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In vivo analysis of cell division during vertebrate development

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In vivo analysis of cell division during vertebrate development

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August, 2009

Dedication

To Mom, Dad and Jason

Acknowledgements

I would like to thank my advisor John Wallingford for his guidance, time, patience, encouragement and knowledge. I am also grateful to my committee members: Jeffrey Gross, Jennifer Morgan, Clarence Chan and Arturo DeLozanne, for their invaluable input during this process. I'd also like to thank the past and present members of the Wallingford lab for the input, the laughs, keeping me in check and the cookies. Also, this would not have been possible with out the support of my partner Jason and best friends Katie and Kristen.

In vivo analysis of cell division during vertebrate development

Publication No._____

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The University of Texas at Austin, 2009

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In this work, we identified and characterized developmentally regulated aspects to cell division in the *Xenopus laevis*. We found that cells in the early neural plate divide in an oriented manner. This orientation is established by Cdc42 controlled maintenance of stable interactions between the spindle and the cell cortex. This role of Cdc42 is developmentally regulated and cells dividing later in a related tissue, the tail epidermis, are not under this control. Moreover, we find that the cell divisions in the early neural plate are further specialized in their mechanisms of cell division. Cells in the early neural plate exhibit exaggerated anaphase-B movements, a delayed onset of cytokinesis, low density of midzone microtubules and a rapid cytokinetic furrow ingression as compared to the late tail epidermis, another ectodermally derived tissue. These modifications to the mechanism of cell division appear to be because of a reduced level of PRC1, a microtubule bundling protein, and thus modifications to the central spindle structure. Finally, we find that cytokinetic mechanisms may be functionally related to the process of ciliogenesis. We find proteins known to localize to the central spindle localized to the rootlet of the basal body of cilia in multiciliated cells of the mucociliary epidermis. This

localization may be related to vesicle transport during both these processes. This work reveals unexpected plasticity to fundamental mechanisms of cell division

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Chapter 1: General Information

1.1 Steps of Cell Division

The phases of the cell cycle are well studied and this process is well conserved between species (Hartwell and Weinert, 1989; Nurse, 1990; Oshima and Campisi, 1991). The process of cell division undergoes a number of modifications during development. Just after fertilization, cells begin a process called cleavage whereby cells divide in a rapid manner, only undergoing DNA replication and mitosis, approximately halving their volume after each division (Etkin, 1988; Masui and Wang, 1998; Philpott and Yew, 2005). Cell divisions are rapid and synchronous during the first 10-13 cell divisions after fertilization (Kane and Kimmel, 1993; Fogarty et al., 1994; Klymkowsky and Karnovsky, 1994). After this cell cycle the cell cycle begins to extend to a classical cell division cycle. The phases of this cycle are interphase and mitosis (Oshima and Campisi, 1991). Interphase is, in fact, three individual phases: a growth period (G1), synthesis (S) and a second growth period (G2). Mitosis is also divided into phases: prophase, prometaphase/metaphase, anaphase and telophase. As embryos develop, the cells begin to differentiate (Figure 1.1). Once fully differentiated, cells generally enter a quiescent state (G0). When required, for example when a tissue is injured, cells can re-enter the cell cycle.

1.1.1 Interphase

Interphase is the longest phase of the mitotic cell cycle. During this period the cell builds up the materials it needs to undergo division and prepares these materials for mitosis. As mentioned above interphase is divided up into three phases, G1, synthesis (S)

and G2. During G1, cells prepare for S-phase by building up the proteins required, such as polymerases and histones (Pardee, 1989). During the synthesis phase, the DNA of the cell is replicated and is prepared for separation during mitosis. Nucleosomes form on newly replicated DNA just behind the replication machinery (Laskey et al., 1989). This is the beginning of chromosome condensation for mitosis. Chromosomes will be further condensed later in the cell cycle. During the second growth phase, G2, the cell ensures that chromosomes are replicated with high fidelity (Laskey et al., 1989) (Figure 1.1).

