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THE FAR C-TERMINUS OF TPX2 CONTRIBUTES TO SPINDLE MORPHOGENESIS

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THE FAR C-TERMINUS OF TPX2 CONTRIBUTES TO SPINDLE MORPHOGENESIS

A Thesis Presented

by

BRETT J. G. ESTES

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

FEBRUARY 2017

Molecular and Cellular Biology
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- Brett
ABSTRACT

THE FAR C-TERMINUS OF TPX2 CONTRIBUTES TO SPINDLE MORPHOGENESIS

FEBRUARY 2017

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M.S., UNIVERSITY OF MASSACHUSETTS AMHERST
Directed by: Patricia Wadsworth

A cell must build a bipolar mitotic spindle in order to faithfully segregate replicated DNA. To do so, multiple microtubule nucleation pathways are utilized to generate the robust spindle apparatus. TPX2, a microtubule binding protein, holds crucial roles in both the Ran-dependent and Augmin-dependent pathways where microtubules are nucleated near the chromosomes and from pre-existing microtubules. However, the exact role TPX2 plays in branching microtubules is less understood. Here, we explored the effect of truncating the essential TPX2 C-terminal 37 amino acids on Augmin localization and branching microtubule activity. First, we depleted LLC-Pk1 cells of the Augmin subunit HAUS6 and show that microtubule nucleation around the chromosomes following a nocodazole washout is strongly reduced leading to exaggerated kinetochore microtubule growth. Next, we depleted endogenous TPX2 in LLC-Pk1 cells harboring full length or truncated TPX2 bacterial artificial chromosome (BAC) DNA. Results show that TPX2 710 LAP cells have reduced Augmin localization on the spindle fibers, which correlates with reduced microtubule regrowth in the chromosomal region. In TPX2 710 LAP cells, regrowth was like Augmin depleted cells. Therefore, we provide evidence that the far C-terminus of TPX2 is required for branching microtubule nucleation and that
kinetochore microtubule growth is Augmin-independent. In addition, we investigated cell cycle regulation of TPX2 by mutating the S738 phosphosite in the C-terminal motor interacting region. We utilized BAC recombineering to create phospho-mimetic and phospho-null mutants. In combination with plasmid DNA knockdown/rescue, overexpression and spindle assembly assays, we show that the phosphorylation of the C-terminal domain contributes to early mitotic events. LLC-Pk1 cells showed a significant increase in aberrant spindle morphology and reduced spindle stability in the presence of 738A and absence of endogenous TPX2. While rescue with the alanine mutant caused in an increase in multipolar spindles, overexpression resulted in a strong dominant negative monopolar phenotype. Therefore, S738 appears to contribute to mitotic force regulation during mitosis. In conclusion, the far C-terminus of TPX2 and its regulation play a role in the formation of a proper mitotic spindle.
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CHAPTER I
INTRODUCTION

Deoxyribonucleic acid (DNA) is the universal blueprint for life. It contains the genetic code that each cell uses to generate the proteome. As life evolved on Earth, organisms developed methods to ensure the delivery of replicated DNA to their progeny. Prokaryotes undergo binary fission – a process in which genomic DNA is replicated, attached to the cell wall and separated during cytokinesis. In contrast, eukaryotic organisms utilize mitosis to properly segregate their genetic material.

Mitosis consists of five phases (prophase, prometaphase, metaphase, anaphase & telophase) in which the goal is to establish a bipolar spindle and properly distribute the chromosomes into each daughter cell. Early mitosis involves the condensing of chromatin into chromosomes, spindle morphogenesis and achieving bi-orientation – a state in which sister chromosomes are attached to opposite poles. Once all kinetochores are properly attached, the spindle elongates and the genetic material is separated (Skibbens & Heider, 1998). The final process during cell division is cytokinesis in which an actin/myosin contractile ring forms and pinches the cytoplasm into two daughter cells (Miller, 2012).

The structure of the spindle apparatus is comprised of a highly-organized network of dynamic microtubules (MT) that exhibit a high turnover rate and poleward flux (Salmon et al., 1984; Mitchison, 1989). Microtubules, which are part of the cell’s cytoskeleton, are hollow cylindrical filaments comprised of α-tubulin and β-tubulin heterodimers that exhibit a distinct polarity (plus and minus ends). These fibers undergo a phenomenon called dynamic instability – a process in which microtubules switch
between phases of polymerization, depolymerization and pausing (Mitchison & Kirschner, 1984; Walker et al., 1988; Shelden & Wadsworth, 1993).

Initially, GTP hydrolysis was shown to govern this process *in vitro*; however, cellular dynamics are more complex due to regulation from microtubule polymerases, depolymerases and microtubule-associated proteins (MAPS) (Michison & Kirschner, 1984; Weisenberg et al., 1968; Howard & Hyman, 2007). These unique properties have recently been recapitulated *in vitro* using a combination of conserved *Drosophila* proteins – all of which have homologs in the mammalian system (Moriwaki & Goshima, 2016).

During spindle formation, MAPS and motor proteins work together to achieve spindle bipolarity. TPX2, an essential mitotic protein, works in synergy with plus-end directed kinesins Eg5 and Kif15, which are partially redundant motors, to stabilize/bundle microtubules in order to generate kinetochore fibers (k-fiber). The C-terminus of TPX2 localizes both kinesins to the spindle and regulates their motor velocities (Wittman et al., 1998; Eckerdt et al., 2008; Ma et al., 2011; Balchand et al., 2015; Mann et al., 2016). Eg5 is a homotetramer that walks and crosslinks anti-parallel microtubules during prophase to separate the centrosomes. In contrast, the partially redundant motor Kif15, which can exist as a tetramer or dimer, crosslinks parallel microtubules (Kashina et al., 1996; Kapetein et al., 2005; Kapetein et al., 2008; Dreschler et al., 2014; Sturgill et al., 2014; Mann et al., 2016).

Molecular motors play a crucial role in generating force within the spindle that establish and maintain bipolarity. The dominant outward force generator, Eg5, counteracts an inward force by the minus-end directed motor dynein (Ferenz et al., 2009; Tanenbaum et al., 2013). Loss of Eg5 before spindle formation results in a monopolar
spindle, although overexpression of Kif15 in mammalian cells can rescue bipolarity despite its non-essential role (Tanenbaum et al., 2009; Raaijmakers et al., 2012; Sturgill & Ohi, 2013; Mann et al., 2016). Contrarily, inhibition of dynein has been shown to result in an increase in metaphase spindle length (Ferenz et al., 2009; Ma et al., 2011). Interestingly, when Eg5 inhibited cells are injected with the CC1 inhibitory fragment of the p150 subunit of dynactin, to inhibit dynein, bipolar spindle formation is observed (Mayer et al., 1998; Ferenz et al., 2009). Therefore, a clear balance of force exists within the mitotic spindle that is interdependent on plus-end and minus-end directed motors (Saunders & Hoyt, 1992).

In interphase mammalian cells, microtubules emanate from a common microtubule-organizing center (MTOC) named the centrosome. Prior to the onset of mitosis, most interphase microtubules are broken down to create a pool of tubulin for spindle formation. During spindle assembly, multiple microtubule nucleation pathways work collectively to establish a bipolar spindle (Figure 1). Initially, microtubule nucleation occurs at the duplicated centrosomes when the nuclear envelope is still intact. At the spindle poles, the γ-tubulin adapter protein NEDD1 recruits a ring-shaped complex named γ-TuRC (Luders et al., 2006; Haren et al., 2006; Petry, 2016) that contains γ-tubulin and other associated proteins involved in microtubule nucleation (Murphy et al., 1998; Fava et al., 1999; Murphy et al., 2001).

Centrosomal microtubules were once thought to be the sole contributor to spindle formation. Kirschner and Mitchison’s “search and capture” hypothesis stated that astral microtubules searched through cellular space ultimately making contact with kinetochores on each chromosome. Once contact occurs, the microtubule becomes
stabilized leading to microtubule bundling and the formation of the k-fiber. However, this hypothesis was inconsistent with mathematical modeling and with spindle formation in acentrosomal systems (Kirschner & Mitchison, 1986; Heald et al., 1996; Wollmann et al., 2005; Hyman & Karesenti, 1998). Moreover, further experiments showed that the mammalian spindle could form after the removal of centrosomes via laser ablation, suggesting that other microtubule nucleation pathways exist and are sufficient for spindle assembly (Khodjakov et al., 2000).

Nucleation of microtubules occurs around the chromosomes in a Ran-dependent manor that requires TPX2 (Heald et al., 1996; Gruss et al., 2001; Kalab et al., 2002; Li & Zheng et al., 2004; Tulu et al., 2006; Torrosantucci et al., 2008). Ran is a small GTPase that is active when bound to GTP and inactive when bound to GDP. The activity of Ran is mediated by the guanine exchange factor RCC1, which exchanges GDP for GTP. When bound to GTP, Ran releases spindle assembly factors from inhibitory importin complexes, which allows for proper spindle morphogenesis. A gradient of Ran exists around the chromosomes that stimulates nucleation via activation of Aurora A, a key mitotic kinase. The underlying mechanism involves the formation of a supracomplex containing TPX2 and the microtubule nucleation duo NEDD1 and γ-tubulin (Scrofani et al., 2015). To stimulate microtubule nucleation, Aurora A must phosphorylate NEDD1 (Pinyol et al., 2013). This process is governed by TPX2, freed from importins, that prevents phosphatase activity in Aurora A’s active site by interacting with the kinase through its N-terminal domain (Kufer et al., 2002; Bayliss et al., 2003). Therefore, Ran activated TPX2 stimulates chromosomal nucleation by sequestering NEDD1 for Aurora A phosphorylation. This phosphorylation event subsequently activates γ-tubulin’s
nucleating activity leading to microtubule growth in the chromosomal region (Scrofani et al., 2015). In addition, the stability of these chromosome-associated microtubules is mediated by the chromosome passenger complex in a MCAK-dependent manor (Sampath et al., 2004; Maresca et al., 2009; Tulu et al., 2006).

