Utilizing S2 Cells to Study the Molecular Mechanisms Regulating Centriole Duplication

by

Jonathan Nye

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Jonathan Nye, titled Utilizing S2 Cells to Study the Molecular Mechanisms Regulating Centriole Duplication and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Gregory Rogers  
Date: July 31st, 2014

Paul Krieg  
Date: July 31st, 2014

Kimberly McDermott  
Date: July 31st, 2014

Lonnie Lybarger  
Date: July 31st, 2014

Ted Weinert  
Date: July 31st, 2014

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director: Gregory Rogers  
Date: July 31st, 2014
STATEMENT BY AUTHOR

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SIGNED: Jonathan Nye
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- Jonathan Nye, Daniel W Buster, and Gregory C Rogers. The use of cultured Drosophila cells for studying the microtubule cytoskeleton. 2014. Methods in Molecular Biology. (c) Humana Press, a part of Springer Science+Business Media, LLC.


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Abstract

Centrosomes are complex organelles consisting of a pair of small microtubule based structures called centrioles embedded in an amorphous cloud of pericentriolar material (PCM). These organelles are critical for proper mitotic spindle assembly and orientation, and can also migrate to the plasma membrane where, as basal bodies, they serve to nucleate cilia. Centrioles are the core duplicating elements of the centrosome and, similar to DNA, duplicate once per cell cycle during S-phase. Errors in this process can lead to a cell that contains too many or too few centrosomes and are thought to promote tumorigenesis by directly promoting genomic instability and loss of polarity in stem cells. Polo-like kinase 4 (Plk4) and Asterless (Asl) are both essential for centriole duplication to occur and overexpression of either of these proteins leads to cells that contain too many centrosomes, a condition known as centrosome amplification. Interestingly, both of these proteins also have the unique ability to promote de novo formation of centrioles in cells that normally lack centrioles.

Plk4 is a member of the Polo-like kinase family of proteins and is named after the founding member Drosophila Polo. In humans, there are four members (Plk1-4) that all share sequence similarity and an N-terminal ser/thr kinase domain. Plk’s 1-3 all contain two characteristic Polo box (PB) motifs, downstream of the kinase domain, that consist of a six stranded β-sheet lying across a C-terminal α-helix. However, Plk4 was thought to be unique among family members since it only contained one C-terminal PB domain and a larger cryptic polo box domain that showed very little sequence similarity to known PBs. However, we performed an in-depth structure/function analysis of this cryptic polo box region and were able to determine, through x-ray crystallography, that Plk4 is unique...
among Plk family members not because it contains one PB domain but, in fact, because it contains three polo box domains. Thus, the cryptic polo box domain contains two previously unidentified polo boxes, termed PB1-PB2, upstream of the C-terminal PB3. Furthermore, we found that PB1-PB2 is necessary for proper localization of Plk4 and that the entire PB1-PB2 cassette is necessary for binding to Asl. Our results also indicate that the PB1-PB2 domain plays a critical role in regulating Plk4 autophosphorylation and degradation, in order to restrict centriole duplication to once and only once per cell cycle.

Previously Asl has been shown to be not only a binding partner of Plk4 but also a substrate for Plk4 kinase activity, however, the functional significance of this phosphorylation has remained elusive. Our work has shown that Asl phosphorylation by Plk4 is conserved from flies to humans and is restricted to the N-terminal (Asl-A) region. In total we identified 13 phosphorylated residues via mass spectrometry. Analysis of phosphorylation mutant constructs revealed that Plk4 and Asl are involved in a novel feedback loop controlling centriole duplication. This feedback loop consists of two important features: first, Asl oligomerization stimulates Plk4 kinase activity and thus promotes centriole duplication and second, the Asl-A region can be phosphorylated by Plk4, preventing further Asl oligomerization, which in turn limits the amount of active Plk4. We propose that this feedback loop is a crucial step in limiting a mother centriole to only one daughter per cell cycle.

This work represents a significant advance in our understanding of the processes that govern the centriole duplication pathway and specifically the structure and function of two critical components, Plk4 and Asl. A detailed understanding of the molecular mechanisms controlling centriole biogenesis is an essential first step in our goal of understanding their role in
tumorigenesis and may serve as a guide for future studies focused on targeting this pathway for the prevention or treatment of cancer.

References


CHAPTER ONE: THE USE OF CULTURED DROSOPHILA CELLS FOR STUDYING THE MICROTUBULE CYTOSKELETON

1.1 Abstract

Cultured *Drosophila* cell lines have been developed into a powerful tool for studying a wide variety of cellular processes. Their ability to be easily and cheaply cultured as well as their susceptibility to protein knockdown via double-stranded RNA-mediated interference (RNAi) has made them the model system of choice for many researchers in the fields of cell biology and functional genomics. Here we describe basic techniques for gene knockdown, transgene expression, preparation for fluorescence microscopy, and centrosome enrichment using cultured *Drosophila* cells with an emphasis on studying the microtubule cytoskeleton.

1.2 Introduction

Since the early 1900’s when Thomas Morgan was performing his ground-breaking work on the role of chromosomes in heredity that eventually won him a Nobel Prize, *Drosophila melanogaster* has proven to be one of the most productive and widely used model systems for the study of a broad range of biological processes. More recently, the use of cultured *Drosophila* cell lines has increased in popularity due to their ability to be cheaply and easily maintained while exploiting the extensive knowledge gained from over a century of fly research. There are over 100 different cell lines available from the Drosophila Genomics Research Center (DGRC), a non-profit repository of *Drosophila* cell lines and DNA clones. The most commonly used cell lines include S2 and Kc cells: these cell lines, derived from spontaneously immortalized cells arising in cultures of disrupted fly embryos, are conveniently maintained at room temperature.
Interestingly, many cultured *Drosophila* cells have the unique ability to take up dsRNA through receptor-mediated endocytosis, meaning that selective knockdown of targeted proteins can be accomplished by simply adding long double-stranded (ds)RNA oligomers directly to the media and “soaking” the cells (1, 2). This eliminates the need for the often costly and toxic short interfering (si)RNA transfection methods used for mammalian cell cultures and provides a more rapid and cost-effective way to study the effects of a gene silencing. Typically, a protein of interest can be depleted from RNAi-treated cells within 3-7 days, although the length of treatment will vary depending on the turnover rate of the target protein and the possible effects of target protein knockdown on the overall health of the cells. These characteristics -- combined with a fully sequenced and well-annotated genome -- have made cultured *Drosophila* cells ideal for the experimental manipulation and analysis of cellular processes. For example, RNAi-based high-throughput genome-wide screens in S2 cells are responsible for significant advances in our understanding of mitotic phenomena, including the identification of fundamental proteins necessary for proper mitotic spindle assembly and function as well as centrosome assembly/duplication (3-5).

In this chapter, we will outline techniques that take advantage of the useful traits of this extraordinary model system including: gene knockdown, transgene expression, and fluorescence microscopy of live and fixed cells. We will focus on the use of cultured fly cells for studying the microtubule cytoskeleton, mitosis, and centrosome biology, including centrosome enrichment, but stress that this system and the protocols described here can be exploited to examine numerous aspects of cell biology. Importantly, most cultured *Drosophila* cells, like many cell types in the fruit fly, display an unconventional cycle of centrosome function as compared to
mammalian cells (Figure 1.1). Centrosomes do not exist during most of the cell cycle in these cells (6). Instead, microtubule nucleation is apparently random and independent of centrioles and γ-tubulin (7). Most cultured cells do contain centrioles though, but these do not nucleate microtubule growth until mitotic entry when they undergo a maturation process and recruit pericentriolar material (PCM). Therefore, bona fide centrosomes only exist during mitosis to assist in mitotic spindle assembly. Upon mitotic exit, centrosomes fragment whereby centrioles shed their PCM (Figure 1.2). Notably, within a culture there exist cells with supernumerary centrioles and cells that completely lack centrioles. However, the presence of too many or two few centrioles has surprisingly little consequence on overall cell health or proliferation save for a subtle increase in the duration of cell division. This is due to centrosome-independent mechanisms of spindle assembly (8), as well as the robust ability of spindles to cluster excess centrosomes and achieve bipolarity (Figure 1.3) (9). Thus, cultured Drosophila cells present an extraordinary system to discover molecular insight into the important processes of microtubule nucleation and spindle assembly in addition to new mechanisms that cope with centrosome amplification, a phenomenon that is frequently observed in cancer cells.

1.3. Materials

2.1. Maintaining Drosophila Cell Lines

1. Tissue culture flasks: Tissue culture grade plastic Petri dishes or culture flasks are suitable. For general maintenance, we use ‘T25’ (25 cm² culture surface area) culture flasks with plug-seal caps containing 5-10 mls of medium. For transfected cells, we use 6-well culture plates, with 1-2 mls of medium per well.

2. Medium: There are a variety of media that can be used for Drosophila cell culture. A number of serum-free media are available such as SF-900 II (Life Technologies),
Insectagro Sf9 (Mediatech), SFX-Insect (Hyclone), and Insect-Xpress (Lonza). These media are a cheap alternative to some that need to be supplemented with heat-inactivated fetal calf serum (FCS) such as Schneider’s medium or Shield and Sang’s M3 medium. Obviously, the specific cell line dictates the medium needed, but some cell lines can be maintained in more than one medium (e.g., SF-900 II or Schneider’s/10% FCS in the case of S2 cells). The specific experimental procedure may also be a factor to consider. For example, Schneider’s/10% FCS medium may be a better option for live cell microscopy experiments since it exhibits less autofluorescence than SF900 II. Also, some serum-free media induce a higher basal level of expression of those transgenes controlled by the inducible metallothionin promoter, compared to Schneider’s/10% FCS medium. Finally, antibiotics may be added to cultures but are not necessary if a researcher’s sterile technique is adequate. Antibiotics are used at the same concentrations for mammalian cell culture: 50-100 U/ml penicillin-G and 50-100 μg/ml streptomycin. We use a commercial cocktail of antibiotics and fungicide (MP Biomedicals).

3. Incubator: An incubator is not needed unless the ambient temperature fluctuates significantly from room temperature (~25°C). If capped culture flasks are used, then it is usually unnecessary to use a humidified chamber.

2.2. Design and Production of dsRNA for RNAi

2.2.1. Production of DNA Template

1. Taq DNA Polymerase.
2. DNA: 1) cDNA, 2) genomic DNA, or 3) aliquot from a previous PCR reaction.
3. 10X PCR Buffer: 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM MgCl₂. (Vary the MgCl₂ concentration as needed.)
4. 10X dNTP mix: 1.25 mM each nucleotide (5 mM total) in nuclease-free H₂O (pH 7.5); sodium salts of dATP, dCTP, dGTP, and dTTP.

5. Primers (see below).

2.2.2. Purification of DNA Template


2.2.3. In vitro Transcription Reaction

1. 5x Transcription Reaction Buffer: 400 mM HEPES-KOH (pH 7.5), 120 mM MgCl₂, 200 mM DTT, 10 mM spermidine (Sigma). Store at -20°C. Optionally, 50 mg/ml PEG-8000 (Sigma) can be added to increase yield.

2. rNTP Solution: mixture containing 62.5 mM of each nucleotide in RNase-free water; pH 8.0; stored at -20°C. Sodium salts of ATP, CTP, GTP, and UTP. Since sodium, potassium, and ammonium reportedly inhibit T7 RNA polymerase, then using the lithium or Tris salts of the nucleotides could increase the speed or yield of the reaction.

3. Pyrophosphatase: 0.1 U/µl. (ThermoFisher).


5. RNase-free DNase: 1 U/µl. (Promega).

6. Nuclease-free H₂O.

2.3. Nucleofection of Cultured Drosophila Cells

1. Nucleofection Solution: 5 mM KCl, 15 mM MgCl₂, 60 mM Na₂PO₄, 60 mM NaH₂PO₄, 50 mM D-mannitol. Adjust pH to 7.2, if necessary. Sterile filter and aliquot for use. Store at 4°C. (See Note 1)

2. Electroporation Cuvettes: 2 mm gap size, sterile, disposable electroporation cuvettes
(VWR). We re-use cuvettes that have been extensively washed and then sterilized with UV light.

3. Transfer pipets: sterile, fine tip, small bulb, disposable transfer pipets (Samco Scientific).

4. Nucleofection Device: Marketed by Amaxa (now owned by Lonza); the Nucleofector 2b device is currently the most basic model.

2.4. Preparation of Glass-Bottom Dishes

1. 35 mm plastic petri dishes: Dishes do not need to be sterile or tissue culture grade. Verify that the 35 mm dishes will fit on the stage of your inverted microscope. We use 35 x 10 mm polystyrene dishes (BD Biosciences).

2. Cover glass: Either round or square microscope-quality cover glass can be used. Verify that the cover glass is sufficiently large to cover the ¾ inch hole that you will make in the petri dish, but not so large that it extends beyond the edge of the dish when it is glued in place. We use number 1.5, 22 x 22 mm square cover glass (VWR).


5. Electric drill: Variable-speed electric drill, preferably with a lock-on button.

6. Adhesive: Sylgard 184 silicone elastomer kit (Dow Corning). This kit contains 2 components: the base, and the catalyst that cures the adhesive after base and catalyst are mixed in a 10:1 weight ratio.