1.1.2 Mitosis

The phase of "mitosis" is the process by which the newly synthesized DNA is separated into two new cells. Mitosis has been divided into four individual phases: prophase, prometaphase/metaphase, anaphase and telophase. During prophase, DNA continues the condensation started during the synthesis phase and the nuclear envelope begins to break down. This phase ensures that the chromosomes are small enough to separate quickly. The mitotic spindle begins to form by mass assembly of microtubules from the newly replicated centrosomes. These mitotic spindle microtubules become attached to the kinetochores of the chromatid pairs and the spindle positions the chromosomes at the metaphase plate during prometaphase/metaphase (McIntosh and Koonce, 1989). Next, anaphase occurs where sister chromatids are separated from each other and move toward the spindle poles. Anaphase occurs in two steps. First, anaphase-A occurs, where the chromosomes move apart from each other and then the spindle poles separate from one another during anaphase-B (Cande and Hogan, 1989). Telophase then occurs to create two new daughter cells by forming two new nuclei and decondensation of the chromosomes (McIntosh and Koonce, 1989). The process of cytokinesis separates these two new cells. Plasma membrane from each side of the cell ingresses then meets at

the middle of the daughter cell (Rappaport, 1971; Glotzer, 2001). The final step to the cell cycle is the abscission where the plasma membranes of the cytokinetic furrows fuse forming two separate cells (Figure 1.1).

1.2 RATIONALE

Cell division ensures the proper segregation of genetic information and contributes importantly to embryo morphogenesis (Rappaport, 1961; Kaltschmidt et al., 2000; Chalmers et al., 2003; Gong et al., 2004). Most of the analysis of mechanisms of cell division has been conducted on cells in culture. While these studies are fundamental to understanding the process, they do not address how these mechanisms are regulated during development. Basic mechanisms of cell division remain relatively ill defined in early vertebrate embryos. Previous analysis of cell division during development has concentrated on fixed cells and DNA stains, such as BrdU or DAPI, to determine the phase of the cell cycle (Hartenstein, 1989).

Furthermore, time-lapse analysis of cell division during development has focused on if cell division simply occurs with a particular gene mutated (Saka and Smith, 2004). More recently time-lapse analysis has been used to study the orientation of cell division during development and the molecular cues that control this orientation in the fish (Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Gong et al., 2004). While these studies are important they do not address how cell divisions are regulated during development. As embryos develop they go through a number drastic changes. During the cleavage stage of development chromosomes are kept in a highly condensed state for much of the short cell cycle. At the 11^{th} cell division when embryos undergo the midblastula transition and cells begin to have gap phases (G1, G2), with some cells becoming quiescent in the G0 stage. This allows the chromosomes to decondense long

enough for the transcription machinery to build up and transcription begins at the midblastula transition (Patterton and Wolffe, 1996). The initiation of transcription causes cells to begin differentiating and to become different from one another. These differences are easily seen in the adult form but leave the question of how they are different earlier in development. Cell division is a process that may be very similar during development or may be very different. We sought to address these questions in *Xenopus* through analysis of the general mechanisms of cell division and the control of oriented cell divisions, throughout development. Combining data from cells in culture, *Xenopus* and other organisms we wish to formulate a more comprehensive model of the controlling factors of cell division. Understanding how these and other systems differ in the control of cell divisions should eventually lead to a model that will encompass all recent findings.