Recently, a new pathway for microtubule nucleation was discovered. Microtubules were observed to nucleate from pre-existing microtubules in an Augmin and TPX2 dependent manor (Janson et al., 2005; Murata et al., 2005; Kamasaki et al., 2013; Petry et al., 2013). In vitro, branching microtubules require TPX2, Augmin, γ-tubulin and active Ran (Petry et al., 2013). Augmin is a hetero-octameric complex (HAUS1-8) that was first discovered in a Drosophila genome-wide RNAi screen for genes required for spindle assembly (Goshima et al., 2007; Goshima et al., 2008; Lawo et al., 2009). Depletion of any of the subunits results in mitotic defects consisting of reduced spindle microtubule density, pole fragmentation and mitotic arrest (Lawo et al., 2009; Zhu et al., 2008; Uehara et al., 2009). While all 8 subunits are essential for the functionality of the complex, HAUS6 and HAUS8 are known to hold important roles in the complex. The microtubule binding subunit HAUS8 interacts with HAUS6 in the Augmin complex – which recruits NEED1 and subsequently γ-tubulin (Wu et al., 2008; Zhu et al., 2009; Hsia et al., 2014). While recruitment of the branching machinery has been studied, the stimulation of branched microtubule nucleation remains poorly understood.

Despite all that is known, the connection between the Augmin pathway and chromatin-dependent pathway remains a mystery. While evidence showing that Augmin contributes to microtubule generation in both the centrosome and chromatin pathway has
surfaced, it cannot be fully applied to the mammalian system (Hayward et al., 2014). Specifically, the Augmin pathway requires TPX2 and the *Drosophila* system lacks a direct homolog (Petry et al., 2013). In addition, *Drosophila* chromosomal microtubule nucleation is dHURP-dependent while mammalian cells are TPX2-dependent (Hayward et al., 2014). Therefore, the mechanism behind branching microtubules in the mammalian system is less understood. Petry’s work showed that TPX2 is required for branching, however, whether TPX2 is solely needed for Aurora A activation or if it plays a greater role in the branching microtubule pathway is not known (Petry et al., 2013).

In Chapter II, we investigated the role of TPX2 in Augmin-dependent MT branching. First, we utilize siRNA targeting either pig TPX2 or HAUS6 to establish the knockdown phenotype of each condition in our hands. Next, we used LLC-Pk1 cell lines expressing TPX2 bacterial artificial chromosome (BAC) DNA (Ma et al., 2011) to see if the far C-terminus of TPX2 regulates localization of Augmin and microtubule branching activity. We utilized siRNA to deplete endogenous TPX2 in the BAC cell lines and measured protein localization with confocal fluorescence microscopy. To assay for microtubule branching, we performed a nocodazole washout to observe microtubule formation in the chromosome region. Results show that the C-terminal 37 amino acids of TPX2 contribute to the localization of Augmin and robust microtubule generation near the chromosomes. In addition, we provide evidence that kinetochore microtubule nucleation and growth can persist without Augmin activity. In contrast, surrounding chromosomal nucleation appears to require Augmin and the far C-terminus of TPX2 in mammalian cells.
Another important area of spindle assembly research is the regulation of key mitotic proteins themselves. Phosphorylation by protein kinases regulates the activity of proteins throughout the cell cycle. Post-translationally modified phosphorylation sites can act as a protein’s off/on switch and mediate/prevent protein-protein interactions. TPX2 houses many mitotic functions, some of which are governed by phosphorylation (Fu et al., 2015; Shim et al., 2015). The expression of TPX2 is cell cycle-dependent, with expression rising in G2 and rapidly depleting towards the end of M phase (Gruss et al., 2002). date, a small number of phosphorylated residues in the N-terminus have been studied, leaving the regulation of the essential C-terminus unexplored.

Chapter III explores the role of TPX2 S738 in mitosis. This residue is located within the motor domain of TPX2 that regulates mitotic motors Eg5 and Kif15 (Wittman et al., 1998; Balchand et al., 2015; Mann et al., 2016). The removal of this region results in mitotic defects that are thought to result from unregulated Eg5 activity (Ma et al., 2011). Therefore, we hypothesized that S738 holds a role in TPX2 regulation of Eg5 during mitosis. Using BAC and plasmid DNA, we confirm that TPX2 738 is important for proper spindle formation and mitotic force regulation. A knockdown/rescue approach in cells shows that a phospho-null 738A results in a multipolar phenotype that is similar, to a lesser magnitude, to the defects observed with TPX2 710 (Ma et al., 2011). However, 738A does not seem to affect cellular Eg5 localization. Lastly, we show that overexpression of the phospho-null mutant affects the prophase centrosome pathway and further forces LLC-Pk1 cells to utilize the prometaphase pathway to establish spindle bipolarity. Overall, the results from both studies reinforce the idea that the far C-terminus of TPX2 holds important mitotic functions.
Figure 1. Schematic of microtubule nucleation pathways after microtubule regrowth. Microtubules are generated at the centrosomes and the chromatin region. Multiple microtubule nucleating pathways are revealed following a release from nocodazole. (A) Microtubules nucleating at the centrosome. (B) Ran-dependent microtubule nucleation in the chromosomal region. (C) Augmin-dependent branching microtubule from a parental microtubule. (D) Microtubule nucleation from the kinetochore. Microtubules are shown in green, chromosomes in blue, centrosomes in grey and centrioles in red.
CHAPTER II
TPX2 C-TERMINUS SUPPORTS ROBUST MICROTUBULE GROWTH IN THE CHROMOSOMAL REGION THROUGH INTERACTION WITH AUGMIN

Introduction

Human TPX2 is a proto-oncogene that codes for a 747-amino acid microtubule associated protein (MAP). This protein has been well studied in both biochemical and cellular settings. In some cancer lines, TPX2 has unregulated expression causing mitotic defects that are thought to contribute towards the survival of abnormal cells. Therefore, understanding the role of TPX2 in the cell holds great value towards translational research. The full structure of TPX2 has been poorly understood, however, a recent structural analytical study has shown that the C-terminus consists of a strong alpha helical content (Sanchez-Pulido et al., 2016). Consequently, the structure of the C-terminus is a good candidate to mediate interactions between TPX2 and other proteins through the alpha helical regions (Andrade et al., 2001).

In the past 15 years, crucial roles of TPX2 have been discovered. The N-terminus holds an essential function of activating Aurora A kinase, a key mitotic regulator. TPX2 is able to interact with Aurora A through amino acids 1-42 (Kufer et al., 2002). This event leads to complete activation of the kinase because TPX2 binds to Aurora’s active site, which prevents phosphatase activity that would inactivate the protein (Bayliss et al., 2003). This activity of TPX2 is governed by the small GTP binding protein Ran, which exhibits a strong gradient around the chromosomes (Kalap et al., 2002). The guanine exchange factor RCC1 mediates the activity of Ran by exchanging its GDP for GTP. This event leads to the dissociation of importin bound nuclear proteins, such as TPX2, HURP
and NuMA, that hold crucial roles during spindle formation and maintenance. Specifically, TPX2 activated Aurora A has been shown to phosphorylate NEDD1, which stimulates microtubule nucleation in the chromosome region through \(\gamma\)-tubulin (Pinyoll et al., 2013; Scrofani et al., 2015).

hTPX2’s C-terminal 37 amino acids (711-747), known as the motor interacting region, hold roles in the regulation of kinesins 5 and 12 (Wittman et al., 1998; Eckerdt et al., 2008; Ma et al., 2011; Balchand et al., 2015; Mann et al., 2016). Previous work from our lab has shown that cells lacking this region undergo mitotic failure due to an imbalance of forces generated by molecular motors (Ma et al., 2011). However, how TPX2 directly contributes to Eg5 activity in the cell is not fully understood. The working hypothesis states that forces generated by both dynein and Eg5 counteract one another to form and maintain a bipolar spindle. In this model, TPX2 C-terminal region contributes to a proper magnitude of force generated by Kinesin 5 through regulation of the motor’s activity (Ma et al., 2011; Gable et al., 2012). In the absence of TPX2’s C-terminal 37 amino acids (TPX2 710), Eg5 levels are reduced on microtubules \textit{in vitro} and in the cell, leading to increased outward force and an overall imbalance of force within the spindle (Ma et al., 2011; Balchand et al., 2015). In the cell, TPX2 710 results in a multipolar phenotype consisting of extra microtubule foci around the spindle (Ma et al., 2011).

Recently, it was discovered in \textit{Xenopus} egg extract that microtubule-based microtubule nucleation, which requires the Augmin complex and results in branched microtubule arrays, requires a large C-terminal fragment of \textit{Xenopus} TPX2 (Petry et al., 2013). This process also requires components involved in the Ran pathway. However, the exact region within the C-terminus that governs this activity has not been determined.
Work performed in different model systems suggests that the molecular requirements of the Ran pathway have diverged amongst organisms. Chromosomal microtubule generation in mammals and *Xenopus* requires TPX2 (Gruss et al., 2001; Tulu et al., 2006). In the *Drosophila* system, a direct TPX2 homolog is absent and Ran-dependent microtubule nucleation depends on a homolog of HURP, whose role in the mammalian system is mediating k-fiber stability (Hayward et al., 2014; Silljé et al., 2006). Like *Drosophila*, *Xenopus* extracts exhibit lack of chromosomal microtubule growth in the absence of HURP (Koffa et al., 2006; Casanova et al., 2008). Another interesting discrepancy is that lack of HURP in mammalian cells has shown to delay not abolish growth at the chromosomes, which contrasts with both *Drosophila* and *Xenopus* (Wong & Fang, 2006). Despite this evidence, the role of HURP in chromosome microtubule generation in the mammalian system has not been extensively explored.

The role of Augmin in chromatin-mediated microtubule generation has also been shown to vary amongst different systems. In *Drosophila* embryos, the removal of Augmin activity eliminates chromosomal microtubule regrowth and reduces centrosomal microtubules (Hayward et al., 2014). This suggests that Augmin is required for microtubule nucleation through the Ran pathway in *Drosophila*. However, depletion of human HAUS6, a subunit of Augmin, has been shown to reduce not abolish microtubule regrowth at the chromosomes (Zhu et al., 2008). Although the effect of residual HAUS6 after siRNA depletion cannot be ruled out, it appears that Augmin depletion in mammalian cells does not abrogate all microtubule nucleation near the chromosomes, rather it strongly reduces the robust regrowth seen in untreated cells. In summary, the roles of TPX2, HURP and Augmin in the microtubule nucleation pathways appear to
vary within different systems; therefore, it appears that the coordination between the mammalian Augmin and Ran pathway acts with a slightly different mechanism that is not fully understood.