7. Drilling surface: The surface used for drilling should not offer much resistance to the bit, but should be firm enough to support the weight of the drill. We use a thick-walled styrofoam box whose surface has been covered with a layer or two of wide-diameter shipping tape (e.g., Scotch packaging tape) to prevent bits of styrofoam from being
dislodged while drilling.

8. Device for gripping the dish: Pliers or vise grips with short pieces of Tygon tubing slipped over the jaws provide a good way to grip the dish while drilling. We use Irwin GV6 pliers.

2.5. Immunofluorescence and Live-Cell Imaging of Cultured Cells

1. Concanavalin-A: 0.5 mg/ml in distilled H₂O. If to be used for imaging live cells, then sterile filter the solution and aliquot. Store at -20°C.

2. Fixation Buffer: Many different fixation buffers are mentioned in the literature; we describe two buffers below. The first is preferred when the microtubules of fixed cells are to be visualized; the second is often adequate for generally preserving epitopes and is a starting point when developing a new fixation protocol. Including a mild detergent (e.g., 1 mg/ml saponin) may improve fixation.
   a. PME: 100 mM Pipes (pH to 6.2 with KOH), 2.5 mM MgCl₂, 0.5 mM EDTA, 5 mM EGTA. The composition of BRB80, another microtubule-preserving buffer, is similar: 80 mM Pipes (pH 6.8), 1 mM MgCl₂, 1 mM EGTA.
   b. PBS: 6.13 mM NaH₂PO₄, 3.87 mM Na₂HPO₄, 4 mM KCl, 130 mM NaCl, pH 7.0.

3. Fixative: Optimal fixation conditions are empirically determined. Some guides to selecting the appropriate fix method are mentioned further below.
   a. Aldehyde Fix: Commonly, a solution of 10% formaldehyde in buffer is used for fixation. However, mixtures of formaldehyde (EM Science) and glutaraldehyde (EM Science) are often used (typically, 2.5-5% formaldehyde, 0.1-0.5% glutaraldehyde), but then a subsequent wash with NaBH₄ (0.5% in PBS, 5 min.) is needed to reduce
residual glutaraldehyde to prevent autofluorescence. Make aldehyde solutions fresh.

Buffers with free amines (e.g., Tris) should not be used for aldehyde fixation.

b. Methanol Fix: Cold (-20°C), anhydrous, premium-grade methanol is preferred; traces of water can be removed by adding drying beads (3Å pore size molecular sieves; Sigma) to the methanol storage container.

c. Methanol/Formaldehyde Fix: 90% methanol / 10% formaldehyde (by volume), 5 mM sodium bicarbonate (pH 9.0), -70°C, 10 min. This fix is not optimal for microtubule preservation, but has been used to preserve a variety of epitopes.

4. PBS/Triton X-100: 0.1% (by volume) Triton X-100 in PBS.

5. Blocking Buffer: 5% (by volume) normal goat serum (Sigma) in PBS/Triton X-100. Sterile filter (or add 0.02% [w/v] sodium azide) and store at 4°C.

6. Mounting Medium: 90% (by volume) glycerol, 10% PBS (10X), 0.1 M propyl gallate (Sigma). Rock overnight at room temperature to completely dissolve propyl gallate. Protect from light and store at -20°C.

7. Commonly Used Stains and Antibodies: For microtubule immunostaining, the anti-α-tubulin mouse monoclonal, DM1A (Sigma; use at 2000x dilution), is excellent. Fluorophore conjugates (e.g., FITC) of DM1A are also available, but must be used at low (~5 fold) dilution. For centrosome immunostaining: anti-γ-tubulin mouse monoclonal, GTU-88 (Sigma). Centriole immunostaining: polyclonal anti-D-PLP (14). Mitotic chromatin: anti-phospho-Ser10-histone H3 (rabbit polyclonal; mouse monoclonal Millipore). DNA: Hoechst 33258 (Sigma).

2.6. Purification of Centrosomes
1. PBS: 6 mM NaH$_2$PO$_4$, 4 mM Na$_2$HPO$_4$, 4 mM KCl, 130 mM NaCl, 0.05% NaN$_3$. (see Note 2) (Need ~20 ml / prep.)
   a. x PBS/8% sucrose: 8% (w/v) ultrapure sucrose in 0.1 x PBS, 0.05% NaN$_3$. (Need ~15 ml / prep.)
2. 8% sucrose: 8% (w/v) ultrapure sucrose in ddH$_2$O, 0.05% NaN$_3$. (Need ~20 ml/prep.)
3. Tris Buffer: 1 mM Tris-HCl, pH 8.0, 8 mM β-mercaptoethanol (β-ME; add fresh).
4. Tris/Igepal CA-630: 0.5% (v/v) Igepal CA-630 in Tris buffer; if necessary, warm to 30°C to dissolve detergent; 8 mM β-ME (add fresh). (Need ~50 ml/prep.)
5. PE/Igepal CA-630: 0.1% (v/v) Igepal CA-630 in 1x PE, 0.05% NaN$_3$. This is only used to make the Ficoll cushion below.
6. Ficoll cushion: 20% (w/w) Ficoll 400 (average M$_W$ = 400,000) in PE/Igepal CA-630.
   [To 10 g of Ficoll, add PE/Igepal CA-630 until reaching 50 g. Stir at room temperature until Ficoll dissolves. Just before use, add 8 mM β-ME.] (Need 3 ml/prep.)
7. 50 x PE: 500 mM PIPES (pH to 7.2 with KOH), 50 mM EDTA, 0.05% NaN$_3$, 400 mM β-ME (add β-ME fresh). (Need < 1 ml/prep.)
8. PE/TX-100: 0.1% (v/v) TX-100 in 1 x PE, 0.05% NaN$_3$. (Need ~45 ml/prep.)
9. 20% sucrose gradient solution: 20% (w/w) ultrapure sucrose in PE/TX-100 solution. [To 20g of sucrose, add PE/TX-100 until mass is 100 g; make 0.05% NaN$_3$. Just before use, add 8 mM β-ME.] (Need ~18 ml / prep.)
10. 70% sucrose gradient solution: 70% (w/w) ultrapure sucrose in PE/TX-100 solution. [To 70 g of sucrose, add PE/TX-100 until mass is 100 g; make 0.05% NaN$_3$. Just before use, add 8 mM β-ME.] (Need ~20 ml/prep.)
11. 20-70% sucrose gradient: To make a 32 ml gradient in a Beckmann centrifuge tube
(344058; capacity of these tubes is ~38 mls), follow the recipe in Table 2. For each layer, mix the indicated amounts of 20% and 70% solutions, then gently add the mixture to the top of the gradient. When all layers have been added, let stand 1 hr, room temperature, without agitation. Then move to 4°C long enough to chill the gradient.

1.4. Methods

3.1. Maintaining Drosophila Cell Lines

These protocols have been optimized for S2 and Kc cell lines, however they should be useful guides for culturing other Drosophila cell lines. For info on other experimentally useful cell lines see Table 1.

1. Cultured Drosophila cells are incubated at room temperature (~25°C) with atmospheric CO₂ levels in non-ventilated tissue culture treated flasks or petri dishes. The frequency of passage naturally depends on the cell line’s doubling time (which in turn is affected by culture temperature and medium) (Table 1). In the case of S2 cells, passaging is required approximately every 4-5 days (see Note 3).

2. Dislodge the cells using a method appropriate for the specific cell line (see below). Then disperse any clumps of cells using gentle trituration (see Note 4).

For loosely adherent lines like S2 or Kc cells, dislodging cells is accomplished by pipetting medium onto cells to release them from the bottom of the flask.

For more tightly adherent lines like S2R+, Kc167, or D17-c3 cells, several techniques can be attempted to dislodge cells: 1) Fully confluent cultures may dislodge as a sheet when medium is pipetted onto them. 2) Scrape the cells from the surface. 3) Cells can be released using commercial cell disassociation solutions which can contain proteases (e.g.,
ICT’s Accutase, or Gibco’s trypsin/EDTA) or can be enzyme-free (e.g., Invitrogen). When using trypsin, cells must first be washed to remove FCS, then trypsinized, and finally treated with medium/FCS to inactivate the trypsin.

3. Remove a portion of the cell suspension and transfer to a new flask at a dilution ratio in the range of 1:3 to 1:5 (cell suspension : fresh media). S2 cells are most adherent when transferred to new tissue culture treated plastic; however, once confluent they can begin to grow in suspension and in large colonies.

3.2 Design and Production of dsRNA for RNAi

These protocols can serve as a less expensive alternative to other commercially available dsRNA synthesis kits and can be very useful if large amounts of dsRNA are needed.

3.2.1. Primer design

In order to produce dsRNA using in vitro transcription, a DNA template containing T7 promoter sequences must first be made by PCR amplification. Primers should be designed to amplify a ~500 bp region of the gene encoding the target protein, although dsRNAs of 150-3000 bp have been shown to work. The chosen template sequence can span one or more exons of the target gene, but a stringent requirement is that the sequence should be free of any ≥19 bp stretches present in the cDNA sequence for any other protein. Be aware that a single protein can have isoforms that may vary by sequence and that the template sequence must be chosen appropriately. ‘Snapdragon’ is a simple primer design program, available through the Drosophila RNAi Screening Center, which will identify primer sequences appropriate to generate long dsRNAs while also minimizing off-target effects (http://www.flyrnai.org/snapdragon_doc1.html). While this method targets exon sequences for knockdown, it may be advantageous to
target the 5’ or 3’ UTR of a gene. dsRNA generated from a gene’s UTRs is used in gene replacement experiments to knock-down endogenous protein (because mRNA contains the UTRs and so is depleted by RNAi) while sparing the exogenous replacement protein (because the transgene lacks the UTRs). Finally, after selecting the gene-specific template sequences for both primers, the T7 promoter sequence (5’-TAATACGACTCACTATAGGG) must be added to the 5’ end of each primer to allow in vitro transcription by T7 RNA polymerase. Custom synthesis of primers is commercially available from many vendors (e.g., Operon, Invitrogen).

3.2.2. Production of the DNA Template

1. PCR Amplification: DNA template for in vitro transcription reactions is generated using standard PCR procedures. We first perform a small-scale PCR reaction with new primers and confirm by agarose gel that a band of expected size has been amplified. Then five identical 100 µl PCR reactions are performed using low-cost Taq polymerase (we sacrifice perfect fidelity for economy), many reaction cycles (35), and an initial template that is determined by availability: (1) 50 ng (per 100 µl reaction) of cloned EST cDNA obtained from a Drosophila cDNA library (e.g., DGRC, https://dgrc.cgb.indiana.edu/index.html), (2) 1 µg (per reaction) of S2 cell genomic DNA (be aware that intron sequences are present in the amplified product if the target sequence spans multiple exons), or least preferably (3) 0.5-2 µl (per reaction) of a previous PCR reaction which amplified the same target sequence.

3.2.3. Template Purification

1. Combine all of the PCR reactions in a single RNase-free 1.7 ml plastic microcentrifuge tube. Add an equal volume phenol/chloroform/isoamyl alcohol and mix well by hand. Centrifuge for 5 min at 20000 x g, room temperature.
2. Carefully and slowly remove the upper aqueous phase while avoiding the lower organic phase and interface, and transfer to a new 1.7 ml tube. Add an equal volume of chloroform/isoamyl alcohol and mix well. Centrifuge as before.

3. Carefully remove the upper aqueous phase and transfer to a new tube. Add 1/10 the volume 3 M sodium acetate, mix, and then add 2 volumes cold (-20°C) 100% ethanol and mix. Incubate at -80°C for 30 min. (Use only explosion-proof freezers.)

4. Centrifuge for 10-20 min 20000 x g at 4°C.

5. A white pellet should be visible at this point. Discard the supernatant. Add 1 ml of 70% ethanol. (The pellet will not dissolve in this wash, but may dislodge or fragment.) Centrifuge for 5 min, 20000 x g, at 4°C.

6. Remove the supernatant as completely as possible. Then add 50 µl of nuclease-free H₂O to dissolve the pellet.

7. Analyze by agarose gel to confirm the quality of product; determine its concentration by measuring the OD₂₆₀ of a diluted aliquot (µg/ml concentration = OD₂₆₀ x 50 x dilution). Store the stock solution at -20°C.

### 3.2.4. dsRNA Synthesis

Thaw all stock solutions to room temperature; keep only the enzymes on ice. Assemble the *in vitro* transcription reaction at room temperature to avoid precipitating spermidine in the reaction buffer.

<table>
<thead>
<tr>
<th></th>
<th>Volume (µl)</th>
<th>Final Quantity</th>
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<tbody>
<tr>
<td>Nuclease-free H₂O</td>
<td>54</td>
<td>(to bring total reaction volume to 100 µl)</td>
</tr>
<tr>
<td>5x Reaction buffer</td>
<td>20</td>
<td>(1x)</td>
</tr>
</tbody>
</table>
rNTP solution    12    7.5 mM each nucleotide
DNA template (1mg/ml)    10    10 µg
Pyrophosphatase    3    0.3 U
T7 RNA polymerase    1    80 U

1. Carefully mix the components (avoid shearing the DNA) and incubate at 37°C for 24-72 hrs (yield often improves with longer incubation).

