathrm{CuSO}_{4}$ | copper sulfate |
| Da | Dalton |
| DIC | Dynein intermediate chain |
| D.m. | Drosophila melanogaster (fruit fly) |
| DMEM | Dulbecco's modified eagle medium |
| DRE | downstream regulatory element |
| eGFP | enhanced green fluorescent protein |
| FBS | fetal bovine serum |
| FL | full-length |
| G1 | cell cycle gap phase 1 |
| G2 | cell cycle gap phase 2 |
| GFP | green fluorescent protein |
| H.s. | Homo sapiens (human) |
| IDL | inter-domain linker |


| IPTG | isopropyl $\beta$-D-1-thiogalactopyranoside |
| :---: | :---: |
| kDa | kiloDalton |
| L | liter (or leucine) |
| LC8 | Dynein light chain 8 |
| M | molar (or mitosis, or methionine; context-dependent) |
| mg | milligram |
| min | minute |
| mL | milliliter |
| M.m. | Mus musculus (mouse) |
| mM | millimolar |
| NaCl | sodium chloride |
| nm | nanometer |
| PAGE | polyacrylamide gel electrophoresis |
| PB | Polo Box |
| PBS | phosphate-buffered saline |
| D-PLP | Pericentrin-Like Protein (Drosophila melanogaster) |
| PCM | pericentriolar material |
| Pctn | Pericentrin |
| PDB | Protein Data Bank (pdb.org) |
| PEG | polyethylene glycol |
| Plk | Polo-like Kinase |
| RMSD | root-mean-square deviation |
| resi | residues |


| RNAi | RNA interference |
| :--- | :--- |
| RPE1 | Human retinal pigment epithelial cells |
| S | cell cycle synthesis phase |
| SAS | Spindle Assembly Abnormal Protein |
| SDS | sodium dodecyl sulfate |
| SEC-MALS | Size Exclusion Chromatography coupled with Multi-Angle Light Scattering |
| SeMet | selenomethionine |
| $\mu \mathrm{m}$ | microgram |
| $\mu \mathrm{l}$ | microliter |
| $\mu \mathrm{M}$ | micromolar |
| WT | wild type |
| ZYG-1 | Zygotic lethal protein-1 |

## CHAPTER 1: INTRODUCTION

## Centrioles are microtubule-based cylindrical structures essential in nucleating polarized microtubule networks

Centrioles are cylindrical subcellular structures observed in nearly all metazoans. Centrioles take species- and cell type-specific dimensions; human somatic cells undergoing mitotic divisions typically have centrioles 250 nm in diameter and 500 nm long (Winey and O'Toole, 2014). Centriolar structure consists of a cartwheel-like formation with 9 spokes that emanate radially (Figure 1.1). This cartwheel forms the basis of the 9 -fold radial symmetry of the microtubule triplet blades (van Breugel et al., 2011; Kitagawa et al., 2011), which decorate the length of the centriolar barrel and are evident in electron microscopic images of centrioles (Guichard et al., 2013; Li et al., 2012). Centrioles play critical roles in centrosomes and cilia, organelles that nucleate and organize dynamic polarized microtubule networks (Figure 1.1).

In cells that have exited the cell cycle (in $\mathrm{G}_{0}$ ), centrioles are recruited to the cell cortex, where they become modified centrioles called basal bodies. Basal bodies then nucleate either a single cilium (called a primary cilium) or many cilia (motile cilia), depending on the tissue type and centriole number (Scholey and Anderson, 2006). The primary cilium emanates from the cell in a finger-like projection, and employs its extended surface area to sense environmental cues and facilitate cell-to-cell communication. The formation of the primary cilium requires exactly two centrioles, which form the physical base of the single cilium in each cell. In other contexts, many centrioles are needed to nucleate
formation of multiple motile cilia, which aid in various tissues (including the fallopian tubes, airway, and brain of human cells (Bylander et al., 2013)) to create movement and propagate signals.

In actively dividing cells, however, centrioles play an important role in forming the centrosome, an organelle that nucleates and organizes the bipolar mitotic spindle (Figure 1.1). Centrosomal centrioles play a key role in regulating these activities in most vertebrate somatic cells, though centrioles are interestingly absent in higher fungi, higher plants, and many oocyte types (Carvalho-Santos et al., 2010), in which acentriolar structures govern mitotic spindle formation. While centrioles themselves do not nucleate microtubule growth, data from several labs in recent years suggest that centrioles are central in recruiting, stabilizing, and spatially organizing the pericentriolar material complex (PCM), which contains such microtubule nucleators as the $\gamma$-TuRC complex (Mennella et al., 2012). A centrosome consists of a pair of centrioles and their associated shells of PCM components. The centriole pair is connected orthogonally, forming an " $L$ " shape between an older ("mother") centriole and a nascent ("daughter") centriole (Rodrigues-Martins et al., 2007). The centrioles maintain a physical connection, with proteinaceous tethers including C-Nap1, LRRC45 and Rootletin, within the mature centrosome through mitosis (Bornens et al., 1987; He et al., 2013). The centrosome seeds growth of the microtubule polymers, and maintains secure connections with the minus ends of microtubules (both astral and spindle microtubules), while the plus ends extend towards the kinetochores. During mitosis, exactly two centrosomes (and therefore a total of exactly four centrioles) form the bipolar mitotic spindle. Supernumerary centrosomes have been implicated in forming multi-polar spindles, a situation that leads to aneuploidy, a hallmark of tumorigenesis (Godinho and Pellman, 2014).

Misregulated centriole duplication is also associated with diseases including dwarfism, male sterility, and primary microcephaly (Chavali et al., 2014; Nigg and Raff, 2009; BettencourtDias et al., 2011), underlining the importance of learning how cells "count" their centrioles and regulate their biogenesis.


Figure 1.1. Centrioles form the basis of centrosomes and cilia. (A) Cross-section view of the proximal end of a centriole (see Figure 1.3 for greater molecular detail). Microtubule triplet blades are depicted in green, surrounding the inner barrel. (B) Side view of a vertebrate centriole. Microtubules are depicted as single green tubes for simplicity. (C) Mother-daughter centriole pairs (shown as connected green barrels) form the basis of either centrosomes (cycling cells) or cilia $\left(\mathrm{G}_{0}\right)$. Top, centrosomes consist of two centrioles (green) and an associated ordered cloud of PCM components (yellow), and nucleate the mitotic spindle to accurately segregate sister chromatids (magenta) during cell division. Bottom, cilia employ centrioles to nucleate inner microtubules of various lengths and multiplicity (purple) to form a finger-like protrusion from the cell.

## Centrioles undergo duplication in a cell-cycle- and Plk4-dependent manner

Despite the fact that cell biologists have studied centrioles for more than a century (Boveri 1909), the manner by which cells create their centrioles has only recently begun to come to light. Using microscopic and molecular techniques, researchers have discovered a couple of mechanisms by which cells undertake centriole biogenesis. In the less understood de novo mechanism, nascent, unconnected centrioles are made in the cytoplasm due to the presence of excessive duplication components. The de novo mechanism has largely been described in oocyte systems (Rodrigues-Martins et al., 2007; Peel et al., 2007; Eckerdt et al., 2011), in which ectopic overexpression of key components leads to the formation of nascent centrioles incapable of PCM recruitment. De novo centriole biogenesis is thought to play a biological role in cells that have exited the cell cycle, differentiated, and require motile cilia (Zhao et al., 2013). To meet this requirement, the cell must make hundreds of modified centrioles to undergo multiciliogenesis; thus, the de novo pathway is an important mechanism through which differentiated cells create centrioles. However, it is not the dominant pathway through which most centrioles are made in dividing cells; therefore, I will focus herein on the axiomatic "templating" duplication mechanism.

In the canonical templating mechanism, a nascent centriole must grow orthogonally to a preexisting centriole; thus, the new (daughter) centriole templates from an older (mother) centriole (Figure 1.2). The mother and daughter centrioles are connected by the physical tether proteins which includes Rootletin; this physical connection is thought to play an important role in limiting the number of daughters able to nucleate from a single mother centriole (Tsou and Stearns, 2006). As a cell exits from cytokinesis, it contains one complete centrosome: a connected mother-daughter pair surrounded by PCM components. As the cell
enters $G_{1}$, the cell-cycle-dependent enzyme Separase and signaling kinase Plk1 function to sever the connection between mother and daughter centrioles. The loss of physical connection "resets" the identities of the centrioles, making both new mothers to begin centriole duplication anew.

By the end of $G_{1}$, the kinase Plk4 (Polo-like Kinase 4) is recruited to the site of centriole duplication to act as the "master licenser" of centriole duplication. While little is known about its mechanism of action, Plk4 is known to be required for centriole duplication; additionally, its licensing activity requires its catalytic activity (Bettencourt-Dias et al., 2005). After Plk4 licenses centriole duplication by its unknown mechanism, daughter centrioles begin to grow orthogonally to their respective mother centrioles. The formation of the initial structures is commensurate with DNA replication during $S$ phase (Figure 1.2). As the cell progresses through $G_{2}$ and prepares for mitotic entry, the daughter centriole completes elongation and the two mature mother-daughter pairs each finish recruiting the PCM components required to build and organize the mitotic spindle. The cell undergoes mitosis, and each new cell undergoes the process again.

## Centriole duplication is driven by a conserved set of proteins

As aforementioned, centrioles are not present in higher fungi and higher plants; however, it is of important note that centrioles are present in basal fungi and plants, suggesting that centrioles were present in common eukaryotic ancestors (Carvalho-Santos et al., 2010; Firat-Karalar and Stearns, 2014). Accordingly, the components responsible for centriole duplication are largely conserved, functionally if not through primary sequence,


Figure 1.2. Canonical centriole duplication requires cell-cycle-dependent cues and mother centriole templating. Clockwise from top, a mitotic cell contains two centrosomes and four centrioles; when it completes cytokinesis, each resulting (daughter) cell receives one centrosome and two centrioles (forest green cylinders). As the cell enters the first Gap phase, Separase (red) and Plk1 (not shown) cleaves the linkage between mother and daughter centrioles, resetting the identity of both to "mother" (Tsou et al., 2009). By the end of $\mathrm{G}_{1}$, Plk4 (blue) licenses centriole duplication. During Synthesis phase, nascent daughter centrioles (chartreuse) begin to grow orthogonally to their respective mothers (forest green). As the cell completes $\mathrm{G}_{2}$ and prepares for mitosis, the daughter centrioles elongate, are capped to pre-determined lengths, and the mother-daughter centriole pair recruits a cloud of PCM proteins (yellow). As the cell builds the mitotic spindle, the two mature centrosomes are separated on opposite sides, and the cyclical process begins again.
within centriolar eukaryotes. Shown in Figure 1.3 (A) is a comparative list of the components required for centriole duplication (Goshima et al., 2007) in the fruit fly Drosophila melanogaster (D.m.), humans (Homo sapiens, H.s.), and the nematode Caenorhabditis elegans (C.e.). While many more proteins contribute to procentriole assembly, elongation, and capping (length determination), shown in Figure 1.3 (A) are the five basic players necessary for centriole duplication.

While Plk4 (also known as SAK/ZYG-1 in D.m. and C.e., respectively) is considered the "master licenser" of centriole duplication (Cunha-Ferreira et al., 2009; Rogers et al., 2009), it requires centriole scaffolding components to localize to the site of nascent centrioles. In D.m., Asterless is the scaffolding protein responsible for binding and recruiting Plk4 to nascent centrioles (Dzhindzhev et al., 2010). However, humans have developed a redundant system to recruit Plk4 to centrioles, presumably due to its essential function in centriole duplication; in humans, either Cep152 (the Asterless homolog) or Cep192 (the SPD-2 homolog) is sufficient for Plk4 localization (Hatch et al., 2010; Cizmecioglu et al., 2010). Interestingly, C.e. have lost the Asterless homolog, and make use of SPD-2 alone to recruit Plk4, indicative that the C.e. Plk4 and SPD-2 proteins have coevolved to form their specific interactions (Shimanovskaya et al., 2014).


Figure 1.3. Centriole duplication requires a set of proteins functionally and spatially conserved across species. (A) Schematic pathway of conserved components. Three species are compared: Drosophila melanogaster (D.m.), Homo sapiens (H.s.), and Caenorhabditis elegans (C.e.), with the resulting structures shown to the left. See text for details. (B) Map of components as they are known to localize to the cartwheel structure (omitting Asl for clarity and Plk4, which is transient). Sas-6 oligomerizes into an 18 -mer and builds the central cartwheel (shown in dark (N-terminus) and light (C-terminus) blue. The localization of Ana2 is unknown, but its N-terminus binds Sas-4 (highlighted in top orange box, Cottee et al., 2013) and its C-terminus binds Sas-6 (highlighted in bottom orange box, Stevens et al.,

2010a). Sas-4 (red) forms a scaffold along the outer wall of the centriole to recruit and stabilize the triplet microtubule blades (Cottee et al., 2013).

It remains unknown exactly what Plk4's function is at the centriole after its recruitment; it is only known that centriole duplication requires catalytically active Plk4, though recent evidence suggests that a direct interaction between Plk4 and Sas-6 is sufficient to recruit Sas-6 to the site of the nascent centriole (Lettman et al., 2013).

The first structure observed through cryotomography in the emerging daughter centriole is the cartwheel with 9-fold symmetry. Structural breakthroughs occurred in 2011, when several labs demonstrated that a higher-order oligomer of the conserved Sas-6 (spindleassembly abnormal-6 (Leidel et al., 2005)) creates cartwheels with an inherent 9-fold radial symmetry in vitro (van Breugel et al., 2011; Kitagawa et al., 2011). It has since been shown (van Breugel et al., 2014) that 18 Sas- 6 protomers associate in two ways to create the 9spoked cartwheel: first, the helical region at the Sas-6 C-terminus interacts with other protomers to form a high-affinity coiled-coil dimer. Second, the globular Sas-6 N-terminus interacts weakly and laterally with the same domain in other protomers, forming the hub of the cartwheel; the coiled-coil regions emanate out from the hub, forming the spokes of the wheel (Figure 1.3 B, shown in blue).

Another component, called Anastral Spindle 2 (Ana2/STIL/SAS-5 in D.m./H.s./C.e., respectively (Stevens et al., 2010a)), is recruited at the same time as Sas-6 and is required for duplication. However, much less is known about the role of Ana2 in centriole duplication. In D.m. cells, overexpressed Sas-6 and Ana2 assemble into tubules reminiscent of Sas-6 cartwheels (Stevens et al., 2010b), a phenotype that requires Plk4 as a permissive cue. Furthermore, Sas-6 N-terminal globular domains have a weak affinity for each other (~110
$\mu \mathrm{M}$ in C.e. (Kitagawa et al., 2011)). Thus, while Ana2 likely promotes Sas-6-based cartwheel structures, the mechanism by which Ana2 does so remains unknown. Ana2 does contain several known binding motifs (presented in greater detail in Chapter 5), including a Sas-6and a Sas-4-binding domain, each of which has been mapped using biochemical techniques and yeast two-hybrid screens (Stevens et al., 2010a; Cottee et al., 2013). The presumptive binding sites between Ana2 and its partners Sas-6 and Sas-4 are mapped in Figure 1.3 B, as well as all currently known protein positions relative to the daughter cartwheel (Guichard et al., 2013; Mennella et al., 2012).

Finally, Sas-4 aids in elongating the nascent centriole, mostly through recruiting additional PCM components required for building the surrounding microtubule triplet blades (Pelletier et al., 2006; Dammermann et al., 2008; Gopalakrishnan et al., 2011; Cottee et al., 2013). At the distal end of the centriole, further components including Poc5, CP110 and Klp10A cooperate to regulate centriole length and prevent centrosomal centrioles from converting to basal bodies (Azimzadeh et al., 2009; Delgehyr et al., 2012).

## The Plk4 phosphorylation targets include itself, cell cycle regulator proteins, and unknown centriole components

Plk4 is a conserved serine/threonine kinase that plays a critical role in centriole duplication. Initial experiments in 2005 determined that Plk4 is required for centriole duplication in both D.m. and H.s. systems (Habedanck et al., 2005; Bettencourt-Dias et al., 2005; Kleylein-Sohn et al., 2007). Further work established that Plk4's phosphorylation activity is paramount to its licensing activity (Guderian et al., 2010). Plk4 has been shown to autophosphorylate (Rogers et al., 2009; Cunha-Ferreira et al., 2009; Holland et al., 2010; Sillibourne et al., 2010) in addition to phosphorylating FBXW5 (Puklowski et al., 2011;

Pagan and Pagano, 2011) and Chk2 (Petrinac et al., 2009), though loss of either of these targets has no direct effect on centriole number in D.m. (Rogers et al., 2009). In C.e., earlier experiments indicated that SAS-6 is a phosphorylation target of the Plk4 ortholog ZYG-1 (Kitagawa et al., 2009), though further work demonstrated that ZYG-1 directly binds SAS-6 for its recruitment to nascent centrioles in a phosphorylation-independent manner (Lettman et al., 2013). Thus, the identity of the direct Plk4 phosphorylation target(s) remains unknown and contested. Of specific interest to the field is whether Plk4 even directly targets a centriole duplication player, or whether Plk4 indirectly licenses centriole duplication by preventing degradation of daughter centriole components (Puklowski et al., 2011; Pagan and Pagano, 2011).

## Plk4 is a divergent member of the Polo-like family of kinases

Plk4 is a member of the widely conserved Polo-like family of serine/threonine kinases (Figure 1.4), which collectively regulate cell cycle progression and proliferation (Park et al., 2010). The most widely studied member of the Plk family, Plk1 is a ubiquitous kinase in cycling cells with roles in mitotic entry and exit, centrosome maturation, spindle assembly, chromatin segregation and cytokinesis, and oncogenic transformation (Xu et al., 2013; Park et al., 2010). Plk2/Snk, Plk3/Prk/Fnk, and Plk5 are less studied, though each is known to have distinct functions. Plk2, though not essential, is associated with centriole maintenance and avoiding mitotic catastrophe (van de Weerdt et al., 2008; de Carcer et al., 2011). Plk3 is required for S phase entry and cyclin E production (Zimmerman and Erikson, 2007). Interestingly, Plk5 is the only member of the Plk family that has lost catalytic activity, as a premature stop codon prevents expression of the kinase domain in the human protein
(Figure 1.4); furthermore, it is only expressed in fully differentiated tissue types including the central nervous system, underlining its evolutionary departure from the other Plk's roles in proliferative tissues (de Carcer et al., 2011). Plk4 is likewise divergent from the Plk4 family, though in different measures: its kinase domain more closely resembles that of the Aurora family of kinases than the Plk family (personal communication, Yao Liang Wong and Karen Oegema), and unlike all other Plk members, Plk4 contains three structurally defined Polo Box (PB) domains rather than two (Slevin et al., 2012). Plk4, like Plk1, is largely expressed throughout embryonic development and in adult proliferative tissues in humans, including bone marrow and the male testis (de Carcer et al., 2011).


Figure 1.4. The architectural map of the Plk family of kinases highlights the divergence of Plk4. Each Homo sapiens major isoform is shown here, with each cartoon drawn to scale. All Plks share an N-terminal kinase domain (black), though Plk4's kinase domain more closely resembles Aurora kinases. Plks 1, 2, 3, and 5 share a C-terminal pair of Polo Box domains (PB1-PB2, light and dark purple) that generally regulate the activity and targeting of the full-length molecule (see the following sections for in-depth discussion regarding PB function). Plk1 contains an inter-domain linker (IDL, tan) that binds Plk1's kinase domain in cis to inhibit kinase activity in a PB1-PB2-dependent fashion (Xu et al., 2013). Plk4 contains a downstream regulatory element (DRE, green) immediately C-terminal to its kinase domain, used to regulate protein levels preceding centriole duplication (see the following section for in-depth discussion of the DRE). Plk4 also differs from the Plk family by containing a Cterminal array of three distinct PB domains (Slevin et al., 2012; Park et al., 2014;

Shimanovskaya et al., 2014). PB1-PB2 (red and orange) collectively bind scaffolding proteins, localizing Plk4 to its centriolar targets, and homodimerize in trans to afford DREdependent polyubiquitination (Dzhindzhev et al., 2010; Slevin et al., 2012). *Plk5's kinase domain is truncated and non-functional, followed by another start site that initiates the remainder of the Plk5 polypeptide.

## The mechanism of Plk4's licensing activity followed by swift degradation is conserved among opisthokonts

Despite the lack of conservation between Plk4 and the other Plk family members, Plk4 remains an essential centriole duplication component within all animals and most eukaryotes, excluding higher plants and fungi (Shimanovskaya et al., 2014). It has been hypothesized that Plk1, present in the last eukaryotic common ancestor, was once responsible for many more cell cycle functions, including centriole duplication (Carvalho-Santos et al., 2010). Over several gene duplication events, Plk1 eventually gave rise to Plks 2, 3, 4, and 5, a hypothesis supported by the fact that heterokonts, alveolates, higher plants and amoebozoa still rely on Plk1 for functions supported by the collective Plk family in animals. This gene duplication event allowed for uncoupling between centriole duplication and mitotic functions; such uncoupling and differentiation events are thought to be an integral part of creating higher-complexity organisms throughout evolution (Carvalho-Santos et al., 2010; Ohno, 1970). Plk4 has been widely studied in human, mouse, and fly homologs, with a tentative ortholog in C. elegans called ZYG-1. Although ZYG-1 lacks apparent sequence homology with all other Plk4 homologs, it is also a kinase that regulates centriole assembly in nematodes (Dammermann et al., 2004; Pelletier et al., 2006; Delattre et al., 2006). However, recent work using x-ray crystallography has demonstrated that ZYG-1 contains a pair of tandem Polo Box (PB) domains, a feature common to all Plk4 homologs (Shimanovskaya et al., 2014). These new data suggest that, contrary to former belief, ZYG-1
represents a new class of bona fide Plk4 orthologs.
Biochemical and structural techniques as well as functional studies in cell culture have revealed the architecture and modules within both human and Drosophila Plk4 (Leung et al., 2002; Habedanck et al., 2005; Dzhindzhev et al., 2010; Slevin et al., 2012; Sonnen et al., 2013; Shimanovskaya et al., 2014). Immediately C-terminal to the conserved kinase domain is the downstream regulatory element (DRE) (Figure 1.5), a phospho-regulated region with important roles in down-regulating the full-length molecule (Cunha-Ferreira et al., 2009; Rogers et al., 2009). Active Plk4 autophosphorylates the DRE in trans, priming the region for F-box binding ( $\beta-\mathrm{TrCP}$ in H.s.; Slimb in D.m.) , polyubiquitination, and proteolytic degradation. This degradation event is essential to limit Plk4 levels in cells, as ectopic Plk4 constructs that are phosphorylation-resistant in both H.s. and D.m. cells become stabilized, leading to centriole amplification (Cunha-Ferreira et al., 2009; Rogers et al., 2009; Holland et al., 2010).


Figure 1.5. Plk4 is functionally conserved and maintains specific structural modules. An architectural comparison of the functional Plk4 orthologs reveals conservation within the domain architecture. H.s., Homo sapiens (human); M.m., Mus musculus (mouse); D.m., Drosophila melanogaster (fruit fly); C.e., Caenorhabditis elegans (nematode). All Plk4 homologs share a similar N-terminal kinase domain (black), while most orthologs (excluding C.e. ZYG-1) share the DRE (green), a region used to regulate FL protein stabilization. A central pair of PB domains (PB1-PB2, red and orange) collectively regulate localization and degradation of the FL molecule. The function of the C-terminal PB3 (blue) remains debated
and undefined. *Asterisks indicate PB modules that have been identified via sequence similarity, but not structurally verified as PB domains. The other PB structures and their references are as follows: (H.s. PB1-PB2: Park et al., 2014; M.m. PB3, Leung et al., 2002; D.m. PB1-PB2: Slevin et al., 2012 and Shimanovskaya et al., 2014; D.m. PB3: author's own data, unpublished; C.e. PB1-PB2: Shimanovskaya et al., 2014).

The C-terminus of Plk4 comprises an array of three PBs. PB1-PB2 (shown in red and orange, respectively, in Figure 1.5) together bind Asterless (Asl), affording localization of the full-length molecule to the site of daughter centriole assembly (Slevin et al., 2012; Shimanovskaya et al., 2014). Additional work from our lab has shown that PB1-PB2 forms a homodimer in trans, effectively dimerizing the full-length molecule and allowing for F-box binding and, ultimately, polyubiquitination and degradation (Figure 1.6) (Slevin et al., 2012). The PB1-PB2 cassette is a conserved feature of Plk4s across the species commonly studied (shown in Figure 1.5). Unique to Plk4 is the presence of the C-terminal PB3, a domain with unknown function in the context of the FL molecule. Early work determined that the M.m. PB3 also forms a dimer; however, work from our lab as well as other labs demonstrated that PB3 is not sufficient for robust centriole localization (Leung et al., 2002; Slevin et al., 2012). The role of PB3 in Plk4 function remains unclear.

A


B


Figure 1.6. Plk4 depends on the PB1-PB2 homodimerization interface to autophosphorylate in trans and bind F-box proteins for its degradation. (A) Active Plk4 (activity notated by a yellow star in the kinase domain) dimerizes via a strong homodimerization interface between PB1-PB2 cassettes in two protomers (Slevin et al., 2012), allowing for phosphorylation of the DRE in trans. (B) Schematic of the FL molecules following dual phosphorylation events in trans. The phosphorylated DRE region is able to then bind an F-box protein (Slimb in D.m., purple; $\beta-\operatorname{TrCP}$ in H.s.) for its degradation mechanism. Note that 1) both diagrams refer to information gleaned in D.m. Plk4, 2) the molecules are translated horizontally relative to each other in (B), and 3) the oligomerization state of PB3 remains contested in D.m. The author's unpublished data suggest that PB3 is a monomer in D.m., thus the schematic presents a monomeric form.

## The Polo Box ( $\mathbf{P B}$ ) domain is used to regulate Plk activity and subcellular targeting by employing a structurally defined fold

The PB domain takes a conserved, simple structural fold: a 6 -stranded anti-parallel $\beta$ sheet packed perpendicularly against a single $\alpha$-helix (Figure 1.7 A and B). Though the sequences and binding partners vary greatly among Plks (Yun et al., 2009; Slevin et al., 2012; Xu et al., 2013; Park et al., 2014; Shimanovskaya et al., 2014), serial structural alignments of single PB domains reveal little structural divergence among the individual domains, with RMSD values ranging from 1.5 to $4 \AA$ (Figure 1.7 C ). However, three trends emerge when comparing both structural alignments as well as their sequence conservation (Figure $1.7 \mathrm{C}, \mathrm{D}): 1) \mathrm{PB}$ structures share greater primary and tertiary structure elements within the same spatial orthologs (i.e., PB1s are more similar in both structure and sequence to other PB1s, PB2s are more similar to other PB2s, etc.); 2) As predicted, C.e. ZYG-1 is the least similar in primary structure for each respective PB than any other species; 3) PB2 architecture deviates from the prototypical PB structure in that its $\beta 6$ strand is parallel to its neighbor and occurs C-terminal to the single $\alpha-1$ helix (Figure 1.7 E , blue arrow pointing to $\beta 6$ within C.e. ZYG-1 PB2). Despite these trends, the average RMSD difference between any
known single PB structure is $2.55 \pm 0.62 \AA$, indicating that the wide variety of PB functions arises not due to large structural differences among individual PB domains, but due to the overall differences among varied PB oligomeric arrays and possibly different conserved residues across similar folds.

A


B



E

1.5 A RMSD 44\% identity

Figure 1.7. The structure of each solved PB domain takes on a conserved fold. (A) The structure of a single PB domain (D.m. Plk4 PB1, PDB accession code 4G7N, Slevin et al., 2012) reveals a 6 -stranded antiparallel $\beta$-sheet packed (cyan, labeled $\beta 1-\beta 6$ ) orthogonally against a single $\alpha$-helix (olive, labeled $\alpha 1$ ). Loops are shown in dark gray and have been smoothened for simplicity. (B) A schematic depicting the architecture of a typical PB domain, mimicking the geometry of the PB domain shown in A . Note that the N -terminus of the protein begins at $\beta 1$, the middle forms the serpentine $\beta$-sheet, and then the C -terminus consists of the final $\alpha 1$. The dotted lines indicate that the loop connecting $\beta 6$ and $\alpha 1$ goes back into the plane of the field of view. (C) A series of structural alignments (using the DALI pairwise alignment server, Holm and Rosenström, 2010) reveals little differences among the solved PB domain structures, regardless of species or Plk origin. The species, Plk, and PB number are noted for each PB tested; each was aligned to every other PB within the grid, and the RMSD and percent identity is reported within the corresponding box. Each box within the grid is colored according to a green/red scale, with green indicating the lowest RMSD (best alignment) and red indicating the highest RMSD (poor alignment). (D) A grid showing the sequence conservation between the same individual pairs as in C, with green indicating high conservation and red indicating low conservation. (E) Alignment between D.m. Plk4 PB1 (shown in eggplant) and either H.s. Plk4 PB1 (PDB accession code 4N9J (Park et al., 2014), shown in bronze) or C.e. ZYG-1 PB2 (PDB accession code 4NKB (Shimanovskaya et al., 2014), shown in blush) reveals conservation among Plk4 PB1 homologs and structural differences between Plk4 PB1 and PB2 homologs.