Another area of interest that remains unclear is the potential difference in requirements between microtubule nucleation near the chromosomes and at the kinetochore, which are typically grouped together as chromosomal microtubules. Nucleation of microtubules at the kinetochores have been directly observed in both mammalian and *Drosophila* cells, suggesting that this pathway can act independently of microtubule nucleation near chromosomes in mitosis (Khodjakov et al., 2003; Maiato et al., 2004; Tulu et al., 2006; Torrosantucci et al., 2008). Like microtubules generated around chromosomes, kinetochore microtubule nucleation is mediated by Ran GTP, which accumulates at kinetochores in complex with RanGAP1 and cmr1 (Torrosantucci et al., 2008). The RanGTP/RanGAP1/cmr1 complex is hypothesized to suppress microtubule growth at the kinetochore because inhibition of cmr1 results in abolishment of RanGTP and RanGap1 resulting in exaggerated growth from the kinetochore (Torrosantucci et al., 2008). Furthermore, depletion of TPX2 eliminates nucleation at both the kinetochore and the surrounding region (Tulu et al., 2006). This suggests that TPX2’s function in kinetochore microtubule nucleation is most likely the same as nucleation around chromatin, where it activates Aurora A and sequesters nucleation machinery for phosphorylation-dependent stimulation. However, the exact mechanism at play has yet to be resolved.

In summary, the role of the Augmin-dependent pathway in Ran-mediated microtubule nucleation at the chromosome region and the molecular mechanisms that
govern this process is unclear in mammalian cells. Here, we sought to elucidate the roles of TPX2, HURP and Augmin in our LLC-Pk1 pig kidney epithelial cells. We provide direct evidence that lack of Augmin activity abrogates most microtubule regrowth near chromatin, which suggests that the Augmin pathway in mammalian cells is required for chromatin-dependent microtubule generation. Our data shows that cells depleted of HAUS6 have minimal microtubule nucleation near the chromosomes following a nocodazole washout, which results in the growth of long microtubule sticks from kinetochores. We also identify a region of TPX2 that contributes to Augmin-dependent branching activity in mammalian cells. Moreover, depletion of HURP did not prevent microtubule generation near the chromosomes or affect microtubule branching, which contrasts with studies performed in other systems. Together, our data suggests that mammalian Ran-dependent microtubule growth requires Augmin and the far C-terminus of TPX2 but does not require the activity of HURP. We show that branching microtubule activity in the cells requires the far C-terminus of TPX2. In addition, kinetochore microtubule nucleation is most likely Augmin-independent.

**Methods**

**Cell culture**

LLC-Pk1 pig kidney epithelial parental, TPX2 FL LAP and TPX2 710 LAP cell lines were cultured in F10 HAMS nutrient mix containing optimum, HEPES, NaHCO₃, 7.5% fetal bovine serum, 1% antibiotic/antimycotic within a humid 37°C environment containing a 5% CO₂ atmosphere. HeLa cells were cultured in DMEM media containing the same components as F10 HAMS but with 10% FBS and 0.5% antibiotic/antimycotic.
Parental cells were plated on 22x22 mm sterile glass coverslips within a sterile 35 mm petri dish, containing the media listed above, 48 hours prior to experiments.

**Mammalian cell nucleofection**

Nucleofections were performed according to Lonza instructions. LLC-Pk1 or HeLa parental cells were plated at a density of 1x10^6 in a 25 cm³ tissue culture flask 18-24 hours prior to the nucleofections of TPX2, HAUS6 or HURP siRNA. At 18-24 hours after plating, cells were subjected to a trypsin digest, diluted in cold media and collected in a sterile 15mL conical tube. The suspended cells were pelleted using a clinical tabletop centrifuge at the lowest setting (1) for 4 minutes. The supernatant containing trypsin and media was removed without disturbing the pellet. 100 µL of Mirus nucleofection reagent and the appropriate amount of siRNA were added to the cells. Cells were gently flicked to dislodge the pellet followed by gentle pipetting up and down using a p1000 pipette in order to create a homogenous solution. The cell solution was transferred to an amaxa nucleofector cuvette and nucleofected using the X-001 (LLC-Pk1) or I-013 (HeLa) program. The cuvette was removed and the cells were immediately transferred to 500uL of pre-warmed media. The media containing the nucleofected cells was distributed to 35 mm petri dishes containing 22x22 mm sterile coverslips.

**RNAi**

Human TPX2 expression was depleted using the siRNA duplex oligonucleotide 5’ GAAUGGAACUGGAGGGCUU 3’ targeting amino acids 144-162 of TPX2 mRNA sequence (Gruss et al., 2002). Pig TPX2 expression was depleted with the siRNA duplex
oligonucleotide 5’ GAAUGGUACAGGAGGGCUU 3’ targeting a sequence within the mRNA sequence (link to sequence). For depletion of TPX2 expression in both LLC-Pk1 and HeLa, cells were incubated for 40 hours after siRNA treatment. Pig HAUS6 expression was depleted using the siRNA duplex oligonucleotide 5’ CAGUUAACAAAGUACGAGA 3’ targeting amino acids 590-608 in the mRNA sequence. LLC-Pk1 cells were incubated for 72 hours before performing experiments. Pig HURP expression was depleted using the siRNA duplex oligonucleotide 5’ CGAATAGACACUUUGGUU 3’ that targets amino acids 121-139 in the mRNA sequence. LLC-Pk1 cells were incubated for 27 hours prior to experiments.

**Nocodazole release assay**

Cells were incubated in complete culture media with 3.3 µM nocodazole for 3 hours at 37°C and 5% CO₂. At the 3-hour time point, the nocodazole media was removed and cells were washed 4 times with 1X PBS, leaving the last rinse inside the petri dish. Microtubule regrowth was allowed in the last 1X PBS rinse. At 5 or 10 minutes post nocodazole washout, cells were fixed in paragluteraldehyde (see methods: Fixation / Immunofluorescence).

**Fixation / Immunofluorescence**

For HAUS6 staining, cells were washed with 1X PBS and fixed on ice inside a coplin jar containing -20°C 100% methanol for 10 minutes. Methanol fixed coverslips were rehydrated in PBS-tween-azide overnight. For all other staining, cells were washed with 1X PBS and fixed for 10 minutes in paragluteraldehyde (1X PBS containing 3.7%
paraformaldehyde, 0.5% triton X-100 and 0.1% gluteraldehyde). Coverslips fixed in paragluteraldehyde were treated with NaBH₄ solution for 10 minutes to remove any remaining fixative and rehydrated in PBS-tween-azide overnight. Coverslips were incubated with primary antibody and 2% bovine serum albumin (BSA) for 1 hour at 37°, washed in PBS-tween-azide and incubated with secondary antibody and BSA at room temperature for 40 minutes. Stained coverslips were washed in PBS-tween-azide, mounted on glass slides with DAPI fluoromount (SouthernBiotech) and sealed with nail polish. See tables 1 and 2 for a list of antibodies used in Chapter II.

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**Expansion microscopy**

Expansion of cells was performed using the protocol described in Chen et al., 2015. LLC-Pk1 cells were fixed in paragluteraldehyde and were stained with anti-tubulin primary antibodies following an hour-long block in boiled donkey. Cells were incubated in antisheep secondary antibody in hybridization buffer (2X SSC, 10% dextran sulfate, 1 mg/mL yeast tRNA and 5% normal donkey serum) overnight. The following day,
coverslips were washed and incubated overnight in hybridization buffer with a tertiary antibody containing trifunctional DNA oligonucleotide complementary to the oligonucleotide on the secondary antibody. Following washes to remove unbound antibodies, cells were covered with ice-cold acrylamide solution and polymerized at 37°C in a dark environment for 2 hours. Gels were carefully removed from the coverslip and digested overnight in digest buffer (50 mM Tris (pH 8), 1 mM EDTA, 0.5% Triton X-100, 0.8 M guanidine HCl). Digested gels were placed in distilled water for expansion, and stored at 4°C until imaging.

**Microscopy**

For the HAUS6 / γ-tubulin localization studies and expansion microscopy, data was collected using a Nikon A1R+ resonant scanning confocal system coupled with a 60X objective lens with a numerical aperture of 1.4. The following laser settings were used for the HAUS6 localization data: 561nm with a laser power of 25% and gain of 0.92, 640nm with a laser power of 73% and gain of 27.0. The following laser settings were used for the γ-tubulin localization data: 561nm with a laser power of 40% and gain of 1.0, 640nm with a laser power of 73% and gain of 27.0. In both studies, z-stacks were acquired with a distance of 0.2 µm between each plane. Data from the rest of this chapter were either acquired from the Nikon instrument mentioned above or a Nikon eclipse Te300 spinning disc confocal coupled with a 100x objective lens with a numerical aperture of 1.4. 488nm and 561nm solid-state lasers were used with the spinning disc microscope.
Image analysis

All analysis was performed on the NIH ImageJ program. For the HAUS6 and γ-tubulin localization data, region of interests (ROI) were drawn on either the spindle pole or within the half spindle. Fluorescence intensity was measured using the same ROI at both locations. In addition, background fluorescence was measured and subtracted from the values obtained from the spindle measurements. The corrected integrated density values for HAUS6 or γ-tubulin from each cell were divided by the corrected integrated density values for α-tubulin to create a HAUS6 : α-tubulin or γ-tubulin : α-tubulin ratio. The half spindle and pole ratios for both data sets were normalized to the average ratio of TPX2 FL LAP. To measure the distribution of protein localization, plot profile intensity values were obtained by drawing a line connecting both spindle poles with a width covering the spindle. Spindle length was normalized to the smallest length obtained in each data set. Fluorescence was normalized to the greatest value within each fluorescence plot (i.e. HAUS6 and α-tubulin). The final plot shows the normalized averaged values for each fluorophore in the normalized spindle length. For the nocodazole release data, histograms representing fluorescence value distribution were created by drawing an ROI encompassing the chromosomal region and using the histogram function on ImageJ. All images were cropped in ImageJ, transferred to Adobe Photoshop to separate the color channels and moved to Adobe Illustrator.
**Results**

**TPX2 710 phenotype resembles Augmin depletion**

We first examined the cellular localization of TPX2 and HAUS6 in LLC-Pk1 cells (Figures 2 & 3). As expected, TPX2 resided in the nucleus during interphase, due to its nuclear localization signal, and started to bind to microtubules in late prophase. TPX2 co-localized with spindle microtubules throughout the rest of mitosis, although dim signals were observed on astral microtubules. In the later stages of mitosis, TPX2 relocalized to each daughter nuclei and was eventually degraded leading to loss of fluorescence signal. In contrast, HAUS6 localized to the centrosomes in interphase. In prophase through metaphase, HAUS6 co-localized with spindle microtubules while retaining a strong association with the centrosome (Figure 3). We did not observe a strong association with central spindle microtubules in anaphase, presumably due to the sensitivity of our antibody, which was raised against human HAUS6. Using the same antibody in HeLa cells, we were able to observe the documented localization in anaphase (data not shown).