2. Hydrolyze the DNA template by adding 1 µl of RNase-free DNase to the solution. Mix thoroughly but not excessively. Incubate at 37°C for 1-2hrs.

3. Quantitate the dsRNA by gel densitometry. Standard TAE-based agarose gels are adequate, presumably because dsRNA is less prone to form intramolecular secondary structures than ssRNA and so denaturing gels are unnecessary. To calibrate the mass of dsRNA from band intensity, one of the following can be run on the same gel: 1) a commercial RNA ladder containing RNA fragments of known concentration (e.g., ThermoFisher SM1833); 2) a previously quantitated dsRNA sample (preferably of a size similar to the newly synthesized dsRNA); 3) a commercial DNA ladder with DNA fragments of known concentration (e.g., ThermoFisher SM1331); however, the fluorescence increase of ethidium bromide when bound to dsDNA is about 1.2x greater than when bound to dsRNA, so this method will slightly underestimate the dsRNA concentration.

4. If desired, dilute the dsRNA with RNase-free water. Aliquot into autoclaved, RNase-free microcentrifuge tubes and store at -20°C. The aliquots will be sterile and ready-to-use if reasonable care has been taken to keep the in vitro transcription reaction free from contamination.
Expected Result: When analyzed on an agarose gel, an aliquot of the synthesized dsRNA should yield a single, robust band with the same apparent size as the DNA template. Any smearing can often be reduced by diluting the gel sample. Lack of product can result if the polymerase is inhibited, for example, by NaCl (>30 mM) or pyrophosphate (a by-product of RNA synthesis; hydrolyzed by pyrophosphatase).

3.3. RNAi of Cultured Drosophila Cells

1. Harvest log-phase cells and transfer them to a well of a tissue culture grade 6-well plate (or 35mm dish), using sufficient cells to reach 50% confluency. (Low confluency may cause cells to stop proliferating or die; high cell confluency will decrease the efficiency of RNAi.) Allow the cells to adhere to the bottom of the well (~10 min for healthy S2 cells) (see Note 5).

2. Once the proper confluency has been achieved and the cells have attached to the bottom of the well. Remove the old medium and add 1 ml of fresh medium. (Usually 1 ml of medium is sufficient for a single well of a 6-well plate.)

3. Prepare dsRNA by adding 10 μg of each dsRNA to a sterile 0.5 ml microcentrifuge tube.

4. Remove a small amount of medium (~100 μl) from the well to be treated and add to the 0.5 ml microcentrifuge tube containing the dsRNA. Mix gently and return the medium/dsRNA mixture back to the well. Gently and briefly swirl the plate by hand as to completely distribute the dsRNA. Length of treatment will depend on a variety of factors. (see Note 6)

5. In the literature, different frequencies of dsRNA application have been used, but our experience is that treating cells every other day with new dsRNA is sufficient to deplete
proteins. Therefore, repeat steps B and C every other day until the treatment time course is completed. If the cells are not stored in a humidified chamber, then the sides of the plate or dish can be wrapped with Parafilm to prevent evaporation of the medium.

6. Treated cells can be removed from the well or dish and cell lysates prepared or transferred to coverglass for live cell imaging or immunofluorescence (see below).

**Cells cultured in serum-containing medium:** J.E. Dixon’s lab (UCSD) has reported that RNAi efficiency in S2 cells is increased 10-100 fold if dsRNA is introduced to cells in serum-free rather than serum-containing medium. Therefore, to RNAi-treat *Drosophila* cells maintained in serum-containing medium, first remove the serum-containing medium, add a mixture of serum-free medium / fresh dsRNA to the cells and incubate 30-60 min., and then add more medium and sufficient FCS to restore the required serum concentration.

**Expected Outcome:** In 2000, RNAi-treatment of cultured *Drosophila* cells was discovered to require only the addition of dsRNA to the culture medium (12). Since then, numerous labs have utilized this technique to knock-down most of the proteins encoded by the fly genome. Endogenous proteins are usually depleted in less than a week of RNAi treatment, but factors like target protein turnover rate, toxicity, and characteristics of the cell line can impact the efficiency of RNAi. In short, the details of the protocol should be optimized for each target.

### 3.4. Nucleofection of Cultured *Drosophila* Cells

This method has proven to be the most cost effective and efficient way of transfecting S2 cells. Our protocol is optimized for the Nucleofector (Lonza) electroporation device, however use of other devices may be possible. *(see Note 7)*

1. Resuspend cells by pipetting media onto cells to dislodge them from the tissue culture
flask. (*See Note 4*)

2. Determine cell density by counting an aliquot of cell resuspension.

3. Centrifuge ~2-5 x 10^6 cells at 1500 x g for 2 min, room temperature. Substantial cell death may occur if too few cells are transfected (*see Note 8*).

4. Carefully aspirate away the medium. Gently resuspend the cell pellet in a solution containing 2 µg of plasmid DNA in sufficient transfection solution to make 100 µl total volume.

5. Transfer this DNA/cell mixture to a clean, sterile electroporation cuvette and close with cap.

6. Place cuvette in the Nucleofector device, select program G-030 (G-30 for Nucleofector I; G-030 for Nucleofector II and 2b), and press start to electroporate cells.

7. Immediately add 1 ml of fresh medium to the cell mixture in the cuvette. Transfer the cuvette contents to a well of a 6-well tissue culture plate using a sterile transfer pipette.

8. Though cells may begin expressing the transgene (e.g., a constitutively-expressed GFP-tagged protein) within hours of transfection, we generally allow the transfected cells 24 hrs to recover before using them.

*Expected Result*: Nucleofection will successfully transfect 70-90% of treated cells, in our experience. In addition, the health of nucleofected cells is usually good, if a sufficient number of healthy cells is used and if the expressed protein is not toxic.

**3.5. Preparation of Glass-Bottom Dishes**

This section briefly describes the preparation of glass-bottom cell culture dishes that can be used to visualize live or immunostained cells on an inverted microscope.
3.5.1. Drilling Culture Dishes

1. Place the bottom half of the 35 mm dish on the taped surface of the styrofoam; the dish should be oriented bottom-side-down. Grip the sides of the dish with the pliers.

2. Drill a hole in the dish bottom using the ¾ inch spade bit and the electric drill.

3. After drilling a dish, the edge of the hole may be rough. The bottom surface of the dish needs to be smooth so that the cover slip that will be attached to this surface will lie flat. Remove any rough edges on the dish bottom using the coarse grinding bit and the electric drill. It is convenient to immobilize the drill, use the lock-on button to run the grinder continuously, and then manipulate the dish by hand over the grinder to remove any roughness on the bottom surface near the hole.

4. Remove most of the loose plastic debris from the dishes by briefly rinsing the dish bottoms and then allowing them to dry.

3.5.2. Attaching the Cover Slip

1. Prepare the adhesive by mixing the Sylgard 184 base with the curing agent at a 10:1 weight ratio (base:catalyst). Mix thoroughly using a disposable transfer pipet, ignoring any small bubbles that appear. 8 g of the adhesive mix should be enough for 160 dishes.

2. To apply the adhesive, try cutting the tip of a 3ml transfer pipet at an angle to create an enlarged, oblong opening. Apply the adhesive from this pipet by depositing a small ring of adhesive around the hole on the bottom surface of the dish.

3. Position a cover slip over the hole. The surfaces of the cover slip and dish bottom should make full contact. The adhesive will slowly spread between the surfaces. Store the dish bottom-side-up for 48hrs while the adhesive cures; the curing rate can be increased by storing the dish at 37°C.
4. If necessary, the glass-bottom dishes can be sterilized by placing them in a tissue-culture hood and turning on the sterilizing UV light for several hours. Separate the dish bottoms and lids and lay them out in the hood so that their interior surfaces face the UV light source.

3.6. Immunofluorescence and Live-Cell Imaging of Cultured Cells

3.6.1. Concanavalin-A Treatment

In order to get the best results from immunofluorescence microscopy, it is necessary to coat the surface of the glass slide with the lectin concanavalin-A (ConA). It has been found to induce extensive cell spreading and dramatically improves fixed or live-cell imaging (1).

1. Add sufficient ConA solution to completely cover the upper side (i.e., lid-facing side) of the attached cover glass on the glass bottom dish.
2. After spreading, immediately remove any excess ConA and let dry.
3. ConA-coated dishes can be sterilized by separating the dishes and their lids, placing them in a tissue culture hood so that the surfaces to be sterilized face the UV lamp, and leaving them for at least 1 hr with the UV lamp on. They can then be stored at room temperature for months.

3.6.2. Fixation and Staining

Some proteins require a fast fixation and/or a harsh extraction for successful immunostaining. These include some kinetochore and centriole proteins as well as +TIP proteins, such as EB1. In these cases methanol fixation is optimal. However, a formaldehyde fixative should be used for preserving microtubules (interphase or mitosis). It is important to test different fixation conditions when immunostaining an uncharacterized protein.
1. Apply a small amount of fresh media (~150 µl) to the coverslip of the glass-bottom dish.

2. Resuspend cells by pipetting a stream of media onto the cells to dislodge them from the tissue culture-treated flask. Add cell suspension to the coverslip in a dropwise manner and gently swirl to get an even distribution. For best results cells should be ~50% confluent. Cells will adhere to the coverslip within 5-10 min. Cells will continue to spread and flatten out on the coverslip for about 1 hr.

At this point, cells can be used for live-cell imaging or processed for immunostaining as described below.

3. **Formaldehyde Fixation:** (1) Rapidly remove all media from dish. (2) Briefly wash with appropriate buffer (e.g., PME to preserve microtubules) by gently adding room-temperature buffer to dish and immediately removing. (3) Gently add 2 ml of fixation solution, and incubate for 12 min., room temperature.

**Methanol Fixation:** For this fixation it is necessary to submerge the specimen in cold methanol. (1) Pre-chill 300 ml of anhydrous methanol in a covered 1 L glass beaker in an explosion-proof -20°C freezer. (2) Rapidly remove the medium from the dish. (3) Use long forceps to grip the dish with adhering cells and rapidly plunge it into the beaker with pre-chilled methanol. (4) Return the beaker to the freezer and leave for 15 min.

4. Dump the fixation solution into an appropriate waste container and permeabilize the cells by adding 2 ml of PBS/Triton X-100. Immediately dump the buffer and repeat two more times for a total of three quick washes.

5. Remove the PBS/Triton X-100 and add 2 ml of blocking buffer. Incubate cells at room temperature for 15 min.

6. Prepare primary antibody solution by diluting to the appropriate concentration in
blocking buffer and add ~150 µl to the coverslip. Incubate cells at room temperature for 1 hour or overnight at 4°C in a tupperware container with a wet towel so the samples do not dry out.

7. Remove antibody solution and wash out excess antibody by adding 2 ml of PBS/Triton X-100 for 5 min a total of three times.

8. Dilute secondary antibody in blocking buffer and add to coverslip. Incubate cells for 30 min at room temperature.

9. Wash cells with 2 ml of PBS Triton/X-100 three times for 5 min. If you require DNA labeling with a Hoechst (or DAPI) dye, then dilute the dye in PBS/Triton X-100 and apply to the cells for 5 min. Remove the dye and wash two more times with PBS/Triton X-100, 5 min.

10. Dump the last buffer and add ~200 µl of mounting medium to the coverslip. This mounting medium will not harden and, if the cells need to be re-stained or labeled with a different antibody, can be removed with gentle washing in PBS/Triton X-100. Store dishes at room temperature, protected from the light.

3.6.3. Live-Cell Imaging

Live *Drosophila* cells can be imaged if plated on glass-bottom dishes, using an inverted microscope at room temperature (23-28 °C), in ambient atmosphere. No special considerations are required for imaging live fly cells, other than those that apply to all cells. The usual cautions are required to minimize photobleaching of fluorescently-tagged proteins and to prevent phototoxicity.

3.7. Purification of Centrosomes

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This procedure to enrich centrosomes from cultured *Drosophila* S2 or Kc cells was derived primarily from a procedure to purify centrosomes from cultured mammalian cells (15). (see Note 9)

### 3.7.1. Cell Production and Harvesting

1. Grow up ten T150 flasks of healthy cultured cells to a high confluency.
2. Dislodge cells from flasks and gently pellet the cells. To pellet, we (1) fill four disposable 50 ml conical tubes with cell suspension and (2) centrifuge in swing-bucket rotor at 500 x g, 4°C, 3 min, brake setting = 0. (3) Discard supernatants by aspiration. (4) Then add more cell suspension to the tubes and centrifuge again. Repeat steps 3 and 4 until all of the suspension has been processed.
3. Gently resuspend all cell pellets in ~12 ml (total) cold PBS and transfer to a 15 ml conical tube. Centrifuge in swing-bucket rotor at 500 x g, 4°C, 2 min, brake setting = 0.
4. Discard the supernatant and rapidly wash the pellet by gently resuspending the cell pellet in each of the wash solutions listed below, and then centrifuging. For each wash, use ~12 ml of ice-cold wash solution, then pellet the cells by gently centrifuging in a swing-bucket rotor at 1000 x g, 2 min., and decelerate with a weak brake; use no braking if the resulting supernatant is cloudy with cells. Aspirate off and discard each supernatant.
   Washes: (1) 0.1 x PBS/8% sucrose. (2) 8% sucrose. (3) Tris buffer (remember to add fresh β-ME). (see Note 10)

### 3.7.2. Centrosome Enrichment

1. Lyse the cells by resuspending a total of 20 mls of ice-cold Tris buffer/Igepal CA-630 (+ fresh β-ME) in a disposable 50 ml conical tube and rocking gently at 4°C, 10 min.
2. Add sufficient 50 x PE (remember to add fresh β-ME) to the lysate to make the PE a 1 x
final concentration. (The required volume of 50 x PE will probably be ~0.4 ml.) Mix gently.