Illustrating the differences in PB function in a Plk context-dependent manner is a recent study illuminating the mechanism by which Plk1's PB1-PB2 array regulates FL catalytic activity (Xu et al., 2013). This study employed x-ray crystallography to show that Plk1's PB1-PB2 bind each other in cis, forming a structural unit that collectively binds and stabilizes the kinase domain within the same molecule (Figure $1.8 \mathrm{~A}, \mathrm{~B}$ ). In binding the kinase domain, the PB1-PB2 heterodimer collectively prevents the flexibility needed for the kinase to accept substrates; thus, in binding the kinase, the PB1-PB2 heterodimer effectively autoinhibits the FL molecule. Interestingly, the mechanism of activating the kinase and localizing the FL molecule to the right place at the right time is one and the same: the PB1PB2 heterodimer binds primed (phosphorylated) targets at subcellular structures including the kinetochore, relieving the PB1-PB2-kinase interaction, activating the kinase, and
localizing it to its catalytic targets (Elia et al., 2003; Kang et al., 2006; Yun et al., 2009). More specifically, the PB heterodimer recognizes the consensus motif Pro/Phe-X-X-Thr/Gln/His/Met-Ser-pThr/pSer-Pro/Y (Elia et al., 2003) (where X is a hydrophobic residue and Y is any residue) within its binding targets, which are known to include at least CENP50 (also known as PBIP1/KLIP1/MLF1IP (Lee et al., 2014)) at kinetochores and hCenexin1, an ODF2 splice variant that localizes to somatic centrosomes (Soung et al., 2009). Plk1 targets can either be primed by other cell cycle-related kinases (i.e., Cdk1) or Plk1 itself, indicating that Plk1 may have a positive feedback regulation feature: the more active Plk1 is, the more it phosphorylates its targets, and thus the more frequent the binding events between PB1-PB2 and primed targets. The structure of human Plk1 PB1 bound to a phospho-peptide representative of primed CENP-50 reveals that the PB heterodimer collectively binds the target, relieving autoinhibition of the kinase activity (Yun et al., 2009; Xu et al., 2013) (Figure $1.8 \mathrm{~B}, \mathrm{C}$ ). Both PB1 and PB2 determinants contribute to binding phospho-CENP-50, underlining the importance of the PB1-PB2 array.


Figure 1.8. Plks employ PB domains to take on differential dimeric configurations for cellular function. (A) A list of the compared Plks and their known PB oligomeric states. Those states labeled with asterisks $\left({ }^{*}\right)$ indicate predicted oligomeric states based on the conservation between mouse and human Plk4. (B) Plk1 PB1-PB2 take on a multidomain architecture to bind the kinase and allosterically inhibit its catalytic activity (Xu et al., 2013). Following phosphorylation events of centrosomal and/or centromeric targets, the PB1-PB2 structural unit binds the phospho-primed targets, releasing the interaction with the kinase domain. (C) The crystal structure of PB1-PB2 bound to a phospho-peptide representative of CENP-50 (PDB accession code 3FVH, Yun et al., 2009) with the same color scheme as B, shown in two orientations.

Importantly, PB domains also act in arrays within Plk4 PB pairs; however, instead of interacting as a heterodimer in cis, Plk4 PB1-PB2 function as a homodimer in trans (compare Figure 1.8 B to Figure 1.6 B). Additionally, extensive studies in both D.m. and H.s. cultured cells have demonstrated the phospho-independence of Plk4-Asl/SPD-2 binding, indicating that the mechanism by which Plk4 PB1-PB2 binds targets and attains subcellular localization differs from that of Plk1 PB1-PB2 target binding, which is phospho-dependent (Dzhindzhev et al., 2010; Hatch et al., 2010; Shimanovskaya et al., 2014). To date, many of the PB structures that have been solved have included pairs of PB domains. Consistent with the idea that PB pairs are employed differently by each Plk, the structures of PB pairs display much more sequence variance than individual PB domains (Figure 1.9 A). Structural alignments of all known PB1-PB2 pairs reveals that Plk1's PB1-PB2 heterodimer also takes a different conformation than Plk4 PB1-PB2 pairs (Figure 1.9 B , bottom left). Plk1 PB1-PB2 collectively create a composite clamshell fold, which acts as a pincer used to bind phosphotargets (Xu et al., 2013). In this conformation (Figure 1.9 B, purple), Plk1 PB1-PB2 each contributes their six-stranded $\beta$-sheet to interface in the center of the clamshell, with the single $\alpha$-helix flanking on either side. In contrast, Plk4 PB1-PB2 pairs form a head-to-tail conformation in which PB1's C-terminal $\alpha 1$ helix leads directly into PB2's $\beta 1$ strand, restricting the distance between the two PB domains and preventing them from creating a $\beta$ clamshell like that of Plk1's PB1-PB2. The head-to-tail formation of D.m. Plk4's PB1-PB2 is a conserved fold, as it has also been described in both C.e. ZYG-1 and H.s. Plk4 (Figure 1.9 B, bottom right; Shimanovskaya et al., 2014; Park et al., 2014). The novelty of the Plk4 PB1PB2 homodimer formation, as well as its unusual head-to-tail conformation, indicates that Plk4 PB1-PB2 performs a distinct function in the context of the FL molecule (see Chapter 2
for a thorough discussion).


Figure 1.9. PB1-PB2 pairs diverge in both sequence and structure. (A) Pairwise alignment (Holm and Rosenström, 2010) of all structurally characterized PB1-PB2 pairs reveals large differences between Plk1 PB pairs and Plk4 PB pairs (top row, ranging from 12-18\% sequence identity). All known Plk4 homolog PB1-PB2 pairs align much more closely, with sequence identities ranging from $18-40 \%$. Sequence similarity is shown on a color-code sliding scale, with white squares representing low similarity and cobalt squares representing complete identity. The structures of 3 specific PB1-PB2 pairs (D.m. Plk4, PDB accession code 4G7N, in oranges; H.s. Plk1, PDB 4J7B, in purples; H.s. Plk4, PDB 4N9J, in
greens) are shown in the sidebar. (B) Structural alignments between PB1-PB2 pairs demonstrate large differences between Plk1 and Plk4 PB arrays. Plk4 PB1-PB2 from both D.m. and H.s. align well with similar folds and primary sequences, while Plk1 PB1-PB2 collectively form a "pincer," interacting side-to-side rather than head-to-tail.

## PB3: The odd one out

Much of the work completed regarding Plk4's PB domains has focused on the central PB1-PB2 array (Slevin et al., 2012; Park et al., 2014; Shimanovskaya et al., 2014), leaving the centriole duplication with a fascinating question: what is the purpose of Plk4's C-terminal PB domain (PB3)? As discussion in-depth in Chapter 2, Plk4 PB1-PB2 collectively play an integral role in binding centriole targets, localizing the FL molecule to the site of nascent centriole formation, and regulating the stability of the FL molecule (Slevin et al., 2012). In Plk1, PB1-PB2 fulfills all of these important roles, but with one additional job: it also directly regulates kinase activity by allosteric inhibition. At press time, Plk4 is unknown to have such autoinhibition mechanism. It is tempting then to speculate that PB3 may play a role in regulating Plk4 catalytic activity, though this is yet to be shown. Interestingly, despite Plk4's PB1-PB2 forming a structurally and sequence-conserved domain throughout all studied animals, PB3 appears to be more divergent; in humans and mice, PB3 forms a stable dimer in solution (author's work, unpublished; Leung et al., 2002, respectively), while in fruit flies, it remains a monomer in solution (author's work, unpublished). Whether PB3 plays different roles in different Plk4 homologs remains an interesting question, and is the focus of the work described in Chapter 3. The future promises to hold exciting discoveries concerning polo box domains, which will shed light not only on their direct functions in a cellular context, but also on the evolutionary development of mechanisms to limit centriole duplication to a single, cell cycle-dependent event.

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# CHAPTER 2: THE STRUCTURE OF THE PLK4 CRYPTIC POLO BOX REVEALS TWO TANDEM POLO BOXES REQUIRED FOR CENTRIOLE DUPLICATION ${ }^{1}$ 

## Summary

Centrioles are key microtubule polarity determinants. Centriole duplication is tightly controlled to prevent cells from developing multipolar spindles, a situation that promotes chromosomal instability. A conserved component in the duplication pathway is Plk4, a polo kinase family member that localizes to centrioles in M/G1. To limit centriole duplication, Plk4 levels are controlled through trans-autophosphorylation that primes ubiquitination. In contrast to Plks 1-3, Plk4 possesses a unique central region called the "Cryptic Polo Box". Here, we present the crystal structure of this region at 2.3-A resolution. Surprisingly, the structure reveals two tandem, homodimerized polo boxes, PB1-PB2, that form a unique, winged architecture. The full PB1-PB2 cassette is required for binding the centriolar protein Asterless as well as robust centriole targeting. Thus, with its C-terminal polo box (PB3), Plk4 has a novel, triple polo box architecture that facilitates oligomerization, targeting, and promotes trans-autophosphorylation, limiting centriole duplication to once per cell cycle.

## Introduction

Centrioles are cylindrical, microtubule-based structures that form the core

[^0]microtubules to form the mitotic spindle and cilia (Bornens 2012). Centriole number is precisely controlled with centriole duplication restricted to a single cell cycle event (Tsou and Stearns, 2006; Tsou et al., 2009; Nigg and Stearns, 2011). Centrioles exist as pairs composed of an older (mother) centriole and a daughter centriole, assembled on the mother centriole in the preceding cell cycle. G1-phase cells contain a single mother-daughter centriole pair. During S-phase, centrioles separate and duplicate, generating two motherdaughter pairs that facilitate bipolar spindle assembly. The mechanisms underlying centriole duplication define a critical step in cellular biology, as misregulation of centriole number is linked to chromosome instability and diseases including ciliopathies, male sterility, primary microcephaly, and tumorigenesis (Bettencourt-Dias et al., 2011; Rosario et al., 2010; Nigg and Raff, 2009).

Several proteins are required for centriole biogenesis. Among these are the conserved proteins Polo-like kinase 4 (Plk4/Sak), Asterless/Cep152, SAS-6, SAS-5/Ana2/STIL, Cep135, and SAS-4/CPAP (Song et al., 2008; Azimzadeh and Marshall, 2010). The order of subunit addition suggests a hierarchical centriole assembly pathway conserved across phyla. Asterless (Asl), a scaffolding protein, initially recruits Plk4 to the site of daughter centriole assembly (Hatch et al., 2010; Dzhindzhev et al., 2010; Cizmecioglu et al., 2010). Plk4 activity is upstream of the SAS proteins and primes the mother centriole for S-phase duplication (Pelletier et al., 2006; Kleylein-Sohn et al., 2007; Kitagawa et al., 2009). Daughter centriole (procentriole) assembly begins at the proximal end of the mother centriole with the formation of a nine-fold symmetric cartwheel structure composed of SAS-6 homodimers (Kitagawa et al., 2011; van Breugel et al., 2011). How Plk4 initiates centriole assembly is not well defined. In Caenorhabditis elegans, ZYG-1 (the Plk4 homolog)
phosphorylates SAS-6, triggering centriole formation (Kitagawa et al., 2009), although this has not been shown in other systems. In humans, Plk4 inactivates FBXW5, a SCF (Skp, Cullin, F-box) component used to degrade SAS-6, suggesting that Plk4 initiates centriole duplication by stabilizing SAS-6 (Puklowski et al., 2011). However, Drosophila FBXW5 has no role in controlling centrosome number (Rogers et al., 2009). Thus, while species show some divergence in the duplication pathway, Plk4 has emerged as a master-regulator of centriole assembly.

Plk4 and its binding partner Asl are required for centriole duplication (BettencourtDias et al., 2005; Habedanck et al., 2005; Varmark et al., 2007) and studies in human, Drosophila and Xenopus systems show that Plk4 or Asl overexpression promotes centriole amplification as well as de novo centriole assembly (Rodrigues-Martins et al., 2007; Peel et al., 2007; Dzhindzhev et al., 2011; Eckerdt et al., 2011). Plk4 is regulated by the $S^{\text {SF }}{ }^{\text {Slimb/ß- }}$ ${ }^{\mathrm{TrCP}}$ ubiquitin ligase which recognizes Plk4 after homodimer-dependent transautophosphorylation of the phosphodegron known as the Downstream Regulatory Element (DRE) (Guderian et al., 2010, Cunha-Ferreira et al., 2009; Rogers et al., 2009; Brownlee et al., 2011; Holland et al., 2010). In cultured Drosophila cells, Plk4 is degraded throughout most of the cell cycle to prevent centriole amplification (Peel et al., 2007; Kleylein-Sohn et al., 2007). During M-phase however, Plk4 is dephosphorylated by Protein Phosphatase 2A, thereby stabilizing Plk4, allowing a brief mitotic debut that restricts centriole duplication to a single event per cell cycle (Brownlee et al., 2011).

Plk4 is a member of the Polo-like kinase family. Plk members 1-4 share sequence similarity to the founding member, Drosophila Polo (Plk1) (Sillibourne and Bornens, 2010). Like Polo, Plk members regulate cell-cycle events that collectively include spindle
formation, the metaphase-to-anaphase transition, mitotic exit, cytokinesis, and DNA damage checkpoints. To perform these critical functions, Plk gene expression, protein expression, localization, kinase activity, and destruction are tightly regulated throughout the cell cycle (Archambault and Glover, 2009) as aberrant Plk activities contribute to chromosome instability and oncogenesis.

Plks share an amino-terminal serine/threonine kinase domain, as well as one or more ~100-residue polo box (PB) domains. Plk members 1-3 contain two carboxy-terminal PBs (Figure 2.1 A ) that interact in cis to bind phosphorylated targets, mediate localization, and activate the kinase (Lowery et al., 2005). The architecture of a PB domain consists of an antiparallel 6-stranded $\beta$-sheet that lies across a C-terminal $\alpha$-helix (Leung et al., 2002). Plk1's tandem PBs (PB1-PB2) clamp around a phosphopeptide target with each PB contributing binding determinants (Elia et al., 2003; Cheng et al., 2003). Intriguingly, Plk4 is structurally divergent. It was annotated as containing only a single, carboxy-terminal PB, which confers homodimerization and moderate centriole localization by binding an unidentified target (Leung et al., 2002). The structure of the Plk4 PB is homodimeric and adheres to a general PB architecture, though it is formed through swapped chains of the homodimer. The homodimeric arrangement of this Plk4 PB is distinct from the tandem arrangement of the Plk1 PB1-PB2 pair, indicative that PBs adopt differential spatial arrangements.

Plk4 also contains a conserved central domain, hitherto called the "Cryptic Polo Box" (CPB), which bridges the kinase domain and the carboxy-terminal PB. This region was initially identified as a centriole-targeting component, capable of binding the kinase domain in trans (Leung et al., 2002). Based on these properties, the region was named the "Cryptic Polo Box" though it showed no apparent sequence homology to canonical PBs (Swallow et
al., 2005). Recent work has identified a CPB binding partner, Asterless (Asl)/Cep152, which targets Plk4 to centrioles (Dzhindzhev et al., 2010; Hatch et al., 2010, Cizmecioglu et al., 2010). To date, the CPB has largely remained an enigma, with questions concerning its structure, function in centriole localization, and role in Plk4 activity outstanding.

Here, we present the crystal structure of the CPB, determined to a resolution of 2.3
A․ Surprisingly, this structure reveals that the CPB comprises two structurally unique PB domains, PB1 and PB2. Cellular localization and biochemical studies indicate that the entire tandem PB1-PB2 cassette is required for robust centriole localization and Asl binding. The PB1-PB2 cassette also mediates Plk4 oligomerization, and when expressed as a trans cassette, protects endogenous Plk4 from trans auto-phosphorylation and subsequent degradation. Thus, the Plk4 PB1-PB2 cassette is a unique architectural component required for Plk4 function.

## Experimental Procedures

## Cloning and Protein Purification

Drosophila melanogaster Plk4 (DG7186) PB1-PB2, residues 382-602, was subcloned into pET28b (Novagen), engineering a thrombin-cleavable N -terminal $\mathrm{His}_{6}$ tag. Protein was expressed in BL21 DE3 E. coli cells under kanamycin selection and induced with $100 \mu \mathrm{M}$ IPTG for 16 hr at $20^{\circ} \mathrm{C}$. Cells were harvested, resuspended in lysis buffer ( 25 mM Tris pH $8.0,300 \mathrm{mM} \mathrm{NaCl}, 10 \mathrm{mM}$ Imidazole, $0.1 \% \beta-\mathrm{ME}$ ) supplemented with 1 mM PMSF, and sonicated to lyse. Lysate was clarified at $23,000 \mathrm{xg}$ for 45 min and supernatant applied to a $\mathrm{Ni}^{2+}$-NTA resin (Qiagen). Plk4 was eluted using a $250 \mathrm{ml}, 10-300 \mathrm{mM}$ Imidazole gradient in lysis buffer. Fractions containing Plk4 were pooled, supplemented with 1 mM CaCl 2 and
digested for 12 hr at $4^{\circ} \mathrm{C}$ with $1 \mu \mathrm{~g} / \mathrm{ml}$ bovine $\alpha$-thrombin. Digested Plk4 was filtered through 1 ml benzamidine sepharose (GE Healthcare) and exchanged into 100 ml of 25 mM HEPES, $\mathrm{pH} 7.0,0.1 \% \beta-\mathrm{ME}$, loaded onto a SP-sepharose column (GE Healthcare), and eluted over a $250 \mathrm{ml}, 0-1 \mathrm{M} \mathrm{NaCl}$ gradient in 25 mM HEPES, $\mathrm{pH} 7.0,0.1 \% \beta$-ME. Fractions containing Plk4 were pooled, exchanged into protein storage solution ( 50 mM Tris $\mathrm{pH} 9.5,300 \mathrm{mM}$ $\mathrm{NaCl}, 0.1 \% \beta-\mathrm{ME}, 10 \%$ glycerol), concentrated to $7.2 \mathrm{mg} / \mathrm{ml}$, and frozen in liquid nitrogen. Selenomethionine (SeMet)-substituted Plk4 (aa 382-602) proved insoluble. We hypothesized that one of the four methionine residues in the PB1-PB2 cassette was affecting solubility when substituted by SeMet, so we systematically mutated each of the four methionines to leucine or alanine and screened for solubility when only three selenomethionines were incorporated into the region spanning aa 382-602. Point mutants were generated using the Quikchange method (Stratagene). SeMet-substituted Plk4 M517A was expressed in B834 DE3 E. coli cells using SeMet minimal media (Leahy et al., 1994). SeMet-substituted Plk4 (aa $382-602$ ) M517A proved marginally soluble and could be concentrated to $3.2 \mathrm{mg} / \mathrm{ml}$ before precipitating out of solution, in contrast to wild-type Plk4 (aa 382-602) that could be concentrated to $7.2 \mathrm{mg} / \mathrm{ml}$ before starting to precipitate out of solution. Expression and purification protocols for SeMet-substituted Plk4 (aa 382-602) M517A were identical to wild-type Plk4 (aa 382-602) except for the difference in final concentrations. The PB2 residues M514 and M517 reside on the two $\beta$-strands that are bridged by the C511-C566 disulfide bond. The cystine likely limits the degree of change in packing caused by SeMetsubstitution. In addition, both M514 and M517 form interactions with neighboring $\beta$-strands that contribute to domain stability. We hypothesize that SeMet-substitution at these residues disrupted these interactions, and in turn the stability of the PB2 domain fold, leading to
insolubility for the SeMet wild-type construct as well as decreased solubility for the SeMet M517A construct.

Crystallization, Data Collection, and Structure Determination
Native Plk4 was crystallized by the hanging drop method using a mother liquor (1ml) containing $1.2 \mathrm{M} \mathrm{Li}_{2} \mathrm{SO}_{4}, 100 \mathrm{mM}$ HEPES, pH 7.5 and a drop containing $2 \mu \mathrm{l} 7.2 \mathrm{mg} / \mathrm{ml}$ protein stock and $2 \mu \mathrm{l}$ mother liquor. Native crystals were transferred to Fomblin oil (Sigma) and flash frozen in liquid nitrogen. SeMet-substituted M517A Plk4 was crystallized using a mother liquor ( 1 ml ) containing $1.4 \mathrm{M} \mathrm{Li}_{2} \mathrm{SO}_{4}, 100 \mathrm{mM}$ HEPES pH 7.5 and a drop containing $2 \mu \mathrm{l}$ of $3.2 \mathrm{mg} / \mathrm{ml}$ protein stock and $1 \mu \mathrm{l}$ mother liquor. SeMet-substituted crystals grew on the same time scale and to the same dimensions as native crystals. SeMet M517A Plk4 crystals were transferred to fomblin oil and flash frozen in liquid nitrogen. Diffraction data were collected at the Advanced Photon Source SER-CAT beamlines 22-ID (native data, $1.00890 \AA$ ) and 22-BM (M517A Se peak SAD data, $0.97980 \AA$ ). Crystals belong to the space group $\mathrm{P} 2_{1} 2_{1} 2$ with two molecules in the asymmetric unit. Data were processed and scaled using the HKL2000 suite (Otwinowski and Minor, 1997). The Phenix program suite (Adams et al., 2010) was used to find selenium sites, phase, build, and refine the structure with reiterative building in Coot (Emsley et al., 2010). Refinement was monitored using $10 \%$ of the data randomly excluded from the refinement and used to calculate an $R$ free (Brünger, 1992). Initial refinement employed a MLHL target function and SeMet phases followed by a ML target function against native data to $2.3 \AA$. The SeMet-substituted M517A crystals and the native crystals were isomorphous, indicative that the M517A mutation, and the incorporation of SeMet at the remaining methionine positions did not affect the overall structure of the PB1-PB2 cassette. The model includes two Plk4 protomers: chain A (residues

382-596) and chain B (residues 382-548, 553-597) each preceded by a four residue (Gly-Ser-His-Met) N-terminal cloning artifact, 186 water molecules, and six sulfate ions. Electrostatics were calculated using APBS (Baker et al., 2001). Structural alignments and rmsd values were calculated using the Dali server (Hasegawa and Holm, 2009). Because the Plk4 PB3 domain uses two molecules to form a canonical PB domain, residues from PB3 chains A and B were selected to form a single chain PB3 domain for comparative purposes.

Size Exclusion Chromatography and Multi-angle Light Scattering (SEC-MALS)
D.m. Plk4 PB1-PB2 was purified without cleaving the N -terminal $\mathrm{His}_{6}$ tag ( $\mathrm{MW}=$ $27,558 \mathrm{Da})$ and exchanged into running buffer ( $300 \mathrm{~mm} \mathrm{NaCl}, 25 \mathrm{~mm}$ HEPES, $\mathrm{pH} 7.0,0.1 \%$ $\beta$-mercaptoethanol, and $0.2 \mathrm{~g} /$ liter sodium azide). A Superdex 200 10/300 GL gel filtration column (GE Healthcare) was equilibrated in the running buffer and runs containing $100 \mu \mathrm{l}$ of protein were injected onto the column. Eluate was passed in tandem through a Wyatt DAWN HELEOS II light scattering instrument and a Wyatt Optilab rEX refractometer. The light scattering and refractive index data were used to calculate the weight-averaged molar mass of each peak using the Wyatt Astra V software program (Wyatt Technology Corp.)(Wyatt, 1993). Two Plk4 PB1-PB2 runs were performed, the first with $100 \mu \mathrm{l}$ of $19 \mu \mathrm{M}$ protein, the second with $100 \mu \mathrm{l}$ of $28 \mu \mathrm{M}$ protein. A control run was also performed using $100 \mu \mathrm{l}$ of 90 $\mu \mathrm{M}$ bovine serum albumin $(\mathrm{MW}=66463 \mathrm{Da})$ which gave monomer, dimer and trimer peaks with experimentally determined molecular weights of $63 \pm 1 \mathrm{kDa}, 134 \pm 9 \mathrm{kDa}$ and $207 \pm 43$ kDa respectively (Figure $2 . \mathrm{S} 1 \mathrm{D}$ ).

## Dynamic Light Scattering

Purified Plk4 PB1-PB2 protein with the N-terminal $\mathrm{His}_{6}$ tag removed (MW 25,675 Da, concentrated to $3.6 \mathrm{mg} / \mathrm{ml}$ in 50 mM Tris $\mathrm{pH} 9.5,300 \mathrm{mM} \mathrm{NaCl}, 0.1 \% \beta-\mathrm{ME}, 10 \%$
glycerol) was analyzed in a Wyatt DynaPro dynamic light scattering plate reader. Measurements are reported in Table 2.2.

Centriole Localization Assay
S2 cell culture was performed and maintained as described in Rogers and Rogers (2008). In brief, S2 cells were cultured in SF900II serum-free media (Invitrogen) supplemented with 1\% Antibiotic-Antimycotic (Invitrogen). Plk4 constructs were subcloned into D-TOPO/pENTR (Invitrogen), and shuttled via Gateway technology (Invitrogen) into a modified pMT destination vector that engineers a C-terminal EGFP tag (D. Roberts). Constructs included PB1-PB3 (aa 382-741), PB1-PB2 (aa 382-602), PB1 (aa 382-500), PB2 (aa 500-602), PB1+ (382-525), and Linker-PB3 (L-PB3, aa 603-741). S2 cells were transfected (Amaxa Cell Line Nucleofector Kit V, Lonza), induced with $\sim 100 \mu \mathrm{M} \mathrm{CuSO}_{4}$, plated on Concanavalin A (ConA)-coated coverslips, and washed with HL3 media (70 mM $\mathrm{NaCl} ; 5 \mathrm{mM}$ trehalose; 115 mM sucrose; 5 mM HEPES; pH 7.2 ). Cells were then fixed in HL3 with $10 \%$ formaldehyde for 15 minutes, washed in phosphate buffered saline containing 0.1\% Triton X100 and TWEEN (PBST), and blocked with 5\% normal goat serum (NGS, Invitrogen) in PBST. Primary rabbit anti-D-PLP antibody was diluted 1:3000 and applied to cells for 30 minutes. Following PBST rinses, cells were treated with a Cy3 labeled secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:500 for 30 minutes and rinsed with PBST. Samples were mounted in a glycerol-based medium ( $90 \%$ glycerol, $10 \%$ PBS, $3 \% \mathrm{w} / \mathrm{v}$ n-propyl gallate) and imaged using a 100x objective (NA 1.49) Apochromatic TIRF objective (Nikon) mounted on an inverted microscope (Nikon Eclipse Ti) equipped with a cooled charge-coupled device camera (CoolSNAP HQ, Roeper Scientific), an excitation and emission wheel (LUDL), and emission filters (Chroma). All microscope hardware was
controlled by Nikon NIS-Elements. All images were processed and prepared for publication using Photoshop (CS5 version 12.0). Cells were processed and binned into one of three categories: strongly co-localized, weakly co-localized, or not co-localized. Co-localized cells displayed GFP signal at every centriole labeled using anti-D-PLP antibody; weakly colocalized cells displayed weak GFP signal at some, but not all centrioles; and not colocalized cells displayed no GFP punctae that overlapped with centrioles. All cells were blindly scored in at least two independent trials. Only cells with sufficient GFP expression levels were scored.