While phenotypes resulting from the depletion of TPX2 and HAUS6 have been examined in other studies, we wanted to determine the effect of depleting either protein in LLC-Pk1 cells. To do so, we used siRNA targeting the pig mRNA sequence of both genes. As expected, silencing expression of TPX2 resulted in a dramatic phenotype, which consisted of ~90% collapsed spindles (Chapter III, Figure 18) (Ma et al., 2011). We also depleted endogenous expression of TPX2 in HeLa cells, which showed a similar but less robust effect (Figure 4). Next, we designed siRNA against pig HAUS6 (adapted from Uehara et al., 2009) and silenced expression in LLC-Pk1 cells. The reduction of
HAUS6 expression resulted in short/weak and multipolar spindle phenotypes (Figure 5). In some cases, we observed the retention of centrosomal localization after depletion, which could result from different populations of HAUS6 having different protein turnover rates. We were unable to perform a western blot to determine the knockdown efficiency because our antibody did not recognize pig HAUS6 from cell extract. However, we confirmed that our siRNA had an effect on HAUS6 expression using immunofluorescence (Figure 5).

Because the large C-terminus of TPX2 is required for branched microtubule nucleation, and the last 37 amino acids of TPX2 hold important roles in the cell, we wanted to compare the phenotype of Augmin depletion to the spindle phenotype of cells lacking the far C-terminus of TPX2 (TPX2 710) to see if that region of TPX2 is implemented in Augmin activity. If two proteins exist in the same pathway, depletion or manipulation of the proteins can give similar spindle phenotypes. We used a cell line expressing truncated TPX2 BAC DNA, lacking the last 37 amino acids, tagged with a C-terminal localization and affinity purification tag (LAP) (Ma et al., 2011; Cheeseman and Desai, 2005; Poser et al., 2008). Following depletion of endogenous TPX2 expression in the TPX2 710 LAP cell lines, we observed the predicted collapsed and multipolar phenotypes seen in previously published work (Figure 6A for image, Figure 18 for data) (Ma et al., 2011). Interestingly, we found that the phenotype of HAUS6 depleted cells and TPX2 710 cells were similar, suggesting that the motor interacting region of TPX2 might regulate Augmin activity.
TPX2 C-terminal 37 amino acids contributes to Augmin localization

Since Augmin interacts with TPX2 in *Xenopus* extracts (Petry et al., 2013) and that HAUS6 depletion phenotype resembles the TPX2 710 phenotype, we asked if the TPX2 C-terminal 37 amino acids were important for Augmin localization in the mammalian spindle. To do so, we analyzed HAUS6 localization in either TPX2 FL LAP or TPX2 710 LAP cells depleted of endogenous TPX2 (Ma et al., 2011). Using fixed cells, we quantified the levels of HAUS6 and α-tubulin at both the spindle pole and half spindle. Strikingly, we observed a ~40% reduction in the half spindle HAUS6 signal in TPX2 710 LAP cells and ~50% in cells lacking TPX2 (Figure 6). In contrast, localization at the pole was unaffected in each condition.

Cells lacking HAUS6 result in a loss of γ-tubulin localization on the spindle fibers (Goshima et al., 2007; Goshima et al., 2008; Zhu et al., 2008). Therefore, we examined γ-tubulin levels in TPX2 710 LAP cells to confirm the reduction of HAUS6 on the spindle observed in our previous experiment. In the half spindle, γ-tubulin levels were reduced by 20% in TPX2 710 cells and 30% in the absence of TPX2 (Figure 7). Taken together, these results show TPX2 contributes to the spindle localization of Augmin and subsequently γ-tubulin through its C-terminal motor interacting region.

Augmin and TPX2 C-terminus contribute to robust microtubule regrowth around the chromosomes

*In vitro*, Augmin-dependent microtubule branching requires the C-terminal half of TPX2 (Petry et al. 2013). In addition, cells lacking TPX2 fail to generate microtubules at the kinetochores and around the chromosomes (Tulu et al. 2006). Because we observed a significant decrease of HAUS6 on spindle fibers in TPX2 710 LAP cells, we asked if
TPX2 710 affects chromosomal microtubule growth in the cell. To examine this issue, we performed a nocodazole release assay that allows observation of microtubule regrowth from both the centrosome and chromosome region (Tulu et al., 2006). We disassembled microtubules in nocodazole, washed the cells in PBS, allowed 10 minutes of microtubule regrowth, fixed the cells and scored the level of regrowth (Figure 8). In control cells, microtubule regrowth was robust near the chromosomes and resulted in a fan like appearance (Figure 9). To determine if the fan like structure of the microtubule regrowth consisted of branched microtubules we performed expansion microscopy, which physically expands the cell resulting in an increase in optical resolution (Chen et al., 2015). With this approach, we determined that the fan-like microtubule regrowth near the chromosomes contained microtubule-based microtubule nucleation (Figure 10). In addition, we did not observe branched nucleation from the centrosomes in expanded cells.

To further confirm that these fan-like structures require Augmin for their formation, we performed a nocodazole release in cells treated with siRNA against HAUS6 (Lawo et al., 2008; Zhu et al., 2008; Uehara et al., 2009). Consistent with previous studies, microtubule generation in the chromosome region was severely diminished. Interestingly, fan-like microtubule regrowth was abolished. Instead, we observed stick-like microtubules projecting from the chromosome region (Figure 9) that are reminiscent of kinetochore microtubule formation (Tulu et al., 2006; Torrsantucci et al., 2008). Augmin depleted cells stained for microtubules and HEC1, an outer kinetochore component, revealed stick-like microtubules emanating from HEC1 positive
foci (Figure 9E). Therefore, Augmin depletion results in a suppression of fan-like, branched arrays, but not kinetochore associated microtubules.

Since depleting Augmin resulted in a substantial reduction in microtubule generation in the chromosome region and resulted in exaggerated growth of kinetochore-associated microtubules, we could use our previous results as an assay for branching activity in the cell. Based on our data, we conclude HAUS6 dependent, fan-like arrays in the chromosome region are composed of branched microtubules. Since a decrease of HAUS6 localization was seen in TPX2 710 LAP cells, we wanted to determine if this region of TPX2 contributes to microtubule branching. TPX2 FL and 710 LAP cells, depleted of endogenous TPX2, were subjected to a nocodazole release and the pattern and level of microtubule regrowth near the chromosomes was examined (Figure 11). We found that cells expressing TPX2 FL LAP showed robust chromosomal regrowth, with abundant fan-like arrays, like LLC-Pk1 control cells. In contrast, regrowth in TPX2 710 LAP cells appeared diminished and exhibited stick-like regrowth, which was remarkably similar to Augmin-depleted cells (Figure 9). Taken together, our data suggests that TPX2 contributes to microtubule branching activity in the mammalian system through its far C-terminus. Additionally, the data show that kinetochore associated microtubules do not require HAUS6 or the far C-terminus of TPX2, suggesting that these microtubules are bundles of parallel microtubules.

Previous work has shown that HURP is required for chromosomal microtubule regrowth and for stabilization of kinetochore fibers, but its role in microtubule branching has not been tested. First, we depleted HURP in LLC-Pk1 cells and confirmed a strong chromosomal alignment phenotype in ~50% of cells (Figure 12A). Next, we used HURP
depleted cells in the nocodazole release assay using a 5-minute time point to compare nucleation defects with prior studies (Wong and Fang, 2006). We found that total microtubule regrowth was reduced in a majority of cells, however fan-like organization of MT regrowth persisted in most cells (Figure 12B). This suggests that the Augmin pathway in LLC-Pk1 cells can still act when HURP activity is diminished, but overall regrowth near the chromosomes is suppressed at a 5-minute recovery time point.

**Discussion**

Branched microtubules do not occur at the mammalian centrosome

The visualization of Augmin-dependent branched microtubules in mammalian cells is not well documented. While images exist from both plant cells and *Xenopus* extract, images of branched microtubules in the mammalian system are solely from electron tomography (ET) of metaphase U2OS spindles (Murata et al., 2005; Petry et al., 2013; Kamasaki et al., 2013). In this work, we provide the first direct visualization of mammalian-branched microtubules during spindle formation using light microscopy. In contrast to work from *Drosophila*, which shows that Augmin acts on centrosomal microtubules, we did not observe branched microtubule nucleation from the centrosomal arrays during our regrowth assay. In fact, depleting HAUS6 lead to increased length of astral microtubules in our cells and others (Figure 5) (Zhu et al., 2008). This can be explained through the lack of a TPX2 homolog and the differences in molecular requirements for microtubule nucleation at the chromosomes, which has been shown to be dHURP-dependent (Hayward et al., 2014). Interestingly, the ET augmin study did not identify branching activity at or near the centrosomes in mature spindles, which
complements our observation of branchless centrosomal regrowth (Kamasaki et al., 2013). One scenario that can explain this result is the varying necessity of TPX2 in the different nucleation pathways. Since TPX2 is not required for centrosomal nucleation but is essential for Ran-mediated/branching microtubule activity, it is possible that Augmin is also not involved in the centrosome pathway in mammalian cells because Augmin appears to act downstream of TPX2 in both cases. While NEDD1 and γ-TURC are localized at the centrosome, depletion of TPX2 in human cells does not impair γ-tubulin nucleating activity at the centrosome, which suggests that nucleation and growth at the centrosome is stimulated in an Augmin-independent manner (Tulu et al., 2006). In addition, we do not observe HAUS6 or γ-tubulin localization on astral microtubules, which implies that Augmin lacks activity on the centrosome microtubule population in mammalian cells.