3. Transfer to a centrifuge tube (e.g., polycarbonate, 28.7 mm diameter, Sorvall) and centrifuge at 1500 x g (e.g., 3650 rpm in a Beckman JA-20 rotor), 4°C, 3 min. Save the supernatant and discard the small brown pellet that should be apparent.

4. Add 3 ml of 20% Ficoll (+ fresh β-ME) to a centrifuge tube. Then carefully load the supernatant on top of the Ficoll cushion. Centrifuge at 26000 x g, 15 min, 4°C. (We use Ultra-Clear centrifuge tubes [Beckman] in a swing-bucket SW28 rotor, 14000 rpm. Fixed-angle rotors can be used; e.g. Beckman JA-20 rotor, Sorvall centrifuge tube, 14750 rpm).

5. Carefully aspirate off (and discard) the supernatant until almost reaching the interface with the Ficoll cushion (leave about 2-3 mls above the cushion). Collect and pool the remaining material just above and at the interface until a total volume of about 3 mls has been collected. Mix the collected material with 3 mls Tris buffer/Igepal CA-630 (+ fresh β-ME). (This decreases the Ficoll concentration to 10% or less.) Total volume should not exceed 6 mls.

6. Layer the solution (not more than 6 mls) onto the 20-70% sucrose gradient. Centrifuge for 1.5 hrs, 131100 x g, 2°C in a swing-bucket rotor (e.g., SW28 rotor, 27000 rpm). (see Note 11)

7. Carefully collect 0.5 ml fractions starting from the top of gradient.

8. Fractions can be stored for the short-term at 4°C. (Most fractions will freeze if stored at -20°C.)

9. Western blot every second or third fraction (using 5-10 µls of each fraction) and the
“input” material (i.e., the clarified lysate loaded onto the gradient). Probe with appropriate primary (such as the centriole antibody anti-D-PLP) and secondary antibodies.

**Expected Result:** About 76 fractions (0.5 ml) will be collected from the sucrose gradient. When analyzed by SDS-PAGE, Coomassie-stained bands are visible in about the first 25 fractions only. When analyzed by Western blotting for a centrosomal/centriolar marker, the major peak of centrosome-containing fractions is centered at roughly fraction 50 (~60% sucrose). Western blots of centrosome preps following this protocol can be found in (14, 16). However, some fractions near the top and bottom of the gradient also contain the centrosome marker, indicating that either the centrosomes are not homogeneous and/or that the centrosomes have not reached an equilibrium position within the gradient.

**1.5. Notes**

1. We have not tested solutions with a different pH. Since medium for insect cells usually has a lower pH (~6.6) than for mammalian cells, decreasing this solution’s pH may decrease cell trauma.

2. The original acid/base molar ratio used by Mitchison (15) is changed to generate a pH = 7.0.

3. Cell density can have an important impact on cell viability. For example, S2 cell cultures will decline if passaged at too low a density, presumably because trophic factors released by the cultured cells are too dilute when cells are plated sparsely. By limiting the dilution at each passage, the subcultured cells receive more conditioned medium and have increased viability. As an approximate guideline, S2 cells should be maintained at 1-25 x 10^6 cells/ml. S2 cells
(especially if recently transfected) are healthier if maintained somewhere in the high-end of the
density range.

4. If resuspension is too vigorous or extensive, then cells can be damaged, especially if their
health is compromised by an experimental manipulation like a recent transfection. If cell debris
is apparent in the culture flasks after passaging, then resuspension was too vigorous.

5. During the multi-day treatment period, it may be necessary to sub-culture the cells to a new
well or dish to maintain the optimal confluency, particularly for extended treatments and fast-
growing cells. The most common mistake made by beginners is to allow the cells to become
over-confluent.

6. The length of dsRNA treatment necessary to deplete the target protein depends on the
protein’s turnover rate. For relatively rapid-dividing cells (e.g., S2 cells) depletion can usually be
achieved by treating with dsRNA for 3-7 days, while depletion in slower dividing cells (e.g.,
D17-c3 cells [10]) may require longer treatment times. Treatment may need to be stopped
prematurely if extensive depletion of the target protein is toxic to cells, in which case the partial
knock-down would be experimentally analogous to generating a hypomorphic allele. Knock-
down of multiple proteins is also possible although the efficiency of depletion often decreases
(presumably because the endogenous Dicer/RISC machinery becomes saturated). Therefore, the
RNAi treatment regime must be tailored for each target, and protein depletion should be
confirmed by Western blot, if possible. Finally, successful RNAi depends on cell health; only
use cells that have been grown under optimal conditions for several passages.
7. The characteristics of the voltage pulse generated by the Nucleofector device are proprietary. We do not know if other commercial electroporators could supply the required pulse. However, a pulse method for a non-Lonza electroporator has been developed for mammalian cells that transfects as efficiently as the Lonza nucleofect system (13), and a similar strategy would probably work for cultured insect cells.

8. Successful transfection requires healthy cells. Using cells from declining cultures or recently thawed cells will give disappointing results.

9. ~97-99% of the cells in an asynchronous population will be in interphase of the cell cycle when centrioles do not associate with much pericentriolar material (PCM) and do not nucleate microtubule growth (7). Thus, without modification, this protocol will purify centrioles. To enrich for centrosomes (i.e., centrioles with PCM), cells must be treated with 30 µM colchicine for 8-12 hours to accumulate in mitosis, although this will only increase the mitotic index to 15-25%. In addition, major changes are that (a) the addition of reagents to depolymerize the actin cytoskeleton is not necessary, and (b) a different sucrose gradient is used (20-70% instead of 20-62.5%).

10. Washes need to be done quickly and gently, but decelerating too rapidly causes vortexing in the tube and some cells to remain in suspension.

11. If a balance tube is needed, use another centrifuge tube containing 5 M NaCl or another solution of relatively high density. **1.7 Table**
### Tables 1.6

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Ploidy (Primarily)</th>
<th>Commonly used Medium</th>
<th>Doubling Time (hrs)$^1$</th>
<th>Original Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>late embryo</td>
<td>tetraploid (often &gt;4n)</td>
<td>serum-free (SF-900 II)</td>
<td>24</td>
<td>(18)</td>
</tr>
<tr>
<td>S2R+</td>
<td>from Wg receptor expressing S2</td>
<td>(unknown)</td>
<td>Schneider’s, 10% FCS$^2$</td>
<td>39</td>
<td>(19)</td>
</tr>
<tr>
<td>Kc</td>
<td>late embryo</td>
<td>diploid</td>
<td>Schneider’s, 10% FCS$^2$</td>
<td>18</td>
<td>(20)</td>
</tr>
<tr>
<td>Kc$_{167}$</td>
<td>from Kc at passage 167</td>
<td>tetraploid</td>
<td>serum-free (SFX)</td>
<td>24</td>
<td>(21)</td>
</tr>
<tr>
<td>D17-c3</td>
<td>larval imaginal disc (haltere)</td>
<td>(unknown; likely diploid)</td>
<td>Schneider’s, 10% FCS$^2$, 10µg/ml insulin</td>
<td>70</td>
<td>(22)</td>
</tr>
<tr>
<td>BG2-c6</td>
<td>larval CNS</td>
<td>diploid</td>
<td>M3, BPYE$^3$, 10% FCS, 10µg/ml insulin</td>
<td>29</td>
<td>(11)</td>
</tr>
</tbody>
</table>

**Table 1.1 Characteristics of some common and some specialized Drosophila cell lines.**

$^1$ Times are approximate and vary with growth conditions (temperature and medium). Most of these reported doubling times were determined for cells grown at 25°C.

$^2$ FCS is traditionally heat-inactivated (56°C for 30 min with frequent mixing); however, heat inactivation may be unnecessary [Invitrogen. 1995. Expressions. 2(2):11].

$^3$ BPYE = *bactopeptone (2.5 g/liter) and yeastolate (1 g/liter).*

Commercial sources of media and supplements mentioned in table: (1) Schneider’s: Gibco. Reportedly, Schneider’s from Sigma is not adequate for S2R+ and Kc cells, but others have not found this claim to be true. (2) SF900 II: Invitrogen. (3) SFX-Insect: HyClone. (4) M3: Sigma. (5) FCS: Life Technologies.
Table 1.2. Recipe for a 5-layer step sucrose gradient.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>0 mls</td>
<td>1.56</td>
<td>3.13</td>
<td>4.69</td>
<td>6.25 mls</td>
</tr>
<tr>
<td>70%</td>
<td>7 mls</td>
<td>4.69</td>
<td>3.13</td>
<td>1.56</td>
<td>0 mls</td>
</tr>
<tr>
<td>Final %</td>
<td>70</td>
<td>57.5</td>
<td>45</td>
<td>32.5</td>
<td>20</td>
</tr>
<tr>
<td>Volume¹</td>
<td>7 mls</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25 mls</td>
</tr>
</tbody>
</table>

¹ Total volume of gradient = 32 mls.
1.7 Figures

Figure 1.1 **Illustration of the centrosome cycle in cultured *Drosophila* cells.**

1) Throughout interphase, microtubule (MT) nucleation is apparently random and occurs by a mechanism which is independent of both centrioles and \( \gamma \)-tubulin. Centrioles do not recruit pericentriolar material (PCM) or nucleate/organize MT arrays and exist as singlets (at the light microscope level) during G1. 2) During late G1 or S phase, procentrioles assemble on parent centrioles. 3) During G2, procentrioles elongate and continue to do so into mitosis. Prior to mitosis, centrosomes assemble as mitotic kinases promote PCM recruitment to centrioles and nucleate MT growth. 4) After nuclear envelope breakdown, excess centrosomes cluster to achieve spindle bipolarity. 5) In late anaphase, mother-daughter centriole pairs separate (disengage). Lastly, centrosomes fragment after mitotic exit and release centriole singlets into the cytoplasm that eventually shed their PCM.
Figure 1.2. **Centriole disengagement is followed by centrosome fragmentation as S2 cells exit mitosis.** Using time-lapse fluorescence microscopy, *bona fide* centrosomes were visualized in live stable cells expressing the PCM marker γTub23C-GFP (green) and the centriole marker mCherry-SAS-6 (red, insets). Time-series of a cell exiting mitosis. The use of concanavalin-A to flatten cells prevents the successful completion of cytokinesis. Nevertheless, cell cycle progression continues as nuclear envelopes reform and rotate to position their attached centrosomes in the middle of the cell; each centrosome appears as a large perinuclear γ-tubulin spot containing three disengaged centrioles. Although the expected number of centrioles in each γ-tubulin spot should be two at this final stage of mitosis, for reasons unknown, some cells contain more than expected number. Within 30 min, each centrosome fragmented, releasing the centrioles into the cytoplasm. These then move throughout the cell. As centrosomes fragment, centrioles retained some γ-tubulin. Presumably they eventually shed all of their γ-tubulin because interphase centrioles do not co-localize with γ-tubulin or nucleate microtubule growth during interphase (7). Similar PCM shedding has been observed in live larval dividing neuroblasts (17).
Figure 1.3. **Centrosome clustering suppresses multipolar spindle formation.**

Time-series of *bona fide* centrosomes labeled with the centriole marker mCherry-SAS-6 (red, insets with orange arrowheads) and PCM marker γTub23C-GFP (green) in a mitotic cell beginning with 8 centrosomes (asterisk marks the site of a centrosome positioned above the focal plane). A population of cells in an S2 culture will contain excess centrioles that generate supernumerary mitotic centrosomes. Centrosomes cluster to achieve spindle bipolarity (two centrosomes that cluster at the left pole are marked with purple arrowheads in the upper panel). Some centrosomes cluster by utilizing a novel behavior of motility by moving along the metaphase spindle periphery (white and blue arrows). One remote centrosome (white arrowhead) is delayed in spindle incorporation. Scale, 5 μm.
1.8 References:


CHAPTER 2: THE STRUCTURE OF THE PLK4 CRYPTIC POLO BOX REVEALS TWO TANDEM POLO BOXES REQUIRED FOR CENTRIOLE DUPLICATION.

2.1 Abstract

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 \textit{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 Å 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 \textit{trans}-autophosphorylation, limiting centriole duplication to once per cell cycle.