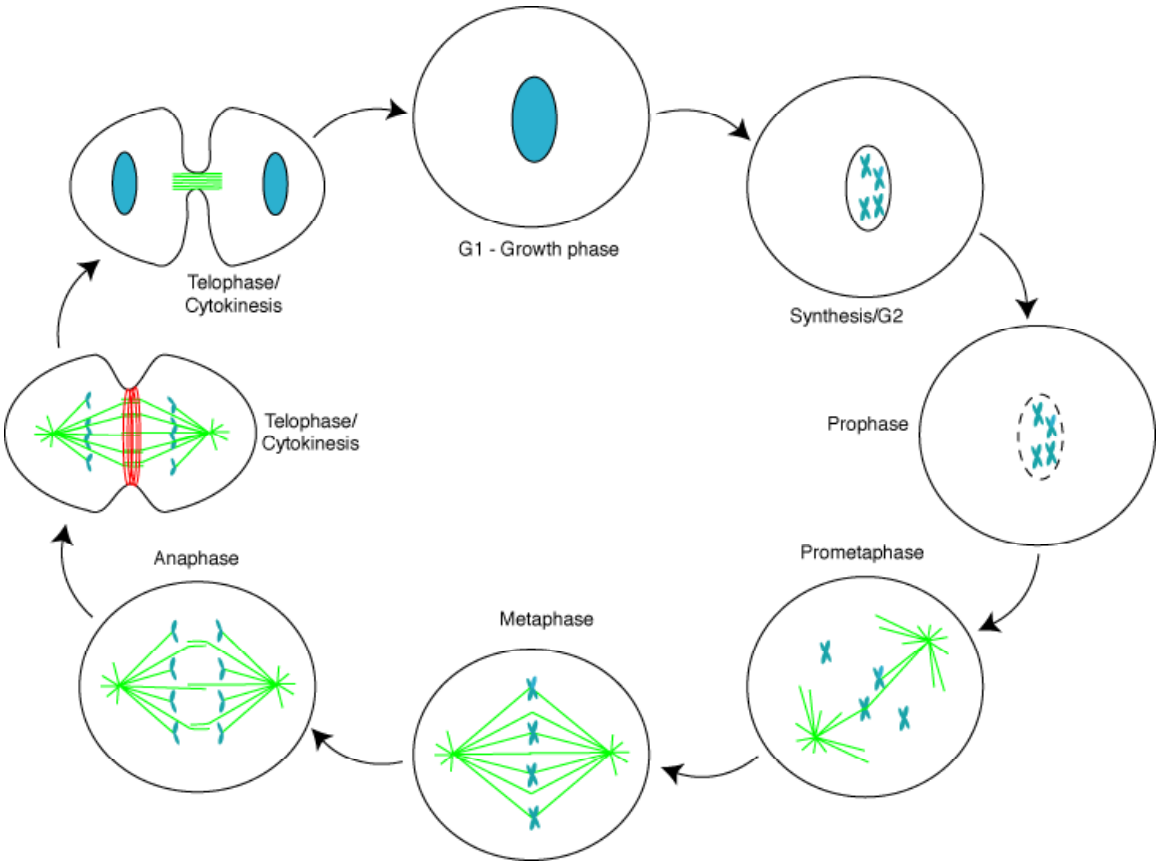

Figure 1.1. The cell cycle

Cells begin the cell cycle in the G1, growth or gap 1, phase where they build up materials for synthesis of DNA. Cells then synthesis their DNA during S-phase and then enter another growth phase, G2, where cells build up the material for mitosis and the chromosomes begin to condense (DNA – blue). Cells then enter mitosis beginning with prophase, where chromosomes continue to condense and the nuclear envelope breaks down (nuclear envelope – black doted line). During prometaphase and metaphase the mitotic spindle begins to form (green). The mitotic spindle attaches to the chromosomes and position the chromosomes to the metaphase plate. The chromosomes begin to separate during anaphase. The central spindle, the bundle of microtubules between the separating chromosomes, sets up the contractile ring (red). The contractile ring causes the cytokinetic furrows to ingress during telophase. Cytokinesis is the process of the ingressing furrows and the separation of the two new daughter cells. Telophase, on the other hand, is the process of the mitotic spindle breaking down and the reformation of the nuclear envelopes. The end result of this cycle is two new daughter cells.

1.3 Neural Tube Closure

Neural tube closure is the process by which a flat sheet of cells, the neural plate, roles into a hollow tube to form the brain and spinal cord. The process of neural tube closure in *Xenopus laevis* is one of large morphogenic movements. The embryo gets becomes thin and lengthens by a process called convergent extension. Convergent extension occurs by a rearrangement of cells. Cells in a tissue elongate along one axis while intercalating between each other along the opposite axis (Figure 1.2A and B) (Keller et al., 1985; Wilson and Keller, 1991; Davidson and Keller, 1999; Zajac et al., 2000; Wallingford et al., 2002). This leads to cells that were once neighbors on one axis are now neighbors along the opposite axis (Figure 1.2C). This process elongates and thins the tissue they are in (Figure 1.2, compare C to A).