**Chromosomal microtubule growth requires Augmin in mammalian cells**

Previous studies have investigated the lack of Augmin activity in human cells through protein depletion but lacked focus on branching activity itself. We provide a system in which microtubule-based microtubule nucleation can be assayed in the cell. By establishing that fan-like growth observed near chromatin in LLC-Pk1 cells is branched (Figure 10), using expansion microscopy, and that Augmin depleted cells show limited regrowth (Figure 9), we argue that the absence of a fan pattern corresponds with diminished branching activity. Moreover, Petry’s branching assay showed a similar fan-like arrangement when branching was stimulated *in vitro* (Petry et al., 2013).
Previously, others have observed a strong reduction in microtubule growth at the chromosomes in mammalian cells lacking HAUS6 (Zhu et al., 2008). However, these images provided difficulty in resolving the appearance of the resulting chromosome regrowth. Our microtubule regrowth in cells depleted of HAUS6 also exhibited a significant reduction in chromosome microtubules. It is possible that Augmin somehow acts as a stabilizing agent for Ran-dependent chromosomal microtubule growth and in its absence microtubule generation cannot proceed correctly.

**Kinetochore microtubules do not require Augmin**

Kinetochore microtubule nucleation requires the recruitment of γ-TURC by NUP107-160 and stimulation by Ran GTP (Torrosantucci et al., 2008; Mishra et al., 2010). Our data supports a model in which γ-tubulin microtubule nucleation at the kinetochore is stimulated in an Augmin-independent manor. In HAUS6 depleted cells and in TPX2 710 LAP cells, regrowth around the DNA was severely suppressed. In both cases, we also observed linear, not fan-like, regrowth. Immunofluorescence revealed that these microtubules emanate from kinetochores and are therefore nascent kinetochore fibers (Figure 9). This is an interesting observation because it suggests that nucleation around the chromosomes requires different molecular mechanisms than at the kinetochore. In *Drosophila*, Hayward et al. showed that Augmin is not essential for the formation of kinetochore microtubules (Hayward et al., 2014). However, they did not distinguish whether Augmin is required for kinetochore microtubule nucleation or not. In the absence of Augmin, astral microtubules would still retain the ability to “search and
capture” kinetochores. This process would lead to the formation of kinetochore microtubules and is most likely the observation seen in flies.

Overall, it is still likely that branching microtubule nucleation occurs on nascent k-fibers and these fibers can continually grow without branching microtubule activity (Figure 9). One explanation behind our data is that growth from the kinetochore can overcome disassembly due to present microtubule stabilizing factors such as HURP and γ-TURC, both of which have been shown to increase microtubule stability and interact with plus and minus ends of microtubules (respectively) (Li et al., 1995; Silljé et al., 2006; Torrosantucci et al., 2008; Anders & Swain, 2011). It would be interesting to deplete both HAUS6 and HURP followed by the regrowth assay to see if the kinetochore fiber protrusions are eliminated.

Our observation of exaggerated kinetochore microtubule growth also fits a model in which multiple microtubules nucleating pathways compete for a limited pool of tubulin (Cavassa et al., 2016). Normally, growth from the kinetochore would be suppressed due to centrosomal and surrounding chromosome nucleation having greater tubulin capturing ability (Torrosantucci et al., 2008). In a normal washout, we occasionally see stick-like microtubules, however, polymerization surrounding the chromosomes is substantial. In the case of HAUS6 depleted and TPX2 710 LAP cells (depleted of TPX2), surrounding chromosomal regrowth is suppressed which would give the kinetochores a greater amount of tubulin access leading to the exaggerated microtubule polymerization observed at the kinetochore.
TPX2 far C-terminus is required for branched microtubules

In our cells, depletion of Augmin resulted in multipolar and short/weak spindle phenotypes previously documented in the literature (Lawo et al., 2008; Zhu et al., 2008; Uehara et al., 2009). We noticed that cells lacking the last 37 amino acids of TPX2 showed a similar phenotype that consisted of multipolar and shortened spindles. These observations lead us to believe that Augmin activity could require the C-terminal motor interacting region of TPX2. Interestingly, we found that TPX2 710 LAP cells exhibit reduced Augmin and γ-tubulin localization on the spindle fibers. This implies that that this region of TPX2 is important for Augmin localization on the mitotic spindle. Even though the HAUS8 subunit of Augmin has been shown to be a microtubule binding protein, it is possible that the far C-terminus regulates spatial control of Augmin by further stabilizing the complex on spindle microtubules. TPX2 has been shown to interact with Augmin, although whether the interaction is direct or indirect remains a mystery (Petry et al., 2013). We argue that the far C-terminus of TPX2 contributes to this overall interaction between TPX2 and the Augmin complex.

Strikingly, our localization data strongly correlates with TPX2 710 LAP results in the branching assay. Like our HAUS6 depletion results, these cells showed stick like regrowth with substantial reduction in overall chromosome-associated microtubules. Therefore, it seems probable that the C-terminal 37 amino acids are not only regulating motor activity but also contributing to branching activity. This was a surprising result because this region has never been implemented in microtubule generation before. Our data raise an important question, why are these amino acids of TPX2 important in the microtubule branching pathway? Indeed, TPX2 710 still contains the N-terminal Aurora
A activation region and therefore can retain its role in the stimulation of Ran-regulated microtubule nucleation (Scrofani et al., 2015). However, we show the depletion of Augmin and the truncation of the C-terminal region of TPX2 strongly reduces robust microtubule regrowth around the chromosomes in mammalian cells, which suggests either the impairment of Ran microtubule nucleation, lack of stimulation of microtubule nucleation or deficiency in microtubule regrowth stability.

Augmin pathway does not require HURP

We and others have now shown that depletion of HURP in mammalian cells does not completely abolish chromosomal microtubules, rather its absence slows down the rate of accumulation. This contrasts with observations in *Xenopus* and *Drosophila*, which showed abrogated microtubule generation near the chromosomes. Therefore, HURP may hold varying roles in spindle formation within different systems (Koffa et al., 2006; Casanova et al., 2008; Hayward et al., 2014). Immunoprecipitation experiments in *Xenopus* have shown that HURP is part of a larger Ran-dependent complex consisting of TPX2, XMAP215, EG5 and Aurora A (Koffa et al., 2006). However, this has not been confirmed in mammalian cells and could explain the differences observed here. While our branching regrowth assay showed an overall decrease in microtubule generation in HURP depleted cells compared to control, we still observed the presence of fan shaped arrays around the chromosomes. We suggest that in LLC-Pk1 cells, HURP is not required for microtubule branching activity but rather it enhances the growth of microtubules at the chromosomes through stabilization, which has been observed in other mammalian cells (Silljé et al., 2006; Wong and Fang et al., 2006; Torrosantucci et al., 2008).
In summary, we provide evidence in a mammalian system that Augmin-dependent microtubule branching requires the C-terminus of TPX2 and does not require the stabilizing factor HURP. In addition, kinetochore MT regrowth occurs in the absence of Augmin activity. While the far C-terminus of TPX2 contributes to Augmin localization on the spindle, the exact mechanism behind why branching microtubule is suppressed in its absence will need to be further investigated. We speculate that the mechanisms of Ran-mediated microtubule nucleation and the Augmin pathway in mammals are interdependent (Figure 13), which has been shown in Drosophila, because loss of Augmin nearly reduced all regrowth at the chromosomes. Our results also raise the question of whether kinetochore MT nucleation harbors an entirely different mechanism than the surrounding chromosome nucleation.
Figure 2. Pig TPX2 localizes to the mitotic spindle during mitosis. Representative images of LLC-Pk1 cells fixed and stained for TPX2 (green), α-tubulin (red) and DNA (blue). Images show maximum projections of acquired z-stack series. Scale bar = 5µm.
Figure 3. Pig HAUS6 localizes to the centrosomes in interphase and to the spindle during mitosis. Representative images of LLC-Pk1 cells fixed and stained for HAUS6 (red), α-tubulin (green) and DNA (blue). Images show maximum projections of acquired z-stack series. Scale bar = 2µm.
Figure 4. Depletion of hTPX2 causes mitotic defects in HeLa cells. Representative images of HeLa cells 40 hours after treatment with siRNA targeting TPX2 fixed and stained for α-tubulin (red) and DNA (blue). Images show maximum projections of acquired z-stack series. N = 283 for control and 213 for siTPX2. **, P = 0.002. Bars = standard deviation, scale bar = 5 µm.
Figure 5. Depletion of HAUS6 causes mitotic defects in LLC-Pk1 cells. Cells were treated with siRNA targeting HAUS6 for 72 hours or with a mock nucleofection, fixed and stained for α-tubulin (red) or HAUS6 (green). Images show maximum projection from acquired z-stack series. N = 150 + each. **, P = 0.001. Scale bars = 5 µm.
Figure 6. TPX2 C-terminus contributes to Augmin localization on spindle fibers. A) TPX2 FL and 710 LAP cells were treated with TPX2 siRNA, fixed and stained for α-tubulin (green), HAUS6 (red) and DNA (blue). B) Normalized HAUS6 to α-tubulin intensity ratios from measurements at the spindle pole and fibers (N= 32, 42 & 32 respectively). Average spindle plots profiles representing the distribution of HAUS6 and α-tubulin in each experimental condition (n = 10 ea.). Plots show normalized fluorescence intensities to normalized spindle length. ***, P = 3.26x10^{-6}. Bars represent SEM. Scale bar = 5 µm.
Figure 7. TPX2 C-terminus contributes to γ-tubulin localization on spindle fibers. 
A) TPX2 FL and 710 LAP cells were treated with TPX2 siRNA, fixed and stained for α -tubulin (green), γ-tubulin (red) and DNA (blue). B) Normalized γ-tubulin to α-tubulin intensity ratios from measurements at the spindle pole and fibers (N = 22, 33 & 11 respectively). Average spindle plots profiles representing the distribution of γ-tubulin and α-tubulin in each experimental condition (n = 10, 10 & 6 respectively.). Plots show normalized fluorescence intensities to normalized spindle length. **, P = 0.01. Bars represent SEM. Scale bar = 5 μm.
Figure 8. Microtubule regrowth classification for the nocodazole release assay. Microtubule regrowth in the chromosomal region were classified into four tiers, with tier + being the least amount of regrowth and ++++ representing the greatest regrowth. Green represents microtubules, blue represents chromosomes and red signifies centrioles within the centrosome.
Figure 9. Augmin depletion diminishes regrowth around the chromosomes and enhances kinetochore microtubule growth. LLC-Pk1 cells were either treated with siRNA targeting HAUS6 or used as control and were subjected to nocodazole microtubule depolymerization for 3 hours followed by washouts and fixation. A) Cells were stained for α-tubulin (green) and DNA (Blue) and (B) the stage of regrowth at the chromosomes was classified (N = 50 ea.). C) Plot profiles representing fluorescence intensity of α-tubulin along a ROI line in the chromosome region (zoomed image). D) Histogram of α-tubulin fluorescence intensity distribution in the chromosome region. E) HAUS6 depletion and nocodazole washout out. Cells were fixed and stained for α-tubulin (green), HEC1 (red) and DNA (Blue). Top image is a max projection and bottom image represents zoomed area of red box seen in full image. Scale bar = 5 μm, Panel E zoomed image scale bar = 1 μm.
Figure 10. Microtubule regrowth contains branched microtubules. Expansion microscopy of LLC-Pk1 cells after a nocodazole washout. Cells were fixed and stained for alpha tubulin followed by expansion. Image shows standard deviation projection of z-stack. Top right shows a trace of branched microtubules. Bottom montage represents zoomed image of red box in the top left image.
Figure 11. TPX2 710 regrowth resembles HAUS6 depletion. LLC-Pk1 TPX2 FL and 710 LAP cells were either treated with siRNA targeting TPX2 and were subjected to nocodazole microtubule depolymerization for 3 hours followed by washouts and fixation. A) Cells were stained for α-tubulin (green) and DNA (Blue) and (B) the stage of regrowth at the chromosomes was classified (N = 100 ea.). C) Plot profiles representing fluorescence intensity of α-tubulin along a ROI line in the chromosome region (zoomed image). D) Histogram of α-tubulin fluorescence intensity distribution in the chromosome region. Scale bar = 5 μm.
Figure 12. Branching microtubules do not require HURP. A) LLC-Pk1 cells were either treated with siRNA for targeting HURP or siGLO for control (N = 125 ea.) and chromosome alignment (C.A.) was scored. Green is α-tubulin and red is DNA. Image shows maximum projection. B) LLC-Pk1 or siHURP cells were subjected to nocodazole microtubule depolymerization for 3 hours followed by a 5-minute washout and fixation. B) Cells were stained for α-tubulin (green) and DNA (Blue). **, P < 0.01. Scale bars = 5 µm.
Figure 13. Model for observed microtubule regrowth. In the presence of Augmin and TPX2, microtubules nucleate in the chromosome region (i.e. around the chromatin region and at the kinetochores) and grow using microtubule branching (left). In the absence of Augmin activity (siHAUS6) or the lack of TPX2 C-terminal 37 amino acids, microtubules nucleate from the kinetochores while overall growth around the chromosomes is suppressed. Kinetochore microtubule nucleation has greater access to the tubulin pool leading to the exaggerated growth observed under these conditions (right). Blue = DNA, green = MT, red = centrioles.
CHAPTER III