2.2 Introduction

Centrioles are cylindrical, microtubule-based structures that form the core components of centrosomes and basal bodies, organelles that nucleate and spatially organize 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 motherdaughter centriole pair. During S-phase, centrioles separate and duplicate, generating two mother-daughter 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 (Bettencourt-Dias 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 SCFSlimb/β-TrCP ubiquitin ligase which recognizes Plk4 after homodimer-dependent trans-autophosphorylation 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 carboxyterminal PBs (Figure 2.1A) 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 anti-parallel 6-stranded β-sheet that lies across a C-terminal α-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
Å. 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.

2.3 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 Plk4\textsubscript{382-602} was bacterially expressed, purified and crystallized as described in Experimental Procedures. Crystals belonged to the space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}. A native dataset was collected on a single crystal to a resolution of 2.3 Å. 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 Å. The structure was built and refined to R and R\textsubscript{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 1.

*The Cryptic Polo Box is Composed of Two Structurally Unique Polo Box Domains*
Plk\textsubscript{382-602} forms a multi-domain structure, surprisingly composed of two tandem, structurally unique PBs (Figure 2.1B-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 Plk4\textsubscript{382-602} 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 antiparallel β-sheet that packs against a C-terminal helix that runs diagonal to the β-strands. PB1 has a sequential anti-parallel β-sheet composed of the six strands 1β1-1β2-1β3-1β4-1β5-1β6 that pack against the 1α1 helix. PB1 contains two unique structural attributes. First, the 1β3-1β4 strands form an extended hairpin off the β-sheet giving PB1 a winged structure (Figure 2.1C-D, green arrows). Second, a sixteen-residue segment that we term the “stirrup” bridges 1β5 and 1β6 and blankets one face of the β-sheet, effectively sandwiching the PB1 β-sheet between 1α1 and the stirrup (Figure 2.1C-D, red arrows). The 1β4 portion of the winged β-hairpin buttresses the N-terminal flank of the stirrup, implicating a structural co-dependence between these unique PB elements. The 1α1 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 1α1 helix by forming a hydrogen bond between the T499 γ-hydroxyl and the K496 backbone carbonyl (Figure 2.1E). The invariant proline P500 makes a jog in the backbone, offsetting the first strand of the PB2 sheet, 2β1, from the 1α1 axis.
Plk4 PB2 also contains unique features that deviate from the canonical PB fold, including secondary structure elements that flank the β-sheet as well as an extended 2α1 helix. The PB2 β-sheet proceeds in an anti-parallel fashion, consecutively snaking through strands 2β1-2β2-2β3-2β4-2β5. Instead of forming a sixth consecutive β-strand, as is the case with PB1, PB2 2β5 is followed by an ordered loop that connects 2β5 with the 2α1 helix (Figure 2.1C-D, grey arrows). The 2β5-2α1 loop is stabilized by a hydrogen bond between the conserved D542 δO and the M546 backbone amine as well as Van der Waals interactions between hydrophobic residues in the loop, the 2β5 strand, and the 2α1 helix. The C-terminal region of this loop is variable across species and contains a seven-residue insert in higher eukaryotes. The 2α1 helix runs diagonal to the β-sheet, spanning 40 Å and extending 13 Å past the 2β1 strand (Figure 2.1C, orange arrow). The helix-sheet interaction is stabilized both by a hydrophobic core as well as a disulfide bond between C511 in 2β2 and C566 in 2α1 (Figure 2.1F). The 2α1 helix leads into a loop that curls back towards 2β1, effectively stabilizing the extended 2α1 helix by packing F588 against the β-sheet-2α1 hydrophobic core. The loop terminates at an invariant proline, P589, that facilitates a bend in the backbone, leading into PB2’s final β-strand, 2β6, which runs parallel to 2β1 and completes the PB2 β-sheet and the PB1-PB2 cassette (Figure 2.1C, blue arrow). The location and polarity of the Plk4 2β6 strand is unique, normally running antiparallel to β5 in other PB structures.

Collectively, PB1 and PB2 form a composite structure, with a conserved core interface that buries 329 Å2 and limits molecular flexibility to peripheral loop regions. Relative to PB1, PB2 is rotated approximately 120°. The protomers align well with 1.2 Å rmsd across 214 Ca atoms. PB1 and PB2 individually align to their dimeric mate with a Ca rmsd equal to 1.1 and 1.2 Å respectively. Structural differences between the protomers, indicative of molecular flexibility,
localize to loop regions, specifically the stirrup’s C-terminal region, the 2β4-2β5 loop, and the 2β5-2α1 loop (Figure 2.2A, red arrows). As a second metric for structural flexibility, we mapped B factors on the PB1- PB2 structure. The structure’s Ca B-factors range from 14-105 Å², with increased levels in the stirrup, the 2β4-2β5 loop, the 2β5-2α1 loop, and the 2α1-2β6 loop, correlating with structural differences noted in the alignment of the protomers (Figure 2.2B, arrows). 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 PB2 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 1α1 helices (Figure 2.1D). A translational component along the pseudo two-fold axis shifts protomer A approximately 5 Å relative to protomer B. The homodimerization interface is mediated by PB1-PB1 contacts as well as PB2-PB2 contacts (Figure 2.2C,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 1α1 with additional contributions from neighboring residues in the β-sheet. The PB2-PB2 interface involves residues from 2β4, the 2β4-2β5 loop, 2β5, and 2α1. The PB1-PB1 and PB2-PB2 dimerization interfaces bury 1511 Å² and 1065 Å² of solvent accessible surface area respectively, collectively totaling 2576 Å². While crystallographic interfaces between protomers exist (Figure S2.1A-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 multi-angle static light scattering was conducted at pH 7.0. His<sub>e</sub>-Plk4 PB1-PB2 protein (MW 27.6 kDa) was injected at initial concentrations of 19 and 28 µM. Single elution peaks had experimentally determined molecular weights equal to 56.3 ± 4 kDa and 54.3 ± 2 kDa respectively (Figures 2.2E, S1D). We also investigated the oligomeric state using batch dynamic light scattering at pH 9.5 using 140 µM Plk4 PB1-PB2 (MW 25.7 kDa) which yielded an experimental mass of 66 ± 16 kDa (Table S1). Collectively, 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).

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 2.3A). The Plk1 PB1 1β1 strand forms key contacts with the phosphopeptide target. The Plk4 PB1 domain aligns to Plk1 PB1 with an rmsd of 2.0 Å across 76 aligned Ca atoms (Figure 2.3B). Structural divergence occurs at the Plk4 PB1 1β3-1β4 hairpin extension, the stirrup, the positioning of 1β6 and the elongated 1α1 N-terminal region. While there is structural divergence, the 1β1 strand, used in Plk1 PB1 to bind phosphopeptide targets, is accessible and may facilitate target binding as observed in Plk1 PB1 (Figure 2.3C).
Plk4 PB2 diverges from the Plk1 PB1 structure, with differences in loops, a helix extension, and the positioning of 2β6. Plk4 PB2 aligns to Plk1 PB1 with a 2.3 Å rmsd over 72 structurally aligned Cα atoms (Figure 2.3B). Plk4 PB2 differs from Plk1 PB1 in the positioning of the 2β2-2β3 and 2β4-2β5 loops. Plk4 PB2 contains an ordered loop between 2β5 and 2α1 that substitutes for the Plk1 PB1 1β6 strand. Plk4 PB2 forms a C-terminal extension on the 2α1 helix. The Plk4 PB2 2β6 strand does not occur between 2β5 and 2α1, but resides C-terminal to 2α1 and runs parallel to 2β1, a site occupied by the phosphopeptide target in Plk1 PB1. Thus, 2β6 occludes the Plk4 PB2 domain from interacting with a phosphopeptide in a manner equivalent to Plk1 PB1.

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 PB1-PB2 is monomeric, with PB1 and PB2 positioned to form a collective β-sandwich. In contrast, Plk4 PB3 is homodimeric and dimerizes across a symmetric β-sandwich (Leung et al., 2002). Like Plk1 PB1-PB2, the Plk4 PB3 β-sheets are sandwiched orthogonal to one another; however, PB3 dimerizes across the opposite face of its PB β-sheets. Plk4 PB1-PB2 adopts a third, unique PB domain arrangement (Figure 2.3C,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 2.3C). 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 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 2.4A, 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 1β1-1β2 and PB2 2β1-2β4 (Figure 2.4B,C, green arrows). The majority of conserved sequences in PB1 1β2-1β5 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 2.4B,C, blue arrows). When Plk1 PB1 with bound phosphopeptide is aligned with Plk4 PB1, the phosphopeptide is positioned at the Plk4 PB1-PB2 conserved junction (Figures 2.3C; 4B,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 β-strands in PB2 (2β1-2β3). While 2β1-2β3 is conserved and contributes to the composite PB1-PB2 conserved patch, it is unlikely that 2β1-2β3 would fold into an ordered β-sheet in the absence of 2α1, 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 co-expressing 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 2.4D). 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 2.4E). Intriguingly, Plk4 lacking PB1 (Plk4 ΔPB1) expressed to a high level, implicating a possible role for PB1 in Plk4 degradation (Figure 2.4D). However, Plk4 ΔPB1 failed to co-immunoprecipitate Asl. Strikingly, expression of only PB1-PB2 (residues 382-602) robustly coimmunoprecipitated 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.

*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 S2 cells containing endogenous Plk4, as Plk4 depletion causes dramatic centriole loss. We transiently expressed a series of inducible Plk4 PB-GFP constructs (Figure 2.5A). After transgene induction, cells were immunostained for pericentrin-like protein (D-PLP) to mark centrioles, and colocalization was scored as strong, weak, or no centriole localization (Figure 2.5B-H). Only two constructs, PB1-PB3 (aa 382-741) 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 co-localized with centrioles in 88% and 96% of cells assayed respectively (Figure 2.5B-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 2.5B,E,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 colocalization as compared to PB1-PB2 (Figure 2.5B, 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 Δ[PB1-PB2]).
SBM Δ[PB1-PB2] showed strong centriole co-localization in only 6% of cells and weak co-localization in 49% of cells (Figure 2.5B,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 2.5B,I).

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

Previous work has revealed an auto-regulatory mechanism in Plk1 whereby its PB1-PB2 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 PB1-PB2 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 S2.2). 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 2.6A-B, S3). Other Plk4 constructs including Plk4-ΔPB1, Plk4-Δ[PB1-PB2], and PB1+ did not alter the centriole count from the GFP control, indicating that the entire PB1-PB2 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 S2.4).

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-Δ[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-Δ [PB1-PB2]-GFP or GFP alone, demonstrating that PB1-PB2 promotes Plk4 stability in trans (Figure 2.6C). To test if PB1-PB2-GFP could bind Plk4-FL-myc in trans, we cotransfected 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 2.6D). 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 PB1-PB2 (Plk4-Δ[PB1-PB2]-GFP) and compared its levels to wild-type Plk4-GFP and a stable, full-length Plk4 kinase-dead mutant (Figure 2.6E) (Brownlee et al., 2011). Wild-type Plk4-GFP protein levels were extremely low. In contrast, Plk4-Δ[PB1-PB2] 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-Δ[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 2.6F). 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 Slimb-binding 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-Δ[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 2.6G).

2.4 Discussion

The Plk4 Cryptic Polo Box is Two Architecturally Distinct Polo Box Domains

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.

Plk4 PB1-PB2 has a Novel Inter-domain Arrangement and Dimerization Interface

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
The PB1-PB2 Cassette Mediates Centriole Targeting and Asterless Binding

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 PB1-PB2 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 β-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 1β1 and PB2 2β1-2β3 (Figure 2.4B,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 co-immunoprecipitates 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 PB1-PB2 Inter-domain Groove and Implications for Asterless Binding

The conserved PB1-PB2 inter-domain groove contains the PB1 1β1 strand, which in Plk1 PB1 is primarily responsible for phosphopeptide binding. It is possible that the Plk4 PB1 1β1 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.

The PB1-PB2 Cassette Regulates Plk4 Stability and Concomitant Centriole Number

Expression of the PB1-PB2 cassette caused interesting dominant effects. First, PB1-PB2 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 down-regulates 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 2.6H).