## Centriole Count Assay

Plk4 constructs not cloned through Gateway technology (Invitrogen), were subcloned into the inducible metallothionein-promoter pMT vector (Life Technologies) and tagged with either a C-terminal EGFP or a V5 epitope tag. S2 cell transient transfections were performed using the Nucleofector II (Amaxa) according to manufacturer's instructions, using $1 \mu \mathrm{~g}$ of the specified Plk4 construct. Constructs include full-length Plk4, Plk4-SBM (Rogers et al., 2009), Plk4 $\Delta \square \mathrm{PB} 1-\mathrm{PB} 2]$ (amino acids 382-602 deleted), Plk4 $\Delta \mathrm{PB} 1$ (amino acids 382-500 deleted), Plk4 PB1-PB2 (amino acids 382-602), Plk4 PB1+ (amino acids 382-500), and Asterless N-terminus (1-374). To count centriole numbers in transfected cells, we cotransfected Plk4 constructs with $0.2 \mu \mathrm{~g}$ of Nlp-EGFP (a constitutively expressed nuclear protein) (Rogers et al., 2009). Centrioles were then counted in cells with GFP-positive nuclei. Expression of all constructs was induced by addition of $0.5-2 \mathrm{mM}$ copper sulfate to the media. The PB1-PB2-GFP construct was also systematically analyzed using $0.25,0.50$ and 1.00 mM copper sulfate to differentially drive expression of the construct. Cells were fixed and processed exactly as described (Rogers and Rogers, 2008) by spreading on concanavalin

A coated, glass-bottom dishes and fixing with $10 \%$ formaldehyde. Affinity-purified rabbit anti-D-PLP antibody was used to stain centrioles, diluted to a concentration of $1 \mu \mathrm{~g} / \mathrm{ml}$. Secondary antibodies (conjugated with Cy2, Rhodamine Red-X, or Cy5 (Jackson ImmunoResearch Laboratories)) were used at manufacturer recommended dilutions. Hoeschst 33342 (Life Technologies) was used at a final dilution of $3.2 \mu \mathrm{M}$. Cells were mounted in $0.1 \mathrm{M} n$-propyl galate, $90 \%$ (by volume) glycerol, $10 \%$ PBS solution. Specimens were imaged at room temperature using a DeltaVision Core system equipped with an Olympus IX71 microscope, a 100x objective (NA 1.4), and a CoolSnap HQ ${ }^{2}$ cooled-CCD camera (Photometrics). Images were acquired with SoftWorx ${ }^{\mathrm{TM}} \mathrm{v} 1.2$ software (DeltaVision). At least 200 cells were counted per construct. Statistical analyses of centriole counts were performed using two-tailed two-sample t-tests, assuming equal variances.

## Immunoblotting

S2 cell extracts were produced by lysing cells in PBS, $0.1 \%$ TritonX-100. The Bradford protein assay (BioRad; manufacturer's instructions) was used to measure lysate protein concentrations. Laemmli sample buffer was then added and samples boiled for 5 minutes. For comparative Western blots, equal amounts of total protein from each sample was loaded and the integrated densities of chemiluminescent bands (measured with ImageJ (NIH)) were normalized relative to the integrated densities of endogenous $\alpha$-tubulin (loading control). Antibodies used for Western blots include monoclonal anti-GFP JL8 Living Colors (Clontech), anti-Slimb (Brownlee et al., 2011), anti-V5 (Life Technologies), anti- $\alpha$ tubulin DM1A (Sigma-Aldrich), diluted 1:1000. HRP-conjugated secondary antibodies (SigmaAldrich) were prepared according to the manufacturer's instructions, diluted 1:1500. Slimb RNAi and control RNAi was performed as described (Brownlee et al., 2011).

## Immunoprecipitation

For GFP immunoprecipitations, GFP-binding protein (GBP) (Rothbauer et al., 2008) was fused to the Fc domain of human IgG (pIg-Tail) (R\&D Systems), tagged with $\mathrm{His}_{6}$ in pET28a (EMD Biosciences), expressed in E. coli and purified on Talon resin (Clontech) according to manufacturer's instructions. GBP was bound to Protein A-coupled Sepharose, cross-linked to the resin using dimethylpimelimidate by rocking for 1 hour at $22^{\circ} \mathrm{C}$, and quenched in 0.2 M ethanolamine ( pH 8.0 ) by rocking for 2 hours at $22^{\circ} \mathrm{C}$. Antibody or GBPcoated beads were washed 3 x with 1.5 ml of cell lysis buffer (CLB; 100 mM Tris pH 7.2 , $125 \mathrm{mM} \mathrm{NaCl}, 1 \mathrm{mM}$ DTT, $0.1 \%$ TritonX-100, 0.1 mM PMSF). Transient-transfected S2 cells were induced to express recombinant Asl or Plk4 constructs with 1-2 $\mathrm{mM} \mathrm{CuSO}_{4}$, lysed in CLB, pre-cleared and diluted to $2-5 \mathrm{mg} / \mathrm{ml}$ in CLB. Antibody-coated beads were mixed with lysate for 40 minutes at $4^{\circ} \mathrm{C}$, washed 3 x with 1 ml of CLB, and boiled in SDS-PAGE sample buffer.

In vitro Kinase Assay
For in vitro kinase assays, His $_{6}$-tagged Plk4 kinase domain plus the downstream regulatory element (Kin-DRE) (Drosophila Plk4 residues 1-317) was cloned into the pET28a vector, expressed in BL21(DE3) E. coli, and purified on Talon resin (Clontech) according to manufacturer's instructions. Kinase assays were conducted in reaction buffer ( 40 mM Na HEPES, $\mathrm{pH} 7.3,150 \mathrm{mM} \mathrm{NaCl}, 5 \mathrm{mM} \mathrm{MgCl} 2,0.5 \mathrm{mM} \mathrm{MnCl}_{2}, 0.1 \mathrm{mM}$ dithiothreitol, 0.1 mM PMSF) supplemented with $10 \%$ (v/v) glycerol. Varying amounts of $\mathrm{His}_{6}$ and GSTtagged Plk4 domains (bacterially-expressed and purified on glutathione sepharose 4 fast flow resin, GE Healthcare, or Talon resin, Clontech) were also added. The tagged Plk4 domain constructs were either $\mathrm{His}_{6}-\mathrm{Plk} 4-\mathrm{PB} 1-\mathrm{PB} 2$ (Plk4 residues 382-602) or GST-Plk4-PB3
(residues 660-769). Finally, $85 \mu \mathrm{M} \gamma^{32} \mathrm{P}$-ATP was added and the reactions were incubated at $25^{\circ} \mathrm{C}$ for 1-2 hours. Reactions were terminated by the addition of Laemmli sample buffer and boiling. Samples were resolved on SDS-PAGE, Coomassie stained, dried, and the gels exposed to X-ray film, or a phosphorimaging screen (Molecular Dynamics) to detect radiolabeled bands. Band intensity was quantitated using the Image J program (NIH).

## Results

## Crystallization and Structure Determination of the Plk4 Cryptic Polo Box

Plk4's central region, termed the "cryptic polo box," was analyzed using secondary structure prediction algorithms in parallel with conservation to delineate the boundary residues for structural, biochemical and cellular analysis. D. melanogaster $\mathrm{Plk}_{382-602}$ was bacterially expressed, purified and crystallized as described in Experimental Procedures. Crystals belonged to the space group $\mathrm{P} 2_{1} 2_{1} 2$. A native dataset was collected on a single crystal to a resolution of $2.3 \AA$. To obtain phasing information, selenomethionine (SeMet)derivatized protein was produced but proved insoluble. We found that a SeMet- M517A mutant was marginally soluble and produced isomorphous crystals. Single wavelength peak anomalous dispersion data to 2.9 Å resolution was collected. Phases were calculated and extended to $2.3 \AA$. The structure was built and refined to R and $\mathrm{R}_{\text {free }}$ factors of 18.5 and $25.5 \%$, respectively. The final model contains two molecules of Plk4 in the asymmetric unit, comprising residues 382-596 (chain A) and 382-548, 553-597 (chain B). Crystallographic information is presented in Table 2.1.

## The Cryptic Polo Box Comprises Two Structurally Unique Polo Box Domains

Plk4 ${ }_{382 \text {-602 }}$ forms a multi-domain structure, surprisingly composed of two tandem, structurally unique PBs (Figure 2.1 B,C,D). This contrasts with the prediction that Plk4 is composed of a single C-terminal PB. In contrast to Plk1-3 that each contain two PBs, Plk4 was designated the "odd one out" (Sillibourne and Bornens, 2010), which holds true, though not because it contains one PB , but three PBs. We henceforth demarcate the $\mathrm{Plk} 4_{382-602} \mathrm{PBs}$ and assign them the sequential numbering PB1 (aa 382-499) and PB2 (aa 500-602), and propagate this scheme to the ultimate C-terminal PB , not included in our structure, assigning it PB3 (aa 660-741). We refer to the structure reported here as PB1-PB2. Both PB1 and PB2 adopt a canonical PB fold, delineated by an N-terminal anti-parallel $\beta$-sheet that packs against a C-terminal helix that runs diagonal to the $\beta$-strands. PB1 has a sequential antiparallel $\beta$-sheet composed of the six strands $1 \beta 1-1 \beta 2-1 \beta 3-1 \beta 4-1 \beta 5-1 \beta 6$ that pack against the $1 \alpha 1$ helix. PB1 contains two unique structural attributes. First, the $1 \beta 3-1 \beta 4$ strands form an extended hairpin off the $\beta$-sheet giving PB1 a winged structure (Figure $2.1 \mathrm{C}, \mathrm{D}$, tan arrowheads). Second, a sixteen-residue segment that we term the "stirrup" bridges $1 \beta 5$ and $1 \beta 6$ and blankets one face of the $\beta$-sheet, effectively sandwiching the PB1 $\beta$-sheet between $1 \alpha 1$ and the stirrup (Figure $2.1 \mathrm{C}, \mathrm{D}$, purple arrowheads). The $1 \beta 4$ portion of the winged $\beta$ hairpin buttresses the N -terminal flank of the stirrup, implicating a structural co-dependence between these unique PB elements. The $1 \alpha 1$ helix completes PB1, followed by a two amino acid linker that bridges PB1 and PB2: threonine 499 and proline 500. T499 is conserved across species and caps the $1 \alpha 1$ helix by forming a hydrogen bond between the T499 $\gamma$ hydroxyl and the K496 backbone carbonyl (Figure 2.1 E). The invariant proline P500 makes a jog in the backbone, offsetting the first strand of the PB2 sheet, $2 \beta 1$, from the $1 \alpha 1$ axis.

Plk4 PB2 also contains unique features that deviate from the canonical PB fold, including secondary structure elements that flank the $\beta$-sheet as well as an extended $2 \alpha 1$ helix. The PB2 $\beta$-sheet proceeds in an anti-parallel fashion, consecutively snaking through strands $2 \beta 1-2 \beta 2-2 \beta 3-2 \beta 4-2 \beta 5$. Instead of forming a sixth consecutive $\beta$-strand, as is the case with PB1, PB2 $2 \beta 5$ is followed by an ordered loop that connects $2 \beta 5$ with the $2 \alpha 1$ helix (Figure 2.1 C,D, green arrowheads). The $2 \beta 5-2 \alpha 1$ loop is stabilized by a hydrogen bond between the conserved D542 80 and the M546 backbone amine as well as Van der Waals interactions between hydrophobic residues in the loop, the $2 \beta 5$ strand, and the $2 \alpha 1$ helix. The C-terminal region of this loop is variable across species and contains a seven-residue insert in higher eukaryotes. The $2 \alpha 1$ helix runs diagonal to the $\beta$-sheet, spanning $40 \AA$ and extending $13 \AA$ past the $2 \beta 1$ strand (Figure 2.1 C, grey arrowhead). The helix-sheet interaction is stabilized both by a hydrophobic core as well as a disulfide bond between C511 in $2 \beta 2$ and C566 in $2 \alpha 1$ (Figure 2.1 F). The $2 \alpha 1$ helix leads into a loop that curls back towards $2 \beta 1$, effectively stabilizing the extended $2 \alpha 1$ helix by packing F588 against the $\beta$-sheet- $2 \alpha 1$ hydrophobic core. The loop terminates at an invariant proline, P589, that facilitates a bend in the backbone, leading into PB2's final $\beta$-strand, $2 \beta 6$, which runs parallel to $2 \beta 1$ and completes the PB2 $\beta$-sheet and the PB1-PB2 cassette (Figure 2.1 C , blue arrowhead). The location and polarity of the Plk4 $2 \beta 6$ strand is unique, normally running anti-parallel to $\beta 5$ in other PB structures.


Figure 2.1. The Plk4 Cryptic Polo Box is Composed of Tandem PB domains, PB1 and PB2. (A) Polo-like kinase family domain architecture. Plk1-3 contain two PB domains, Plk4 contains three PB domains. Plk4 levels are regulated by the DRE (green). (B) Secondary structure topology diagram of Plk4's conserved central domain: PB1 ( $\beta$-strands in red, $\alpha$ helix in pink, stirrup in lavender, loops in black) and PB2 ( $\beta$-strands in orange, $\alpha$-helix in yellow, loops in black). (C) Tertiary structure of the Plk4 PB1-PB2 monomer colored as in B. (D) Quaternary structure of homodimeric Plk4 PB1-PB2, rotated $90^{\circ}$ relative to C. (E) Stick representation of the junction between PB1 $1 \alpha 1$ and PB2 where the T449 hydroxyl caps the $1 \alpha 1$ helix. (F) Stick representation of the PB2 intra-domain disulfide formed between $2 \alpha 1$ C566 and $2 \beta 2$ C511.

Collectively, PB1 and PB2 form a composite structure, with a conserved core interface that buries $329 \AA^{2}$ and limits molecular flexibility to peripheral loop regions. Relative to PB1, PB2 is rotated approximately $120^{\circ}$. The protomers align well with $1.2 \AA$ rmsd across $214 \mathrm{C} \alpha$ atoms. PB1 and PB2 individually align to their dimeric mate with a $\mathrm{C} \alpha$ rmsd equal to 1.1 and $1.2 \AA$ respectively. Structural differences between the protomers, indicative of molecular flexibility, localize to loop regions, specifically the stirrup's Cterminal region, the 2 $2 \beta 4-2 \beta 5$ loop, and the $2 \beta 5-2 \alpha 1$ loop (Figure 2.2 A , black arrows). As a second metric for structural flexibility, we mapped B factors on the PB1-PB2 structure. The structure's C $\alpha$ B-factors range from 14-105 $\AA^{2}$, with increased levels in the stirrup, the 2 $2 \beta 4$ $2 \beta 5$ loop, the $2 \beta 5-2 \alpha 1$ loop, and the $2 \alpha 1-2 \beta 6$ loop, correlating with structural differences noted in the alignment of the protomers (Figure 2.2 B, arrowheads). The core regions of PB1 and PB2 as well as the T499-P500 bridge exhibit low temperature factors and little structural divergence when protomers are compared, indicative that the relative arrangement of PB1 and PB 2 is static.

## Plk4 PB1-PB2 Forms a Pseudo-symmetric Homodimer

The two Plk4 PB1-PB2 molecules in the asymmetric unit form a homodimer. The two PB1-PB2 molecules are related by a pseudo-symmetric two-fold axis that runs parallel to the $1 \alpha 1$ helices (Figure 2.1 D). A translational component along the pseudo two-fold axis shifts protomer A approximately $5 \AA$ relative to protomer B. The homodimerization interface is mediated by PB1-PB1 contacts as well as PB2-PB2 contacts (Figure 2.2 C,D). Due to the translational component, non-equivalent sets of residues are involved in the asymmetric
dimerization. The PB1-PB1 interface primarily involves residues from $1 \alpha 1$ with additional contributions from neighboring residues in the $\beta$-sheet. The PB2-PB2 interface involves residues from $2 \beta 4$, the $2 \beta 4-2 \beta 5$ loop, $2 \beta 5$, and $2 \alpha 1$. The PB1-PB1 and PB2-PB2 dimerization interfaces bury $1511 \AA^{2}$ and $1065 \AA^{2}$ of solvent accessible surface area respectively, collectively totaling $2576 \AA^{2}$. While crystallographic interfaces between protomers exist (Figure 2.S1 A,B,C), the homodimer in the asymmetric unit buries the largest surface area and is the only interaction that involves both PB1 and PB2; thus it likely represents the biological dimer.

To verify that Plk4 PB1-PB2 forms an oligomeric species in solution, we analyzed the oligomeric state using light scattering. Size exclusion chromatography coupled to multiangle static light scattering was conducted at pH 7.0 . $\mathrm{His}_{6}-\mathrm{Plk} 4 \mathrm{~PB} 1-\mathrm{PB} 2$ protein (MW 27.6 $\mathrm{kDa})$ was injected at initial concentrations of 19 and $28 \square \mathrm{M}$. Single elution peaks had experimentally determined molecular weights equal to $56.3 \pm 4 \mathrm{kDa}$ and $54.3 \pm 2 \mathrm{kDa}$ respectively (Figures 2.2 E, 2.S1 D). We also investigated the oligomeric state using batch dynamic light scattering at pH 9.5 using $140 \square \mathrm{M} \mathrm{Plk} 4$ PB1-PB2 (MW 25.7 kDa ) which yielded an experimental mass of $66 \pm 16 \mathrm{kDa}$ (Table 2.2). Collectivley, the static and dynamic light scattering values indicate that Plk4 PB1-PB2 exists as a homodimer with potential higher order oligomers forming at elevated concentration and pH . This is supported by prior work showing that the mouse Plk4 PB1-PB2 region self-associates (Leung et al., 2002).


Figure 2.2. Plk4 PB1-PB2 is an Asymmetric Homodimer with Plastic Stirrups and Loops. (A) Superposition of Plk4 PB1-PB2 protomers A and B showing plasticity in the stirrup as well as the $2 \beta 4-2 \beta 5$ and $2 \beta 5-2 \alpha 1$ loops. Plk4 colored as in Figure 2.1 B, shown in cartoon format. (B) Plk4 PB1-PB2 protomer A backbone colored and scaled according to $\mathrm{C} \alpha$ B-factor values ranging from 14 (dark blue) to 105 (red) $\AA^{2}$. High B-factors correlate with the structurally plastic loop segments between protomers A and B (shown in A, black arrows). The region bridging PB1 and PB2 shows little structural divergence and is dominated by low B-factor values. Plk4 PB1-PB2 protomer A (C) and protomer B (D) shown in sphere format; oriented as shown in the inset. Residues involved in homodimerization from both protomers, unique to protomer A, and unique to protomer B, are colored dark grey, purple, and raspberry, respectively. (E) Size exclusion chromatography - multi-angle light scattering analysis of $\mathrm{H}_{6}-\mathrm{Plk} 4 \mathrm{~PB} 1-\mathrm{PB} 2$ injected at $19 \mu \mathrm{M}$ (red trace) and $28 \mu \mathrm{M}$ (green trace $)(100 \mu \mathrm{l})$. Y-axis at left displays molecular weight $(\mathrm{kDa}), \mathrm{Y}$-axis at right displays normalized differential refractive index, X -axis displays time component of the run. (See Figure 2.S1).

## Unique Features of Plk4 PB1 and PB2 and Implications for Target Binding

While Plk4 PB1 and PB2 contain characteristic features of a PB domain, each diverges from PB structures determined to date, yielding implications for target binding. To highlight these differences, we compare Plk4's PB1 and PB2 domains with Plk1’s PB1 domain bound to a phosphopeptide target (Figure 2.3 A). The Plk1 PB1 $1 \beta 1$ strand forms key contacts with the phosphopeptide target. The Plk4 PB1 domain aligns to Plk1 PB1 with an rmsd of $2.0 \AA$ across 76 aligned $\mathrm{C} \alpha$ atoms (Figure 2.3 B). Structural divergence occurs at the Plk4 PB1 1 $\beta 3-1 \beta 4$ hairpin extension, the stirrup, the positioning of $1 \beta 6$ and the elongated $1 \alpha 1 \mathrm{~N}$-terminal region. While there is structural divergence, the $1 \beta 1$ strand, used in Plk1 PB1 to bind phosphopeptide targets, is accessible and may facilitate target binding as observed in Plk1 PB1 (Figure 2.3 C).

Plk4 PB2 diverges from the Plk1 PB1 structure, with differences in loops, a helix extension, and the positioning of $2 \beta 6$. Plk4 PB2 aligns to Plk1 PB1 with a $2.3 \AA$ rmsd over 72 structurally aligned $\mathrm{C} \alpha$ atoms (Figure 2.3 B). Plk4 PB2 differs from Plk1 PB1 in the positioning of the $2 \beta 2-2 \beta 3$ and $2 \beta 4-2 \beta 5$ loops. Plk4 PB2 contains an ordered loop between $2 \beta 5$ and $2 \alpha 1$ that substitutes for the Plk1 PB1 $1 \beta 6$ strand. Plk4 PB2 forms a C-terminal extension on the $2 \alpha 1$ helix. The Plk4 PB2 $2 \beta 6$ strand does not occur between $2 \beta 5$ and $2 \alpha 1$, but resides C-terminal to $2 \alpha 1$ and runs parallel to $2 \beta 1$, a site occupied by the phosphopeptide target in Plk1 PB1. Thus, $2 \beta 6$ occludes the Plk4 PB2 domain from interacting with a phosphopeptide in a manner equivalent to Plk1 PB1.


Figure 2.3. Plk4 PB1 and PB2 Diverge from Plk1 PB Domain Structures and Form a Unique Inter-domain Interaction. (A) Structural alignment of Plk4 PB1 and Plk4 PB2 (colored red and orange, respectively) with human Plk1 PB1 bound to a phosphopeptide target ( $3 \mathrm{FVH}, \mathrm{PB} 1$ in green, peptide in slate). (B) Matrix showing the rmsd ( $\AA$ ) between structures of human Plk1 PB1 and PB2, fly Plk4 PB1 and PB2, and mouse Plk4 PB3. (C) Superposition of Plk4 PB1-PB2 homodimer and the Plk1 PB1-PB2-phosphopeptide structure, aligned over Plk4 PB1 and Plk1 PB1, highlighting the differential organization of Plk1 PB1-PB2 as compared to Plk4 PB1-PB2. The Plk4 PB1 stirrup overlaps with the corresponding binding site of Plk1 PB2. The location where Plk1 PB1 binds its phosphopeptide target is accessible on Plk4 PB1. (D) Superposition of Plk4 PB1-PB2 and the Plk4 PB3 homodimer, aligned over single PB1 and PB3 domains. Insets show the orientation of each independent structure (C,D).

## Plk4 PB1-PB2 has a Novel Inter-domain and Homodimeric Arrangement

PB structures determined to date show diverse intra- and inter-molecular PB-PB interactions. Here we compare Plk4 PB1-PB2 with Plk1 PB1-PB2 and Plk4 PB3. Plk1 PB1PB2 is monomeric, with PB1 and PB2 positioned to form a collective $\beta$-sandwich. In contrast, Plk4 PB3 is homodimeric and dimerizes across a symmetric $\beta$-sandwich (Leung et al., 2002). Like Plk1 PB1-PB2, the Plk4 PB3 $\beta$-sheets are sandwiched orthogonal to one another; however, PB3 dimerizes across the opposite face of its PB $\beta$-sheets. Plk4 PB1-PB2 adopts a third, unique PB domain arrangement (Figure 2.3 C,D). To highlight the differential inter-domain arrangements across these paired PB domain structures, we superimposed the Plk4 PB1-PB2 structure and the Plk1 PB1-PB2 structure after a least squares fit of their respective PB1 domains (Figure 2.3 C ). Relative to PB1, the Plk4 PB2 domain is positioned dramatically different than Plk1 PB2, each engaging a distinct, non-overlapping face on their respective PB1 partner. In Figure 2.3D, the Plk4 PB1-PB2 structure is superimposed on the Plk4 PB3-PB3 structure after a least squares fit of individual Plk4 PB1 and PB3 domains. The position of the PB3 dimeric mate does not correlate with the relative positioning of PB2, or the PB1 dimeric mate. Overall, all PB-PB structures exhibit non-homologous domain arrangements. One consistent feature across Plk4 PB structures is homodimerization of the individual PB domains. However, while the PB3-PB3 interface is symmetric, the PB1-PB1 and PB2-PB2 interfaces are asymmetric.

The Conserved Plk4 PB1-PB2 Inter-domain Groove Corresponds to the Plk1 PB1 Target Binding Site

To highlight conserved determinants across the Plk4 PB1-PB2 structure, we generated an alignment of PB1-PB2 across ten diverse species and contoured identity at $100 \%$ and $80 \%$ (Figure 2.4 A , green and yellow, respectively). When mapped onto the Plk4 PB1-PB2 homodimer structure, the prime cluster of invariant residues occurs at a composite site formed at the PB1-PB2 junction, with contributions by PB1 $1 \beta 1-1 \beta 2$ and PB2 2 $\beta 1-2 \beta 4$ (Figure 2.4 B,C, green arrows). The majority of conserved sequences in PB1 1 $\beta 2-1 \beta 5$ are occluded by the stirrup and accessibility would thus require a dramatic rearrangement of the stirrup which we do not rule out. The PB1-PB2 junction is formed on a single protomer, thereby constituting two independent sites on the homodimer. The PB1-PB2 junction is concave with both hydrophobic and basic determinants (Figure $2.4 \mathrm{~B}, \mathrm{C}$, green arrows). When Plk1 PB1 with bound phosphopeptide is aligned with Plk4 PB1, the phosphopeptide is positioned at the Plk4 PB1-PB2 conserved junction (Figures 2.3 C; 2.4 B,C). Whether this site on Plk4 binds targets remains to be determined.

The Full Plk4 PB1-PB2 Cassette is Required for Asterless Binding
A fragment of Plk4's PB1-PB2 region has been shown to bind the centriole component Asl in vitro (Dzhindzhev et al., 2010). The Asl-binding region spans Plk4 residues 376-525, while a shorter fragment spanning residues 376-500 (encompassing only PB1), lacks Asl-binding activity. The Plk4 PB1-PB2 structure spans residues 382-602, with proline P500 defining the PB1-PB2 bridge and residues 501-525 encoding the first three contiguous anti-parallel $\beta$-strands in PB2 (2 $2 \beta 1-2 \beta 3$ ). While $2 \beta 1-2 \beta 3$ is conserved and contributes to the composite PB1-PB2 conserved patch, it is unlikely that $2 \beta 1-2 \beta 3$ would
fold into an ordered $\beta$-sheet in the absence of $2 \alpha 1$, though it may fold upon Asl binding. To test the ability of Plk4 PB1-PB2 to bind Asl, taking structural insight into construct design, we immunoprecipitated various Plk4-GFP constructs from S2 cell lysates transiently coexpressing the Asl Plk4-binding domain, V5-Asl (residues 1-300), and immunoblotted for these proteins. As expected, full-length Plk4 did not express at high levels, due to its ubiquitin-mediated degradation, and thus did not co-immunoprecipitate detectable levels of Asl (Figure 2.4 D). To ensure that Plk4 was capable of immunoprecipitating Asl, we examined a full-length Plk4 Slimb Binding Mutant (SBM), S293A/T297A, that prevents phospho-dependent Slimb binding and concomitant Plk4 ubiquitination, yielding stable Plk4 (Rogers et al., 2009). Plk4-SBM-GFP was stably expressed and co-immunoprecipitate Asl (Figure 2.4 E). Intriguingly, Plk4 lacking PB1 (Plk4 $\Delta \mathrm{PB} 1$ ) expressed to a high level, implicating a possible role for PB1 in Plk4 degradation (Figure 2.4 D). However, Plk4 $\Delta \mathrm{PB} 1$ failed to co-immunoprecipitate Asl. Strikingly, expression of only PB1-PB2 (residues 382602 ) robustly co-immunoprecipitated Asl. In contrast, a construct containing the previously described Plk4 Asl-binding domain, Plk4 PB1+ (residues 382-525), exhibited low expression and failed to co-immunoprecipitate detectable levels of Asl, suggesting that the full PB1-PB2 structural cassette is required for robust Asl binding in vivo.

## A




Figure 2.4. Plk4 PB1 and PB2 Form a Composite Inter-domain Groove Delineated by Conserved and Basic Residues. (A) Plk4 sequence alignment across ten species. Protomer A solvent accessible surface area (ASA) ( $\AA^{2}$ ) is indicated. $100 \%$ identity is highlighted in green, $80 \%$ identity in yellow (homologous residues also highlighted in yellow where the $80 \%$ identity criteria is met). Human Plk1 PB1 sequence is aligned against Plk4 PB1 and PB2, based on structural alignment. Residues involved in homodimerization are indicated below the alignment, colored as in Fig 2.2 C,D. (B) Plk4 PB1-PB2 homodimer structure shown in sphere format with conserved residues colored as in A (left) and in surface representation (right) showing electrostatics contoured from -2.0 to $+2.0 \mathrm{kT} / \mathrm{e}$. (C) Protomer A rotated $45^{\circ}$ relative to the orientation shown in B showing conservation and electrostatics as in B. The phosphopeptide from the Plk1 PB1-PB2 structure (3FVH) shown in stick format
and colored blue, is docked onto the Plk4 structure in both B and C, based on the structural alignment of Plk1 PB1 and Plk4 PB1 shown in Fig 2.3 C. (D-E) Anti-GFP immunoprecipitates from S2 cell lysates transiently-expressing N-terminal Asl-V5 and the indicated Plk4-GFP construct or control GFP, probed for GFP and V5.