PHOSPHORYLATION OF TPX2 C-TERMINUS CONTRIBUTES TO BIPOLAR SPINDLE ASSEMBLY THROUGH REGULATION OF SPINDLE FORCE

Introduction

Mitosis is governed by many essential proteins. The microtubule associated protein TPX2 has proven to be a master regulator during cell division by holding key roles in the production and maintenance of the mitotic spindle. While TPX2 contributes to the function of other mitotic proteins, it too must be regulated in order to ensure mitotic progression. TPX2 contains over 40 putative phosphorylation sites (Hornbeck et al., 2012; Hornbeck et al., 2015). To date, only the function of conserved N-terminal phosphorylated residues has been investigated leaving C-terminal phosphorylation unexplored.

One of TPX2’s most important functions is the activation of Aurora A through its amino acids 1-42. Previously it was discovered that the phosphorylation of Tyr 8 and Tyr 10 mediate this interaction, and TPX2 cannot activate Aurora A without their modification (Eyers et al., 2002). This absence of Aurora A activity leads to the lack of phosphorylation at TPX2 S121, 125. Without these post translation modifications, TPX2 is unable to regulate the spindle length due to lack of contribution towards spindle microtubule flux, leading to an abnormally short spindle length (Fu et al., 2013). Furthermore, TPX2 is phosphorylated by CDk1/2 at Thr 72 in a cell cycle-dependent manor. Removal of this phosphorylation activity results in a significant increase in multipolar spindles, which is hypothesized to result from hyperactive Aurora A activity (Shim et al., 2015). Taken together, regulation of TPX2 N-terminus has proven to be important for key kinase activity during mitosis.
The far C-terminus of hTPX2 contains an evolutionarily conserved Serine at amino acid position 738 (Figure 14). Previously, Mass spectrometry and biochemical assay data have shown that CDk1 phosphorylates TPX2 S738 (Blethrow et al., 2008; Shim et al., 2015). CDk1 activity is cell cycle-dependent, with peak activity occurring in M-phase. Interestingly, the phosphorylation activity of S738 appears to be cell cycle dependent, with its modification accumulating in G2 (unpublished data: Tony Ly, Lamond lab). Since pS738 accumulates prior to the onset of mitosis, it is most likely important for cell division.

S738 lies in the C-terminal motor interacting region of TPX2 (711-747). Without this region, cells experience mitotic failure due to unregulation of Kinesin 5 (Ma et al., 2011). This region of TPX2 has shown to inhibit Eg5 and Kif15 in vitro and regulate the localization of these motors in the cell (Ma et al., 2011; Balchand et al., 2015; Mann et al., 2016). In addition, it has been proposed that the regulation of Eg5 occurs in a cell cycle dependent manor mediated by TPX2 and Dynein, a minus-end directed motor (Gable et al., 2012). Previous studies have observed Eg5 transported poleward within the spindle during early mitosis (Uteng et al., 2008; Gable et al., 2012). In the absence of TPX2, Eg5 exhibited plus end directed transportation while the inhibition of dynein resulted in static motility (Gable et al., 2012). This suggests that TPX2 links Eg5 to dynein through its C terminus, which carries both proteins poleward.

Since Eg5’s transportation switches from minus to plus end at anaphase and S738 phosphorylation should steadily decrease after metaphase, due to cell cycle activity of CDk1, we hypothesize that this phosphorylation activity of TPX2 is involved in the cell cycle regulation of Eg5 activity. We also ask a broader question of whether S738 is
important for spindle assembly, because cells lacking the motor interacting region of TPX2 exhibit aberrant spindle morphology (Ma et al., 2011). To test these two hypotheses, we mutated TPX2 S738 in traditional plasmid DNA and BAC DNA. BAC DNA contains the genomic mouse sequence with the endogenous promoter and regulatory regions (Poser et al., 2008). BACs are more suited for experiments because their expression is usually closer to endogenous levels. Traditional plasmid DNA is under the expression of a generic promoter, which leads to under or overexpression of your gene that could lead to misinterpretation of data.

In combination with expressing different forms of TPX2 738 mutants, we also performed cell based assays and an overexpression study to explore the role of pS738. We show that the removal of phosphorylation of 738 leads to an increase in a multipolar phenotype with both traditional knockdown/rescue and BAC expression with siRNA-mediated TPX2 depletion. Furthermore, TPX2 738A LAP also exhibited reduced spindle stability and appeared to have normal distribution of Eg5 on the spindle microtubules. When expressing the TPX2 738A plasmid in cells, we observed a strong dominant-negative monopolar phenotype that results from lack of initial pole separation. Taken together, these results showed that this site contributes to the proper formation of a metaphase spindle by contributing to the regulation of forces within the mitotic spindle.

**Methods**

**BAC recombineering**

A bacteria stab harboring mouse TPX2-BAC clone RPE-24-E011 was obtained from CHORI BAC Resources and streaked on to chloramphenicol (CAM) LB agar plates. We
fused TPX2 homologous arms, containing either the 738A or 738D point mutation, to a localization and affinity purification (LAP) cassette using the following primers:

738A forward (BAC10A) - 5’AGCTGCCTCTGACTGTGCCGGTGGCTCCTAAGTTCTCCACTCGGTTCAGGA TTATGATATTCCAACACTTG 3’

738D forward (BAC10D) – 5’AGCTGCCTCTGACTGTGCCGGTGGATCCTAAGTTCTCCACTCGGTTCAGGA TTATGATATTCCAACACTTG 3’

738A/D reverse (BAC11) - 5’GGGTGAGTCTGAAGACGCGTGCTCCATGCTTGTGATATAGATGCAGCTTCT CAGAAGAACTCGTCAAGAAG 3’

We used the 738A and 738D LAP cassettes with TPX2 homologous arms as a source of DNA for homologous recombination during the recombineering process. All cloning steps were followed according to Gene Bridges – version 3.1 (March 2008). Preparation of cells for electroporation was performed at 4°C and electroporation voltage was set to 1.8 kV. mTPX2 738A LAP and mTPX2 738D LAP were purified using the Nucleobond BAC 100 (Clontech) protocol (Figure 16).

Mammalian cell transfection / Generation of BAC cell lines

LLC-Pk1 cells were plated at a density >50% in a 6 well plate 24 hours prior to transfection. Mirus Transit X-2 system or Clontech Xfect transfection reagents and 2.5 µg of BAC DNA were used during the transfection processes. BAC positive cells were selected with G418 using the method described in Poser et al., 2008. The day after transfection, cell media was replaced with fresh media. The following day, 0.05 g/L of G418 was added to cells. Five days after, the concentration of G418 was increased to 0.4g/L. Cells were kept in selection for ~3 weeks until individual colonies formed. The
colonies were screened for GFP expression and expanded in 25cm$^3$ tissue culture flasks. Tissue culture was performed as described in chapter II.

**Western blot analysis**

LLC-Pk1 mTPX2 738A or 738D LAP whole cell extracts were prepared via lysis in 0.5% SDS, 1mM EDTA, aprotinin, leupeptin and pefabloc. Extract solutions were sonicated and mixed with SDS sample buffer followed by 5 minutes of boiling. Extracts were run on an 8% polyacrylamide gel, transferred to Amersham Hypbond-P membrane (GE Healthcare) and stained for TPX2. Membranes were probed for TPX2 using a Rabbit α TPX2 primary antibody (1:1000, Novus Biologicals) in 5% non-fat dry milk dissolved in TBS-tween for 1 hour. Blots were washed with TBS-tween, probed with a Goat α Rabbit IgG HRP secondary antibody (1:5000, Jackson Immunoresearch Laboratories, Inc.) in 5% non-fat dry milk dissolved in TBS-tween for one hour at room temperature, and visualized using chemiluminescence.

**Cold stability assay**

Cells were incubated in 5 µM MG132 for 1.5 hours to arrest them in metaphase. Next, cells were washed in ice cold PBS twice, placed in ice cold Non-CO$_2$ medium and incubated on ice for 10 minutes to depolymerize unstable MT. Cells were immediately fixed at 10 minutes.
**Overexpression**

2 µg of GFP-TPX2 FL, 738A or 738D DNA were nucleofected into LLC-Pk1 cells using the Amaxa nucleofector (see Chapter II methods). Cells were maintained with tissue culture and plated for experiments when needed.