2.5 Conclusion

Our results reveal unique structural and functional determinants in Plk4 (Figure 2.6H). 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.
2.6 Figures

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 (orange). B) Secondary structure topology diagram of Plk4’s conserved central domain: PB1 (β-strands in green, α-helix in
yellow, stirrup in red, loops in black) and PB2 (β-strands in blue, α-helix in orange, 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° relative to C. E) Stick representation of the junction between PB1 1α1 and PB2 where the T449 hydroxyl caps the 1α1 helix. F) Stick representation of the PB2 intra-domain disulfide formed between 2α1 C566 and 2β2 C511. Final 2Fo-Fc electron density contoured at 1.5σ (E,F).
**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 2β4-2β5 and 2β5-2α1 loops. Plk4 colored as in Figure 2.1B, shown in cartoon format. B) Plk4 PB1-PB2 protomer A backbone colored and scaled according to Cα Bfactor values ranging from 14 (dark blue) to 105 (red) Å2. High B-factors correlate with the structurally plastic loop segments between protomers A and B (shown in A). 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 H6-Plk4 PB1-PB2 injected at 19 μM (red trace) and 28 μM (green trace)(100 μl). 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. (See Figure S2.1).
**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 as in Figure 2.1B) with human Plk1 PB1 bound to a phosphopeptide target (3FVH, β-strands in purple, α-helix in grey, peptide in blue). B) Matrix showing the rmsd (Å) 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).
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) (Å²) 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 2.2C,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 kT/e. C) Protomer A rotated 45° 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 2.3C. 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.
**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 (green), induced to express for 24 hours, and immunostained with anti D-PLP antibody to mark centrioles (red). Centriole co-localization was classified as strong, weak, or no co-localization. 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 colocalization. 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, 5µm.
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 (green), induced for 3 days, fixed, and stained for centrioles (PLP,
red) and DNA (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 S2.3, 2.4). 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 S2 cell lysates showing that overexpression of PB1-PB2-GFP (but not Plk4-Δ[PB1-PB2]-GFP or GFP) stabilizes wild-type full-length Plk4-GFP. Tubulin, loading control. D) PB1-PB2-GFP co-immunoprecipitates Plk4-FL-myc. Immunoblots of anti-GFP immunoprecipitates from S2 cells co-transfected with Plk4-FL-myc and PB1-PB2-GFP or GFP (control). E) Anti-GFP immunoblot of Plk4-GFP constructs transiently expressed in S2 cells showing differential stability. Tubulin, loading control. F) Plk4-FLGFP 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 PB1-PB2 cassette shows reduced autophosphorylation of the DRE as assayed by Slimb binding. Immunoblots of anti-GFP immunoprecipitates from S2 cells transfected with Plk4-FL-GFP or Plk4-Δ[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 SCFSlimb binding and ubiquitination.
### 2.7 Supplementary Data

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Values in parentheses are for the highest resolution shells unless otherwise denoted.

<sup>†</sup>R<sub>sym</sub> = \(\frac{\sum_{h} I(h) - \langle I(h) \rangle}{\sum_{h} I(h)}\) where \(I(h)\) is the integrated intensity of the \(h\)th reflection with the Miller Index \(h\) and \(\langle I(h) \rangle\) is the average over Friedel and symmetry equivalents.

<sup>‡</sup>Log-likelihood gain value as determined by Phenix.

<sup>§</sup>Figure of merit is the weighted mean of the cosine of the deviation from \(\alpha_{best}\).

<sup>##</sup>R value = \(\frac{\sum_{h} |I(h)| - |F_{calc}(h)|}{\sum_{h} |F_{calc}(h)|}\).

<sup>‡</sup>R<sub>free</sub> is calculated using a 10% subset of the data that is removed randomly from the original data and excluded from refinement.

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Table 2.1 Crystallographic data, phasing and refinement
Figure S2.1. 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 2.2C,D.

D) Size exclusion chromatography – multi-angle light scattering (SEC-MALS) analysis of H₆-Plk4 PB1-PB2 injected at 19 µM (red trace) and 28 µM (green trace).
trace)(100 µl). Bovine serum albumin control, 100 µl injected at 90 µM (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 Da). This data supplements the SEC-MALS data presented in Figure 2.2E.
**Figure S2.2.** 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 His\textsubscript{6}-tagged Plk4 PB1-PB2 were incubated with constant amounts of purified His\textsubscript{6}-tagged Plk4-Kin-DRE and γ\textsubscript{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 \textsuperscript{32}P into Plk4-Kin-DRE is not affected by Plk4 PB1-PB2. To calculate \textsuperscript{32}P incorporation, bands corresponding to Plk4-Kin-DRE in the autoradiograph and gel shown in A were measured by densitometric scanning. Each \textsuperscript{32}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\textsubscript{6}-tagged Plk4-Kin-DRE and γ\textsuperscript{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 \textsuperscript{32}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 S2.3.** 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 2.6B.
Figure S2.4. Titration 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 µM) 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 2.6B-G, showing that a PB1-PB2 construct can bind full length Plk4 and protect it from trans-autophosphorylation and subsequent Slimb binding which would promote its ubiquitin

2.8 Methods

Cloning and Protein Purification

D.m. Plk4 PB1-PB2 (residues 382-602) was subcloned into pET28 (Novagen), expressed in BL21 DE3 E. coli and induced with 0.1 mM IPTG for 16 hr at 20°C. Protein was purified using
successive Ni\textsuperscript{2+}-NTA (Qiagen) and SP-sepharose (GE Healthcare) chromatography. The His\text{6} tag was proteolytically removed. Protein was exchanged into 50 mM Tris pH 9.5, 300 mM NaCl, 0.1% β-ME, 10% glycerol and concentrated to 7.2 mg/ml. The SeMet-M517A mutant was generated using standard protocols (Leahy et al., 1994).

Structure Determination and Analysis

Native Plk4 was crystallized by the hanging drop method using a mother liquor (1 ml) containing 1.2 M Li\text{2}SO\text{4}, 100 mM Hepes, pH 7.5 and a drop containing 2 μl 7.2 mg/ml protein stock and 2 μl mother liquor. Native crystals were transferred to Fomblin oil (Sigma) and frozen in liquid nitrogen. SeMet-M517A Plk4 was crystallized using a mother liquor containing 1.4 M Li\text{2}SO\text{4}, 100 mM Hepes pH 7.5 and a drop containing 2 μl 3.2 mg/ml protein stock of 1 μl mother liquor. Diffraction data were collected at the Advanced Photon Source SER-CAT beamlines 22-ID and 22-BM. HKL2000 (Otwinowski and Minor, 1997), Phenix (Adams et al., 2010) and Coot (Emsley et al., 2010) were used to integrate and scale diffraction data, solve and refine, and build the structure respectively.

Centriole Localization and Count Assays

S2 cell culture was performed as described in Rogers and Rogers (2008). Primary rabbit anti-D-PLP antibody (1:3000) and Cy3 labeled 2° antibody (Jackson ImmunoResearch Laboratories, 1:500) were used to label centrioles for colocalization studies. To count centriole numbers, we co-transfected S2 cells with Plk4 constructs and Nlp-EGFP (a constitutively expressed nuclear protein) (Rogers et al., 2009), and analyzed cells with GFP-positive nuclei. Centrioles were labeled with anti-D-PLP and a 2° antibody (conjugated with Cy2, Rhodamine Red-X, or Cy5
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.

### 2.9 References


Elia, A.E.H., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J, Hoepker, K., Mohammad, D.,
phosphodependent substrate targeting and regulation of Plks by the Polo-Box Domain. Cell 115, 83-95.


transautophosphorylation regulates centriole number by controlling βTrCP-mediated
degradation. J. Cell Science 123, 2163-2169.


Chapter 3: Centriole Duplication is Regulated Through a Novel Feedback Loop Between Asterless and Plk4.

3.1 Abstract

Defects in centriole duplication can lead to a variety of diseases including Primary Microcephaly, Seckel Syndrome, and can also lead to chromosomal instability, a hallmark of cancer\textsuperscript{1-7}. The proteins Asterless (Asl) and Polo-like kinase 4 (Plk4) are essential centriolar proteins and overexpression of either of these can lead to centrosome amplification and even \textit{de novo} formation of centrioles\textsuperscript{8-14}. Asl and Plk4 are thought to be involved in the earliest steps of centriole duplication; in fact, recent work suggests that Asl may be a ‘licensing factor’, since stable incorporation of Asl into the centriole is essential for daughter centrioles to function as a mother centriole capable of duplication\textsuperscript{15}. Here we describe a novel mechanism to regulate centriole duplication involving a feedback loop between Asl and Plk4. We show that this feedback loop has two important features: first, Asl oligomerization stimulates Plk4 kinase activity and thus promotes centriole duplication and second, the N-terminal domain of Asl (Asl-A) can be phosphorylated by Plk4, preventing further Asl oligomerization, which in turn limits the amount of active Plk4. We propose that this feedback loop is a crucial step in limiting a mother centriole to only one daughter per cell cycle.
3.2 Results/Discussion

During mitosis, centrosomes are necessary for proper chromosome segregation and orientation of the mitotic spindle and, during interphase, serve as the major microtubule organizing center of animal cells\textsuperscript{16}. They can also migrate to the plasma membrane where, as basal bodies, they serve as a platform for the assembly of cilia and flagella, and in this way are essential regulators of cell signaling and motility\textsuperscript{17}. Centrioles are the core duplicating elements of the centrosome and, like DNA, duplicate once per cell cycle during S-phase. Errors in this process can generate cells containing either too many or too few centrosomes, increasing the frequency of incorrect spindle microtubule attachments to kinetochores and thus causing genomic instability in the form of chromosome mis-segregation and rearrangements. Indeed, centrosome amplification is often seen in many types of cancer and has also been observed in pre-malignant lesions\textsuperscript{18,19}. Furthermore, recent work has shown that centrosome amplification can increase the invasiveness of transformed cells and therefore may play a key role in cancer progression\textsuperscript{20}.

Asl is a conserved coiled-coil protein and is thought to function as a scaffold to bind and recruit two essential centriolar proteins Plk4 and Sas-4/CPAP to the centriole, allowing the assembly of a procentriole. In \textit{Drosophila}, Asl is sufficient to recruit Plk4 to the centriole, however in humans it is functionally redundant with another protein Cep192 in its ability to localize Plk4\textsuperscript{9,21-24}. Interestingly, \textit{in vitro} kinase assays using the human orthologue of Asl, Cep152, have shown that the N-terminal region (amino acids 1-217) of Celp152 is phosphorylated by Plk4\textsuperscript{21}. However, the functional significance of this phosphorylation event in centriole duplication is still unknown. Furthermore, it is unclear whether this phosphorylation
event is conserved in *Drosophila*, which, if true, would suggest that Asl phosphorylation is an important event in the centriole biogenesis pathway.

In order to determine if Plk4 phosphorylates Asl in flies, we first co-expressed V5-tagged Asl with either GFP, a hyperstable mutant Plk4 (Plk4-SBM-EGFP whose Slimb-binding site is mutated and thus is resistant to SCF<sup>Slimb</sup>-mediated destruction), or a kinase dead mutant of Plk4 (Plk4-KD-EGFP) in cultured *Drosophila* S2 cells (Fig. 3.1B). Lysates of transfected cells were analyzed by Western blotting to evaluate the electrophoretic mobility of Asl which serves as an indicator of its phosphorylation state. When co-expressed with Plk4-SBM, Asl migrates as a lower mobility smear on a Western blot, suggesting that Asl has been phosphorylated to a variable extent. In contrast, Asl migrates as a focused higher mobility band, indicating a uniform non- or low-phosphorylation state, when co-expressed with either the control GFP or the inactive Plk4 mutant Plk4-KD. Therefore, our results are consistent with the hypothesis that Asl can be phosphorylated by Plk4 in fly cells, and that phosphorylation is indeed a conserved modification in flies as well as humans.

Next we set out to determine where Plk4 phosphorylation occurred within the Asl protein since previously only the N-terminus of Cep152 had been tested in *in vitro* kinase assays. In order to determine which domains of Asl can be phosphorylated by Plk4, we bacterially expressed and purified three GST-tagged Asl constructs for our own *in vitro* kinase assays: Asl-A (aa 1-374), Asl-B (aa 375-630) and Asl-C (aa 631-901). Incubating these Asl fragments with purified Plk4 kinase domain revealed that, aside from Plk4 autophosphorylation, only Asl-A is phosphorylated, as seen by P<sup>32</sup> incorporation, and not Asl-B, Asl-C, or GST alone (Fig. 3.1A).

In order to identify which specific residues of Asl-A are phosphorylated, we chose two different approaches. First, purified GST-Asl-A fragment was incubated with either wild
type(WT) or kinase dead (KD) Plk4 kinase domain and was then analyzed using differential tandem mass spectrometry (MS). This resulted in identification of 6 residues that were labeled when incubated with WT and not KD Plk4 (Fig 3.1C). Our next approach was to co-express Asl-GFP with either Plk4-Myc or Plk4-KD-Myc in S2 cells. MS analysis of immunoprecipitated Asl-GFP from these cells identified an additional 7 residues that were phosphorylated when co-expressed with WT but not KD Plk4. In total 13 phosphorylated residues were identified within Asl-A using a combination of in vitro and in vivo analyses.

These data support the hypothesis that phosphorylation of Asl by Plk4 is indeed a conserved event from flies to humans and suggest that this may be a critical event in centriole duplication. In order to understand the functional significance of Asl phosphorylation, we created a non-phosphorylatable Asl mutant construct (13A mutant) in which all 13 residues phosphorylated by Plk4 were mutated to non-phosphorylatable Ala. In addition, a phosphomimetic mutant construct (13D mutant) was made in which the same 13 residues were mutated to a negatively charged Asp or Glu (Fig 3.2A). Since it had been shown previously that Cep152 protein levels fluctuate in a cell cycle dependent manner in human cells, we reasoned that phosphorylation may play a role in regulating Asl protein levels. To test this hypothesis, we expressed full length 13A and 13D mutant constructs in cells and examined protein levels via quantitative Western blots. (Fig 3.2B) Importantly, in order to minimize the effects of endogenous Asl on our experiments we treated cells with dsRNA containing the 5’and 3’ UTR of the endogenous Asl transcript. In this way we were able to effectively do a replacement assay by knocking down the endogenous protein and expressing our mutant constructs. Our results indicated that the protein levels of the non-phosphorylatable 13A and the phosphomimetic 13D mutants were unchanged when compared to WT Asl (Fig 3.2B). Furthermore, we also found that
Asl localization to the centriole was unchanged as well (Fig 3.2C). Therefore, phosphorylation does not appear to affect stability or centriolar targeting of the Asl protein.