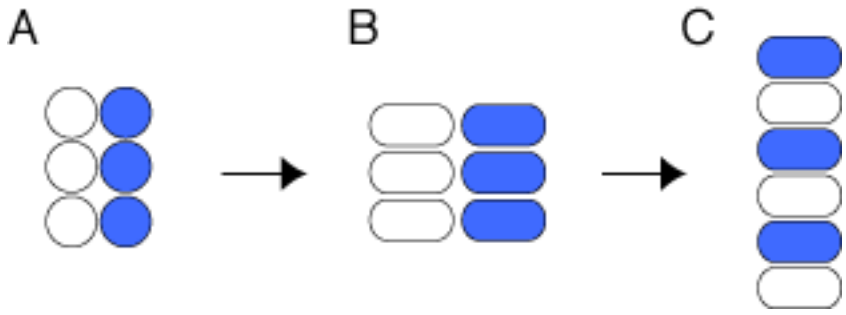

Figure 1.2. Model of convergent extension

(A) Cells that will undergo convergent extension begin in a non-extended state with each axis being about the same length. (B) Cells elongate one axis by extension of lamellipodia on each side of the cells. (C) Cells uses these lamellipodia to crawl between each other. This process causes the tissue to be longer and thinner than it was.

To form the brain and spinal cord a tube of ectoderm is formed and internalized by invagination of the neural tissue and the medial movement of the epidermis (Figure 1.3). Two neural folds form on the lateral edges of the neural plate (Zohn et al., 2003). These folds are formed by the changes to the shape of the cells in this region (Jacobson and Gordon, 1976). Cells in the folds constrict their apical surface while keeping their basal surface the approximate original size. This causes the kinking of the tissue and the formation of the fold (Figure 1.3B) (Lee et al., 2007). These types of cell shape change occur also at the midline to begin the folding of the tissue forming a medial hinge point (Figure 1.3B) (Zohn et al., 2003). The epidermis that flanks the neural plate also aids in the folding/rolling of the neural tube by its medial movement (Figure 1.3B and C) (Alvarez and Schoenwolf, 1992). Finally, dorsal lateral hinge points form between the neural folds and the medial hinge point (Figure 1.3C) (Zohn et al., 2003). These motions bring the neural folds close to each other and the folds can then fuse together forming a tube of internalized ectoderm. The neural plate of *Xenopus* has two cell layers, a deep layer giving rise to the primary neurons and a superficial layer that only differentiates much later and continues to proliferate dramatically during general tube closure (Hartenstein, 1989; Chalmers et al., 2002). This superficial layer of the *Xenopus* neural plate allows for easy analysis of cell division during neural tube closure in this organism.