**Fixation / Immunofluorescence**

Immunofluorescence was performed as described in Chapter II. Cover slips were stained with Rabbit α Eg5 (1:1000, abcam) and goat α rabbit CY3 to visualize Eg5. For microtubule staining, coverslips were stained with mouse α DM1A and goat α mouse CY5 (see Chapter II methods).

**RNAi**

Endogenous pig TPX2 expression was depleted as described in Chapter II.

**Microscopy**

Images and time-lapse series were acquired using the microscope systems mentioned in Chapter II. For the Eg5 localization experiment, 561nm (20% laser power, 0.5 gain) and 640nm (81% laser power, 5.83 gain) were used. Images for the GFP expression analysis were obtained on a Nikon TI-S inverted microscope with a 100X objective lens (NA 1.3) using a blue filter. Live imaging of GFP-738A expressing cells was performed on the Nikon inverted microscope using a 20X objective lens with a NA of 0.5.
**Image analysis**

Image analysis was performed using NIH ImageJ. Plot profiles of Eg5 and α-tubulin were obtained by drawing a line connecting both spindle poles with a width of the spindle. To obtain total GFP expression in the overexpressing cells, signal intensities were measured in ROIs encompassing each cell, corrected by subtracting background intensity and binned based on total fluorescence values. To quantify centrosome separation in prophase cells, the distance between centrosomes was divided by the area of the nucleus for each cell to create a distance: nuclear area ratio.

**Results**

**Phosphorylation of TPX2 S738 contributes to bipolar spindle formation**

Because TPX2 738 phosphorylation accumulates in G2 (unpublished data: Tony Ly, Lamon lab), we asked if this modification is important for proper spindle formation during mitosis. To do so, we performed knockdown and rescue experiments using GFP tagged TPX2 mutants and siRNA targeting pig TPX2. We generated GFP tagged TPX2 full-length, S738D (phospho-mimetic) and S738A (phospho-null) siRNA-resistant constructs. LLC-Pk1 cells were co-nucleofected with siRNA and the indicated TPX2 plasmids and incubated for 40 hours. GFP-TPX2 FL and 738D substantially rescued bipolarity (~65%) while 738A showed a significant increase in multipolar spindles (Figure 15B). Live imaging revealed mitotic rescue in FL and 738D and spindle pole fragmentation in 738A (Figure 15A). Therefore, our data suggest that S738 phosphorylation plays a role in spindle formation and/or maintenance.
To further investigate S738, we generated BAC cell lines with the 738A and 738D mutations (Figure 16). Western blot analysis revealed that the expression of mTPX2 was roughly 30% of endogenous pig TPX2 expression, which was abnormally low, but we proceeded with experiments (Figure 17). The BAC cell lines did not exhibit any overexpression defects and progressed through mitosis normally (data not shown). To confirm our 738A multipolar phenotype, we depleted TPX2 expression in the BAC lines and scored spindle phenotype (Figure 18). TPX2 FL and 738D LAP rescued bipolarity in ~80% of mitotic spindles. In contrast, TPX2 738A LAP resulted in significant increase in multipolar spindles (~25% vs. ~10% in FL and 738D LAP). These phenotypes were confirmed using live-cell imaging (Figure 19). Based on results from the knockdown/rescue and BAC experiments, phosphorylation of the TPX2 far C-terminus contributes to proper spindle formation and/or maintenance.

Eg5 distribution is normal in TPX2 738A LAP multipolar spindles

Since the S738 resides in the Eg5 interacting region of TPX2 and Eg5 is regulated in a cell cycle dependent manor, we asked if the localization pattern of Eg5 was affected in 738A LAP cells with the multipolar phenotype. Using fixed BAC cells depleted of pTPX2, we stained for Eg5 and analyzed the distribution of the fluorescence signal along the spindle. We did not observe any difference in localization pattern of Eg5 amongst TPX2 FL, 738D or 738A LAP cells (Figure 20). Therefore, it appears that Eg5 can bind to the spindle microtubules independently of the phosphorylation status of TPX2 S738.
TPX2 738A LAP decreases the stability of kinetochore fibers

Unlike other spindle microtubules, kinetochore fibers can resist catastrophe and depolymerization following a short 4°C incubation (Rieder, 1981). Since TPX2 contributes to cold stable kinetochore fiber formation, through its C-terminus (Ma et al., 2011), we asked if the formation of kinetochore fibers was disrupted in 738A LAP cells. TPX2 FL, 738D and 738A LAP cells, depleted of endogenous TPX2, were incubated on ice for 10 minutes followed by fixation. As expected, cells lacking TPX2 expression resulted in no detectable k-fibers (Ma et al., 2011). Control LLC-Pk1, TPX2 FL and 738D LAP cells showed strong retention of kinetochore fibers after cold treatment. In contrast, TPX2 738A LAP showed a significant reduction in cold stable fibers (Figure 21). Therefore, the lack of phosphorylation at S738 contributes to lack of cold stable kinetochore fibers.

Expression of 738A interrupts initial centrosome separation

Another method to study the function of a protein is to express DNA constructs in cells and ask if the presence of excess protein disrupts any cellular functions. When expressing GFP-TPX2 FL and 738D in our cells, we observed normal mitotic progression with mostly bipolar spindles and a small population of multipolar and monopolar spindles (Figure 22). However, expression of GFP-TPX2 738A resulted in a strong dominant-negative monopolar phenotype (Figure 23). We measured total GFP expression in live cells expressing each of the TPX2 constructs and scored spindle phenotype (bipolar or monopolar). While we saw small numbers of multipolar spindles in each condition, we did not quantify this phenotype. Remarkably, GFP-TPX2 738A expression caused a
significant increase monopolar spindles (~40%) while FL and 738D resulted in ~5-10% monopoles within the same expression range (Figure 23). Therefore, the presence of 738A seems to affect either centrosome separation, bipolar spindle maintenance or both.

During spindle formation, cells separate their centrosomes in prophase, when the nuclear envelope is still intact, or in prometaphase when the NE has broken down (Tanenbaum & Medema, 2010). These two mechanisms are called the prophase and prometaphase pathways (respectively). LLC-Pk1 cells mostly separate their centrosomes in prophase (data not shown). Therefore, we asked if 738A monopolar spindles initially formed bipolar spindles and collapsed, or if the centrosomes failed to properly separate in prophase. We fixed overexpressing cells and measured prophase centrosome distance. To account for varying size of the nucleus, we calculated the ratio of distance between the centrosomes to the area of the nucleus in each cell (CD:NA). We found that 738A overexpressing cells were deficient in prophase centrosome separation, resulting in a ~50% reduction in CD:NA compared to FL overexpression (Figure 24).

When culturing 738A cells, we noticed that the cultures continued to proliferate. This was a surprising result because 738A expression was observed to have a negative effect on spindle morphology. We live imaged 738A cells in mitosis and observed monopolar spindles transitioning to bipolar spindles after ~3 hours. These cells eventually progressed through mitosis and resulted in the formation of micronuclei in 75% of cells (Figure 25). Therefore, these cells utilize the prometaphase pathway to separate centrosomes, but experience defects in the segregation of genetic material, as evidenced by the formation of micronuclei.
Discussion

During prometaphase and metaphase, Eg5, a plus-end directed kinesin, is transported toward microtubule minus ends. In anaphase, however, plus-end directed motion of Eg5 is observed (Gable et al., 2012). Eg5 poleward movement requires dynein and in cells depleted of TPX2 motion is plus-end directed. These results lead to a model in which TPX2 links Eg5 to dynein for poleward transport in early mitosis and that this interaction is regulated throughout mitosis. It is not yet known why a plus-end directed motor, Eg5, is moved to microtubule minus ends in mitosis. One hypothesis is that to generate force for centrosome separation in early mitosis, Eg5 is required at microtubule minus ends where microtubules from the two centrosomes interact. However, in prophase only diffuse motion of Eg5 was observed, suggesting that minus motion requires TPX2, which is predominantly nuclear in prophase cells (Gable et al., 2012). Following centrosome separation, Eg5 functions on antiparallel microtubules located between the separated centrosomes. Poleward motion of Eg5 may function to limit the number of motors in the midzone and thus prevent excessive outward force generation seen in 738A LAP cells (Figure 18).

Earlier work demonstrated that the C-terminal 37 amino acids of TPX2 contribute to the interaction of Eg5 with TPX2. Cells expressing TPX2 lacking this region (TPX2 710) experience severe pole fragmentation and defects in chromosomal alignment (Ma et al., 2011). In addition, TPX2 710 is less effective in inhibiting Eg5 motility in vitro and cells expressing TPX2 710 fail to generate cold stable kinetochore microtubules in the absence of endogenous TPX2 (Ma et al., 2011; Balchand et al., 2015). These results lead to a model in which TPX2 acts as a tether or brake to regulate Eg5 in the cell by
inhibiting its motility. When this interaction is disrupted, Eg5 generates excess outward force, which causes the poles to fragment leading to the extra microtubule foci seen in TPX2 710 cells.

Phosphorylation of serine, threonine and tyrosine residues in proteins is a widespread mechanism to regulate protein-protein interactions. Previous work has shown that CDk1 phosphorylates TPX2 738 in both cell extract and in *in vitro* settings (Blethrow et al., 2008, Hornbeck et al., 2012; Hornbeck et al., 2015). Since CDk1 activity peaks in early mitosis, TPX2 S738 phosphorylation could play an important role during mitosis. Additionally, this site lies in the region of TPX2 that has been shown to contribute to the interaction with both Eg5 and Kif15 motors (Wittman et al., 1998; Eckerdt et al., 2008; Ma et al., 2011; Balchand et al., 2015; Mann et al., 2016). Moreover, loss of phosphorylation at serine 738 as cells progress into anaphase could disrupt the Eg5 TPX2 interaction, resulting in the observed switch from minus transport by TPX2 and dynein to plus end directed motion (Gable et al., 2012).