Interestingly, since Asl is not necessary for proper Plk4 localization in humans and Xenopus, but its co-localization is necessary for centriole duplication to occur, it has been suggested that Asl does not function merely as a scaffold but in fact may affect Plk4 activity\textsuperscript{25}. To test this hypothesis, we knocked down endogenous Asl using RNAi and then co-expressed Plk4 along with our Asl-A constructs including the 13A and 13D mutants. (Fig 3.3A) Asl RNAi increased Plk4 protein levels almost two-fold, which was expected since Asl has been shown to stabilize the Plk4 homodimer allowing for more efficient autophosphorylation and subsequent degradation of Plk4 via the SCF\textsuperskimub ubiquitin ligase pathway. Notably, expression of WT Asl-A can rescue this RNAi phenotype, decreasing Plk4 protein levels to the same extent as control RNAi. Furthermore, we found that the phosphorylation state of Asl regulates this activity, since expression of Asl-A 13A can also rescue the Asl RNAi phenotype, however expression of Asl-A 13D did not. In fact, Plk4 protein levels were stabilized to the same extent as Asl RNAi alone, in the presence of Asl-A 13D.

Interestingly, expression of the Asl phospho-mutant constructs had an unexpected effect on the phosphorylation state of Plk4 in cells. Using electrophoretic mobility shifts as an indicator of phosphorylation state, Plk4 expressed in cells appears to be variably autophosphorylated since it normally runs as a smear on a Western blot. However, when Asl-A 13D was co-expressed, Plk4 shifted to a higher mobility band, indicating that Plk4 is less phosphorylated; in contrast, co-expression of the 13A mutant led to an exaggerated phosphorylation phenotype. To confirm that this difference in phosphorylation was indeed due to increased autophosphorylation and not due to the activity of another kinase we performed this
same experiment with Plk4-KD (Fig 3.3B). In this case, no change in the phosphorylation state of Plk4-KD was observed with expression of any of our Asl-A constructs. In every case Plk4-KD was seen as a single non-phosphorylated band and protein levels were uniformly high in all cases, as expected if Plk4 cannot autophosphorylate. Together these data suggest that phosphorylation of Asl may play a role in modulating Plk4 activity.

To further test this hypothesis, we transfected S2 cells with either vector alone, Asl-A WT, Asl-A 13A, or Asl-A 13D and then induced expression for 3 days. Cells were immunostained for the centriolar marker D-PLP and the number of centrioles per cell was counted. (Fig 3.3C) Compared to control cells expressing Asl-A WT, expression of Asl-A 13A significantly increased the percentage of cells with >2 centrioles. However expression of Asl-A 13D seemed to act as a dominant negative, significantly increasing the percentage of cells with <2 centrioles. In order to rule out the possibility that the observed changes in centriole number were due to mislocalization of Plk4, instead of changes in Plk4 kinase activity, we co-expressed our Asl-A constructs in cells along with Plk4-EGFP (Fig 3.3D). Immunostaining revealed that expression of these constructs did not affect Plk4 recruitment to the centriole. Together these data are consistent with the hypothesis that Asl phosphorylation can regulate Plk4 kinase activity and suggests that a novel feedback loop between Asl and Plk4 may play a critical role in limiting centriole duplication to one event per cell cycle.

Previous work has shown that Asl can stabilize the Plk4 homodimer, leading to more efficient Plk4 autophosphorylation\(^26\). In order to determine if Asl phosphorylation affects its ability to promote Plk4 dimerization, we first knocked down endogenous Asl via RNAi and then co-transfected both Plk4-EGFP and Plk4-Myc along with our Asl-A phospho-mutant constructs. After inducing expression, Plk4-GFP was immunoprecipitated (IP) and the amount of
Plk4-Myc that co-IP’d was measured using quantitative Western blots as a readout for Plk4 homodimerization. As expected, expression of Asl-A WT resulted in an increase in the amount of Plk4-Myc that co-IP’d with Plk4-EGFP when compared to cells lacking Asl. (Fig 3.4A) Notably, expression of either of our phospho-mutants, Asl-A 13A or Asl-A 13D, did not affect the ability of Plk4 to homodimerize when compared to Asl-A WT. This result was unexpected since we see clear differences in the electrophoretic mobility and therefore phosphorylation state of Plk4 which we thought may be explained by differences in the ability of Plk4 to homodimerize in the presence of the phospho-mutants. Indeed, expression of Asl-A 13A resulted in a shift to a lower mobility hyper-phosphorylated band compared to Asl-A WT. However in contrast to Asl-A 13A, expression of Asl-A 13D once again resulted in a higher mobility low or non-phosphorylated band (Fig 3.4A,B). These data suggest that the changes in Plk4 phosphorylation state are due to the ability of the Asl phospho mutants to effect Plk4 kinase activity and not due to the ability of Asl to stabilize the Plk4 homodimer.

To further confirm our results that there is indeed a change in the phosphorylation state of Plk4 we wanted to determine the amount of slimb that co-IP’d with Plk4-GFP in the presence of our phospho-mutants (Fig 3.4A). Slimb is a subunit of the SCF<sup>slimb</sup> ubiquitin ligase complex that specifically binds to phosphorylated Plk4. Our results showed that expression of Asl-A 13A increased the amount of slimb that co-IP’d with Plk4 and as expected, expression of the Asl-A 13D mutant lead to a decrease in slimb binding, when compared to Asl-A WT. Our observations suggest the changes we observe in Plk4 phosphorylation state with expression of our phospho-mutant constructs are due to changes in kinase activity and not a change in the ability of Plk4 to homodimerize.
Interestingly, we observed an increase in the amount of Asl-A 13A that co-IP’d with Plk4-EGFP when compared to either Asl-A WT or Asl-A 13D. Since Asl is known to be able to bind to itself, this raised an interesting possibility that this change in the amount of Asl that IP’d with Plk4 may reflect an increased ability of Asl to oligomerize. In order to test this intriguing hypothesis we once again performed binding assays by expressing WT or phospho-mutant forms of Asl-A-GFP and Asl-A-V5 in cells, and performed GFP IP’s to determine the amount of the V5-tagged form that co-IP’d. Strikingly, the extent of binding between the two 13A constructs was dramatically increased compared to what we observed with either WT or 13D (Fig 3.4B) and this result may explain why we previously observed an increased amount of the 13A mutant bound to Plk4-EGFP (Fig 3.4A). These data support the following model: non-phosphorylated Asl can oligomerize and bind to Plk4, leading to activation of the kinase. Upon activation, the A region of Asl can be phosphorylated on multiple residues, decreasing the ability of Asl to oligomerize and inhibiting further Plk4 activation.

Finally, expressing our phospho-mutant constructs in cells revealed an interesting phenotype. When Asl-A-GFP 13A was overexpressed in cells we observed that almost 92% of cells formed large aggregates, however, these aggregates were only very rarely seen when expressing Asl-A WT, 6.7% and they were never observed upon expression of GFP alone. (Fig 3.5A,B) Presumably these aggregates are a result of the oligomerization of Asl and the inability of Plk4 to phosphorylate the 13A mutant and disassemble these complexes. As expected, expression of our Asl-A 13D mutant led to a decrease in the formation of these aggregates, 1.3%, when compared to WT. To further test this hypothesis, we co-expressed Asl-A WT with either Plk4 or kinase dead Plk4. Our data show that expression of kinase dead Plk4 lead to a significant increase in the formation of aggregates, 88.2%, compared to Plk4, 10.3%. (Fig 3.5B) These data
support our hypothesis that in the absence of phosphorylation Asl can oligomerize and form large aggregates and furthermore that phosphorylation of Asl regulates the disassembly of these oligomers.

Our study has uncovered a novel feedback loop between two important regulatory proteins, Plk4 and Asl (Fig 3.5C). We propose a model in which upon entry to mitosis, levels of Plk4 begin to rise, however Plk4 activity remains low as a result of autoinhibition. As a consequence of low Plk4 activity, our data suggest that a non-phosphorylated Asl can bind to Plk4 and recruit it to the centriole where Asl can oligomerize. We speculate that Asl oligomerization can lead to a high local concentration of Plk4 and a highly activate state of the Plk4 kinase, presumably through autophosphorylation of its regulatory T-loop. Furthermore, we have shown that active Plk4 can then inhibit oligomerization through the phosphorylation of the N-terminal region of Asl. This feedback loop would place strict limits on the amount of active Plk4 and in theory may represent a mechanism to confine active Plk4 to a single spot on the centriole, therefore marking the sight where a future daughter centriole will form in S-phase. Our model predicts that the first spot to form and activate Plk4, can inhibit any further kinase activation through the phosphorylation of Asl, in turn preventing the necessary oligomerization step important for kinase activation. Indeed, previous work has shown that Plk4 forms a single asymmetric spot on the mother centriole and active Plk4 within this spot is thought to modify this site, thus making each centriole competent to duplicate\textsuperscript{10,27}. Our model also suggests an intriguing possibility for the role of Asl in \textit{de novo} formation of centrioles as well. For example, it is possible that in the absence of a mother centriole, Asl oligomerization may serve as a platform for Plk4 and other centriolar proteins to assemble into a new centriole. In the future, it
will be important to understand the roles that the phosphatase PP2A and the C-terminus of Asl play in these processes since both have been shown to protect Plk4 from degradation\textsuperscript{26,28}.

### 3.3 Figures

**Figure 3.1.** Phosphorylation of Asterless by Plk4 is Conserved in *Drosophila*.

**a)** *In vitro* kinase assays using bacterial expressed and purified GST-Asl-A (aa 1-374), GST-Asl-B (aa 375-630), GST-Asl-C (aa 631-901), or GST alone. Incubated with Plk4 kinase domain (aa
1-317) where indicated. Comassie staining (left) showing the indicated proteins and the corresponding autoradiograph (right) indicating proteins with incorporated $\text{P}^{32}$. b) S2 cells were transiently transfected with Asl-V5 and either GFP, Plk4-SBM-EGFP, or Plk4-KD-EGFP and NLP-GFP as a transfection and loading control and harvested for western blots. c) Diagram of the Asl-A region showing the residues that were identified as phosphorylated by Plk4 in vivo and in vitro, via mass spectrometry.
**Figure 3.2.** Asterless phosphorylation mutants exhibit normal protein levels and localization.  

**a)** Diagram of the residues that were identified via mass spectrometry as being phosphorylated by Plk4 and the mutations that were made creating a non-phosphorylatable 13A mutant and a phosphomimetic 13D mutant.  

**b)** Western blots showing the levels of either wild type Asl-V5, Asl-13A-V5, or Asl-13D-V5 in S2 cells transiently expressing these constructs along with NLP-GFP as a transfection/loading control. This experiment was performed while knocking down endogenous Asterless protein using the indicated dsRNA.  

**c)** RNAi against endogenous Asl was performed and cells were transiently transfected with the indicated Asl-GFP constructs. Cells were then fixed and immunostained using antibodies against D-PLP, a centriolar marker.
Figure 3.3. Asterless phosphorylation regulates Plk4 kinase activity.