The PB1-PB2 Cassette is Necessary and Sufficient for Robust Centriole Targeting
In light of our finding that the "cryptic polo box" is composed of two bona fide PB domains, we set out to determine whether individual PBs could mediate centriole localization or if the full PB1-PB2 structure was required for centriole targeting. Localization experiments were conducted in interphase S 2 cells containing endogenous Plk4, as Plk4 depletion causes dramatic centriole loss. We transiently expressed a series of inducible Plk4 PB-GFP constructs (Figure 2.5 A). After transgene induction, cells were immunostained for pericentrin-like protein (D-PLP) to mark centrioles, and co-localization was scored as strong, weak, or no centriole localization (Figure 2.5 B-H). Only two constructs, PB1-PB3 (aa 382741) and PB1-PB2 (aa 382-602), containing the entire PB1-PB2 cassette defined in our crystal structure, displayed robust centriole localization. PB1-PB3 and PB1-PB2 strongly colocalized with centrioles in $88 \%$ and $96 \%$ of cells assayed respectively (Figure 2.5 B-D). Examination of constructs lacking the full PB1-PB2 cassette showed significantly reduced centriole co-localization. Expression of the single PB domains that compose the PB1-PB2 cassette, individually displayed no centriole localization in the majority of cells scored (Figure $2.5 \mathrm{~B}, \mathrm{E}, \mathrm{F}$ ). We also scored the centriole targeting activity of the previously described Plk4 Asl-binding region (PB1+) (Dzhindzhev et al., 2010). PB1+ displayed a dramatic reduction in centriole co-localization as compared to PB1-PB2 (Figure $2.5 \mathrm{~B}, \mathrm{G}$ ), suggesting
that the full PB1-PB2 cassette is necessary to bind Asl and target Plk4 to centrioles. To determine if the PB1-PB2 cassette is necessary for centriole targeting, we designed a full length, SBM construct with the PB1-PB2 cassette deleted (SBM $\Delta[\mathrm{PB} 1-\mathrm{PB} 2]$ ). SBM $\Delta[\mathrm{PB} 1-$ PB2] showed strong centriole co-localization in only $6 \%$ of cells and weak co-localization in $49 \%$ of cells (Figure $2.5 \mathrm{~B}, \mathrm{H}$ ). [PB1-PB2]-independent centriole association is likely conferred by PB3 as mouse PB3 has weak centriole targeting activity (Leung at al., 2002). In agreement, we found that a Linker-PB3 construct (L-PB3; aa 602-741) showed some level of centriole co-localization in $49 \%$ of cells (Figure 2.5 B,I).

## PB1-PB2 Scaffolds Plk4 Trans-autophosphorylation to Limit Centriole Duplication

Previous work has revealed an auto-regulatory mechanism in Plk1 whereby its PB1PB2 cassette binds the kinase domain in trans and inhibits kinase activity in vitro. It has also been shown that the mouse Plk4 central region (encompassing PB1-PB2) can bind the Plk4 kinase domain in trans (Leung et al., 2002; Leung et al., 2007). To determine whether inhibition of kinase activity is a conserved PB feature, we incubated a fly Plk4 construct containing the kinase domain + DRE (Kin-DRE) with increasing molar ratios of the PB1PB2 cassette or the PB3 domain and assayed Plk4 autophosphorylation. No change in autophosphorylation was detected upon titration with PB1-PB2 or PB3 in trans (Figure 2.S2).


Figure 2.5. The Plk4 PB1-PB2 Cassette is Required for Robust Centriole Localization. (A) Schematic of PB-GFP containing constructs assayed for centriole localization. (B) S2
cells were transiently-transfected with the constructs shown in A (GFP), induced to express for 24 hours, and immunostained with anti D-PLP antibody to mark centrioles (Cy3). Centriole co-localization was classified as strong (green), weak (chartreuse), or no colocalization (purple). Total number of cells analyzed and independent experiments performed noted at right. (C, D) Expression of PB1-PB3 (B) or the PB1-PB2 cassette (D) results in strong centriole co-localization. Cell margins are indicated (white dashed lines). (E-I) Single or incomplete PBs primarily display weak or no centriole co-localization. Representative images of differential localization are shown. Boxed regions are magnified in the lower panels. Scale bar, $5 \mu \mathrm{~m}$.

Endogenous Plk4 levels are tightly regulated through trans-autophosphorylation of the DRE. Trans-autophosphorylation should be promoted by Plk4 oligomerization. Given that our PB1-PB2 structure is homodimeric, we tested whether expression of the PB1-PB2 cassette could inhibit Plk4 degradation by heterodimerizing with full-length Plk4 and preventing trans-autophosphorylation. We first analyzed Plk4 stabilization by assessing centriole number, as increased Plk4 stabilization promotes centriole amplification. Cells transfected with full-length Plk4 or non-degradable Plk4-SBM resulted in centriole amplification, with Plk4-SBM generating a stronger effect (Rogers et al., 2009). Strikingly, cells transfected with PB1-PB2 displayed centriole amplification on par with Plk4-SBM (Figures 2.6 A,B, 2.S3). Other Plk4 constructs including Plk4- $\Delta \mathrm{PB} 1$, Plk4- $\Delta[\mathrm{PB} 1-\mathrm{PB} 2]$, and PB1+ did not alter the centriole count from the GFP control, indicating that the entire PB1PB2 cassette is required to stimulate centriole amplification, presumably by hyper-stabilizing endogenous Plk4. While low PB1-PB2 induction levels caused centriole amplification, this effect varied slightly at higher induction levels, potentially due to PB1-PB2 homodimers saturating centriole binding sites (Figure 2.S4).

To directly test if PB1-PB2 promotes centriole amplification through stabilization of Plk4, we co-transfected full-length Plk4-GFP with either PB1-PB2-GFP, Plk4- $\Delta$ [PB1-PB2]-

GFP or GFP and analyzed Plk4-GFP levels. We found that full length Plk4-GFP levels increased dramatically when co-transfected with PB1-PB2-GFP but not with Plk4- $\Delta[$ PB1-PB2]-GFP or GFP alone, demonstrating that PB1-PB2 promotes Plk4 stability in trans (Figure 2.6 C). To test if PB1-PB2-GFP could bind Plk4-FL-myc in trans, we co-transfected these constructs, immunoprecipitated PB1-PB2-GFP and probed for Plk4-FL-myc. We found that PB1-PB2-GFP was able to immunoprecipitate Plk4-FL-myc, indicative of an interaction mediated by PB1-PB2 (Figure 2.6 D). Both PB1-PB2 and PB3 independently confer dimerization. We asked whether Plk4 homodimerization, as mediated by PB1-PB2, is required for efficient trans-autophosphorylation and degradation or whether PB3-mediated dimerization suffices. To test this, we expressed a construct containing PB3 but lacking PB1PB2 (Plk4- $\Delta$ [PB1-PB2]-GFP) and compared its levels to wild-type Plk4-GFP and a stable, full-length Plk4 kinase-dead mutant (Figure 2.6 E) (Brownlee et al., 2011). Wild-type Plk4GFP protein levels were extremely low. In contrast, Plk4- $\Delta[\mathrm{PB} 1-\mathrm{PB} 2]$ protein levels were dramatically stabilized and on par with kinase-dead Plk4. To ensure that Plk4-GFP was expressed in these cells and could be compared to Plk4- $\Delta$ [PB1-PB2], we depleted Slimb via RNAi and immunoblotted for GFP. Slimb depletion yielded detectable Plk4-GFP, confirming Plk4 expression and its Slimb-mediated degradation (Figure 2.6 F). These findings indicate that PB1-PB2 plays a key role in Plk4 degradation beyond homodimerization and may extend to scaffolding the kinase for trans-autophosphorylation (Guderian et al., 2010). To test if PB1-PB2 plays a role in auto-phosphorylation of the DRE, we examined Slimbbinding as a read-out for DRE phosphorylation. Plk4-FL-GFP ran as a broad band on SDS PAGE and was able to co-immunoprecipitate Slimb, however Plk4- $\Delta$ [PB1-PB2]-GFP ran as a tight doublet and co-immunoprecipitated comparatively reduced levels of Slimb, indicative
that the PB1-PB2 cassette enhances auto-phosphorylation, priming the DRE for Slimb binding (Figure 2.6 G).


Figure 2.6. Plk4 PB1-PB2 Promotes Centriole Amplification and Protects Full-length Plk4 in trans. (A) S2 cells were transiently-transfected with either inducible GFP, Plk4 PB1-PB2-GFP, or non-degradable Plk4-SBM-GFP (GFP), induced for 3 days, fixed, and stained for centrioles (PLP, Cy3, red) and DNA (DAPI, blue). Arrowheads mark centrioles. Boxed regions are magnified in the insets and highlight centriole clusters not observed in controls. (B) Histograms of centriole counts were measured from S2 cells transiently expressing the indicated constructs after 3 days of induction (see Figures 2.S3,4). The percentage of cells
with a centriole count per cell <2, 2, and >2 is indicated. (C) Ectopic Plk4 PB1-PB2-GFP expression is sufficient to stabilize full-length Plk4-GFP. Immunoblots of S 2 cell lysates showing that overexpression of PB1-PB2-GFP (but not Plk4- $\Delta$ [PB1-PB2]-GFP or GFP) stabilizes wild-type full-length Plk4-GFP. Tubulin, loading control. (D) PB1-PB2-GFP coimmunoprecipitates Plk4-FL-myc. Immunoblots of anti-GFP immunoprecipitates from S2 cells co-transfeted with Plk4-FL-myc and PB1-PB2-GFP or GFP (control). (E) Anti-GFP immunoblot of Plk4-GFP constructs transiently expressed in S 2 cells showing differential stability. Tubulin, loading control. (F) Plk4-FL-GFP is expressed but rapidly degraded by Slimb-mediated ubiquitination. Cell lysates from S2 cells transfected with Plk4-FL-GFP and treated with control or Slimb dsRNA. Tubulin, loading control. (G) Plk4 lacking the PB1PB2 cassette shows reduced auto-phosphorylation of the DRE as assayed by Slimb binding. Immunoblots of anti-GFP immunoprecipitates from S2 cells transfected with Plk4-FL-GFP or Plk4- $\Delta$ [PB1-PB2]-GFP and blotted for GFP and Slimb. (H) Model of the Plk4 homodimer. PB1 and PB2 mediate homodimerization. PB1 and PB2 form a composite Asl/Cep152 binding site, recruiting Plk4 to the centriole. PB1-PB2 homodimerization scaffolds Plk4 trans-autophosphorylation, priming the DRE for $\mathrm{SCF}^{\text {Slimb }}$ binding and ubiquitination.

## Discussion

Plk4's essential role in centriole duplication is well established, but a fundamental understanding of its mechanism has been lacking. Here, we have determined the structure of the Plk4 CPB and found that it is actually composed of two PB domains, PB1-PB2. This finding recalibrates the number of PB domains in Plk4 from one to three. Both the Plk4 PB1 and PB2 domains have unique structural features that distinguish them from Plk1 PB1, PB2 and Plk4 PB3.

The spatial arrangement of Plk4 PB1 and PB2 differs from the arrangement observed in Plk1 PB1-PB2 structures. Plk4 PB1 and PB2 pack end-to-end, linked by a short, ordered threonine-proline segment, flanked by hydrophobic residues that anchor PB1 on PB2. The PBs are rotated relative to their $\beta$-sheet planes and translated, placing the $1 \alpha 1$ helix axis in line with the PB2 $2 \beta 2$ strand. In contrast, Plk1's tandem PBs are packed around a pseudo 2fold axis, forming a $\beta$-sandwich. Thus, Plk4 and Plk1 each have distinct, non-homologous
arrangements of their PB1-PB2 domains. The Plk1 PB1-PB2 structure is monomeric in contrast to the homodimeric Plk4 PB1-PB2 structure. Plk4 PB1-PB2 has extensive pseudosymmetric dimerization interfaces across PB1-PB1 and PB2-PB2. Our light scattering data confirm a Plk4 PB1-PB2 homodimeric state, in agreement with previous work showing that the CPB confers self-association (Leung et al., 2002). The Plk4 PB3 structure is also homodimeric (Leung et al., 2002), indicating that full-length Plk4 has at least three dimerization interfaces mediated by its three PB domains. Overall, the three Plk4 PBs are unique among themselves and distinct from Plk1 PB1 and PB2 in their structure, multidomain arrangement, and oligomeric state.

Plk4 PB1-PB2 localizes robustly to centrioles in S2 cells while the individual PB1 and PB2 domains display only weak centriole co-localization, indicating that the full PB1PB2 cassette collectively confers strong centriole targeting. Previous work examining Plk4's interaction with Asl in vitro mapped the Plk4 determinants to a segment embodying PB1 and the first three $\beta$-strands of PB2 (PB1+) (Dzhindzhev et al., 2010). In the same study, the Plk4 PB1 region failed to bind Asl in vitro. This maps key Plk4 Asl-binding determinants to the conserved inter-domain, concave junction defined by PB1 $1 \beta 1$ and PB2 2 $\beta 1$-2 23 (Figure 2.4 $\mathrm{B}, \mathrm{C})$. We found that while PB1+ enhances centriole localization over PB1 alone, it is not as effective as the full PB1-PB2 cassette. In support we found that PB1-PB2 coimmunoprecipitates Asl while PB1+ does not. Collectively, our work and previous work can be interpreted in light of our PB1-PB2 structure. While PB1+ contains prime Asl-binding determinants, the remaining PB2 elements are likely required to complete the domain fold and stabilize the determinants that bind Asl and afford robust centriole targeting.

The conserved PB1-PB2 inter-domain groove contains the PB1 $1 \beta 1$ strand, which in

Plk1 PB1 is primarily responsible for phosphopeptide binding. It is possible that the Plk4 PB1 $1 \beta 1$ strand is used to bind Asl, but Plk1 and Plk4 target binding will have significant differences. First, bacterially expressed Asl binds Plk4 in vitro, indicative that the interaction, in contrast to Plk1, is not phospho-dependent. Second, Plk4 binds to a minimal, 300 residue N-terminal segment of Asl (Dzhindzhev et al., 2010). This is in contrast to the short, phosphorylated motifs Plk1 recognizes and suggests that Plk4 binds a domain in Asl rather than a short motif. In support, Plk4 PB1-PB2 forms a large, concave surface that could accommodate domain binding. This contrasts with Plk1, where PB1 and PB2 clamp around a phosphopeptide target.

Expression of the PB1-PB2 cassette caused interesting dominant effects. First, PB1PB2 amplified centriole levels on par with the non-degradable Plk4-SBM construct. Second, we found that full-length Plk4 was stabilized in trans by the PB1-PB2 construct. Plk4 downregulates its own protein level by trans-autophosphorylating its DRE to promote Slimb binding (Holland et al., 2010; Guderian et al., 2010). We found that the PB1-PB2 construct heterodimerizes with endogenous Plk4 and protects it from trans-autophosphorylation. Trans-autophosphorylation is not simply mediated by oligomerization because a Plk4 construct lacking the PB1-PB2 cassette but retaining the PB3 homodimerization domain was itself dramatically stabilized. This indicates that PB1-PB2 dimerization positions the kinase domains and DREs optimally for trans-autophosphorylation in order to restrict centriole duplication to once and only once per cell cycle (Figure 2.6 H ).

Our results reveal unique structural and functional determinants in Plk4 (Figure 2.6 H ). In contrast to Plk1-3, Plk4 is a unique polo kinase member, containing three PBs that mediate centriole localization as well as homodimerization. The PB1-PB2 cassette collectively binds

Asl and affords robust centriole localization, optimally positioning the kinase domain for trans-autophosphorylation. While PB1-PB2 affords centriole localization via Asl, we do not rule out the possibility that PB1-PB2 interacts with additional centriole factors. While PB3 does not bind Asl in vitro (Dzhindzhev et al., 2010), it does mediate centriole localization, albeit weakly, implicating a non-Asl PB3-binding factor at the centriole. Further structural studies are needed to illuminate the Plk4/Asl interaction, understand how the PB1-PB2 cassette regulates trans-autophosphorylation, and identify additional Plk4 centriole targets.

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TABLES

| Crystal | Native | SeMet M517A |
| :---: | :---: | :---: |
| Beamline | APS 22-ID | APS 22-BM |
| Space group | $\mathrm{P} 21_{2}{ }_{1}$ |  |
| Cell: a,b,c ( $\mathbf{A}$ ) | 86.5, 136.4, 46.5 | 87.1, 136.5, 46.6 |
| Wavelength (A) | 1.00890 | 0.97980 |
| $\mathbf{d}_{\text {min }}(\mathbf{A})$ | 2.3 (2.38-2.30) | 2.9 (3.00-2.90) |
| \% Complete | 90.1 (85.2) | 100.0 (100.0) |
| I/G | 21.3 (5.2) | 16.3 (3.2) |
| No. of observations | 114559 (10620) | 101205 (9570) |
| No. of independent observations | 22630 (2082) | 23604(2333) |
| Multiplicity | 5.1 (5.1) | 4.3 (4.1) |
| $\mathbf{R}_{\text {sym }}$ (\%) ${ }^{\text {² }}$ | 5.9 (35.9) | 8.5 (37.9) |
| Resolution (A) at which anomalous completeness exceeds 85\% for $l / \sigma I>5,>3,>2$ |  | 4.1, 3.9, 3.4 |
| Overall log-likelihood gain ${ }^{\star}$ / figure of merit ${ }^{\dagger}$ |  | $1.4 \times 10^{5} / 0.44(0.35)$ |
| Figure of merit ${ }^{\dagger}$ Centrics / Acentrics |  | 0.25(0.25) / 0.47(0.36) |
| Figure of merit ${ }^{\dagger}$ after density modification Centrics / Acentrics |  | 0.66(0.42) / 0.73(0.52) |
| Refinement (A) | 50-2.3 (2.38-2.30) |  |
| R value ${ }^{\text {d }}$ | 18.5 (19.8) |  |
| $\mathrm{R}_{\text {free }}{ }^{\ddagger}$ | 25.5 (30.7) |  |
| Rmsd bond lengths ( $\mathbf{A}$ ) | 0.008 |  |
| Rmsd bond angles ( ${ }^{\circ}$ ) | 1.09 |  |
| Mean B (min / max) ( $\AA^{2}$ ) | 35.9 (9.4 / 141.6) |  |
| No. atoms: protein / water / $\mathrm{SO}_{4}^{-}$ | 3478 / 184 / 6 |  |

Table 2.1. Plk4 PB1-PB2 crystallographic data, phasing, and refinement. Values in parentheses are for the highest resolution shells unless otherwise denoted.
$* R_{\text {sym }}=\Sigma_{\mathrm{h}} \Sigma_{\mathrm{i}}\left|\mathrm{I}_{\mathrm{i}}(\mathrm{h})-\langle\mathrm{I}(\mathrm{h})\rangle\right| / \Sigma_{\mathrm{h}} \Sigma_{\mathrm{i}} \mathrm{I}_{\mathrm{i}}(\mathrm{h})$, where $\mathrm{I}_{\mathrm{i}}(\mathrm{h})$ is the $\mathrm{i}^{\text {th }}$ measurement and $\langle\mathrm{I}(\mathrm{h})\rangle$ is the mean of all measurements of $I(h)$ for Miller indices $h$.
*Log-likelihood gain value as determined by Phenix.
${ }^{\dagger}$ Figure of merit is the weighted mean of the cosine of the deviation from $\alpha_{\text {best }}$.
${ }^{\square} \mathrm{R}$ value $=\Sigma(\mid$ Fobs $|-k|$ Fcalc $\mid) / \Sigma \mid$ Fobs $\mid$.
${ }^{\ddagger} \mathrm{R}_{\text {free }}$ is calculated using a $10 \%$ subset of the data that is removed randomly from the original data and excluded from refinement (Brünger 1992).

| Run Number | \% Polydispersity | MW (kDa) | \% Intensity | \% Mass |
| :--- | :--- | :--- | :--- | :--- |
| $\mathbf{1}$ | 52.9 | 80 | 71.5 | 99.9 |
| $\mathbf{2}$ | 59.5 | 88 | 68.4 | 99.9 |
| $\mathbf{3}$ | 35.2 | 43 | 56.4 | 99.9 |
| $\mathbf{4}$ | 45.2 | 63 | 67.4 | 99.9 |
| $\mathbf{5}$ | 31.4 | 59 | 81.8 | 99.9 |
| $\mathbf{6}$ | 29.7 | 65 | 50.9 | 94.0 |
| Mean $\pm$ S.D. |  | $\mathbf{6 6} \pm \mathbf{1 6}$ |  |  |

Table 2.2. Plk4 PB1-PB2 dynamic light scattering. Plk4 PB1-PB2 at $3.6 \mathrm{mg} / \mathrm{mL}$ in 50 mM Tris $\mathrm{pH} 9.5,300 \mathrm{mM} \mathrm{NaCl}, 0.1 \% ~ \beta-\mathrm{ME}, 10 \%$ glycerol, measured on a Wyatt DynaPro dynamic light scattering plate reader (Wyatt 1993). The Plk4 PB1-PB2 monomeric molecular weight is $25,675 \mathrm{Da}$.

## SUPPLEMENTAL FIGURES



Figure 2.S1. Plk4 PB1-PB2 forms numerous crystallographic interfaces but is a homodimer in solution. (A-C) Cartoon diagram of Plk4-PB1-PB2 molecular interfaces involved in crystal packing. The total buried surface area for each interaction is indicated. This data supplements the homodimeric interface observed in the asymmetric unit, presented in Figure 2.2 C,D. (D) Size exclusion chromatography - multi-angle light scattering (SECMALS) analysis of $\mathrm{H}_{6}$-Plk4 PB1-PB2 injected at $19 \mu \mathrm{M}$ (red trace) and $28 \mu \mathrm{M}$ (green trace) ( $100 \mu \mathrm{l}$ ). Bovine serum albumin control, $100 \mu \mathrm{l}$ injected at $90 \mu \mathrm{M}$ (blue trace). Y-axis at left displays molecular weight ( kDa ), Y-axis at right displays normalized differential refractive index, X -axis displays time component of the run. Plk4 constructs showed a single peak with molecular weight of that peak concordant with homodimerization. The bovine serum albumin control produced three peaks with the respective molecular weights of those peaks concordant with monomer, homodimer and homotrimer formation (bovine serum albumin molecular weight $=66,463 \mathrm{Da}$ ). This data supplements the SEC-MALS data presented in Figure 2.2 E.

A


B


C


Figure 2.S2. The presence of either the PB1-PB2 or PB3 domain of Plk4 does not inhibit the in vitro kinase activity of Plk4-Kin-DRE in trans. (A) Increasing amounts of purified $\mathrm{His}_{6}$-tagged Plk4 PB1-PB2 were incubated with constant amounts of purified His ${ }_{6}$-tagged Plk4-Kin-DRE and $\gamma^{32}$ P-ATP. The Coomassie-stained SDS-PAGE gel of the resolved in vitro reactions is shown above its corresponding autoradiograph. The calculated molar ratios of Plk4 PB1-PB2 to Plk4-Kin-DRE are shown at top. (B) Incorporation of ${ }^{32} \mathrm{P}$ into Plk4-KinDRE is not affected by Plk4 PB1-PB2. To calculate ${ }^{32} \mathrm{P}$ incorporation, bands corresponding to Plk4-Kin-DRE in the autoradiograph and gel shown in A were measured by densitometric scanning. Each ${ }^{32} \mathrm{P}$ measurement was then normalized to the measure of its corresponding Coomassie-stained band. (C) A similar in vitro assay was performed with GST-Plk4 PB3: increasing amounts of purified Plk4 PB3 were incubated with constant amounts of His $6^{-}$ tagged Plk4-Kin-DRE and $\gamma^{32}$ P-ATP. In this case, the autophosphorylation of Plk4-Kin-DRE could not be measured by densitometry because of the similar mobilities of Plk4-Kin-DRE and Plk4 PB3. However, the presence of Plk4 PB3 does not change the level of ${ }^{32} \mathrm{P}$ incorporation relative to the control. This data supports the findings reported in the Results and Discussion section: "PB1-PB2 Scaffolds Plk4 Trans-autophosphorylation to Limit Centriole Duplication."


Figure 2.S3. Expression of Plk4 PB1-PB2 is sufficient to promote centriole amplification. Graphs and histograms of centriole counts measured from S2 cells transiently expressing the indicated constructs after 3 days of induction. (A-G) Centriole count distribution shown for cells transfected with a GFP control construct or the Plk4-GFP construct indicated. Mean and median values are shown including standard error of the mean. This data supplements the binned histograms and values reported in Figure 2.6 B.


Figure 2.S4. Titrated expression of Plk4 PB1-PB2 causes differential centriole amplification. Histograms of centriole counts measured from S2 cells transiently expressing PB1-PB2-GFP after 3 days of induction using three different concentrations of copper sulfate (250, 500 and $1000 \mu \mathrm{M}$ ) to differentially induce PB1-PB2-GFP expression under the metallothionein promoter. Centriole counts per cell are binned under <2, 2, and $>2$. Mean and median values are indicated. At least 179 cells were examined in each of the copper sulfate treatments. This data supplements the observations reported in Figure 2.6 B-G, showing that a PB1-PB2 construct can bind full length Plk4 and protect it from transautophosphorylation and subsequent Slimb binding, which would promote its ubiquitinmediated degradation.

# CHAPTER 3: PLK4 PB3 IS A DIVERGENT PB DOMAIN WITH SPECIES-DEPENDENT ROLES AND A VARIABLE STRUCTURE 

## Summary

Prior work from our lab demonstrated that Plk4 contains three PB domains as defined by the canonical PB structural fold. This finding highlights Plk4 divergence from the Plk family of kinases, which usually contain exactly two PB domains to attain subcellular localization and regulate catalytic activity. Plk4 PB1-PB2 homodimerizes, allowing for centriole localization via interactions with centriole scaffold components and dimerizationdependent trans autophosphorylation that primes subsequent downregulation, begging the question: what is the purpose of PB3? In this study, we employ x-ray crystallography to determine the structure of D.m. Plk4 PB3 and find that it surprisingly forms a different structure than that of the previously reported M.m. Plk4 PB3. Specifically, D.m. PB3 does not undergo chain-swapping to complete two PB folds, as does M.m. PB3; instead, each of the two D.m. PB3 chains in the ASU produces an independent PB domain. Consistent with this finding, D.m. PB3 exists solely as a monomer in solution, while M.m. (Leung et al., 2002) and H.s. PB3 (this study) exist primarily as a dimer in a concentration-independent manner. Expression of an mCherry-tagged H.s. PB3 in human RPE1 cells confirms the inability of PB3 to localize to centrioles. Ongoing work in our lab is discussed here, in which we examine the complementation of D.m. and H.s. PB3 in a centriole amplification assay in human cell lines. Our work has important implications for the divergent role of PB3 in a
species-dependent Plk4 context and conjures questions regarding the evolutionary history of the third Plk4 PB domain.

## Chapter 1 serves as an introduction to this chapter-

## Experimental Procedures

## Cloning and Protein Purification

Drosophila melanogaster Plk4 (DG7186) PB3, residues 657-745, was subcloned into pET28b (Novagen), engineering a thrombin-cleavable N-terminal His ${ }_{6}$ tag. Protein was expressed in BL21 DE3 E. coli methionine auxotrophic cells under kanamycin selection with SeMet media (Leahy et al., 1994) and induced with $100 \mu \mathrm{M}$ IPTG for 16 hr at $20^{\circ} \mathrm{C}$. Cells were harvested, resuspended in lysis buffer ( 25 mM Tris $\mathrm{pH} 8.0,300 \mathrm{mM} \mathrm{NaCl}, 10 \mathrm{mM}$ Imidazole, $0.1 \% \beta-\mathrm{ME})$ supplemented with 1 mM PMSF, and sonicated to lyse. Lysate was clarified at $23,000 \mathrm{xg}$ for 45 min and supernatant applied to $\mathrm{a}_{\mathrm{Ni}}{ }^{2+}$-NTA resin (Qiagen). Plk4 PB3 was eluted using a $250 \mathrm{ml}, 10-300 \mathrm{mM}$ Imidazole gradient in lysis buffer. Fractions containing Plk4 were pooled, supplemented with $1 \mathrm{mM} \mathrm{CaCl} 2_{2}$ and digested for 12 hr at $4^{\circ} \mathrm{C}$ with $1 \mu \mathrm{~g} / \mathrm{ml}$ bovine $\alpha$-thrombin. Digested Plk4 was filtered through 1 ml benzamidine sepharose (GE Healthcare) and exchanged into 100 ml of 25 mM HEPES, $\mathrm{pH} 7.0,0.1 \% \beta$ ME, loaded onto an SP-sepharose column (GE Healthcare), and eluted over a $250 \mathrm{ml}, 0-1 \mathrm{M}$ NaCl gradient in 25 mM HEPES, pH 7.0, $0.1 \% \beta-\mathrm{ME}$. Fractions containing Plk4 SeMet PB3 were pooled, exchanged into protein storage solution ( 25 mM HEPES at $\mathrm{pH} 7.0,100 \mathrm{nM}$ NaCl , and $0.1 \% \beta-\mathrm{ME}$ ), concentrated to $15 \mathrm{mg} / \mathrm{ml}$, and frozen in liquid nitrogen. Selenomethionine (SeMet)-substituted Plk4 (aa 657-745) proved soluble and yielded
diffractable crystals; however, these crystals provided a very weak anomalous signal, preventing phase information. We hypothesized that the single methionine residue in PB3 (M696) may reside in a disordered loop, consistent with both secondary structure prediction algorithms and lack of phase information from SeMet-substituted protein-derived crystals. Therefore, we systematically mutated several hydrophobic residues at the end of predicted secondary structure features to methionine (L675M, V692M, and V723M) in order to provide structured sites of SeMet incorporation. Point mutants were generated using the Quikchange method (Stratagene). Following solubility tests and crystallization trials, only V692M yielded protein crystals.