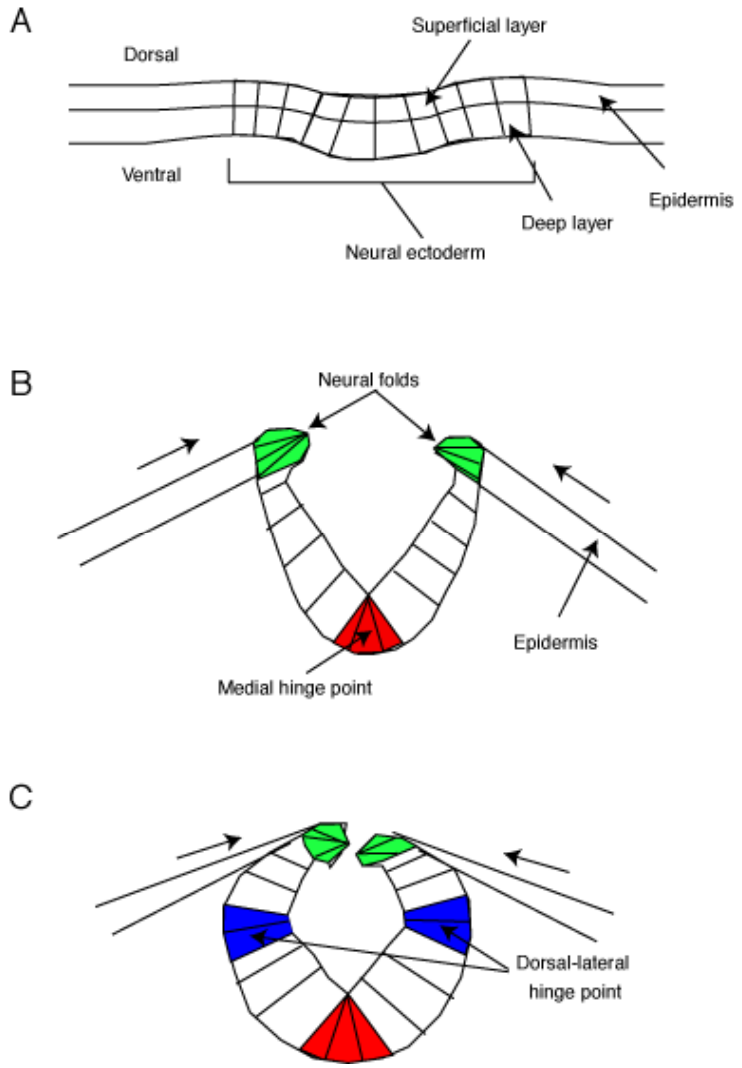

Figure 1.3. Diagram of neural tube closure

(A) Diagram of the neural plate prior to the initiation of neural tube closure. In *Xenopus* the neural plate is made of two layers, the superficial layer and the deep layer, and is flanked on each side by non-neural ectoderm, which will form the epidermis of the animal. (B) At the boarder between the neural ectoderm and epidermis cells undergo apical constriction to form the neural folds. Apical constriction also occurs at the midline of the neural plate, forming the medial hinge point. This process begins the folding of the neural plate (C) At a position between the medial hinge point and the neural folds the dorsal-lateral hinge points, again by apical constriction. This brings the neural folds in close proximity to one another. These neural folds will eventually fuse completing the neural tube closure.

1.4 ORIENTED CELL DIVISION

The proper completion of the phases of cell division is not the only aspect that is important to cells dividing in a tissue. The orientation of division has been found to be an attribute crucial for proper morphogenesis and embryonic development. Oriented cell division is the process in which cells in a tissue all divide in a particular orientation thereby contributing to the elongation or general shape of that tissue (Strutt, 2005). These types of divisions are found in many organisms and control different processes during development. In *C. elegans* oriented cell divisions determine the anterior/posterior axis of the animal (Gonczy and Rose, 2005), while in the fly these types of divisions contribute to the elongation of kidney tubules (Fischer et al., 2006; Saburi et al., 2008) and the distribution of determination factors in neuroblasts (Bellaiche et al., 2001a; Bellaiche et al., 2001b). Analysis of how these types of divisions are controlled throughout development is lacking (Geldmacher-Voss et al., 2003).

1.4.1 Oriented Cell division and development

Developmentally regulated cell division is a central facet of embryogenesis. Precisely oriented divisions are important for controlling determination of the embryonic axes in *C. elegans* (Gotta et al., 2001; Gonczy and Rose, 2005), for axis elongation and epithelial tube elongation in vertebrates (Concha and Adams, 1998; Gong et al., 2004; Fischer et al., 2006; Saburi et al., 2008) and for the diversification of cell types in the embryonic nervous systems of *Drosophila* and *Xenopus* (Bellaiche et al., 2001a; Bellaiche et al., 2001b; Chalmers et al., 2003; David et al., 2005).

Oriented cell divisions have been thoroughly described in more mature vertebrate nervous systems as well. Early in development, the hollow neural tube forms by the intricate folding of an initially flat neural plate during a process termed neural tube

closure (Wallingford, 2005). The formation of neurons occurs in the closed neural tube via oriented cell divisions. Divisions that occur parallel to the plane of the neural epithelium, dividing the apical membrane in half equally, are generally proliferative, while perpendicular divisions, one daughter cell adopts more of the apical membrane, tend to be neurogenic. The daughter cell that adopts more of the apical membrane remains proliferative while its sister cell differentiates into a neuron (Haydar et al., 2003; Gotz and Huttner, 2005; Wilcock et al., 2007).

Furthermore, proliferative divisions occurring within the plane of the neural epithelium can display stereotypical orientations with respect to the anteroposterior axis. During neural tube closure in chick embryos, the axis of cell division is preferentially parallel to the long axis of the embryo and help to elongate the neural tube along this axis (Sausedo et al., 1997), while in zebrafish, divisions are initially parallel to the long axis and shift to a perpendicular orientation as neurulation proceeds (Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Gong et al., 2004). The *Xenopus* embryo represents a special situation, however. The neural plate of *Xenopus* has two cell layers, a deep layer, giving rise to the primary neurons, and a superficial layer that only differentiates much later and continues to proliferate dramatically during general tube closure. Previous analysis has shown that cell divisions occur in both these layers (Hartenstein, 1989; Chalmers et al., 2002). Because of imaging constraints we focused our work on the superficial layer. *Xenopus* eggs and embryos contain a large amount of yolk, which makes them opaque. This makes imaging more than ten-to-twenty microns past the surface of the embryo difficult (Becker, 2006). This means that the superficial layer can be imaged easily but the deep layer cannot.