**a)** RNAi against Asl was performed and cells were transfected with Plk4-EGFP as well as the indicated Asl-A-V5 constructs. NLP-GFP was used as the transfection/loading control. Graph showing levels of Plk4-EGFP adjusted for the amount loaded and levels were normalized against Asl RNAi alone (Lane 2). **b)** Same experiment as in **a** except that Plk4-KD-EGFP was used in place of wild type Plk4-EGFP. Graph showing levels of Plk4-KD-EGFP adjusted for the amount loaded and levels were normalized against control RNAi (Lane 1). **c)** Centriole counts showing the percent of cells with the indicated amount obtained from S2 cells transiently transfected with either vector alone, Asl-A-V5, Asl-A-V5 (13A), or Asl-A-V5 (13D) and NLP-GFP as an indicator of transfected cells. Expression of these constructs was induced for 3 days and then
these cells were fixed and immunostained using a D-PLP antibody. (100 cells counted per experiment / 3 experiments total). Asterisks indicate a significant difference (** Percent cells with >2 centrioles Asl-A-V5 vs. Asl-A-V5 (13A) P= <0.05; * percent cells with <2 centrioles Asl-A-V5 vs. Asl-A-V5 (13D) P= < 0.001) Error bars indicate SEM. d) Cells were transiently transfected with Plk4-GFP and the indicated Asl-A-V5 construct. Cells were then fixed and immunostained for the centriolar marker D-PLP.
Figure 3.4. Phosphorylation controls Asterless oligomerization state and Plk4 activity.

a) S2 cells were treated with Asl dsRNA and then transiently transfected with Plk4-EGFP and Plk4-Myc as well as the indicated Asl-A-V5 constructs. Anti-GFP IPs were performed and immunoblots of the input lysates and IPs were probed for α-tubulin, GFP, Myc, and slimb. Graph showing the amount of Plk4-Myc that co-IP’d, adjusted for the amount of Plk4-EGFP, as a measure of Plk4 dimerization.

b) S2 cells were treated with Asl dsRNA and then transiently transfected with the indicated Asl-A constructs as well as the Plk4-Myc. Anti-GFP IPs were performed and immunoblots of the input lysates and IPs were probed for GFP, Myc, and V5. Graph showing the amount of Asl-A-V5 that co-IP’d, adjusted for the amount of Asl-A-GFP, as a measure of Asl oligomerization.
Figure 3.5. Asterless oligomerization is regulated by phosphorylation.

a) Immunofluorescence images of S2 cells transiently transfected with the indicated constructs.

b) Graph showing the quantitation of the percent of cells with GFP aggregates. (200 cells counted per transfection/ 3 experiments per treatment). Asterisk indicates a significant difference (Percent cells with aggregates Error bars indicate SEM. Asterisks indicate a significant difference (** Percent cells with aggregates Asl-A-GFP vs. Asl-A-GFP (13A) P= <0.001; * percent cells with aggregates Asl-A-GFP vs. Asl-A-GFP (13D) P= < 0.05; *** percent cells with aggregates Asl-A-GFP + Plk4-Myc vs. Asl-A-GFP + Plk4-KD-Myc P=<0.0001) c) Model illustrating the feedback loop between Asl and Plk4.
3.4 Materials and Methods

Cell culture and double-stranded RNAi.- Drosophila S2 cell culture, in vitro dsRNA synthesis, and RNAi treatments were performed as previously described. Briefly, cells were cultured in Sf900II SFM media (Life Technologies). RNAi was performed in 6 well plates. Cells (50-90% confluency) were treated with 5μg of dsRNA in 1ml of media and replenished with fresh media/dsRNA every day for 4-7 days. Control dsRNA was synthesized from control DNA template amplified from a non-GFP sequence of the pEGFP-N1 vector (Clontech) using the primers 5’-CGCTTTTCTGGATTCATCGAC and 5’-TGAGTAACCTGAGGCTATGG (all primers used for dsRNA synthesis begin with the T7 promote sequence 5’-TAATACGACTCACTATAGGG). DsRNA was synthesized from cDNA using the primers against the following genes: Asl-C targeting exon 5’-CGTCTGATCCATCGCC and 5’-CATCGCCTCTTCGTGGG. DsRNA targeting the Asl UTR was synthesized from an EST template by first removing the Asl cDNA, joining 76 bp of 5’UTR with 114 bp of 3’UTR, and amplified using the primers 5’-GTTGCCTACGAAAATAGCGCC and 5’-TTTTGTAGGAATGTACAGCG.

Immunofluorescence microscopy.- For immunostaining, S2 cells were fixed and processed exactly as previously described29 by spreading S2 cells on concanavalin A–coated, glass-bottom dishes and fixing with 10% formaldehyde. Primary antibodies were diluted to concentrations ranging from 1 to 20 μg/ml. They included rabbit anti–PLP and guinea pig anti-Asl. Secondary antibodies (conjugated with Cy2, Rhodamine red-X, or Cy5 [Jackson ImmunoResearch Laboratories, Inc.]) were used at manufacturer-recommended dilutions. Hoechst 33342 (Life
Technologies) was used at a final dilution of 3.2 μM. Cells were mounted in 0.1 M n-propyl galate, 90% (by volume) glycerol, and 10% PBS solution. Specimens were imaged using a DeltaVision Core system (Applied Precision) equipped with an Olympus IX71 microscope, a 100× objective (NA 1.4), and a cooled charge-coupled device camera (CoolSNAP HQ2; Photometrics). Images were acquired with softWoRx v1.2 software (Applied Science).

**Immunoblotting**- S2 cell extracts were produced by lysing cells in cold PBS and 0.1% Triton X-100. Laemmli sample buffer was then added and boiled for 5 min. Samples of equal total protein were resolved by SDS-PAGE, blotted, probed with primary and secondary antibodies, and scanned on an Odyssey imager (Li-Cor Biosciences). Care was taken to avoid saturating the scans of blots. Transfected Nlp-EGFP (a constitutively-expressed nuclear protein) was used as loading control and transfection marker. Antibodies used for Western blotting include anti-V5 (Invitrogen), anti-Slimb, anti-Asl, anti-GFP monoclonal JL8 (Clontech), anti-alpha tubulin monoclonal DM1A (Sigma-Aldrich), anti-myc (Cell Signaling Technologies) and used at 1:1,000 dilutions. IRDye 800CW secondary antibodies (Li-Cor Biosciences) were prepared according to the manufacturer’s instructions and used at 1:2,000 dilutions.

**Constructs and Transfection**- Full-length cDNAs of *Drosophila* Asl and Plk4 were subcloned into a pMT vector containing in-frame coding sequence for EGFP, V5, or myc and the inducible metallothionein promoter. Phusion polymerase (ThermoFisher) was used according to manufacturer’s instructions to generate the various Asl point mutants. Transient transfections of S2 cells were performed using a Nucleofector II and nucleofector kit V (Lonza) according to
manufacturer’s instructions. Expression of all Plk4 and Asl constructs (and GFP control) was induced by addition of 50 μM–2 mM copper sulfate to the culture medium.

*GFP Immunoprecipitation Assays*- GFP-binding protein (GBP)\(^{29}\) was fused to the Fc domain of human IgG (pIg-Tail) (R&D Systems), tagged with His6 in pET28a (EMD Biosciences), expressed in E. coli and purified on HisPur resin (ThermoFisher) according to manufacturer’s instructions. Purified GBP was bound to Protein A-coupled Sepharose, and then cross-linked to the resin by incubating with 20mM dimethyl pimelimidate dihydrochloride in PBS, pH 8.3, 2 hours at 22°C, and then quenching the coupling reaction by incubating with 0.2 M ethanolamine, pH 8.3, 1 hour at 22°C. Antibody-coated beads were washed three times with 1.5 ml of cell lysis buffer (CLB; 50 mM Tris, pH 7.2, 125 mM NaCl, 2 mM DTT, 0.1% Triton X-100, and 0.1 mM PMSF). Transfected cells expressing recombinant proteins were lysed in CLB, and the lysates clarified by centrifugation. GBP-coated beads were rocked with lysate for 1 hour at 4°C, washed two times with 1 ml CLB, and then boiled in Laemmli sample buffer.

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

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

3.5 References


Conclusions/Future Directions

My thesis work has focused on the structure, function, and interaction of two essential centriolar proteins, Polo-like kinase 4 (Plk4) and Asterless (Asl), in Drosophila S2 cells. In chapter 1 I describe methods perfected in our lab for using Drosophila cell culture to study the microtubule cytoskeleton including RNAi knockdown, transgene expression, and fluorescence microscopy. I have also highlighted methods to preserve the potentially fragile microtubule cytoskeleton and also centrosome enrichment techniques that are extremely useful for identifying novel centrosomal components.

My work has also contributed to a greater understanding of the structure and function of Plk4’s cryptic polo box domain. Previously it was thought that Plk4 was unique among Polo-like kinase family members because it contained only one conserved polo box (PB) domain, in contrast to all other Plk family members which have two PB domains. Our work has changed the way that we think of Plk4, since we now know that it is unique because it actually contains three PB domains. In fact, the cryptic polo box region encodes
tandem PB domains, PB1 and PB2, upstream of the C-terminal PB3. The presence of additional PB domains opens the door to the possibility of increased complexity in the features and processes affecting Plk4 function and regulation. For example, I show that the entire PB1-PB2 cassette is necessary for binding to Asl and is necessary and sufficient to target Plk4 to the centriole. Interestingly, we found that expression of PB1-PB2 can stabilize Plk4 in \textit{trans} by binding endogenous Plk4; this alone has a large impact on both Plk4 function and regulation because it has the consequence of blocking Plk4 autophosphorylation and subsequent degradation by the SCF$^{\text{Simb}}$ ubiquitin ligase pathway. Therefore the PB1-PB2 domain plays a critical role in restricting centriole duplication to once and only once per cell cycle.

Plk4’s PB1-PB2 domain is multi-functional. It is not only necessary for Plk4 homodimerization but also for binding to the centriolar protein Asl. Previous work has shown that in flies, Asl is necessary for localization of Plk4 to the centrosome and that the human orthologue of Asl (Cep152) is phosphorylated by Plk4. However, the functional significance of Asl phosphorylation in the centriole duplication pathway remained a mystery. In chapter 3, I show that Asl is phosphorylated in its N-terminal (Asl-A) region and mapped the phosphorylated residues. That work laid the foundation for the next step, the analysis of the impacts of phosphorylation on Asl function in cells. The creation of Asl phosphorylation mutants, including a non-phosphorylatable and a phospho-mimetic form revealed the existence of a novel feedback loop between Asl and Plk4. We show that this feedback loop has two important features: first, Asl oligomerization stimulates Plk4 kinase activity and thus promotes centriole duplication and second, the N-terminal domain of Asl (Asl-A) can be phosphorylated by Plk4, preventing further Asl oligomerization, which in turn limits
the amount of active Plk4. We propose that this feedback loop is a crucial step in limiting a mother centriole to only one daughter per cell cycle.

Altogether my work has shed light on the mechanisms regulating centriole duplication, specifically the interaction between two proteins involved in the earliest steps of centriole formation. For instance, the feedback loop I describe in chapter 3 likely plays a critical role in limiting the amount of active Plk4 and therefore controlling centriole duplication. Previous work has shown that upon entry to mitosis Plk4 covers the entire surface of the centriole, however, upon mitotic exit centriolar Plk4 is reduced to a single asymmetric spot. According to the current model, this spot is then licensed for duplication and determines the site where a daughter centriole will assemble in the following S-phase. The mechanism by which this occurs remains unclear, but an intriguing hypothesis is that this feedback loop between Asl and Plk4 is not only necessary to limit Plk4 kinase activity, but also can serve to confine Plk4 activity to one specific spot. Indeed, our model suggests active Plk4 at one specific spot may be able to inhibit the activation of Plk4 anywhere else on the centriole through the phosphorylation of Asl. In support of this model my data suggests that once Asl is phosphorylated by Plk4 it lacks the ability to oligomerize and therefore can no longer serve as an activator of Plk4. In fact, expression of a phosphomimetic construct acted as a dominant negative and inhibited centriole duplication in S2 cells. Using super resolution microscopy to visualize the dynamic process of Plk4 remodeling in live mitotic cells expressing Asl phosphorylation mutants would be an informative approach. It may be possible to identify multiple spots, or possibly a much larger spot, forming in the presence of a non-phosphorylatable Asl mutant. In contrast, our model suggests that Plk4 may not be able to assemble into a spot in the presence of a phosphomimetic Asl mutant.
Furthermore, it will be important to determine the roles of the phosphatase PP2A and the C-terminus of Asl in the formation of this spot, since both of these are important for stabilization of the Plk4 protein during mitosis. Given that the phosphorylation state of Asl is important for its function, it would be interesting to test the hypothesis that PP2A can also act on Asl as well as Plk4. As I have shown in chapter 3, a non-phosphorylatable Asl mutant construct is able to oligomerize and form large aggregates in cells. It would be interesting to determine if PP2A can stabilize Asl oligomers by keeping Asl in a non-phosphorylated state. This hypothesis predicts that overexpression of PP2A may lead to an increase in Asl aggregates and furthermore knockdown of PP2A should allow for greater phosphorylation of Asl and consequently a decrease in Asl aggregates. Asl immunoprecipitation experiments to determine its ability to oligomerize with and without PP2A present would be very informative. Future work to determine how the phosphorylation state of Asl is regulated will be necessary, as this may be an additional mechanism in place to regulate Plk4 activity and localization on the centriole surface.

Interestingly, recent work has shown that Asl contains two Plk4 binding domains. One at the N-terminus and another located in the C-terminal region. Evidence suggests that these two binding sites play significantly different roles in the centriole duplication pathway. The N-terminus playing an essential role in activation of Plk4 kinase activity and the C-terminus acting to stabilize the Plk4 protein. Future studies will be important to determine how these two binding domains cooperate to allow for centriole duplication to occur. Experiments looking at the competition between these two regions for Plk4 binding will shed light on how and when these domains function during the cell cycle. For example, immunoprecipitating Plk4-GFP from cells co-expressing Asl fragments containing these two binding sites would allow us to identify if one may have a higher affinity for Plk4 than the other based on how much of each domain co-IP’s
with Plk4. Further experiments to determine the effects of different stages of the cell cycle on binding as well as how the phosphorylation state of Asl effects this will also be important steps toward a better understanding of the complex Plk4-Asl interaction. Future studies in this area will give us a better understanding of the mechanisms regulating centriole duplication and will be essential in order to identify key targets that may be exploited to prevent or treat cancer.