## Crystallization, Data Collection, and Structure Determination

SeMet-substituted V692M Plk4 PB3 was crystallized using a mother liquor (1 ml) containing $32 \%$ PEG $4000,200 \mathrm{mM} \mathrm{Li}_{2} \mathrm{SO}_{4}$, and 200 nM Tris at pH 8.5 . Crystals formed in a drop containing $2 \mu \mathrm{l}$ of $15 \mathrm{mg} / \mathrm{ml}$ protein stock and $2 \mu \mathrm{l}$ mother liquor. SeMet V692M Plk4 crystals were transferred to MiTeGen LV CryoOil (Mitegen) and flash-frozen in liquid nitrogen. Diffraction data were collected at the Advanced Photon Source SER-CAT beamline 22-ID (V692M Se peak SAD data, $0.97926 \AA$ ). Crystals belonged to the space group P2 ${ }_{1}$ with two molecules in the asymmetric unit. Data were processed and scaled using the HKL2000 suite (Otwinowski and Minor, 1997). The Phenix program suite (Adams et al., 2010) was used to find selenium sites, phase, build, and refine the structure with reiterative building in Coot (Emsley et al., 2010). Refinement was monitored using 10\% of the data randomly excluded from the refinement and used to calculate an R free (Brünger, 1992). The model includes two Plk4 PB3 protomers: chain A (residues 660-714, 716-743) and chain B (residues 659-693, 700-714, 722-743) and 52 water molecules. Electrostatics were calculated
using APBS (Baker et al., 2001). Structural alignments and rmsd values were calculated using the Dali server (Hasegawa and Holm, 2009).

Size Exclusion Chromatography and Multi-angle Light Scattering (SEC-MALS)
D.m. Plk4 PB3 was purified without cleaving the N-terminal $\operatorname{His}_{6}$ tag ( $\mathrm{MW}=11,942$ Da ), concentrated to either 10 or $27 \mathrm{mg} / \mathrm{mL}$, and exchanged into running buffer ( 25 mM HEPES, $\mathrm{pH} 7.5,300 \mathrm{mM}$ sodium chloride, $0.1 \% \beta$-mercaptoethanol, and $0.2 \mathrm{~g} / \mathrm{L}$ sodium azide). H.s. Plk4 PB3 (resi 884-970, based on alignments with the published M.m. PB3 (Leung et al., 2002); MW $=11,860 \mathrm{Da}$ ) was purified using the same protein expression and purification protocols, and concentrated to either 4.6 or $8 \mathrm{mg} / \mathrm{mL}$ in running buffer. A Superdex 200 10/300 GL gel filtration column (GE Healthcare) was equilibrated in the running buffer and runs containing $100 \mu \mathrm{l}$ of protein were injected onto the column. Eluate was passed in tandem through a Wyatt DAWN HELEOS II light scattering instrument and a Wyatt Optilab rEX refractometer. The light scattering and refractive index data were used to calculate the weight-averaged molar mass of each peak using the Wyatt Astra V software program (Wyatt Technology Corp.) (Wyatt, 1993). Two D.m. Plk4 PB3 and H.s. Plk4 PB3 runs were performed for each purification scheme, with two purifications for each construct, for a total of eight experiments.

Centriole Localization Assay
Human hTERT-immortalized retinal pigment epithelial cells (RPE1) with a stable CSAP-GFP expression background (Backer et al., 2012) were transiently transfected with mCherry-tagged H.s. Plk4 constructs with the intent of assaying for co-localization. However, CSAP-GFP signal was greatly reduced following fixing and staining procedures; therefore, Pericentrin (Pctn) staining was used in the Cy2 channel to enhance centrosome
marker signal. In brief, RPE cells were cultured at $37^{\circ} \mathrm{C}$ with $5 \% \mathrm{CO}_{2}$ in DMEM media (Gibco) supplemented with 1\% Antibiotic-Antimycotic (Gibco) and 10\% FBS (Gibco). H.s. Plk4 PB3 (resi 884-970) was subcloned into pmCherry-N1 using HindIII and BamHI restriction sites (yielding a C-terminal mCherry tag under the constitutively active CMV promoter; Clontech). Stable RPE1 cells were transfected (Lipofectamine LTX with Plus reagent, Invitrogen; following the manufacturer's protocol) with $2 \mu \mathrm{~g}$ DNA directly on glass coverslips and allowed to express PB3:mCherry for 12 hours at $37^{\circ} \mathrm{C}$ with $5 \% \mathrm{CO}_{2}$. Cells were briefly rinsed with PBS and fixed with $4 \% p$-formaldehyde in PBST (PBS with $1 \%$ TWEEN) for 15 min . Cells were then blocked for 30 min at ambient temperature in 1X PBST supplemented with $10 \mathrm{mg} / \mathrm{mL}$ BSA (herein "blocking solution"). Primary rabbit antiPericentrin antibody (Cat. \# ab4448, Abcam) was diluted 1:1000 and applied to cells for 1 hour in blocking solution. Following PBST rinses, cells were treated with a Cy2 labeled antirabbit secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:500 and DAPI diluted to 1:2000 (Molecular Probes, Invitrogen) for 30 min and rinsed with PBST followed by PBS. Samples were mounted in a glycerol-based medium ( $90 \%$ glycerol, $10 \%$ PBS, 3\% w/v n-propyl gallate) and imaged using a 100x objective (NA 1.49) Apochromatic TIRF objective (Nikon) mounted on an inverted microscope (Nikon Eclipse Ti) equipped with a cooled charge-coupled device camera (CoolSNAP HQ, Roeper Scientific), an excitation and emission wheel (LUDL), and emission filters (Chroma). All microscope hardware was controlled by Nikon NIS-Elements. All images were processed and prepared for publication using Photoshop (CS5 version 12.0). Cells were processed and binned into one of three categories: strongly co-localized (interphase cells), weakly co-localized (interphase), not colocalized (interphase), or spindle-enhanced (mitotic cells). Co-localized cells displayed
strong mCherry signal at every centriole labeled using anti-Pctn antibody; weakly colocalized cells displayed weak mCherry signal at marked centrioles; and not co-localized cells displayed no mCherry punctae that overlapped with centrioles.

## Results

## Crystallization and Structure Determination of Plk4 PB3

Plk4's C-terminal PB region, called PB3 (Slevin et al., 2012), was delineated in D.m. and H.s. based on both secondary structure prediction algorithms and protein conservation (Figure 3.1 D). D.m. Plk4 PB3 (resi 657-745) and H.s. Plk4 PB3 (resi S884-H970) were expressed and purified as described in Experimental Procedures. Only D.m. PB3 yielded protein crystals; thus, we pursued SeMet-substituted PB3 crystals to gain experimental phasing. D.m. Plk4 PB3 SeMet crystals diffracted, but produced a weak anomalous signal that failed to provide phasing information. To increase anomalous signal, we designed a point mutant (V692M) predicted to increase SeMet signal in an ordered portion of the protein (see Experimental Procedures). D.m. Plk4 PB3 V692M crystals diffracted to $1.90 \AA$ and belonged to the space group $\mathrm{P} 2_{1}$ (see Table 3.1 for crystallographic data, phasing, and refinement statistics and geometries). The current model of D.m. PB3 V692M contains two molecules in the ASU (Chain A: resis 660-714, 716-743; Chain B: resis 659-693, 700-714, 722-743; Figure 3.1 A,B) and is built and refined to $R$ and $\mathrm{R}_{\text {free }}$ values of 22.8 and 28.9, respectively. Refinement of the model is ongoing to reduce both parameters and converge their values.

The Structure of D.m. PB3 Contains Two Chains in the ASU but Lacks a Dimerization Interface

The D.m. PB3 structure forms a canonical Polo Box (PB) domain, with six consecutive $\beta$-strands folding to form a 6 -stranded anti-parallel $\beta$-sheet ( $\beta 1-\beta 6$; shown in indigo in Figure 3.1 A,D). The C-terminal single $\alpha$-helix ( $\alpha 1$ ) (shown in lavender, Figure 3.1 A,D) packs nearly perpendicularly to the $\beta$-sheet, stabilizing its formation via a shared buried hydrophobic interface. The structure of the single PB3 aligns well to published single PB domains (Slevin et al., 2012; Leung et al., 2012; Shimanovskaya et al., 2014; Park et al., 2014), despite sharing highest sequence homology only with PB3 domains from other species (Figure 1.7; Figure 3.1D). While our structure of the D.m. PB3 contains two PB domains in the ASU (much like the H.s. PB3 structure (Leung et al., 2002)), the two chains do not undergo swapping, with each chain forming a complete PB domain (Figure 3.1 B). Close examination of the contacts made by each chain demonstrates a weak interface between consecutive PBs. The interface between chains varies with the symmetry mate being compared, indicative that there is no conserved interface to suggest biological dimerization in our crystal. Indeed, inspection of the ASU shown in Figure 3.1 demonstrates that there are only two points of potential contact within the ASU between chains: two van der Waalsbased interactions between L735 $\square$ and I693 and S691 (Figure 3.1 C). These interactions are weak, as they nearly exceed the limit of distance between atoms to create a van der Waals interaction; thus, they likely represent a crystallographic interface rather than a biological dimerization interface within our presented ASU.


Figure 3.1. The structure of D.m. PB3 reveals a single PB domain with two protomers in the asymmetric unit. (A) The structure of D.m. PB3 depicts a canonical PB domain, with a 6 -stranded anti-parallel $\beta$-sheet ( $\beta 1-\beta 6$, indigo) packed perpendicularly against a single $\alpha$ helix ( $\alpha 1$, lavender). The domain packs in order of primary structure, with $\beta 1$ at the N terminus and the $\alpha 1$ at the C-terminus. (B) The crystallographic asymmetric unit (ASU) comprises two complete PB3 chains, Protomers A (resis 660-714, 716-743) and B (resis 659-$693,700-714,722-743$ ). The two protomers are shown in two orientations for clarity. Protomer A is shown in the remaining figures. (C) Left, the structure of the ASU encompassing two PB3 protomers for orientation. Right, final $2 \mathrm{~F}_{\mathrm{o}}-\mathrm{F}_{\mathrm{c}}$ density shown in green, demonstrating a high correlation between observed density and the final model. Highlighted in orange are the only three residues between Protomer A (S691 and I693) and Protomer B (L735■) that form contacts to suggest a possible dimerization interface; however, note that the interactions are separated by $3.5 \AA$, indicating a weak interaction, if any. (D) Plk4
sequence alignment across ten species. Protomer A solvent accessible surface area (ASA) $\left(\AA^{2}\right)$ is indicated. $100 \%$ identity is highlighted in green, $80 \%$ identity in yellow (homologous residues also highlighted in yellow where the $80 \%$ identity criteria is met). $D$. mel., Drosophila melanogaster, fruit fly; N. vec., Nematostella vectensis, sea anemone; N. vit., Nasonia vitripennis, wasp; D. rer., Danio rerio, zebrafish; T. nig., Tetraodon nigroviridis, puffer fish; M. mus., Mus musculus, mouse; R. nor., Rattus norvegicus, rat; B. tau., Bos taurus, cow; P. abe., Pongo abelii, orangutan; H. sap., Homo sapiens, human.

Comparison of D.m. and M.m. Structures Reveals both Conserved PB3 Features and Unique

## PB3 Oligomer Configurations

To date, the only other published PB3 structure belongs to mouse Plk4 (M.m. Plk4 PB3, Leung et al., 2002). Our D.m. PB3 aligns well to a single M.m. PB3 domain, with an RMSD value of $2.25 \AA$ (Figure 3.2 A). Interestingly, though the structural elements align well, the identity of each element differs between the two structures: D.m. $\beta 1$ aligns with M.m. $\beta 6$, D.m. $\beta 2$ with M.m. $\beta 1$, etc. such that the M.m. PB3 $\beta$-sheet is offset as compared with D.m. (Figure 3.2 A, bottom, and C, which shows schematics of secondary structural features mapped to primary sequences). Additionally, the single $\alpha 1$ helix aligns well between D.m. and M.m. structures; however, the helix is markedly truncated in M.m. as compared to D.m. (10 resi and 19 resi, respectively, leading to a $16.3-\AA$ difference in helix lengths; Figure 3.2 B,C). As a result, there are considerably more contacts between the D.m. $\alpha 1$ helix and $\beta$ sheet, wherein D.m. $\beta 1$ forms several contacts with the C-terminus of $\alpha 1$ (Figure 3.2 B). M.m. PB3 does not form these contacts due to a shortened $\alpha 1$ helix, and as a result, its equivalent $\beta$-strand (M.m. $\beta 6$ ) splays away from the $\alpha 1$ interface (Figure 3.2 B).
M.m. PB3 forms a unique PB homodimer among known PB homodimers (Leung et al., 2002). In contrast to Plk4 PB1-PB2, which forms a homodimer via PB2-PB2 contacts to
form an extended, central, 12 -stranded $\beta$-sheet to bind acidic partners Asl and SPD-2 (Slevin et al., 2012; Shimanovskaya et al., 2014; Park et al., 2014), the M.m. PB3 homodimer forms via chain-swapping from the two protomers in the ASU (Leung et al., 2002). As a result, the composite M.m. PB3 homodimer contains two PB domains, each of which has contributions from each chain (Figure 3.2 C and D, top right inset. One protomer is shown in dark teal, while the other is in light cyan, with the composite PB domains each delineated). This has proven to remain a unique PB dimerization formation, as no other known PB homodimer forms via chain-swapping. Alignment of our D.m. PB3 ASU with the published M.m. PB3 homodimer reveals poor agreement between the two structures, with the second PB domains (PB $\square$ ) in different positions relative to the aligned PBs (Figure 3.2 D). No crystallographic symmetry mate recapitulates the interactions seen in PB1-PB2, highlighting that PB3 likely takes a different oligomerization state in a species-dependent manner. D.m. PB3 represents an architectural departure from both M.m. PB3 and D.m. PB1-PB2, as PB3 does not display an apparent conserved basic patch as seen in PB1-PB2 to bind centriole targets (Figure 3.2 E). Therefore, D.m. PB3 is a unique PB3 structure that does not form dimerization interfaces in crystal form.


D


E


■ $100 \%$ Identity - $80 \%$ Conservatlon


Figure 3.2. D.m. and M.m. Plk4 PB3 form similar folds but maintain different spatial crystallographic arrangements. (A) D.m. PB3 (indigo) and M.m. PB3 (teal) (PDB accession code 1MBY; Leung et al., 2002) overlay with an RMSD of $2.25 \AA$; however, their individual architectures differ. Note that the M.m. $\beta$-sheet comprises $6 \beta$-strands out of chronological order. (B) An alternative view of the D.m./M.m. PB3 alignment reveals that the M.m. $\alpha 1$ helix is significantly shorter ( $16.3 \AA$ ) than the $D$.m. equivalent. The $D$.m. $\beta$-sheet forms van der Waals contacts between $\beta 1$ (K662, I664, and V666) and the extended Cterminus of $\alpha 1$ (K736, L737, A738, and A740), effectively pulling D.m. $\beta 1$ closer to $\alpha 1$ as compared to the equivalent position of M.m. $\beta 6$. (C) A primary sequence alignment among D.m., H.s., and M.m. reveals conservation among mammalian PB3 sequences, with D.m. PB3 sharing $30 \%$ conservation with H.s. and M.m. PB3. Secondary structural alignments between D.m. PB3 (indigo, top) and M.m. PB3 (teal, bottom) reveal offset secondary structure elements between D.m. and M.m. PB3s despite high residue conservation. Yellow residues notate identity or similarity between two species; green residues notate identity among all three species. Note that the composite M.m. PB3 is formed by the two PB3 chains in the published structure (PDB 1MBY), which undergo swapping to form two inter-dependent PB domains. (D) Structural alignment of the D.m. PB3 asymmetric unit and the published M.m. PB3 dimer reveals a lack of the M.m. dimerization interface in the D.m. PB3. (E) Mapping both conservation (spherical model, top) and electrostatics (surface representation, bottom) indicates the absence of a conserved basic patch as observed in D.m. PB1-PB2 (Figure 6.1). Conservation is contoured to $80 \%$ similarity (yellow) or $100 \%$ identity (green).
D.m. PB3 is a Monomer in Solution, while Mammalian PB3s Exist as Dimers

To determine the oligomerization state of D.m. PB3 as compared with mammalian PB3, we purified both D.m. PB3 (resi 657-745) and H.s. PB3 (resi S884-H970) as described in Experimental Procedures and used SEC-MALS to obtain accurate MW measurements. Both proteins remained soluble throughout purification and concentration, and exceeded 50 $\mathrm{mg} / \mathrm{mL}$ without precipitating out of solution. To avoid detection of oligomerization states forced by exceedingly high concentrations, we performed all SEC-MALS experiments with ranges of concentrations of purified proteins, from 2-27 mg/mL. Both D.m. and H.s. PB3 were purified in two independent rounds, and yielded the same results. Representative traces are shown in Figure 3.3, with the MW reported as the average and standard deviation of all experiments.


Figure 3.3. PB3 takes on a species-dependent oligomerization state in solution. All data shown here are representative profiles of all completed SEC-MALS experiments. The MW for each construct is displayed as the average value $\pm$ standard deviation. (A) An overview of the entire timecourse for each run shown showcases that each protein runs at a characteristic size, with an additional peak at $\sim 15 \mathrm{~min}$ corresponding to elution of a non-specific aggregate. The expected monomeric size of either construct is 11.9 kDa . (B) Gel filtration of either H.s. PB3 (orange) or D.m. PB3 (two independent purification schemes shown in green and blue) yields a single major peak, indicating stability at specific oligomeric states. H.s. PB3 elutes as a dimer $(22.9 \pm 0.2 \mathrm{kDa})$, while D.m. PB3 elutes as a monomer ( $11.6 \pm 0.2 \mathrm{kDa})$.

Consistent with our findings that D.m. PB3 lacks a convincing dimerization interface in the crystal, D.m. PB3 eluted as a single peak (Figure 3.3, mint green and light blue traces) with a measured MW of $11.6 \pm 0.2 \mathrm{kDa}$ (dark green and blue traces; expected monomeric MW=11.9 kDa). The MW of D.m. PB3 was consistent, even when concentrations of purified protein exceeded $27 \mathrm{mg} / \mathrm{mL}$, confirming that D.m. PB3 forms a monomeric species. Previous work from other labs indicated that M.m. PB3 primarily forms a dimer (Leung et al., 2002), suggesting that PB3 oligomerization state might be species-dependent. To confirm this, we tested H.s. PB3, which shares nearly complete identity with M.m. PB3 (>98\% identity over the PB3 regions, as shown in Figure 3.2 C). Similar to D.m. PB3, H.s. PB3 eluted as a single peak (Figure 3.3, peach trace); however, the peak corresponded to a MW value of $22.9 \pm 0.2$
kDa (orange trace; expected monomeric $\mathrm{MW}=11.9 \mathrm{kDa}$ ), corresponding to formation of a dimer. H.s. PB3 consistently eluted as a dimer, regardless of concentration. The H.s. PB3 dimer formation at dilute concentrations $(2 \mathrm{mg} / \mathrm{mL})$ indicates that the dimerization interface between the two protomers is strong, and that PB3 dimerization is a conserved feature of mammalian PB3s.

## H.s. PB3 is Not Sufficient for Centriole Subcellular Localization

Previous work from our lab determined that in D.m. S 2 cultured cells, D.m. PB3 robustly localizes to centrioles in only 23\% of cells (Slevin et al., 2012; see L-PB3, Figure 2.5 B,I). Conversely, PB1-PB2 localizes to centriole markers in ~96\% of cells (Figure 2.5 $\mathrm{B}, \mathrm{D})$, indicating that Plk4 uses PB1-PB2 to bind targets and attain proper localization. To determine whether insufficiency of PB3 for centriole localization is conserved among species, we expressed m-Cherry-tagged H.s. PB3 (resi 884-970) under the constitutive CMV promotor in human RPE1 cells expressing a stable centriole marker, CSAP:GFP (Backer et al., 2012). We then assayed for PB3:mCherry co-localization with Pctn, a centrosomal marker, following fixing and staining protocols (see Experimental Procedures). Consistent with our previous results in D.m. cultured cells, we found that H.s. PB3 lacks robust centriole localization in human RPE cells and is largely cytoplasmic (Figure 3.4 A). A small percentage ( $\sim 25 \%$ ) of cells examined showed a slight mCherry enhanced signal in centriole regions (called "weak centriole localization," Figure 3.4 B), though no cells displayed strong centriole localization. A small number of transfected cells ( $\sim 3 /$ slide) were mitotic, and we noticed that in transfected mitotics, PB3:mCherry signal was enhanced along the spindle

MTs (Figure 3.4 C). There is no precedence for a PB3-MT interaction, whether direct or indirect; whether this observation is indicative of a MT or kinetochore fibre interaction with PB 3 is yet to be determined.


Figure 3.4. H.s. PB3 is not a robust centriole localization domain in cultured RPE1 cells. Scale bar, $10 \mu \mathrm{~m}$. (A, B) Constitutive expression of H.s. PB3 with a C-terminal mCherry tag in a stable RPE1 cell line in interphase cells reveals a lack of centriole localization, as assayed via staining for Pctn (green). $85 \%$ of cells assayed displayed no co-localization with Pctn (cytoplasmic) (A), while $15 \%$ of cells displayed weak enhancement at centrioles (B). (C) Mitotic cells displayed a weak enhancement of PB3:mCherry on the spindle.

## Discussion

Our work with Drosophila PB3 combines x-ray crystallography with biochemistry and cell biology to determine the structure and function of PB3 in the context of FL Plk4. The crystal structure of D.m. PB3 surprisingly reveals a monomeric PB domain with several important differences as compared to the published M.m. PB3 structure (PDB accession code 1MBY; Leung et al., 2002). First, D.m. PB3 contains two chains in the ASU, like M.m. PB3; however, the D.m. structure's two chains each form an independent PB domain, whereas the M.m. structure features two chains that collectively form two inter-dependent PB domains
that undergo chain-swapping (Figure 3.2 D). Second, D.m. PB3 secondary structural elements fold in chronological order, more closely mimicking the known Plk4 PB1 as well as Plk1 PB structures, whereas M.m. PB3 folds out of order with regards to primary sequence (Figure 3.2 A). Third, the single $\alpha 1$ helix in D.m. is approximately twice as long as the M.m. $\alpha 1$, allowing for more stabilizing contacts between the D.m. $\alpha 1$ and the $\beta$-sheet (Figure 3.2 B). These differences are perplexing, as they result in poor alignment between secondary and tertiary structure of the two species (Figure 3.2 C,D), and yet the primary amino acid sequences align very well between M.m. and D.m. (Figure 3.2 C). How similar sequences could yield such different structures, even out of the context of the rest of the protein and in the absence of any binding partners, remains an important question.

Careful examination of the M.m. PB3 crystallization methodology reveals that crystals were obtained with $50 \mathrm{mg} / \mathrm{mL}$ PB3 (Leung et al., 2002). While crystallization requires concentrating protein far beyond biological concentrations, $50 \mathrm{mg} / \mathrm{mL}$ is even higher than typical crystallization conditions, raising questions of whether the published structure involving a unique chain-swapping mechanism represents a true biological protein configuration. Future work with the PB3 domains should include small-angle x-ray scattering to determine a low-resolution envelope of D.m., H.s., and M.m. PB3 domains in order to confirm the differences seen in crystallographic form.

Interestingly, our SEC-MALS data seem to confirm what is known thus far about each crystal structure: that D.m. PB3 forms a monomer in solution (even at higher concentrations), while M.m. PB3 forms a dimer (even at lower concentrations; Figure 3.3 B). Whether the individual PBs take on different oligomerization states depending on the context of the FL protein, stage of the catalytic and regulation cycle, or presence of PB3 binding
partners (as well as determining if there are any), remains a priority to determine in future experiments.

Finally, our lab is currently interested in learning more about the role of PB3 function within Plk, as well as determining whether PB3 function is species-dependent. Others' previous work in cultured cells demonstrated that Plk4 homologs across species cannot complement each other following depletion of endogenous proteins (Carvalho-Santos et al., 2010). However, extensive work has shown that PB1-PB2 maintains similar function and structures across species (Slevin et al., 2012; Shimanovskaya et al., 2014; Park et al., 2014), suggesting that the variation and lack of complementation among species arises specifically from PB3. We are currently undertaking experiments in human HeLa cells to determine whether PB3 has a species-dependent role in Plk4 centriole licensing activity, in which we overexpress mCherry-tagged Plk4 constructs (FL, $\Delta \mathrm{PB} 3$, and a chimeric construct in which D.m. PB3 replaces H.s. PB3) and determine the effects on centriole numbers. Our assay design allows for detecting whether PB3 has a directed role in Plk4-dependent centriole duplication. PB domains in other Plks serve to localize the FL molecule, bind targets, and regulate kinase activity; while PB1-PB2 bind the only known Plk4 PB targets and acts as the centriole localization domain, it remains unknown if any or which PB domain(s) regulates catalytic activity. While more work will need to be done to demonstrate a PB3-kinase interaction, our current experiments will aid in laying the foundation to better understand the role of PB3 in Plk4 centriole duplication licensing.

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## TABLES

| Crystal | SeMet V692M PB3 |
| :---: | :---: |
| Beamline | APS 22-ID |
| Space Group | P21 |
| Cell: a,b,c ( $\AA$ ) ; $\alpha, \beta, \gamma\left({ }^{\circ}\right)$ | 35.3, 52.1, 41.1 ; 90.0, 106.2, 90.0 |
| Wavelength (A) | 0.97926 |
| Resolution (A) | 50.0-1.90 |
| Completeness (\%) | 95.5 (73.2) |
| I/ $\sigma$ | 26.0 (5.4) |
| Redundancy | 6.6 (4.2) |
| No. of observations | 71,961 |
| Rsym (\%)* | 7.6 (30.6) |
| Overall log-likelihood gain** / figure of merit ${ }^{\dagger}$ | 93,634 / 0.361 |
| Refinement (A) | 50.0-1.90 (1.98-1.90) |
| R value | 22.8 (23.2) |
| $\mathrm{R}_{\text {free }}{ }^{\ddagger}$ | 28.9 (31.1) |
| Rmsd bond lengths ( A ) | 0.008 |
| Rmsd bond angles ( ${ }^{\circ}$ ) | 1.3 |
| Mean B-value (min / max) ( $\AA^{2}$ ) | 21.6 (8.3 / 74.3) |
| No. atoms: protein / water | $1151 / 52$ |
| B-factor rmsds (MC / SC) | 2.2 / 3.0 |

Table 3.1. Plk4 PB3 crystallographic data, phasing, and refinement. Values in parentheses are for the highest resolution shells unless otherwise denoted.
$* R_{s y m}=\Sigma_{\mathrm{h}} \Sigma_{\mathrm{i}}\left|\mathrm{I}_{\mathrm{i}}(\mathrm{h})-\langle\mathrm{I}(\mathrm{h})\rangle\right| / \Sigma_{\mathrm{h}} \Sigma_{\mathrm{i}} \mathrm{I}_{\mathrm{i}}(\mathrm{h})$, where $\mathrm{I}_{\mathrm{i}}(\mathrm{h})$ is the $\mathrm{i}^{\text {th }}$ measurement and $\langle\mathrm{I}(\mathrm{h})\rangle$ is the mean of all measurements of $I(h)$ for Miller indices $h$.
**Log-likelihood gain value as determined by Phenix.
${ }^{\dagger}$ Figure of merit is the weighted mean of the cosine of the deviation from $\alpha_{\text {best }}$.
${ }^{\square} \mathrm{R}$ value $=\Sigma(|F o b s|-k \mid$ Fcalc $\mid) / \Sigma \mid$ Fobs $\mid$.
${ }^{\ddagger} \mathrm{R}_{\text {free }}$ is calculated using a $10 \%$ subset of the data that is removed randomly from the original data and excluded from refinement (Brünger 1992).