1.4.2 Role of PCP signaling in oriented cell divisions

Many oriented cell divisions have a few controlling factors in common. In particular, the planar cell polarity (PCP) pathway has been found to be a major player. Cells in an epithelium have one major axis of polarity, apical/basal polarity, which is perpendicular to the plane of the tissue. Some epithelial cells also have another polarity, planar cell polarity, which organizes the cells within the plane of the tissue (Nechiporuk and Vasioukhin, 2006). This polarity can be seen through the organization of cellular structures, such as hairs or cilia. For example, in the *Drosophila* wing, each cell forms a single hair that is formed on the distal most part of the cell and points in a distal direction. The PCP signaling pathway determines all of these aspects, the number, the location and the orientation, of the hair. When genes of the PCP signaling pathway is mutated various alterations to the hairs are seen (Adler and Lee, 2001).

The planar cell polarity pathway is also known as the non-canonical Wnt (Wingless/Int) pathway (Nusse and Varmus, 1982; van Ooyen and Nusse, 1984; Rijsewijk et al., 1987). The canonical Wnt pathway and the non-canonical pathway share a similar gene set but elicit different results. The canonical Wnt pathway occurs when a Wnt ligand binds to a Frizzled receptor and this activates the cytoplasmic Dishevelled protein. Dishevelled then inhibits the degradation of β-catenin by the Axin/APC/GSK3β complex (Cadigan and Nusse, 1997; Korswagen, 2002). This leads to the stabilization of β-catenin and its accumulation in the nucleus. β-catenin then interacts with and stabilizes the TCF/LEF-1 family of transcription factors and induces the transcription of down stream genes which effect cell fate decisions (Struhl and Basler, 1993; Lawrence et al., 1996; Cadigan and Nusse, 1997). The non-canonical Wnt pathway does not induce transcriptional activation. Instead, PCP signaling causes changes in the morphology of cells through changes to the cytoskeleton.

The PCP signaling cascade uses two sets of genes: the core PCP signaling genes and PCP effector genes. Core PCP genes include a number of membrane bound receptors required to activate the signaling pathway. Some of these genes are also involved in canonical Wnt signaling, such as Frizzled receptors and Dishevelled (Shulman et al., 1998). PCP effector proteins are genes that are activated down stream of the core PCP genes and are tissue specific (Eaton, 1997; Adler, 2002; Weber et al., 2008). As mentioned above, the PCP signaling pathway controls the localization, orientation and number of hairs on cells in the *Drosophila* wing. Core PCP genes localize in distinct regions at the cell cortex to direct the growth of hairs. Flamingo, a seven-pass transmembrane cadherin, localizes to the proximal and distal sides of wing cells, while Dishevelled and Frizzled localize only to the distal cortex (Klingensmith et al., 1994; Usui et al., 1999; Adler and Lee, 2001; Malbon, 2004). When one of these core PCP molecules are mutated the other core proteins are mislocalized and alterations to the hair location, orientation and/or number are seen (Adler and Lee, 2001). Core PCP molecules signal downstream to PCP effector proteins. These effector proteins transduce the signal to the cytoskeleton of the cell and control the orientation and number of hairs. When these genes are mutated the core PCP proteins are not mislocalized but defects are seen.

Core PCP and PCP effector proteins also control the orientation of cell divisions in other tissues of the fly. PCP signaling was first shown to regulate spindle rotations that orient cell divisions in *Drosophila* neuroblasts, facilitating the asymmetric distribution of determination factors and the formation of sensory organs (Bellaiche et al., 2001a; Roegiers et al., 2001; David et al., 2005). In the fly, the PI cell divides in the plane of the epithelium to form the PIIa and PIIb cells. A spindle rotation occurs to ensure the proper division orientation of this division. These two cells under go a stereotypical set of oriented cell divisions, each of with is established by mitotic spindle

rotations, to form five cells which then form the sensory organ (Roegiers et al., 2001). For the proper determination of these different neuroblasts Numb must be segregated correctly. Recently, Frizzled has been found to control the correct localization of Numb and partner of numb (Pon) and control spindle rotations associated with the correctly oriented cell divisions (Bellaiche et al., 2001a; Bellaiche et al., 2001b).