In TPX2 depleted and TPX2 710 cells, cold stable kinetochore fiber formation is abolished due to substantial reduction in Eg5 regulation (Ma et al., 2011). In TPX2 710 cells, there is a strong reduction of Eg5 localization on spindle microtubules. This is also true in single molecule assays with microtubules and purified TPX2 (Ma et al., 2011). With decreased regulation of Eg5 activity, microtubule bundling, which helps form a proper kinetochore fiber, could be affected leading to the decreased cold stable kinetochore fibers observed. In 738A LAP cells, we observed a significant reduction in kinetochore fiber stability following cold treatment. This result suggests that there is a reduction in the interaction between Eg5 and TPX2. While we observed pole
fragmentation like TPX2 710, we did not detect a reduction in Eg5 localization on spindle fibers. It is possible that there is a slight decrease in localization that immunofluorescence could not be detect. Therefore, we will need to perform a protein pull down assay with 738D and 738A LAP cells to fully elucidate the role of S738 in the regulation of Eg5. In addition, we cannot rule out that this phosphorylation site is mediating an interaction between TPX2 and an unknown protein that is important for the integrity of the spindle poles and kinetochore fiber stability.

In addition to an increase in multipolar spindles in the knockdown/rescue experiments with 738A, we observed a fourfold increase in monopolar spindles when expressing TPX2 738A in cells. We are confident this is an actual effect of 738A overexpression and not a result of excessive TPX2 levels because FL and 738D expression showed significantly fewer monopolar spindles in the same GFP expression range. In addition, we found that initial centrosome separation in the 738A expressing cells was delayed. These results raise the question of how excess TPX2 738A affects centrosome separation (Gable et al., 2012). We detected TPX2 and Eg5 at the centrosomes in prophase, which suggests that both proteins are interacting during this stage of mitosis. We also observed an increase in prophase cells in 738A LAP cells depleted of endogenous TPX2 (data not shown), which signifies that S738 is mediating some process during early mitosis. Although much TPX2 remains in the nucleus in prophase, a subpopulation associates with the centrosomes where Eg5 is accumulating. Therefore, Eg5 and TPX2 could be interacting during prophase through S738. It would be important to determine if Eg5 levels at the centrosomes in prophase changes when expressing 738A.
With 738A overexpression and knockdown/rescue experiments giving entirely different spindle phenotypes (monopolar and multipolar spindles respectively) the exact mechanism at play remains unknown. Interestingly, previous work has shown that cells depleted of dynein light intermediate chain result in Eg5-dependent spindle pole fragmentation (Jones et al., 2014). This shows that a reduction in dynein force generation gives a similar phenotype as excessive Eg5 force generation. However, this model cannot explain the monopolar spindle phenotype in our overexpressing cells. If dynein force generation was perturbed in 738A expressing cells, we should have observed longer spindle lengths. Therefore, we hypothesize that 738A affects Eg5, not dynein, force generation. Our results also raise the possibility that mitotic forces could be regulated differently in prophase than in prometaphase and metaphase.

Remarkably, we observed 738A monopolar spindles eventually forming bipolar spindles (Figure 25). This observation supports the idea that 738A overexpressing cells can correct the error in initial centrosome separation using a redundant mechanism or by eventually overcoming the inhibitory effect of the alanine mutation. Kif15 could be responsible for the late bipolar spindle assembly seen here. However, Kif15 has only been shown to rescue spindle bipolarity when substantially overexpressed or in the presence of Eg5 rigor mutants (Tanenbaum et al., 2009; Raaijmakers et al., 2012; Sturgill & Ohi, 2013; Mann et al., 2016). In this case, it seems highly unlikely that 738A cells contain upregulated Kif15 expression. Another contributor could be cortical dynein activity on monopolar spindle microtubules, which could pull poles apart and work with Kif15 to establish bipolarity (Tanenbaum & Medema, 2010). In future experiments, we could deplete Kif15 expression or inhibit astral microtubules with a low dose of
nocodazole to see if either influences the ability of 738A monopolar spindles achieving bipolarity.

During mitosis, chromosome missegregation is mediated by lack of proper kinetochore microtubule attachments. In most cases, 738A corrected bipolar spindles formed micronuclei in telophase (Figure 25), suggesting that the presence of the alanine mutation interrupts proper segregation of genetic material. Surprisingly, 738A LAP cells (depleted of TPX2) revealed a ~15% increase in lagging chromosomes compared to FL and 738D LAP cells (data not shown), which is reminiscent of the alignment defects seen in TPX2 710 (Ma et al., 2011).

In summary, we observe phenotypes of dynein light intermediate chain depletion (Jones et al., 2014) and TPX2 710 (Ma et al., 2011) when replacing TPX2 expression with TPX2 738A LAP. Therefore, the multipolar spindle phenotype most likely results from imbalance of forces generated by dynein and Eg5. It is unclear why we see the phenotype of Eg5 inhibition when expressing TPX2 738A. We hypothesize that the presence of 738A in prophase somehow contributes to the reduction of outward force generation in the spindle leading to inward force domination. This would result in a lack of centrosome separation. Taken together, TPX2 S738 most likely contributes to spindle formation by regulating forces within the mitotic spindle.
Figure 14. TPX2 C-terminus is highly conserved. A) Protein schematic of TPX2. B) Alignment of hTPX2 C-terminus with mouse and frog. Serine 738 in the motor interacting domain is evolutionarily conserved. An asterisk indicates a fully conserved residue. A colon (:) represents high conservation in amino acid property. A period (.) indicated low conservation in amino acid property. Colors represent physiochemical properties of indicated residues. Red = small hydrophobic, blue = acidic, magenta = basic, green = hydroxyl + sulfhydryl + amine + G. Arrow indicated hTPX2 S738 within alignment.
Figure 15. TPX2 738A does not rescue TPX2 depletion. A) Time lapse images showing labeled tubulin in untreated, TPX2 siRNA, or knockdown/rescue with siRNA resistant GFP TPX2 constructs. B) Quantification of knockdown/rescue experiments. Expression of GFP-TPX2 738A resulted in increased multipolar spindles. N = 301, 357, 206 and 348 (respectively). **, P = 0.01. Bars represent SEM. Scale bar = 10 µM. (Data by Barbara J. Mann and Cassandra Pelletier).
Figure 16. BAC transgenomics as a tool to study protein function. A) Schematic of the recombineering process used to mutate Serine 738 in the genomic mouse TPX2 sequence. pRED/ET plasmid DNA was electroporated (lightning bolt) into bacteria containing unmodified TPX2 BAC. Translation of the pRED/ET plasmid was activated with L-arabinose and 37°C. A LAP cassette with TPX2 homologous arms, containing the point mutations for 738A or 738D, was electroporated into the bacteria and incorporated into the genomic TPX2 sequence. The modified TPX2 BAC was purified from bacteria and transfected into mammalian cells. B) Depiction of the mTPX2-738A and 738D LAP constructs generated for this study. The LAP tag consists of an S-tag, GFP and neomycin resistance sequences. Genomic sequence depiction by Ensemble. Red boxes in sequence represent exons (gene ID 72119).
Figure 17. Western blot analysis of mTPX2 738A and 738D LAP expression in LLC-Pk1 cells. Whole cell extracts were prepared from each cell line and ran on a polyacrylamide gel. After transferring protein to the membrane, blots were probed for TPX2 and visualized with chemiluminescence. mTPX2 bands are ~30% of pTPX2 signal.
Figure 18. BAC transgenomics confirms 738A multipolar phenotype. BAC cell lines were treated with TPX2 siRNA for 40 hours, fixed (left) and scored for spindle phenotype (right). FL and 738D LAP could mostly rescue spindle bipolarity while 710 and 738A LAP were not. 738A LAP showed a similar phenotype to 710 LAP but to a lesser extent. Images show max projection of a z-stack. N = 185, 304, 310, 301 and 403 (respectively). **, P < 0.05. Scale bar = 5 µm.
Figure 19. BAC cells in mitosis after depletion of endogenous TPX2. The indicated BAC cells were depleted of endogenous TPX2 and imaged live. Images show GFP expression from the LAP tag. Scale bars = 5 µm.
Figure 20. Eg5 distribution is unaffected in 738A LAP multipolar spindles. (Left) Indicated BAC cells were depleted of endogenous TPX2, fixed and stained for α-tubulin (green), Eg5 (red) and DNA (blue). (Right) Plot profiles of representative images show normalized intensity distribution for α-tubulin and Eg5 along the length of each spindle. Images show maximum projections of z-stacks. Scale bar = 5 µm.
Figure 21. Lack of 738 phosphorylation reduces kinetochore fiber stability. LLC-Pk1 (control) and the indicated BAC cells lacking endogenous TPX2 expression were exposed to cold treatment for 10 minutes followed by fixation. FL and 738D LAP rescue fiber stability while 738A LAP cells exhibit a shift in cold-stable microtubule abundance. Images represent maximum projections of z-stacks. \( N = 168, 151, 190 \) and 214 (respectively). **, \( P = 0.001 \). Scale bar = 5 \( \mu \text{m} \).
Figure 22. Mitotic progression is unaffected in FL and 738D expressing cells. Indicated cells were plated and imaged throughout mitosis. Images show single focal plane. Scale bar = 5 µm.
Figure 23. TPX2 738A expression affects spindle bipolarity. Indicated TPX2 constructs were expressed in LLC-Pk1 cells, measured for GFP expression live and scored on spindle phenotype. A) Fixed cell images representing each experiment stained for α-tubulin (red) and DNA (blue). B) Expression of 738A causes a four-fold increase in monopolar spindles. C) Histograms representing binned total GFP fluorescence in each experiment. N = 90, 95 and 150 (respectively). Scale bar = 5 µm.
Figure 24. TPX2 738A expression interrupts initial centrosome separation. A) LLC-Pk1 control cells were fixed and stained for TPX2 (green) and DNA (blue). The overexpressing TPX2 cells were fixed and stained for phosphorylated histone 3 Ser 10 to stain mitotic cells. B) The distance between the centrosomes and the total nuclear area were measured for each cell to form the CD:NA ratio. C) 738A shows reduced centrosome separation in prophase. N = 20, 6, 3 and 18 (respectively). Images show single focal plane. **, P < 0.001. *, P < 0.05. Bars represent SEM. Scale bar = 5 µm.
Figure 25. TPX2 738A expressing cells achieve bipolarity concomitant with chromosome alignment defects. A) Live imaging of GFP-TPX2 738A cells in mitosis. Monopolar spindles were observed to establish two spindle poles and subsequently progress through mitosis. These cells formed micronuclei 75% of the time. B) 100X time lapse of 738A monopoles pushing spindle poles apart. Scale bar = 5 µm.