# CHAPTER 4: A MOTOR-INDEPENDENT FUNCTION OF DYNEIN LIGHT CHAIN IN MITOTIC SPINDLE ORIENTATION <br> An Introduction to Chapter 5 

## Asymmetric divisions play a critical role in tissue specification in the developing embryo

All multicellular eukaryotes undertake the gargantuan task of developing from a single-celled zygote to an elaborate organism with compartmentalized tissue structures and functions. To do so, cells must continually divide; however, simple division is not sufficient to explain the complexity of such organisms. In order to form different organs, cells must be programmed to take on specific traits, including shape, orientation and localization within the context of the whole organism, to perform tissue-specific functions. For instance, the neuronal cells that comprise the vertebrate central nervous system are elongated (with lengths in humans measuring up to 1 m ) and have an inherent polarity in which one end contains the soma, or cell body, while the other, the dendrite (Lodish et al., 2000). The structure of the neuron is designed for its function, which is to communicate information via chemoelectrical signals quickly and unidirectionally over long distances in the body. Throughout development, genetic and biochemical pathways must converge to produce progenitors of differentiating tissues, an undertaking that requires asymmetric cell divisions (ACDs). Each ACD event is critical to create two daughter cells with different fates.

Despite the apparent importance of ACD in generating new cell fates during development, it has historically been difficult to study ACD events due to the symmetry inherent in most cultured cell types. However, one model of ACD that has recently become
routinely studied is the Drosophila melanogaster neuroblast, a stem cell that divides asymmetrically to produce both a stem population and a neuron progenitor within the D.m. larval brain (Jiang and Reichert, 2014). In this ACD event, the neuroblast is initially formed by delaminating from the neuroectoderm epithelial layer within the central region of the developing brain (Figure 4.1). In subsequent steps, the neuroblast maintains apicobasal polarity by maintaining different protein complex populations in the apical and basal ends of the cell, which collectively act as internal cues to determine the differential fates of the two daughter cells resulting from ACD. One daughter inherits the apical complex and retains its "stemness," while the other inherits the basal complex and is terminally fated to give rise to exactly two neuron cells (Vorhagen and Niessen, 2014) (Figure 4.1). The inherent asymmetry in this division event is clearly visible in the daughter cell sizes alone; the neuroblast (NB) daughter remains a large cell, while the fated cell (a Ganglion Mother Cell, or GMC) is much smaller and remains basally located. The ability to clearly observe daughter cell fates, as well as newer technologies that allow for genetic manipulation to study specific proteins in fruit flies, has made the Drosophila developing brain a favorite model to learn more about the mechanisms underlying ACDs (Lerit et al., 2014).


Figure 4.1. The Drosophila larval neuroblast marshals cell fate determinant complexes and the mitotic spindle structure to achieve asymmetric cell division along an apicobasal polarity axis. The D.m. third instar larval brain (bottom, dark blue) contains the neuroectoderm, a tissue fated to become the nervous system. Specific cells delaminate from the layer (top, beige), and take on a rounded shape and are neuroblasts. The mitotic spindle within the neuroblast aligns with apical (purple) and basal (neon blue) protein networks, with a different centrosome, mother or daughter, aligned with the apical and basal sides, respectively. The neuroblast divides asymmetrically to produce two daughters: a larger, apical neuroblast (NB) cell that remains stem cell-like, and a smaller, basal ganglion mother cell (GMC) fated to become exactly two neuron cells (Bowman et al., 2006; Li et al., 2014; Reina and Gonzalez, 2014; Vorhagen and Neissen, 2014; Wang et al., 2011; Slevin et al., 2014). Inset, top right: several molecular complexes have been determined to orient the spindle and direct cell fates: the Par complex, G $\alpha_{i} / P i n s / M u d / D y n e i n, ~ a n d ~ M u d / L C 8 / A n a 2 . ~$

## ACDs require cooperation between the cortex, molecular motors, dynamic cytoskeletal polymers, and the centrosome

Much of the research regarding D.m. NB asymmetry has focused on the mutual regulation of the apical and basal complexes to maintain polarity. Briefly, the apical cortex contains two complexes with both distinct and overlapping functions. One is collectively referred to as the "Par complex", which anchors to the apical cortex through both direct and indirect mechanisms (Figure 4.1). The Par complex negatively regulates proteins associated with neural fate via phosphorylation events, relegating their cortical location to the basal side (reviewed in Vorhagen and Niessen, 2014). Par proteins can also indirectly localize to the apical cortex via Pins and $\mathrm{G} \alpha_{\mathrm{i}}$, scaffolding and signaling components, respectively, that act to orient the spindle within the cell. Importantly, Pins and $\mathrm{Ga}_{\mathrm{i}}$ assert their control over spindle orientation by binding Mud (Mushroom body-defect, D.m.; NuMA, H.s.), which in turn can interact with the molecular motor dynein via dynactin (Figure 4.1) to anchor it to the cortex (Merdes et al., 1996). As a minus-end-directed motor, dynein plays a critical role in exerting pulling forces on the astral microtubules to anchor one spindle pole at the apical end, resulting in a division event in line with polarity cues and segregated fate determinants (Li et al., 2014).

Interestingly, Mud/NuMA plays an essential, complicated role in orienting NB ACDs. In Drosophila null Mud alleles, NB mitotic spindles fail to align properly with polarity cues; additionally, Mud is a conserved component that can bind to dynactin, Pins, and even directly to MTs (Bowman et al., 2006; Merdes et al., 1996). Whether the interaction between Mud and MTs is high-affinity to afford a direct role in Mud/astral MT-based spindle alignment remains to be determined. In addition, it is not known if and how Mud can interact with all of its binding partners. Nevertheless, the requirement of Mud to ensure faithful
asymmetric cell segregation indicates that Mud is a critical hub in directing the axis of division (Figure 4.1).

## An interaction among Ana2, LC8, and Mud regulates divisions within the developing Drosophila brain

While centrioles are essential in building the bipolar mitotic spindle in mammalian cells, recent evidence has suggested that centriolar components play a more direct role in orienting the mitotic spindle during ACDs. Using fluorescent microscopy, in vitro binding assays and yeast two-hybrid tests, Mud was shown to form a complex with a dynein light chain, LC8, and the essential centriole component Ana2 at the daughter centrosome (Wang et al., 2011). This tripartite complex was further shown to be required for faithful spindle alignment in D.m. NBs, as null mutants in either LC8 or Ana2 randomized spindle orientation (Wang et al., 2011) (Figure 4.1). Ana2 remains the only centriolar component known to be directly involved in spindle orientation, indicating a possible role of the centrosome in orienting spindle alignment during ACD. This study did not resolve whether this interaction directs orientation through the known Mud/Pins/G $\alpha_{\mathrm{i}}, \mathrm{Mud} / \mathrm{Dynactin} / \mathrm{Dynein}$, or Mud/MT pathways (inset in Figure 4.1; dotted lines showing the potential modes of action).

Critical to the function of the Mud/Ana2/LC8 complex is its asymmetrical formation, as LC8 and Ana2 are both enhanced in the apical (daughter) centrosome (Wang et al., 2011) according to the apical localization of Mud. The two centrosomes at the mitotic spindle poles are known to be asymmetric, as the mother centrosome recruits high levels of pericentrin-like protein (PLP) to block D.m. Plk1-mediated centrosome maturation; thus, the daughter centrosome is able to mature and recruit higher levels of PCM and centriole
components while the mother remains inactivated (Lerit and Rusan, 2013). The inherent asymmetry of the centrosomes mirrors the asymmetry of the cell, and ensures that older centrioles are inherited by the resulting GMC daughter cells while younger centrioles segregate to the NB (Figure 4.1). The mechanism through which the Mud/Ana2/LC8 complex exerts spindle orientation regulation at the daughter centrosome remains unknown.

## LC8 is a motor-independent dimerization module

LC8 is a dynein light chain conserved among almost all known eukaryotic branches. As its name suggests, LC8 is a regulatory dynein chain that complexes with intermediate chains to enhance their dimerization, effectively acting as a processivity factor (Rao et al., 2013). However, LC8 also acts as a dimerization "hub" in various contexts throughout the cell, binding many targets in a motor-independent fashion (reviewed in Barbar, 2008). Several examples of LC8-mediated dimerization include Nup159 (a nucleoporin component, Romes et al., 2012), Swallow (an mRNA localization factor in the developing D.m. embryo, Kidane et al., 2013), and Pak1 (a kinase that regulates cell motility, Lightcap et al., 2008), highlighting the many subcellular uses of LC8. There are two common motifs that bind LC8: $\mathrm{G}_{-2} \mathrm{I}_{-1} \mathrm{Q}_{0} \mathrm{~V}_{1} \mathrm{D}_{2}$ and $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$, where the glutamine $(\mathrm{Q})$ is held in a constant position relative to the LC8 binding groove and X corresponds to any residue (Rapali et al., 2011). LC8 itself exists as a homodimer in solution (Romes et al., 2012; Slevin et al., 2014), and therefore contains two identical target-binding sites (Figure $4.2 \mathrm{~A}, \mathrm{~B}$ ). Binding targets, though diverse in cellular localization and function, generally conform to the LC8 binding motifs and also contain an adjacent low-affinity dimerization domain (Figure 4.2 C; Barbar, 2008). LC8 binds two identical targets, effectively bringing them closer together and lowering
energy barriers for their own dimerization domains (Figure 4.2 C), often ultimately forming a coiled-coil. These collective binding events increase dimerization of LC8 targets in a motorindependent manner, allowing for increased function of the targets. While work in 2011 initially identified the LC8/Ana2/Mud complex (Wang et al., 2011), little was known about the LC8/Ana2 interaction at the molecular level.


Figure 4.2. LC8 acts to homodimerize its targets in parallel. (A) A cartoon schematic and the crystal structure of the D.m. LC8 homodimer (shown in green and purple for clarity). LC8 forms a homodimer via formation of a central $\beta$-sandwich, flanked on either side by a pair of $\alpha$-helices that support folding. (B) A schematic and the crystal structure of one side of the $\beta$-sandwich, as seen in the middle of the sandwich. Each $\beta$-sheet comprises 4 strands from one LC8 protomer (green), one strand from the other protomer (purple), and the final strand is contributed by a binding target, which forms the last anti-parallel strand (orange). (C) Cartoon of LC8-induced dimerization. Two identical targets (left), containing an LC8binding motif and a low-affinity dimerization domain, bind an LC8 homodimer, creating a stabilized target homodimer in parallel.

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# CHAPTER 5: THE MECHANISM OF DYNEIN LIGHT CHAIN LC8-MEDIATED OLIGOMERIZATION OF THE ANA2 CENTRIOLE DUPLICATION FACTOR ${ }^{1}$ 

## Summary

Centrioles play a key role in nucleating polarized microtubule networks. In actively dividing cells, centrioles establish the bipolar mitotic spindle and are essential for genomic stability. Drosophila Anastral spindle-2 (Ana2) is a conserved centriole duplication factor. While recent work demonstrated that an Ana2-dynein light chain (LC8) centriolar complex is critical for proper spindle positioning in neuroblasts, how Ana2 and LC8 interact is yet to be established. Here we examine the Ana2-LC8 interaction and map two LC8-binding sites within Ana2's central region, Ana2M (residues 156-251). Ana2 LC8-binding site 1 contains a signature TQT motif and robustly binds LC8 ( $\mathrm{K}_{\mathrm{D}}$ of $1.1 \mu \mathrm{M}$ ) while site 2 contains a TQC motif and binds LC8 with lower affinity ( $\mathrm{K}_{\mathrm{D}}$ of $13 \mu \mathrm{M}$ ). Both LC8-binding sites flank a predicted $\sim 34$-residue $\alpha$-helix. We present two independent atomic structures of LC8 dimers in complex with Ana2 LC8-binding site 1 and site 2 peptides. The Ana2 peptides form $\beta$ strands that extend a central composite LC8 $\beta$-sandwich. LC8 recognizes the signature TQT motif in Ana2's first LC8 binding site, forming extensive van der Waals contacts and hydrogen bonding with the peptide, while the Ana2 site 2 TQC motif forms a uniquely extended $\beta$-strand, not observed in other dynein light chain-target complexes. Size-exclusion

[^1]chromatography coupled with multi-angle static light scattering demonstrates that LC8 dimers bind Ana2M sites and induce Ana2 tetramerization, yielding an Ana2M4-LC88 complex. LC8-mediated Ana2 oligomerization likely enhances Ana2's avidity for centriole binding factors and may bridge multiple factors as required during spindle positioning and centriole biogenesis.

## Introduction

Centrioles are cylindrical cellular structures that form the core of centrosomes and basal bodies, organelles responsible for nucleating polarized microtubule networks in the cytoplasm and cilia, respectively. A cell's centriole count largely determines its capabilities, as single centrioles form the base of sensory cilia while multiple centrioles are needed to nucleate motile cilia (Bornens 2012). A centriole pair constitutes the core of the centrosome, needed for bipolar mitotic spindle formation. Centriole structure is largely conserved across metazoans, protists, and some plants, with a characteristic 9 -fold radial symmetry established by an inner, 9-spoked cartwheel structure (Gönczy 2012). While different species have different microtubule arrangements in the surrounding blades (singlets, doublets, or triplets, as shown in Figure 5.1 A) as well as different cartwheel architectures (Guichard et al., 2013), the critical centriole duplication components are conserved. The inner cartwheel recruits centriolar proteins and pericentriolar matrix components to build and elongate the outer centriole wall. During elongation, nine sets of microtubule blades (each a microtubule triplet in the case of Drosophila) form around the centriole perimeter parallel to the longitudinal axis, propagating the organelle's 9-fold radial symmetry.

Canonical centriole duplication is coupled to the cell cycle to limit centriole number (Tsou and Stearns, 2006). A subset of conserved centriole proteins are involved in centriole duplication (Pelletier et al., 2006), as their misregulation leads to increased or decreased centriole counts (Figure 5.1 B). Three key initiation factors include Polo-Like Kinase 4 (Plk4), Spindle assembly abnormal protein 6 (Sas-6), and Anastral spindle 2 (Ana2/STIL/Sas-5, found in D. melanogaster, humans, and C. elegans, respectively). Plk4 is a serine/threonine kinase whose catalytic activity is required for centriole duplication. Plk4 phosphorylates a set of both known and unknown components to transmit the centriole duplication signal (Habedanck et al., 2005; Lettman et al., 2013). Plk4 is recruited to the centriole through an interaction with Asterless (Asterless/Cep-152 found in D. melanogaster and humans, respectively), but the conserved definitive target of Plk4's kinase activity remains unknown (Lettman et al., 2013). Downstream of Plk4, nascent centriole construction involves Sas-6 oligomerization to form the inner, 9-fold symmetric cartwheel (van Breugel et al., 2011; Kitagawa et al., 2011; Hilbert et al., 2013). A third and less-studied centrioleinitiating factor is the Sas-6-binding protein, Ana2, whose role in centriole duplication is unclear.

Ana2 was identified in a genome-wide screen in which Ana2 depletion caused a decrease in centriole count (Goshima et al., 2007). Ana2 is functionally conserved across metazoan species, with orthologs in humans (STIL), D. rerio (STIL), and C. elegans (SAS-5) (Stevens et al., 2010a). However, the Ana2 sequence has diverged among species, with similarity restricted to an N-terminal Sas-4 binding site (Cottee et al., 2013; Hatzopoulos et al., 2013), a central predicted coiled coil, and a C-terminal STAN (STil/ANa2) domain that binds Sas-6 in vitro (Stevens et al., 2010a; Leidel et al., 2005; Qiao et al., 2012) (Figure 5.1

C ; see domain conservation presented in the inset, scored using \% identity and $\%$ similarity between species). In Drosophila oocytes, Sas-6 overexpression results in centriole amplification only when Ana2 is dually overexpressed (Stevens et al., 2010b). In human systems, expression of Ana2 is essential in maintaining centriole count (Arquint et al., 2012). Furthermore, mutations in Ana2 have been linked to primary microcephaly, leukemia, and cancer (Aplan et al., 1991; Izraeli et al., 1997; Erez et al., 2004; Basto et al., 2008; Kumar et al., 2009). How Ana2 and Sas-6 synergistically function remains to be determined. While Ana2's function is poorly understood, recent work demonstrated that Drosophila Ana2 interacts with the dynein light chain, LC8 (Cut up (Ctp)) (Wang et al., 2011), a ubiquitous protein that binds diverse targets throughout the cell to confer or potentiate target dimerization (reviewed in Barbar 2008). The Ana2-LC8 interaction is important for directing spindle orientation during Drosophila larval brain development (Figure 5.1 D). Loss of either Ana2 or LC8 results in aberrant spindle positioning and defective separation of apico-basal polarity determinants during neuroblast asymmetric cell division.

While LC8 acts as a processivity factor for the dynein motor by enhancing motor dimerization, it largely plays a dynein motor-independent role throughout the cell to potentiate dimerization of its binding partners (Lei and Davis, 2003; Liang et al., 1999; Chaudhury et al., 2008; Romes et al., 2012; Nyarko et al., 2013; Purohit et al., 1999; Navarro et al., 2004; Asthana et al., 2012; Nyarko and Barbar, 2011; Benison et al., 2007; Williams et al., 2007; Stuchell-Brereton et al., 2011). It was shown in a yeast two-hybrid screen that LC8 binds two Ana2 fragments: the first fragment spanning residues 1-200, and the second spanning residues 201-274, which includes a predicted $\alpha$-helix highly conserved across fly
species (Figure 5.1 E, F) (Wang et al., 2011; Stevens et al., 2010a). To date, there is no structural insight into the LC8-Ana2 complex.

Here, we use x-ray crystallography, isothermal microtitration calorimetry (ITC), and size-exclusion chromatography with multi-angle static light scattering (SEC-MALS) to characterize the interactions between LC8 and Ana2. Our results demonstrate that LC8 dimers bind Ana2 at two distinct sites, the first of which contains a high-affinity, canonical LC8-binding TQT motif (residues 159-168), while the second contains a non-canonical TQC motif (residues 237-246). We present the structures of LC8 bound to peptides encompassing both of Ana2's LC8 binding sites as well as the apo LC8 dimer, and highlight the conserved Ana2 features that underlie these different interactions with the peptides. SEC-MALS analysis of WT and mutant Ana2M (residues 156-251) in complex with LC8 reveals LC8dependent Ana2M tetramerization in an Ana2M4-LC88 complex. The Ana2 LC8 binding sites flank a predicted $\alpha$-helix likely involved in Ana2 oligomerization. Our findings suggest that LC8 is responsible for enhancing Ana2's oligomerization and structural stability. LC8potentiated Ana2 oligomerization has spatial and avidity implications for Ana2's N-terminal Sas-4 binding motif and its C-terminal Sas-6-binding STAN domain.


FIGURE 5.1. Ana2 contains two conserved LC8 binding sites. (A) The nascent centriole cartwheel with mapped components. The precise location of Ana2 on the cartwheel remains unknown, but it is known to bind the Sas-6 N-terminal region (Stevens et al., 2010a) and the Sas-4 C-terminal region (Cottee et al., 2013). (B) A conserved set of proteins drive centriole duplication. Conserved centriole duplication pathway components from Drosophila
melanogaster (D.m.), Homo sapiens (H.s.), and Caenorhabditis elegans (C.e.) are presented with orthologous proteins listed on the same row. Drosophila Asterless (Asl) recruits SAK/Polo-Like Kinase-4 (Plk4) to the site of nascent centriole formation via a direct interaction (Dzhindzhev et al., 2010; Slevin et al., 2012), where it phosphorylates both a known and unknown set of substrates in the centriole duplication pathway (Lettman et al., 2013; Puklowski et al., 2011). Spindle-assembly Abnormal-6 (Sas-6) oligomerizes to form the first structure observed using electron microscopy; this 9-spoked cartwheel depicted at left. In cells, Sas-6 oligomerization is Anastral Spindle-2 (Ana2)-dependent (Kitagawa et al., 2011; Stevens et al., 2010a; Stevens et al., 2010b). Spindle-assembly Abnormal-4 (Sas-4) is thought to recruit triplet microtubule blades and stabilize centriole elongation and maturation (mature centriole shown at left) (Pelletier et al., 2006; Cottee et al., 2013; Hatzopoulos et al., 2013). (C) Comparison of H.s., Danio rerio (D.r.), D.m., and C.e. Ana2 orthologs reveals diversity in protein structure. While the length of Ana2 orthologs differ, the presence of a Sas-4 binding domain (red), a Sas-6 binding domain (STAN domain, gray), and a predicted central coiled-coil region remain constant (domains shown as determined in Hatzopoulos et al., 2013; Stevens et al., 2010a). Inset, alignments of individual Sas-4 binding domains and STAN domains between D.m. and H.s, D.r., or C.e. reveal high percentages of invariant (first value) and similar (second value) residues. (D) Ana2 and LC8 form a complex with Mud to orient the mitotic spindle during asymmetric divisions in the developing Drosophila neuroblast (Wang et al., 2011). Asymmetry is achieved, in part, via differential maturation of the centrosomes. The daughter centrosome forms the LC8/Ana2/Mud complex that coordinates spindle alignment with cortical polarity cues to maintain a stem population (GMC: ganglion mother cell) (Lerit and Rusan, 2013; Wang et al., 2011). (E) Full-length Drosophila Ana2 has an N-terminal Sas-4 binding region (Cottee et al., 2013; Hatzopoulos et al., 2013) and a C-terminal STAN motif (Stevens et al., 2010a) conserved across functional Ana2 orthologs. The central predicted helical domain is flanked by two LC8 binding sites (Site 1, residues 159-168; Site 2, residues 237-246). Residue identity across Drosophila species is noted below in green. (F) Conservation within the Ana2 central helical domain and LC8 binding sites. Residues with $100 \%$ identity are highlighted in green, while those with $80 \%$ similarity are highlighted in yellow. Note that both the TQT (165-166) and TQC (243244) sites are conserved within the genus.

## Experimental Procedures

Cloning and Expression of full-length LC8
Full-length Drosophila melanogaster LC8 was subcloned into the pGEX-6P-2
expression vector (GE Healthcare). pGEX-6P-2-LC8 was transformed into E. coli BL21 DE3
(pLysS) and grown under ampicillin selection in 6 L of LB media at $37^{\circ} \mathrm{C}$. At an optical density of 0.6 ( 600 nm ), GST-LC8 expression was induced using 0.1 mM isopropyl-1-thio- $\beta$ -

D-galactopyranoside for 16 hours at $18^{\circ} \mathrm{C}$. Cells were harvested by centrifugation at 2100 xg for 10 minutes at $4^{\circ} \mathrm{C}$ and the pellets resuspended in buffer A: 25 mM Tris, $\mathrm{pH} 8.0,300 \mathrm{mM}$ sodium chloride, and $0.1 \% \beta$-mercaptoethanol, and stored at $-20^{\circ} \mathrm{C}$.

## Protein Purification for Crystallization

LC8 was purified as previously described for the yeast homologue Dyn2 (Romes et al., 2012). Briefly, cells expressing GST-LC8 were lysed by sonication, clarified by centrifugation at $23,000 \mathrm{xg}$ for 45 minutes, and the supernatant loaded onto a Glutathione-Ssepharose column (GE Healthcare). The column was washed with buffer A and the GST-LC8 fusion step eluted in buffer A supplemented with 25 mM glutathione. The GST tag was cleaved with PreScission protease (GE Healthcare). LC8 was subsequently purified on an SP Sepharose Fast Flow column (GE Healthcare) and exchanged into MES storage buffer (25 mM MES, $\mathrm{pH} 6.0,50 \mathrm{mM} \mathrm{NaCl}$, and $0.1 \% \beta$-mercaptoethanol). LC8 was concentrated to 0.5 mM , snap frozen in liquid nitrogen and stored at $-80^{\circ} \mathrm{C}$. The final LC8 contains an N terminal five-residue (GPLGS) cloning artifact. Synthesis of Ana2 Peptides

Ana2 peptides were synthesized at the UNC Microprotein Sequencing and Peptide Synthesis Facility and lyophilized peptides were reconstituted in final MES storage buffer. An N-terminal, non-native Asn and Tyr were added to each peptide to facilitate peptide concentration determination (underlined in the sequences presented below). The Ana2 peptide sequences are peptide 1 (pep1): NYTICAGTQTDP (Ana2 residues 159-168) and peptide 2 (pep2): $\underline{\text { NYSSTTGTQCDI (Ana2 residues 237-246). }}$

Crystallization of the LC8/Ana 2 peptide complexes

Final concentrations of 0.5 mM LC8 and 0.6 mM Ana2 pep1 (or 0.75 mM LC8 and 0.9 mM Ana2 pep2) in MES storage buffer were incubated for 30 minutes on ice. For the LC8/pep1 complex, crystallization followed the hanging drop protocol using $2 \mu \mathrm{~L}$ of the LC8-Ana2 pep1 mixture and $2 \mu \mathrm{~L}$ of a 1 mL well solution that contained 0.3 M magnesium acetate, 0.1 M sodium cacodylate, pH 6.5 , and $26 \%(\mathrm{w} / \mathrm{v})$ polyethylene glycol 8000 . The same method was used for LC8/pep2 in a well solution containing 0.19 M ammonium acetate, $27 \%(\mathrm{w} / \mathrm{v})$ polyethylene glycol $4000,0.1 \% \beta$-mercaptoethanol, and 0.1 M sodium acetate, pH 4.6 . For both structures, crystals grew at $20^{\circ} \mathrm{C}$ into rods (pep1) or rounded cubes (pep2) within three days and remained at full size for up to three weeks. Crystals were transferred into fomblin oil (Sigma) cryoprotectant and flash frozen in liquid nitrogen.

## Data Collection, Structure Determination, and Refinement

Diffraction data were collected on LC8-Ana2 crystals (both peptides) at the Advanced Photon Source SER-CAT beamline 22-ID with $1^{\circ}$ oscillations over $180^{\circ}$ from single crystals. Data were indexed, integrated, and scaled using HKL2000 (Otwinowski and Minor, 1997). The LC8-Ana2 peptide structures were determined using the AutoMR molecular replacement program (PHENIX crystallographic suite (Adams et al., 2010)) and a modified 2PG1 (Williams et al., 2007) coordinate file in which a monomeric (for LC8/Ana2 pep1) or dimeric (for LC8/Ana2 pep2) apo Drosophila LC8 search model was used. The models were built using AutoBuild (PHENIX) and refined iteratively through manual builds in Coot (Emsley et al., 2010) followed by refinement runs using phenix.refine against a maximum likelihood target (PHENIX) (Adams et al., 2010). Refinement statistics were monitored using a Free R, calculated using $5.4 \%$ or $5.6 \%$ of the data for pep1 and pep2, respectively, randomly excluded from refinement (Brünger 1992).

## Isothermal Microtitration Calorimetry

ITC experiments were carried out at $26^{\circ} \mathrm{C}$ in MES storage buffer on a MicroCal AutoITC200 (GE Healthcare). Lyophilized peptides were solubilized in MES storage buffer. $19 \times 2 \mu \mathrm{~L}$ injections of 1.0 mM Ana2 pep1 were automatically injected into $200 \mu \mathrm{~L}$ of $50 \mu \mathrm{M}$ LC8 and 2.0 mM pep2 was automatically injected into $200 \mu \mathrm{~L}$ of $100 \mu \mathrm{M}$ LC8. The resulting binding isotherms were analyzed using the Origin 7.0 software package (OriginLab) and were fit to a single-site, independent-binding model. Ana2 peptide control experiments were performed to determine the contribution from each peptide's heat of dilution. These controls involved $19 \times 2 \mu \mathrm{~L}$ injections of 1.0 mM Ana2 pep1 or 2.0 mM Ana2 pep2 into a chamber containing $200 \mu \mathrm{~L}$ of MES storage buffer. The Ana2 pep1 control isotherm did not reveal significant heat of dilution; therefore, the final five injection values (where binding was saturated in the pep1-LC8 isotherm) were averaged and this value was subtracted from each injection in the pep1-LC8 experiment. The Ana2 pep2 control isotherm revealed a significant endothermic heat of dilution (data not shown); therefore, these control values were individually subtracted from the corresponding raw experimental values from the pep2-LC8 binding isotherm. Experiments were conducted in triplicate, the internal or external controls were subtracted, and the resulting heats of dilution were averaged to determine respective mean $K_{D}$ values and standard deviations.

## Cloning and Expression of LC8 and Ana2M Constructs for SEC-MALS

Full-length Drosophila melanogaster LC8 was subcloned into a pET28b expression vector (EMD Millipore) with an engineered PreScission protease (GE Healthcare) cleavage site following the N -terminal $\mathrm{His}_{6}$ tag. The subcloning of SNAP-tag-LC8 (New England Biolabs) into pET28b followed a similar protocol. Drosophila melanogaster Ana2 residues

D156-Q251 (Ana2M) was subcloned into a pGEX-6P-2 expression vector (GE Healthcare). pET28b-LC8 and pGEX-6P-2-Ana2M were separately transformed into E. coli BL21 DE3 (pLysS) and grown individually under kanamycin (LC8) or ampicillin (Ana2M) selection, each in 5 L of LB media at $37^{\circ} \mathrm{C}$. At an optical density of $0.6(600 \mathrm{~nm})$, $\mathrm{His}_{6}-\mathrm{LC} 8$ or GSTAna2M expression was induced using 0.2 mM isopropyl-1-thio- $\beta$-D-galactopyranoside for 16 hours at $18^{\circ} \mathrm{C}$. Cells were harvested by centrifugation at 2100 xg for 10 min at $4^{\circ} \mathrm{C}$ and the pellets of both $\mathrm{His}_{6}$-LC8 and GST-Ana2M were combined and resuspended in buffer A: 25 mM Tris, $\mathrm{pH} 8.0,10 \mathrm{mM}$ imidazole, 300 mM sodium chloride, and $0.1 \% \beta$ mercaptoethanol, and stored at $-20^{\circ} \mathrm{C}$.