PCP signaling also controls proliferative oriented cell divisions in other organisms. PCP signaling in the zebrafish governs orientation of cell divisions along the anterior-to-posterior axis that facilitate elongation of the embryo (Gong et al., 2004). Most recently, PCP signaling has been found to control oriented cell division in the developing mammalian kidney to elongate kidney tubules (Saburi et al., 2008). The mechanism of this process is currently unknown but thought to be similar to what is observed in fly neuroblasts. It should be noted however, that this role for PCP signaling in controlling oriented cell division is not universal. For example, extension of the germband in *Drosophila* also relies on oriented cell divisions, but this orientation is independent of PCP signaling (da Silva and Vincent, 2007).

1.4.3 Role of Cdc42 in oriented cell divisions

Another common player in developmental regulation of oriented cell divisions is the small Rho GTPase, Cdc42. The Rho-GTPases include isoforms of Rho, Rac and Cdc42. These proteins have many various roles in the cell but the major body of research has been on their regulation the cytoskeleton (Boureux et al., 2007). Rho-GTPases can be in two states, active, bound to GTP, or inactive, bound to GDP. Three different sets of proteins regulate these states. Guanine nucleotide exchange factors (GEFs) help exchange a GDP for a GTP and thus activate the GTPase. GAPs, GTPase activating protein, inactivate the protein by promoting the hydrolysis of GTP to GDP. Finally the

guanine nucleotide dissociation inhibitors (GDI) stabilize the GDP bound state making it harder for the GEFs to activate the proteins (Bement et al., 2006). When RhoGTPases are bound to GTP and in their active state they bind to and activate downstream effector proteins (Etienne-Manneville and Hall, 2002). Active effector proteins then cause changes within the cell. These changes range from actin polymerization to endocytosis (Niedergang and Chavrier, 2005; Ridley, 2006).

Cdc42, in particularly, has been shown to have a wide range of activities. Cdc42 has been shown to control cleavage furrow ingression in *Xenopus* eggs (Drechsel et al., 1997) and controls axon guidance (Luo et al., 1996). Cdc42 is associated with the Par3 complex (Par3/Par6/aPKC) and controls spindle orientation during the first cell cycle and thereby is involved in defining the anteroposterior axis in *C. elegans* (Etemad-Moghadam et al., 1995; Tabuse et al., 1998; Hung and Kemphues, 1999; Joberty et al., 2000; Gotta et al., 2001; Gonczy and Rose, 2005). Cdc42 has also been shown to be involved in bud site selection for yeast (Adams et al., 1990). Cdc42 is localized to the bud site in yeast and in the growing bud and appears to direct vesicle traffic to the bud (Ziman et al., 1993). These data indicate that Cdc42 has a clear role in controlling the polarity of cells through its subcellular localization. A clear role for Cdc42 in controlling oriented cell division in vertebrate somatic cells has not been reported as yet, but Cdc42 is essential for proper localization and organization of the meiotic spindle in mouse oocytes (Na and Zernicka-Goetz, 2006). Furthermore, Cdc42 is activated asymmetrically on meiotic spindles in *Xenopus* oocytes, where it links spindle position to the position of the cytokinetic ring (Ma et al., 2006).

Cdc42 has been found to play a role in controlling the polarity of cells and oriented cell divisions (Kuroda et al., 1997; Rojas et al., 2001; Suzuki et al., 2001; Yamanaka et al., 2001). The involvement of Cdc42 in oriented cell division in

developing embryos is particularly intriguing in light of recent findings that suggest that Cdc42 plays cell-type-specific roles in many fundamental aspects of cell division. In some cell lines, Cdc42 has been reported to control microtubule attachment to kinetochores (Yasuda et al., 2004), while in others it governs assembly of the cytokinetic septin cytoskeleton, as it does in yeast (Joberty et al., 2000; Caviston et al., 2003; Garcia et al., 2006). Moreover, biosensors in live cells have revealed differing patterns of Cdc42 activity during division of different cultured cell types (Yoshizaki et al., 2003; Garcia et al., 2006). These differences may reflect the fact that even fundamental aspects of cell division are under cell-type specific developmental control. Recently, Cdc42 has been found to control spindle orientation in HeLa and Caco2 cells (Jaffe et al., 2008; Mitsushima et al., 2009). In HeLa cells, this appears to be through Cdc42 influencing the localization of dynein/dynactin, which then controls the interaction of actin with the cell membrane and microtubules (Mitsushima et al., 2009). The mechanism of Cdc42's role in Caco2 cells has not yet been elucidated but may relate to its role in HeLa cells (Jaffe et al., 2008).