## Ana2M-LC8 Complex Purification for SEC-MALS

The composite pellet of $\mathrm{His}_{6}-\mathrm{LC} 8$ and GST-Ana2M was thawed and lysed by sonication with addition of phenylmethanesulfonylfluoride to a final concentration of 200 $\mu \mathrm{M}$. The supernatant was purified over $\mathrm{Ni}^{2+}$-NTA resin (QIAGEN) followed by PreScission protease (GE Healthcare) treatment to cleave off the His ${ }_{6}$ and GST tags. The LC8:Ana2M complex was subsequently purified over a Superdex 200 size exclusion column (GE Healthcare) and concentrated in SEC-MALS buffer: 25 mM HEPES, pH 7.5, 300 mM sodium chloride, $0.1 \% \beta$-mercaptoethanol. The presence of both components was confirmed by SDS-PAGE. Expression and purification of the Ana2M-SNAP-LC8 complex followed a similar protocol.

## Mutagenesis of Ana2M

An Ana2M site 1 LC8-binding mutant (Q165A/T166A) was created using the QuikChange (Agilent Technologies) method on the wild-type GST-Ana2M construct
according to the manufacturer's instructions. The mutant GST fusion protein was expressed and co-purified with wild-type LC8 as described above.

SEC-MALS
LC8/Ana2M complexes ( $100 \mu \mathrm{~L}$ ) were injected onto a Wyatt WTC-030S5 silicone size exclusion column (for elution of $5-1,250 \mathrm{kDa}$ proteins) in SEC-MALS buffer supplemented with $0.2 \mathrm{~g} / \mathrm{L}$ sodium azide and passed in tandem through a Wyatt DAWN HELEOS II light scattering instrument and a Wyatt Optilab rEX refractometer. The light scattering and refractive index data were used to calculate the weight-averaged molar mass and the mass fraction in each peak using the Wyatt Astra V software program (Wyatt Technology Corp.) (Wyatt 1993).

## Results

## Ana2 Contains Two High-Affinity LC8 Binding Sites

Drosophila Ana2 is a 420-residue centriole duplication component that lacks apparent conservation across species barring an N-terminal Sas-4 binding region (Cottee et al., 2013; Hatzopoulos et al., 2013), a central predicted coiled-coil domain, and the highly conserved C-terminal STAN (STil/ANa2) motif (Figure 5.1 C, E, F) (Stevens et al., 2010a). Previous studies demonstrated a physical interaction between the N-terminal 274 residues of Ana2 and LC8, a dynein light chain, via yeast two-hybrid (Wang et al., 2011). Structure function analysis indicated that Ana2 contained at least two LC8 binding sites, one within the region spanning residues 1-200, and the second within the region spanning residues 201-274. LC8 binds many subcellular targets across species in a cytoplasmic dynein motor-independent mechanism to promote target dimerization (Rao et al., 2013), suggesting that LC8 may
potentiate Ana2 oligomerization. To map the interactions between Ana2 and LC8, we scanned Ana2 $2_{1-274}$ for potential LC8 binding sites. LC8 target motifs comprise up to 11 contiguous residues, which, though diverse in sequence composition, often contain a $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}$ ${ }_{1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ or $\mathrm{G}_{-2} \mathrm{I}_{-1} \mathrm{Q}_{0} \mathrm{~V}_{1} \mathrm{D}_{2}$ motif with the conserved glutamine, $\mathrm{Q}_{0}$, set as the zero reference point (Rapali et al., 2011a). Target peptides with these LC8-binding motifs bind LC8 with $\mathrm{K}_{\mathrm{D}}$ values in the $0.1-100 \mu \mathrm{M}$ range (Rapali et al., 2011a; Benison et al., 2008). We identified two potential binding sites within Ana2, corresponding to residues 159-168 (containing a $\mathrm{T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ sequence) and 237-246 (containing a $\mathrm{T}_{-1} \mathrm{Q}_{0} \mathrm{C}_{1}$ sequence). These two sites flank either end of the conserved predicted coiled-coil (Figure $5.1 \mathrm{E}, \mathrm{F}$ ) and correlate with the two fragments identified via yeast two-hybrid as LC8-binding segments.

To determine whether these sites were conserved, we aligned several Ana2 sequences from ten different Drosophila species. Much of the protein is conserved within the genus, with the largest concentration of identity mapping to the STAN motif and an N-terminal region with no predicted secondary structure, but involved in Sas-4 binding (Figure 5.1 E) (Cottee et al., 2013; Hatzopoulos et al., 2013). Additional identity maps to the central predicted coiled-coil and the flanking regions that contain the tentative LC8-binding sites we identified (Figure 5.1 F ). The linkers that bridge the predicted LC8 binding sites with the central, predicted coiled coil show diversity in both sequence length and composition. When we analyzed the central, predicted coiled-coil domains in Ana2 orthologs: human STIL, zebrafish STIL, and C. elegans Sas-5, only Sas-5 contained a potential QT-motif N-terminal to the predicted coiled coil with the sequence KTVNVSQTVE, suggesting that the LC8Ana2 interaction may be specific to a subset of Ana2 orthologs.

To confirm the ability of Ana2's putative LC8-binding sites to bind LC8, we synthesized peptides corresponding to the two predicted Ana2-LC8 binding sites (Figure 5.1 E,F) and performed ITC, monitoring the heat released as each peptide was titrated into the calorimeter cell containing purified LC8. Experiments were performed in triplicate, with reported values reflecting the average of all trials. The Ana2 peptide 1 (pep1) - LC8 binding isotherm was exothermic and yielded a $\mathrm{K}_{\mathrm{D}}$ value of $1.14 \pm 0.07 \mu \mathrm{M}$ (Figure 5.2 A). Compared to reported LC8-target affinities ( $100-0.1 \mu \mathrm{M}$ ) (Rapali et al., 2011a; Benison et al., 2008), Ana2 pep1 binds LC8 in the higher-affinity range. The Ana2 pep2 binding isotherm was also exothermic, and yielded an experimentally determined $\mathrm{LC} 8 \mathrm{~K}_{\mathrm{D}}$ value of $12.8 \pm 1.5 \mu \mathrm{M}$ (Figure 5.2 B), a weaker binding affinity than pep1, but within the commonly reported range of LC8-target affinities.


FIGURE 5.2. LC8 binds two Ana2 sites with different affinities. ITC isotherms of Ana2 peptide-LC8 interactions. (A) $19 \times 2 \mu \mathrm{~L}$ of $60 \mu \mathrm{M}$ Ana2 peptide 1 was injected into $200 \mu \mathrm{~L}$ of $50 \mu \mathrm{M}$ LC8. (B) $18 \times 2 \mu \mathrm{~L}$ of 2 mM Ana2 peptide 2 was injected into $200 \mu \mathrm{~L}$ of $100 \mu \mathrm{M}$

LC8. Both Ana2 peptides display exothermic binding to LC8. The thermal profiles were integrated (top panels in A, B) and fit to a one-site binding model during iterative fitting until the model best fit the data. Each experiment was run in triplicate, with the $K_{D}$ reported as the average (lower corner of bottom panels) with standard deviation indicated.

## Crystallization of the LC8 Ana2 Pep1 and Pep2 Complexes

To determine the molecular determinants underlying the LC8-Ana2 interaction, we attempted to crystallize LC8 in complex with each synthesized Ana2 peptide. Both LC8peptide complexes were amenable to crystallization, though diffraction-quality crystals formed in different conditions (see Experimental Procedures). Ana2 pep1-LC8 crystals diffracted to $1.83 \AA$ resolution and belonged to the space group $\mathrm{P} 2_{1} 2_{2} 2_{1}$ (Table 1). Ana2 pep2-LC8 crystals diffracted to $1.9 \AA$ resolution and belonged to the space group P1 (Table 1). To solve both structures, we performed molecular replacement using a search model containing a single Drosophila LC8 chain (for Ana2 pep1) or an LC8 dimer (for Ana2 pep2) without bound peptide, derived from PDB 2PG1 (Williams et al., 2007).

## The Structure of LC8 Bound to Ana2 Pep1

Four LC8 chains were found in the asymmetric unit. The LC8 chains are paired to form two independent homodimers, each arranged around non-crystallographic 2-fold axes. Clear electron density was evident in the initial $\mathrm{F}_{\mathrm{o}}-\mathrm{F}_{\mathrm{c}}$ map to build four Ana2 pep1 chains (Figure 5.3 A), two bound to each LC8 homodimer. The structure was built and refined to R and $\mathrm{R}_{\text {free }}$ values of 17.6 and $20.7 \%$, respectively (see Table 1 for refinement statistics).

The LC8 homodimer forms a composite platform for Ana2 pep1 binding (Figure 5.3 B). The homodimeric core is characterized by a central 12 -stranded $\beta$-sandwich, each half of which is formed by four $\beta$-strands from one LC8 chain ( $\beta 1$ from V7-D12, $\beta 4$ from H72-L78, $\beta 5$ from V81-K87, $\beta 2$ from W54-G59), one $\beta$-strand from the LC8 homodimeric mate ( $\beta 3^{\prime}$ from G63'-E69'), and the Ana2 peptide, which contributes the sixth and final $\beta$-strand (Figure 5.3 B). Each $\beta$-sheet is entirely anti-parallel. The $\beta$-sandwich is flanked on either side by two $\alpha$-helices. Ana2 pep1 binding engages determinants in both LC8 chains, with $\beta 3$ forming a key extended interface with the peptide (Figure 5.3 B). Peptide binding is stabilized by backbone/backbone anti-parallel $\beta$-sheet hydrogen bonding (Figure 5.4 A ) as well as several side chain interactions. Ana2's conserved glutamine Q165 (notated as $\mathrm{Q}_{0}$ in reference to its position in the canonical $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ binding motif) forms key contacts including van der Waals interactions with both LC8 chains and hydrogen bonds with the E35' side chain carboxylate group and the K36' backbone amide, serving to cap the $\alpha 2^{\prime}$ helix's $N$-terminal region (Figure 5.4 B).

## The Structure of LC8 Bound to Ana2 Pep2

The Ana2 pep2-LC8 crystal contains three LC8 dimers in the P1 unit cell. One LC8 dimer is bound to two Ana2 pep2 chains (Figure 5.3 A,C), while the other two LC8 dimers are in the apo form with crystal packing sterically occluding the peptide binding sites. The structure was built and refined to R and $\mathrm{R}_{\text {free }}$ values of 18.5 and 23.7 \%, respectively (see Table 1 for refinement statistics).


FIGURE 5.3. Structures of LC8-Ana2 complexes reveal LC8 homodimers bound to two parallel Ana2 peptides. (A) LC8-Ana2 complex structures were determined using peptidefree LC8 search models. Initial $\mathrm{F}_{\mathrm{o}}-\mathrm{F}_{\mathrm{c}}$ electron density for the Ana2 peptides is shown in green and contoured at $2.0 \sigma$ (Pep1) and $1.65 \sigma$ (Pep2). Final $2 \mathrm{~F}_{0}-\mathrm{F}_{\mathrm{c}}$ electron density is shown below in gray with the final Ana2 pep1 and pep2 model included; electron density is contoured at $2.0 \sigma$ (pep1) and $1.0 \sigma$ (pep2). Final models of the respective LC8-Ana2 peptide
complexes are presented in the upper left (LC8-Ana2 pep1) and upper right (LC8-Ana2 pep2) with peptides in the same orientation for reference. (B and C) The final structures of LC8 bound to Ana2 pep1 (orange, B) and Ana2 pep2 (cyan, C) are shown looking down the complex's two-fold axis (left) and after a $90^{\circ}$ rotation about the y-axis (right). Center schematic in $B$ and $C$ summarizes the secondary structure elements that comprise a single $\beta$ sheet in the LC8-peptide complexes. Each $\beta$-sheet is extended by the third $\beta$-strand contributed by the LC8 homodimeric mate (purple) as well as the bound Ana2 peptide (pep1 shown in orange, B ; pep2 shown in cyan, C ). The final $\beta$-sheet comprises a total of six strands is flanked by two $\alpha$-helices (shown in mint, behind the sheet).

Ana2 pep2 binds in a manner similar to Ana2 pep1, extending either side of LC8's core $\beta$-sandwich and making several backbone interactions with LC8 $\beta 3$ (Figure 5.3 C , Figure 5.4 A ). Ana2 pep2's $\mathrm{Q}_{0}$ participates in similar interactions as observed in the LC8Ana2 pep1 structure; however, pep2 contains a non-canonical cysteine residue at the +1 position, C244. To our knowledge, this is the first example of an LC8 target with a cysteine in the +1 position. In contrast to the canonical threonine at the +1 position, Ana2 pep2 C244 is angled into the LC8 peptide binding groove, with its side chain engaging LC8 E35', R60, N61, F62, Y77, and A82. Specifically, the cysteine's terminal sulfhydryl group forms a 3.6A electrostatic interaction with the backbone carbonyl of LC8 R60 (Figure 5.4 C). This shift allows for extended backbone-backbone contacts including interactions between Ana2 C244 and LC8 F62, as well as between Ana2 I246 and LC8 R60 (Figure 5.4 D). As a result, the Ana2 pep2 C-terminal region differentially engages the LC8 dimer as compared to Ana2 pep1, whose respective determinants are positioned upwards of $5 \AA$ away (Figure 5.4 D).


FIGURE 5.4. Ana2's LC8-binding sites 1 and 2 employ both shared and unique LC8binding determinants. (A) Interaction matrix displaying contacts between the LC8 homodimer (y-axis) and Ana2 pep1 (orange, top x-axis) or Ana2 pep2 (cyan, bottom x-axis). Interactions are presented where atoms are less than or equal to $3.5 \AA$ apart (hydrogen bonds and electrostatic interactions; shown in red for pep1 and pink for pep2) and $4.5 \AA$ apart (van der Waals contacts; shown in dark gray for pep1 and light gray for pep2). Boxes completely filled in reflect similar LC8 interaction modes with each peptide while those boxes that are half-filled indicate unique, peptide-specific interactions. (B) Ana2 pep1's conserved Q165
forms hydrogen bonds to LC8' residues E35' and K36'. (C) Ana2 pep2's C244 forms an electrostatic interaction with LC8 residue R60. (D) The Ana2 pep2 (cyan) C-terminal region forms extensive backbone hydrogen bonds with LC8 and is positioned different that Ana2 pep1 (orange) which has been overlayed on the LC8-Ana2 pep2 structure for comparative purposes. In contrast to the Ana2 pep2 C244 backbone carbonyl and the I246 backbone amide that interact with LC8 F62 and R60 respectively, the comparable Ana2 pep1 determinants (indicated with magenta arrows) are splayed and rotated away from LC8.

The LC8 Binding Pocket Undergoes Structural Shifts to Accommodate Ana2 Peptides
In addition to observing an LC8-Ana2 pep2 complex in the P1 unit cell, two sets of apo LC8 homodimers were also present. As previously observed (Benison et al., 2008), the apo LC8 binding pocket is narrower than the peptide-bound cleft observed in both Ana2bound LC8 structures (Figure 5.5 A). Several LC8 residues that directly engage the Ana2 peptides are swung towards the peptide binding pocket in the apo state, including N10, K36', Y65, T67, F73, Y75, and Y77, highlighting the mobility of LC8 side chains upon target binding.

## Ana2 Employs a Unique Tandem Set of LC8 Binding Motifs

LC8 targets vary widely in their binding affinity and motif composition, both within and beyond the canonical $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ or $\mathrm{G}_{-2} \mathrm{I}_{-1} \mathrm{Q}_{0} \mathrm{~V}_{1} \mathrm{D}_{2}$ sequence motifs. Interestingly, both Ana2 LC8 binding motifs combine features from each canonical motif (pep1: A-3G-2T${ }_{1} \mathrm{Q}_{0} \mathrm{~T}_{1} \mathrm{D}_{2}$; pep2: $\mathrm{T}_{-3} \mathrm{G}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{C}_{1} \mathrm{D}_{2}$ ). Both Ana2 LC8 sites have threonine residues at the -1 position as found in the $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ motif, and both have glycine and aspartate residues at the -2 and +2 positions as found in the $\mathrm{G}_{-2} \mathrm{I}_{-1} \mathrm{Q}_{0} \mathrm{~V}_{1} \mathrm{D}_{2}$ motif. Neither Ana2 site employs a basic residue at the -3 position, which is often seen in high-affinity LC8 interactors (Rapali et
al., 2011b) including Nek9 (a kinase that regulates mitotic spindle formation and chromosome separation) (Gallego et al., 2013) and the dynein intermediate chain (DIC, a dynein motor complex component used in cargo recognition) (Williams et al., 2007) (Figure 5.5 A,B,C). Both Nek9 and DIC LC8 target sites contain a lysine at the -3 position (K-3) that interacts with the LC8 D12' side chain carboxyl group, and promotes relatively strong LC8binding interactions ( $\mathrm{K}_{\mathrm{D}} \mathrm{S}$ on the order of 0.1-0.2 $\mu \mathrm{M}$ ) (Williams et al., 2007; Gallego et al., 2013) (Figure 5.5 B,C). Ana2 sites 1 and 2 contain an alanine and threonine, respectively, at position -3 that do not engage the D12' side chain carboxyl (Figure 5.5 A). At the -3 position, Pak1 (a kinase that regulates cell motility and, together with LC8, plays a role in cancer transformation (Lightcap et al., 2008)) is an interesting point of comparison. Pak1 contains the non-canonical LC8 binding sequence $\mathrm{V}_{-3} \mathrm{~A}_{-2} \mathrm{~T}_{-1} \mathrm{~S}_{0} \mathrm{P}_{1} \mathrm{I}_{2}$ and has the weakest affinity for LC8 $\left(K_{D}\right.$ of $\left.42 \mu \mathrm{M}\right)$ of the peptides we use for comparison. Like both Ana2 peptides, Pak1 employs a non-charged residue at the -3 position, but similar to the -3 lysine in Nek9 and DIC, positionally equivalent aliphatic side chain determinants are used to engage LC8, highlighting LC8's ability to accept side chain variability at the -3 position.

We next examined how the conformations of Ana2 pep1 and pep2 compared to other LC8 binding peptides by aligning LC8-peptide complex structures (Figure $5.5 \mathrm{D}, \mathrm{E}, \mathrm{F}$ ). Ana2 pep1 aligns well with other LC8 binding peptides including Nek9, DIC, and Pak1 (Figure 5.5 E). However, the C-terminal region of Ana2 pep2 departs from this common LC8-bound architecture. The aforementioned Ana2 pep2 cysteine, C244, at position +1 is angled into the LC8 peptide-binding groove, effectively positioning the peptide's C-terminal region closer to the LC8 homodimer. In contrast, Ana2 pep1, Nek9, DIC, and Pak1, each of which has a threonine at position +1 , splay away from the LC8 dimer, with their C-terminal regions
positioned $\sim 3-5 \AA$ from the comparative location of Ana2 pep2 (Figure 5.5 E). Underlying the differential position of Ana2 pep2 is comparative placement of the cysteine C244 backbone carbonyl in the same location of the threonine (T1) side chain hydroxyl as found in Ana2 pep1, Nek9, and DIC (Figure 5.5 F).

## LC8 Mediates Ana2M's Solubility and Oligomerization State

Multiple attempts to express various Ana2 constructs containing either or both of the LC8 binding sites yielded insoluble protein, making it difficult to study Ana2's oligomeric state in the absence of LC8. However, co-purification of Ana2M (residues 156-251, encompassing both LC8 binding sites and a central predicted helical domain, Figure 5.1 B) and LC8 yielded a stable, soluble complex that could be purified via LC8's His ${ }_{6}$ affinity tag followed by size exclusion chromatography. LC8's canonical role as a dimerization "hub" led us to predict that the purified LC8-Ana2M complex would form a heterohexamer, with two Ana2M chains forming a central coiled-coil flanked at either end by LC8 homodimers. To experimentally determine the Ana2M-LC8 complex's mass and stoichiometry, we analyzed the complex using SEC-MALS.

A


C
LC8/DYNLL Binding Partners

|  |  |  |
| :---: | :---: | :---: |
|  | Position | $----321012$ |
|  | Canonical 1 | ------GIQVD- |
|  | Canonical | -----КХTQT-- |
| D.m. | Ana2 site | AYTICAGTOTDP |
| D.m. | Ana2 site | -ASSTTGTOCDI |
| H.s. | Nek9 | VGMHSKGTOTA- |
| D.m. |  | IVTYTKETOTP- |
| D.m. | Pak1 | -PTRDVATSPI- |


E

F


FIGURE 5.5. Ana2's two LC8 binding sites differentially bind LC8. (A) A comparison of the LC8 target-binding site among the apo, Ana2 pep1-bound, and Ana2 pep2-bound LC8 structures. Several LC8 residues within the binding pocket show conformational change upon binding peptides and are colored red: N10, Y65, T67, F73, Y75, Y77, and K36'. (B) Comparative panel showing the positioning of other peptides bound to Drosophila LC8:

Nek9 (3ZKE), DIC (2PG1), and Pak1 (3DVP) (Gallego et al., 2013; Williams et al., 2007; and Lightcap et al., 2008, respectively). (C) Alignment of Ana2 peptides with Nek9, DIC, and Pak1, as well as the canonical binding motifs $\mathrm{G}_{-2} \mathrm{I}_{1} \mathrm{Q}_{0} \mathrm{~V}_{1} \mathrm{D}_{2}$ and $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$. Conservation is shown in yellow, contoured to $\geq 70 \%$ similarity. (D) Ana2 peptides 1 and 2 superimposed after aligning their respective, bound LC8 homodimers (not shown), viewed in two orientations. (E) Comparisons of the Ana2 peptides with Nek9 (periwinkle), DIC (lime), and Pak1 (salmon) peptides show that while relative positions of the sidechains are conserved, the Ana2 pep2 C-terminus uniquely bends toward the LC8 homodimer. Above: stick diagram; below, C $\alpha$ trace. Measurements of the pep 2 backbone show a $3.5 \AA$ and $5.6-\AA$ positional shift at the +1 and $+2 \mathrm{C} \alpha$ positions respectively for Ana2 pep2 versus Ana2 pep1. (F) Zoom view of the peptides at position +1 reveals the mechanism of Ana2 pep2's C244 shift: the same position usually occupied by a +1 position threonine side chain hydroxyl is instead occupied by Ana2's C244 backbone carbonyl group. This effectively positions the peptide deeper into the LC8 binding pocket.

As previously reported (Romes et al., 2012), purified LC8 eluted primarily as a dimer (Figure 5.6 A, light green trace indicating the Rayleigh ratio) with a mass of 21.6 kDa (Figure 5.6 A, dark green trace indicating the molecular weight). Surprisingly, Ana2M-LC8 formed a stable complex with a mass of $117.1 \pm 5.9 \mathrm{kDa}$ (average of four experiments from two independent protein purifications, Figure 5.6 A , red traces). This is approximately twice the mass an $\mathrm{Ana} 2 \mathrm{M}_{2}-\mathrm{LC} 84$ heterohexamer would form ( 68 kDa ). Adding excess purified LC8 to the Ana2M-LC8 complex did not shift or increase the mass of the eluted complex, but yielded a second peak that eluted later with an experimentally determined mass of 21 kDa , correlating with excess LC8 homodimers (data not shown). These results led us to postulate the existence of an LC8-mediated Ana2M tetramer comprising four Ana2M molecules and four LC 8 homodimers ( $\mathrm{Ana}^{2} \mathrm{M}_{4}-\mathrm{LC} 88$, Figure 5.7).

To test our predicted stoichiometry, we reasoned that mutating Ana2's first LC8 binding site to compromise LC8 binding would result in an ${\mathrm{Ana} 2 \mathrm{M}_{4}-\mathrm{LC} 84}^{4}$ complex with a corresponding mass of 90.8 kDa . We mutated Ana2M's first LC8 binding site (Q165A and

T166A or "QT/AA") and co-purified the Ana2M $\mathrm{MTTAA}^{-L C 8}$ complex, noting that the second LC8-binding site was sufficient for LC8-mediated Ana2M solubilization. The Ana2 $\mathrm{M}_{\mathrm{QT}^{2} / \mathrm{AA}^{-}}$ LC8 mutant retained its solubility and showed an excess of unbound LC8 during sizeexclusion chromatography. In accordance with forming an $\mathrm{Ana}_{2} \mathrm{M}_{4}$-LC84 heterooctomer (predicted mass of 90.8 kDa ), the complex had an apparent mass of $84.8 \pm 2.5 \mathrm{kDa}$ (Figure 5.6 A, blue traces).

In our experiments, Ana2M and LC8 were approximately the same mass (11.4 and 11.3 kDa , respectively), making it difficult to determine the relative contribution of LC8 and Ana2M to the complex. To independently confirm the composition of the proposed Ana2M ${ }_{4}$ $\mathrm{LC}_{8}$ complex, we purified an N-terminally SNAP-tagged LC8 (monomer mass: 30 kDa ) alone and in complex with Ana2M. The SNAP tag served the sole purpose of increasing the mass of the LC8 construct to see how this change in turn altered the mass of the Ana2M-LC8 complex. An Ana2M4-SNAP-LC88 complex would have a mass of 286 kDa . SNAP-LC8 was expressed with an N-terminal His tag, and behaved similar to wild-type LC8 throughout purification. Purified SNAP-LC8 eluted from the SEC-MALS column as a homodimer with an apparent mass of 59.3 kDa , indicative that the SNAP tag does not interfere with LC8 dimerization (Figure 5.6 B , dark green trace). Ana2M co-purified with SNAP-LC8, suggesting that SNAP-LC8 retained target-binding capabilities. The Ana2M-SNAP-LC8 complex eluted broadly from the SEC-MALS column with experimentally determined masses ranging from 290 kDa (early portion of the elution peak) to 150 kDa (later portion of the elution peak) (Figure 5.6 B , purple trace). The early portion of the elution peak mass correlates with an Ana2M $\mathrm{M}_{4}$-SNAP- $\mathrm{LC}_{8}$ complex while the 150 kDa shoulder suggested that
the SNAP tag may sterically hinder Ana2 tetramerization, yielding an Ana2M ${ }_{2}$-SNAP-LC8 4 subspecies.



FIGURE 5.6. SEC-MALS of Ana2M co-purified with LC8 shows a stable complex corresponding to $\mathbf{L C 8}_{\mathbf{8}}$-Ana2M $\mathbf{4}_{\mathbf{4}}$. Purified Ana2M (residues $156-251,11 \mathrm{kDa}$ ) remained soluble only when co-purified with excess LC8, and behaved as a single species throughout the purification which included affinity tag chromatography followed by two sizing columns. (A) Detection of the LC8/Ana2M complex on a sizing column coupled with multi-angle static light scattering shows a single peak (pink trace, Rayleigh ratio) at $117 \pm 5.9 \mathrm{kDa}$ (red, molecular weight measurement). The same experiments with a $\mathrm{Q}_{0} \mathrm{~T}_{1}$ to $\mathrm{A}_{0} \mathrm{~A}_{1}$ mutation shows a single peak (light blue trace, Rayleigh ratio) at $84.8 \pm 2.5 \mathrm{kDa}$ (dark blue trace, molecular weight measurement). LC8 alone elutes as a dimer with a mass of 21.6 kDa (dark green trace; molecular weight measurement). (B) SNAP-LC8 elutes as a single species (light green trace, Rayleigh ratio) at 59.3 kDa (dark green traces), corresponding to a dimer. Copurification of SNAP-LC8 with Ana2M yielded a complex that eluted from the size exclusion column in a broad peak, with a shoulder characteristic of complex dissociation (light purple trace, Rayleigh ration). Experimentally determined molecular weight across the broad peak indicated complexes of varying size, ranging from 290 to 150 kDa (dark purple traces; different parts of the peak were integrated to determine the contributing sizes). All experiments are consistent with the formation of a stable $\mathrm{LC} 8_{8}-\mathrm{Ana}_{2} \mathrm{M}_{4}$ complex.

## Discussion

Ana2 is an integral component of the centriole duplication pathway, but how it works with Sas-6 and Sas-4, and whether LC8 plays a role in this pathway, remains to be determined. The Sas-6 dimer interactions that facilitate cartwheel formation are very weak $\left(K_{D}\right.$ of $\left.>100 \mu \mathrm{M}\right)$, making it unlikely that Sas-6 could spontaneously build cartwheels in a
cellular context at endogenous levels. Additionally, Sas-6 overexpression promotes centriole amplification only when Ana2 is co-overexpressed, suggesting that Ana2 plays a supporting role in enhancing Sas-6 oligomerization and cartwheel formation (Stevens et al., 2010b). One mechanism by which Ana2 could promote Sas-6 oligomerization is if Ana2 itself were oligomeric. This idea is supported by recent evidence that Ana2 binds LC8, a dynein light chain that plays a ubiquitous role as a dimerization machine (Wang et al., 2011). Our work provides insight into the Ana2-LC8 quaternary structure, and establishes a foundation upon which the Ana2 tetramer's avidity effects on Sas-6 oligomerization can be investigated.

We have identified two LC8 binding sites in Ana2, conserved within the Drosophila genus, that flank a central domain with predicted helical structure (Figure 5.1 A ). While the exact binding sites are not apparent in other metazoan species, the presence of a central predicted coiled-coil is conserved across Ana2 orthologs from C. elegans Sas-5 to human STIL, and suggests a role in oligomerization. This is supported by a report that the C. elegans Sas-5 N-terminal region (containing the central predicted coiled coil) forms a tetramer in solution (Shimanovskaya et al., 2013). While Sas-5 tetramerization in vitro is not LC8dependent, its oligomeric state parallels the LC8-dependent tetramerization we observe with Ana2.

Dynein light chains often bind targets proximal to an endogenous oligomerization domain, potentiating target dimerization. Both of Ana2's LC8-binding sites are an amalgam of the canonical $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ and $\mathrm{G}_{-2} \mathrm{I}_{-1} \mathrm{Q}_{0} \mathrm{~V}_{1} \mathrm{D}_{2}$ LC8-binding motifs. Using ITC, we have shown that Ana2 pep1 binds LC8 with micromolar affinity $\left(K_{D}=1.1 \mu \mathrm{M}\right)$. Our crystal structure of LC8 bound to Ana2 pep1 shows an LC 82 -Ana2 pep1 2 binding mode, with Ana2 pepl's canonical TQT sequence contributing key binding determinants.

Our second identified LC8-binding site (Ana2 site 2, pep2) flanks the central helical domain's C-terminal region and is composed of the sequence $\mathrm{T}_{-3} \mathrm{G}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{C}_{1} \mathrm{D}_{2}$. Ana2 pep2 binds LC8 with lower affinity $\left(\mathrm{K}_{\mathrm{D}}=13 \mu \mathrm{M}\right)$ than Ana2 pep1. Our crystal structure of LC8 bound to Ana2 pep2 also has an $\mathrm{LC}_{2}$-Ana2 pep2 2 binding mode. Interestingly, Ana2 pep2 adopts a unique architecture when bound to LC8 that contrasts with other LC8-peptide structures. The Ana2 pep2 cysteine C244 at position +1 is positioned deeper into the LC8 binding groove. The affinities we report for the LC8-Ana2 peptide interactions likely underestimate the stability of the biological complex involving full length Ana2 and LC8. As our solution studies support interactions between LC8 homodimers and a tetrameric Ana2M region, we anticipate that avidity effects will increase the complex's stability beyond the affinities we report for LC8 and Ana2 pep1 and pep2. This is consistent with the finding that a stable Ana2-LC8 complex can be extracted from Drosophila cell lysate (Wang et al., 2011). We note that within the genus Drosophila, the two segments that bridge the predicted central coiled-coil with the two flanking LC8 binding sites are not conserved in sequence or length. We predict that these segments serve as general spacers that link the LC8 binding sites to the Ana2 coiled-coil oligomerization domain and maintain a general length that enables LC8 homodimers to bind and potentiate Ana2 oligomerization without sterically compromising coiled-coil formation.

Our data support a model in which LC8 stabilizes an Ana2 tetramer (Figure 5.7). An Ana2 tetramer may spatially arrange its conserved C-terminal STAN motifs to interact with Sas-6 and promote the Sas-6 oligomerization that underlies centriole cartwheel formation. Our SEC-MALS analysis of the Ana2M-LC8 complex reveals a stable, single-species complex consisting of four Ana2M molecules and eight LC8 molecules (Ana2M $\mathrm{M}_{4}$-LC88 $)$.

This stable complex was purified over two successive sizing columns, demonstrating its ready formation, and yielded a similar experimental mass in two independent purifications and SEC-MALS assays. Mutating the first Ana2M LC8 binding site as well as adding a SNAP tag to LC8 supported the $\mathrm{Ana}^{2} \mathrm{M}_{4}-\mathrm{LC} 88$ stoichiometry (Figure 5.7).


FIGURE 5.7. A proposed model of LC8-mediated Ana2 oligomerization. Our data indicate the formation of an $\mathrm{LC} 8{ }_{8}$-Ana 24 complex, which may have implications in Ana2's role in centriole duplication by clustering multiple Sas-4-binding (red ellipses) and Sas-6binding (gray ellipses) domains. Each LC8 homodimer locally mediates parallel dimerization of Ana2. The model, as presented, portrays the central, predicted $\alpha$-helix as a tetramerization domain. Whether this domain forms a tetrameric four-helix bundle remains to be determined, but it is presented as a parallel four-helix bundle (above) and an antiparallel four-helix bundle (below).

The Ana2-LC8 interactions that we characterized raise important questions about Ana2's role in centriole duplication. Previous work has shown that Ana2's C-terminal half
binds the N-terminus of Sas-6 in Drosophila (Stevens et al., 2010a), implicating a possible role for Ana2's conserved STAN domain in Sas-6 binding. In our model, LC8 binds and stabilizes an Ana2 tetramer that may structurally organize four trans STAN domains at one end of a parallel tetramerization domain, or two trans STAN domains at either end of an antiparallel tetramerization domain (Figure 5.7). In either configuration, Ana2's oligomeric state, coupled with its ability to bind Sas-6, is predicted to enhance Sas-6 oligomerization and cartwheel formation. This correlates with cellular studies in which Sas-6 and Ana2 dual overexpression was required for cartwheel formation, suggesting that Ana2 potentiates Sas-6 cartwheel formation, potentially through oligomerization (Stevens et al., 2010b). Recent cryotomographic studies of nascent centriole architecture reveal auxiliary protein density connecting the Sas-6-based cartwheel to Sas-4 and the distal microtubule triplets (Guichard et al., 2013). Given Ana2's integral role in Sas-6's cartwheel formation as well as evidence that it binds both Sas-6 and Sas-4, Ana2 is a likely candidate for this density. More work is needed to determine if Ana2 can bridge Sas-6 and Sas-4, and whether the LC8-Ana2 interaction plays a role in this Ana2 function, as it does in neuroblast asymmetric cell division. Our work outlines the structural basis of the LC8-Ana2 interaction, with implications for its role in Ana2 structure and function at the centriole.

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TABLES

| Data Collection | LC8-Ana2 Peptide 1 | LC8-Ana2 Peptide 2/Apo LC8 |
| :---: | :---: | :---: |
| Wavelength (A) | 1.00000 | 1.07426 |
| Space group | $\mathrm{P} 2{ }_{1} 2_{1} 2_{1}$ | P1 |
| Cell dimensions ( $\AA$ ) |  |  |
| $a$ | 51.5 | $36.6 \quad(\alpha=99.3)$ |
| $b$ | 77.9 | $44.8 \quad(\beta=103.0)$ |
| $c$ | 108.9 | $85.9 \quad(\gamma=91.8)$ |
| Resolution (A) | 50.00-1.83 (1.90-1.83) | 50.00-1.90 (1.97-1.90) |
| Reflections |  |  |
| Measured | 108,273 | 70,555 |
| Unique | 37,488 | 36,096 |
| Completeness (\%) | 95.1 (95.2) | 87.5 (47.3) |
| Mean redundancy | 2.9 (2.5) | 2.0 (1.8) |
| I/ $\sigma$ | 13.7 (2.4) | 19.5 (7.0) |
| $R_{\text {sym }}{ }^{\text {m }}$ | 0.08 (0.37) | 0.04 (0.12) |
| Refinement |  |  |
| Resolution ( A ) | 45-1.83 (1.87-1.83) | 36-1.90 (1.95-1.90) |
| $R / R_{\text {free }}$ (\%) | 17.6 (22.1)/20.7 (24.2) | 18.5 (20.1)/23.6 (31.1) |
| No. of reflections, $R / R_{\text {free }}$ | 34,418/1,953 | 33,673/1,991 |
| Total atoms | 3356 | 4580 |
| Protein/Water | 3046/310 | 4320/260 |
| Stereochemical ideality (Rmsd) |  |  |
| Bonds/angles ( $\AA$ / ${ }^{\circ}$ ) | 0.007/0.98 | 0.008/1.07 |
|  |  |  |
| MC/SC/water | 16.5/20.5/31.3 | 15.8/19.9/21.1 |
| $B$-factor Rmsd ( $\AA^{2}$ ) | 3.2 | 4.8 |
| Ramachandran analysis |  |  |
| Favored/allowed (\%) | 98.1/1.9 | 95.7/3.9 |

Table 5.1. LC8-Ana2 Crystallographic Data, Phasing and Refinement. Values in parentheses are for the highest resolution shells unless otherwise denoted. $* R_{s y m}=\Sigma_{\mathrm{h}} \Sigma_{\mathrm{i}} \mid \mathrm{I}_{\mathrm{i}}(\mathrm{h})-$ $\langle\mathrm{I}(\mathrm{h})>| / \Sigma_{\mathrm{h}} \Sigma_{\mathrm{i}} \mathrm{I}_{\mathrm{i}}(\mathrm{h})$, where $\mathrm{I}_{\mathrm{i}}(\mathrm{h})$ is the $\mathrm{i}^{\text {th }}$ measurement and $\langle\mathrm{I}(\mathrm{h})>$ is the mean of all measurements of $I(h)$ for Miller indices $h$.

## CHAPTER 6: DISCUSSION AND FUTURE WORK

## Plk4 employs an assemblage of Polo Box domains to license centriole duplication

Plk4 is a highly conserved serine/threonine kinase required for centriole duplication in nearly all animals, excluding higher plants and fungi (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Rodrigues-Martins et al., 2007; Rogers et al., 2009; Cunha-Ferreira et al., 2009; Carvalho-Santos et al., 2010; Holland et al., 2010; Dzhindzhev et al., 2010). As a member of the Plk family of kinases, Plk4 contains an Nterminal kinase domain and C-terminal "Polo Box" (PB) domains; however, the similarity between Plk4 and its family members stops there. Plk4's kinase domain more closely resembles that of the Aurora family of kinases (personal communication, Yao Liang Wong and Karen Oegema) than the Plk family, indicating that different mechanisms of target recognition and/or kinase regulation may be in place for Plk4 than other Plks. Additionally, Plks 1-3 and 5 contain only two PB domains that are thought to form heterodimers in cis to allosterically downregulate the kinase domain (shown to be true for Plk1 in Xu et al., 2013). In contrast, our work has shown that Plk4 contains three PB domains (Slevin et al., 2012), indicating that Plk4 represents an architectural and evolutionary departure from the other Plk family members. Regardless, Plk4 remains a conserved core member of the centriole assembly pathway, for both canonical duplication mechanisms (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Pelletier et al., 2006) and de novo centriole formation (RodriguesMartins et al., 2007; Peel et al., 2007; Eckerdt et al., 2011).

Due to its essential role in regulating centriole biogenesis, Plk4 misregulation (as well of many of the other pathway elements) is associated with many diseases in humans, including male sterility, primary microcephaly, primordial dwarfism, and other ciliopathies (Nigg and Raff, 2009; Rosario et al., 2010; Bettencourt-Dias et al., 2011; Marthiens et al., 2013; Chavali et al., 2014). Several mutations within specific centrosome components have been identified in these diseases; for instance, the human Ana2 homolog (known as STIL; Stevens et al., 2010a) contains a C-terminal TIM domain, which stands for "truncated in microcephaly" (Bennett et al., 2014). Most of the known mutations within these diseases give rise to centriole and basal body defects or reduced centriole numbers, leading to improper cilia formation required during development. However, some mutations lead to decreased centrosome numbers, mitotic delays, or deregulation of asymmetric cell division (Chavali et al., 2014). Conversely, it has been shown that overexpressed Plk4 in Drosophila is associated with multi-polar mitotic spindles, aneuploidy, and tumorigenesis (Basto et al., 2008), implicating a link between unregulated Plk4 levels and cancer. Indeed, Plk4 is now a target at the forefront of efforts to design new antiproliferative therapeutics. Though no Plk4-specific drugs are currently being tested in clinical trials, several labs within the last couple of years have published drugs said to specifically target Plk4's catalytic activity and reduce centrosome number in tumorigenic cell lines (Laufer et al., 2013; Sampson et al., 2014; Godinho and Pellman, 2014). Much more work regarding Plk4's role in tumorigenic transformation is forthcoming, with data pointing to concurrent mutations in both Plk4 and p53 leading to cancer (Li et al., 2005). It is an exciting time for both cell biologists and pharmacologists, as links between centriole duplication and cancer have revealed Plk4 as a prime target for novel therapeutics.

Of course, Plk4 does not exert its effects on centriole number alone: in humans, Plk4 binds both scaffolding proteins Cep152 (Asl in D.m.) and Cep192 (SPD-2 in C.e.) to attain centriole localization (Sonnen et al., 2013; Shimanovskaya et al., 2014). In human cells depleted of both Cep152 and Cep192, Plk4 is unable to localize to centrioles, underscoring the importance of the binding events between Plk4 and its scaffolding partners (Sonnen et al., 2013). The requirement of binding partners for Plk4's centriole localization is conserved throughout all animals studied; however, Plk4 exclusively uses Asl for its recruitment in D.m., or SPD-2 in C.e. (Dzhindzhev et al., 2010; Pelletier et al., 2006, respectively). Recent work from two labs (Park et al., 2014; Shimanovskaya et al., 2014) has uncovered the mechanism through which Plk4 binds its scaffolds: the Plk4 homodimer forms via lateral interactions formed between PB2-PB2 to create 12 -stranded extended $\beta$-sheet (corresponding to a conformation shown in Figure 2.S1 B, Chapter 2). This is contrary to our published work, which claimed that PB1-PB2 forms a head-to-tail homodimer, with contributions to dimerization made by both PB1-PB1 interactions and PB2-PB2 interactions (Figure 2.2, Chapter 2). However, we presented our chosen dimerization interface (called the " $X$ " dimer) as the most likely biologically relevant dimer due to its extended buried surface area and full use of both PB1-PB2 to create the interface; additionally, the head-to-tail dimerization is consistent with the current model of FL Plk4 dimerization to mediate self-regulation (Rogers et al., 2009). However, the dimerization interface presented in Shimanovskaya et al., 2014, and Park et al., 2014 (called the "Z" conformation) more likely represents the biological dimerization interface for several reasons: it creates a groove that allows for electrostatic interactions between Plk4 and its scaffolding partners, shown in a crystal structure in the Park manuscript; and it better fits a SAXS (small-angle x-ray scattering) envelope of PB1-

PB2 in solution, as shown in the Shimanovskaya manuscript. Therefore, PB1-PB2 forms a Zdimer to interact with Cep152 and Cep192. Further crystal structures of Plk4 in complex with Asl/Cep152 or SPD-2/Cep192 will be essential in learning more about the mechanism through which Plk4 binds its partners, affording centriole localization. These crystallization targets are ongoing in the Slep lab as well as others.

Our work with Plk4's Cryptic Polo Box (CPB) (presented here in Chapter 2 and in Slevin et al., 2012) established PB1-PB2 as bona fide tandem PB domains using structural biology. Despite labeling the seemingly incorrect interface as biological rather than a crystallographic artifact, we also showed that PB1-PB2 forms a dimer consistently in solution via both SEC-MALS and dynamic light scattering. Furthermore, our experiments in Drosophila S2 cells established that the entire PB1-PB2 cassette is required for robust centriole localization, and that removal of the cassette ablated Plk4 localization to centrioles. This work was confirmed using pull-down experiments, in which we established that Plk4 employs PB1-PB2 to bind Asl in D.m. We also found that the homodimerization between PB1-PB2 plays a key role in dimerizing the FL Plk4 molecule, and therefore allows for the established trans autophosphorylation mechanism required to downregulate Plk4 levels to limit centriole duplication to a brief time period. Thus, Plk4 uses its central PB1-PB2 cassette for subcellular targeting, partner binding, and regulating the stability of the FL molecule. More work needs to be conducted to determine whether PB1-PB2 directly affects the catalytic activity of the kinase domain, as it commonly does in Plk1 homologs (Yun et al., 2009; Xu et al., 2013).

Our work characterizing PB1-PB2 led to the observation that PB1-PB2 (whether in the X or Z dimer conformation) displays a "basic patch," or a region with many positive
charges, that is conserved, surface-exposed, and not involved in dimer formation (Figure 6.1 A). Thus, we initially postulated that this basic patch could be employed by PB1-PB2 to bind Asl or other negatively charged binding partners via electrostatic interactions (Slevin et al., 2012). Though the experiments were not completed in time to be included in our 2012 publication, we have since identified a series of residues within the PB1-PB2 basic patch that is responsible for PB1-PB2 subcellular localization (Figure 6.1 B). In our experiments (following the same protocols as outlined for the "Centriole Localization Assay" in Chapter 2), site-directed mutagenesis was used to create charge reversals within targeted surfaceexposed basic residues within PB1-PB2 (Figure 6.1 A). We then assayed construct localization in Drosophila S2 cells using GFP-tagged mutant PB1-PB2 constructs and staining for D.m. D-PLP (pericentrin-like protein). We then identified the minimal set of mutants needed to reduce PB1-PB2 localization to centrioles: K510, R523, K529, and K539 (highlighted in Figure 6.1 A; representative images shown in C). This corresponds very well to the set of mutants used in Shimanovskaya et al., 2014, in which they used in vitro pulldowns and purified proteins to detect the residues important for the Plk4/Asl interaction. They identified Plk4 residues R490, K510, R523, and R594, which collectively overlaps with our identified minimal residue set. Additionally, a recent manuscript detailing the structure of human Plk4 PB1-PB2 in complex with a 60-residue fragment of Cep152 or 58-residue fragment of Cep192 confirmed the importance of electrostatic interactions between Plk4 and its scaffolding partners (Park et al., 2014). Further work is required to determine whether PB1-PB2 only binds scaffolding partners, or if other binding partners (including potential phosphorylation targets) interact with PB1-PB2.


Figure 6.1. Plk4 PB1-PB2 employs a basic patch to localize to centrioles. (A) Left, conservation of PB1-PB2 shown in spherical representation, with $100 \%$ identical residues shown in green and $80 \%$ conserved residues shown in yellow. Right, an electrostatic map of PB1-PB2, displaying the basic patch formed within PB2. Bottom, a surface representation of the minimal four basic-to-acidic residue mutations (all to glutamic acid) required to ablate PB1-PB2 centriole localization. (B) A schematic of wt PB1-PB2 with specific mutations mapped onto the cassette. A total of six point mutations (labeled A-F for clarity) were tested in different combinations. Shown in the bar graph are the localization results for three of the mutant constructs: ABCDEF (all six residues), BCDEF, and CDEF (the minimal K510E, R523E, K529E, and K539E set). All three constructs show a nearly complete disruption in
centriole localization, indicating that PB1-PB2 requires its basic patch for proper localization. (C) Representative images of the centriole localization assay. The protocol is exactly the same as that detailed in Chapter 2, except instead of co-staining for D-PLP, we used a goat anti-Asl serum stained in Cy3 (provided by N. Rusan, NIH). Scale bar, $10 \mu \mathrm{~m}$.

Additionally, the recent atomic structure of Plk4 bound to a Cep152/Cep192 peptide provides compelling evidence for a specific interaction that could potentially mark a new target for developing Plk4-based therapeutics. All of the cancer drugs targeting Plk4 to date have bound its kinase domain, which has many undesired off-target effects due to Plk4's kinase domain sharing high levels of similarity with the Aurora kinases. Making use of new atomic information regarding a different, unique area of Plk4, as well as how it interacts with binding partners to attain localization and license centriole duplication, would be a powerful way to design new drugs with truly high Plk4 specificity levels.

Our work in Chapter 3 (unpublished) details the variation of PB3 structure and oligomerization among species. Interestingly, PB3 takes on both different folding patterns and oligomerization states in a species-dependent manner, indicating that it may not have a conserved role in Plk4 function. Current and future work regarding this divergent domain focuses on three aspects of PB3 function: 1) determining the role of PB3 in human HeLa cells, in which H.s. Plk4 either lacking PB3 or containing D.m. PB3 in its place is examined for centriole overduplication phenotypes; 2) using extensive sequence alignments among PB3 domain homologs to determine conservation and detect a possible evolutionary branching event; 3) collaborating with other labs to discover PB3-specific binding partners. These key experiments are crucial in understanding why Plk4, unlike all other Plk family members, contains three PB domains.

While there is still much work to do to learn how Plk4 employs its array of PB domains to license centriole duplication, there is sure to be exciting implications for those findings. In addition to Plk4 being an important target to develop anti-cancer therapies, future work will focus on a seemingly basic and yet hotly debated topic within the field of centriole biology: what is Plk4's phosphorylation target? Although we know that Plk4 phosphorylates itself (Rogers et al., 2009; Cunha-Ferreira et al., 2009; Holland et al., 2010; Sillibourne et al., 2010), FBXW5 (Puklowski et al., 2011; Pagan and Pagano, 2011) and Chk2 (Petrinac et al., 2009), we do not yet know if it has a direct target within the centriole assembly pathway (Lettman et al., 2013). More information at the structural level and at the cellular level as to the catalytic targets of Plk4, in addition to its binding partners, will be critical in understanding the mechanism of Plk4-dependent centriole biogenesis. It is also paramount to determine, through comparative alignments of Plk4 orthologs throughout all eukaryotic branches, the evolutionary branchpoints that created Plk4, a Plk unlike any other. This information will provide clues as to the presence of an additional PB (PB3), and help answer the ultimate question of how Plk4 employs its PBs collectively to license centriole duplication.

## LC8 potentiates Ana2 oligomerization, with potential implications for the role of Ana2 in asymmetric cell division and centriole duplication

In addition to acting as a key downstream element of centriole duplication (Stevens et al., 2010a), Anastral-2 (Ana2) is an essential factor in maintaining correct spindle orientation in D.m. neuroblast asymmetric divisions (Wang et al., 2010; see Chapter 4). The initial discovery of the LC8/Ana2/Mud complex in D.m. NBs led the authors to postulate that LC8 (also known as Cutup, or Ctp) simultaneously binds Ana2 and/or Mud in addition to
dynein intermediate chain (DIC), which serves as an adaptor to dynein (Wang et al., 2010). However, research from several independent labs have converged to demonstrate that LC8 does not simultaneously bind other dynein chains and its other varied targets, indicating that LC8 likely binds Ana2 and/or Mud independently of the cytoplasmic dynein motor complex (Benison et al., 2007).

Our work regarding the relationship between LC8 and Ana2 (presented in Chapter 5 and in Slevin et al., 2014) identified two LC8 binding sites within Ana2, which very loosely conform to the binding motif $\mathrm{K}_{-3} \mathrm{X}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1}$ : Ana2 159-168 ( $\mathrm{A}_{-3} \mathrm{G}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{~T}_{1} \mathrm{D}_{2}$, called Site (or Peptide) 1) and 237-246 ( $\mathrm{T}_{-3} \mathrm{G}_{-2} \mathrm{~T}_{-1} \mathrm{Q}_{0} \mathrm{C}_{1} \mathrm{D}_{2}$, called Site (or Peptide) 2). We solved two independent structures of the LC8 homodimer bound to each of the Ana2 peptides and used ITC to characterize the binding of each peptide to LC8. We found that, similar to other known LC8 targets, each Ana2 site binds LC8 as a parallel dimer with affinities well within the range of known LC8 interactors. Importantly, the two LC8 binding sites that we determined flank a central predicted coiled-coil domain; furthermore, attempts in our lab to purify different portions of Ana2 were unsuccessful due to protein insolubility. However, we found that co-purification of a middle portion of Ana2 (Ana2M, residues 156-251) with LC8 yielded a stable complex comprising 8 LC 8 protomers and 4 Ana 2 M protomers (LC8 $8_{8}{ }^{-}$ Ana $2 \mathrm{M}_{4}$, Figure 5.6). Thus, LC8 is an important factor that binds Ana2, promotes its stability, and mediates its oligomerization (Slevin et al., 2014).

This work has important implications for Ana2's diverse roles in centriole duplication as well as asymmetric cell division. In the D.m. NB, Ana2 localizes preferentially to the daughter centrosome at the apical cortex, mirroring the apical localization of Mud. The additional presence of LC8 in the daughter centrosome suggests that LC8-mediated Ana2
oligomerization stabilizes it, allowing for enhanced centrosome maturation in an as-yet undetermined mechanism. Whether the stabilized Ana2 tetramer exerts control over spindle orientation via a Mud/Pins/G $\alpha_{i}, \mathrm{Mud} /$ dynactin/dynein, or Mud/MT pathway is yet to be established, and discerning among these mechanisms is an important step in connecting centrosome asymmetry to spindle orientation control in ACDs.

The role of Ana2 in centriole duplication is much more opaque. While Ana2 is largely acknowledged to be one of few proteins actually required for centriole assembly (the main five consisting of Asl, Plk4, Sas-6, Ana2, and Sas-4; Goshima et al., 2007), it is very unclear as to how Ana2 affects centriole assembly. While Asl and Plk4 are considered to be scaffolding and signaling components, respectively, Sas-6 is the first molecule to make a stable structure within the nascent centriole: the 9 -spoked cartwheel (van Breugel et al., 2011; Kitagawa et al., 2011). However, biochemical evidence indicates that lateral Sas-6 interactions are very weak, implying that other factors are needed in cells to faithfully build the 9-spoked cartwheel (Kitagawa et al., 2011). Indeed, Sas-6-based centriole amplification is only observed when Sas-6 is overexpressed in tandem with Plk4 and Ana2, suggesting that Sas-6 and Ana2 are both needed for cartwheel formation (Stevens et al., 2010a). Additionally, co-overexpressed Sas-6 and Ana2 form "tubules" in D.m., a phenomenon that requires Ana2 (Stevens et al., 2010b). Thus, while it is likely Ana2 plays a direct role in stabilizing Sas-6 oligomers, and ultimately cartwheel formation, it has not been definitively shown. Whether the LC8-mediated LC8 tetramer is necessary to build the Sas-6 higher-order structures is yet to be shown. LC8 is not strictly required for centriole duplication (Goshima et al., 2007); furthermore, our work has failed to show an LC8 requirement for centriole duplication in S2 cells (data not shown). However, LC8 is a ubiquitous protein, and its
depletion is expected to have many pleiotropic effects; therefore, it is difficult to dissect its role solely in centriole duplication without observing off-target effects. One of the next steps in the field of centriole biology is an enormous yet very important undertaking in understanding the molecular mechanisms underlying centriole assembly: to create entire centrioles in vitro. Now that we have a thorough understanding of the main components in centriole biogenesis, an in vitro system that allows for high-resolution visualization of centriole morphology is required to determine the effects of depleting individual proteins. This would be invaluable in determining the precise role of Ana2 in centriole formation. In the meantime, it would be very helpful to create different N - and C -terminal nanolabels (e.g. gold particle labeling) for Ana2 to visualize organization and localization of the FL molecule in the context of the centriole, via electron microscopy. Our lab is very interested in the outcome of these types of experiments to better understand the structural role of Ana2 in centriole assembly.

## Concluding remarks

Centrioles are fascinating structures. They form the basis of centrosomes and cilia, organelles responsible for nucleating polarized microtubule networks in eukaryotes. Despite the fact that flies lacking centrioles can develop to adulthood (Basto et al., 2006), centrioles remain highly conserved eukaryotic structures with a characteristic 9 -fold symmetry. Mutant alleles for individual centriole components results in abnormal centriole number and/or morphology, and such mutations are implicated in human diseases such as microcephaly, male sterility, and cancer (Nigg and Raff, 2009; Basto et al., 2008), underlining the importance of regulating exact centriole number. The formation of the centriole, with its
inherent 9-fold radial symmetry, is an intricate and yet faithful process that has fascinated structural and cell biologists alike over the last decade or so. Many fundamental questions remain, including how does a cell detect its centriole number? What are the regulatory mechanisms preventing overduplication? Our work with both Plk4 and Ana2 has highlighted some of the structural-based mechanisms of centriole assembly and licensing thereof, though many questions remain. The future of the centriole biology field is bright, with newer highresolution techniques allowing for more and more questions to be addressed in situ, as we continue to uncover the conserved mechanisms of centriole duplication.

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