1.5 THE CENTRAL SPINDLE

While the orientation of division is important for proper segregation of determination factors and development, the other more fundamental steps of cell division are important to the survival of the animal. As mentioned above the mechanism of cell division is divided into a number of sub-steps: interphase and mitosis (Oshima and Campisi, 1991). Interphase is, in fact, three individual phases: a growth period (G1), synthesis (S) and a second growth period (G2). Mitosis is also divided into phases: prophase, prometaphase/metaphase, anaphase and telophase (Cande and Hogan, 1989; Laskey et al., 1989; McIntosh and Koonce, 1989; Pardee, 1989). Plasma membrane from

each side of the cell ingresses then meets at the middle of the daughter cell (Rappaport, 1971; Glotzer, 2001).

This furrow ingression is called cytokinesis and is an important step to ensuring the fidelity of cell division (Glotzer, 2001). A number of structures are necessary for the completion of this process, one of which is the central spindle (Glotzer, 2001; Severson and Bowerman, 2002). The central spindle is a set of bundled anti-parallel microtubules that originate from the spindle poles but are not associated with kinetochores (Figure 1.2). The region of microtubule overlap concentrates several proteins and protein complexes required for cytokinesis (Glotzer, 2005). Many of these proteins and protein complexes rely on the other central spindle proteins for their localization (Schumacher et al., 1998; Severson et al., 2000; Kurasawa et al., 2004). The position of the central spindle dictates the position of the contractile ring and thus the cleavage furrows (Figure 1.2) (Adams et al., 1998; Gatti et al., 2000; Kimura et al., 2000; Dechant and Glotzer, 2003).

1.5.1 Contractile ring and the central spindle

The contractile ring is a complex structure made of actin and myosin. This actomyosin ring is associated with the plasma membrane by a number of scaffolding proteins. This ring, as evident by its name, contracts inward to the center of the cell. Actin becomes aligned and myosin is able to walk along the actin causing the actin filaments to slide past one another (Figure 1.4) (Piekny et al., 2005; Yuce et al., 2005). The scaffolding proteins allow for the ingression of the membrane furrows (Neufeld and Rubin, 1994; Field and Alberts, 1995). Small Rho GTPases, specifically RhoA, have been shown to be required for the formation of the contractile ring. RhoA, in its active form, activates actin assembly factors and regulates the activation state of myosin light

chain (Werner and Glotzer, 2008). The specific localization and activation of RhoA requires the central spindle. As mentioned above many proteins required for proper completion of cytokinesis are recruited to the region of microtubule overlap at the central spindle. Many of these proteins and protein complexes rely on the other central spindle proteins for their localization (Schumacher et al., 1998; Severson et al., 2000; Kurasawa et al., 2004). A RhoGEF, ECT2 (epithelial cell transforming sequence 2 oncogene) is localized to the central spindle by its association with the centralspindlin complex, a motor proteins, MKLP1 (mitotic kinesin-like protein 1) and a RhoGAP, CYK-4 (cytokeratin-4), (Watanabe et al., 1997; Prokopenko et al., 1999; Tatsumoto et al., 1999). The association of ECT2 with centralspindlin activates ECT2 specifically at the central spindle (Solski et al., 2004; Yuce et al., 2005; Chalamalasetty et al., 2006; Nishimura and Yonemura, 2006). Activation of ECT2 causes the localized activation of RhoA at the central spindle (Saint and Somers, 2003). Plk1 (polo-like kinase 1) promotes the localized activation of ECT2 by CYK-4 (Petronczki et al., 2007; Santamaria et al., 2007; Wolfe et al., 2009) and the localization of Plk1 to the central spindle requires the microtubule bundling protein PRC1 (protein regulator of cytokinesis 1) (Neef et al., 2007).

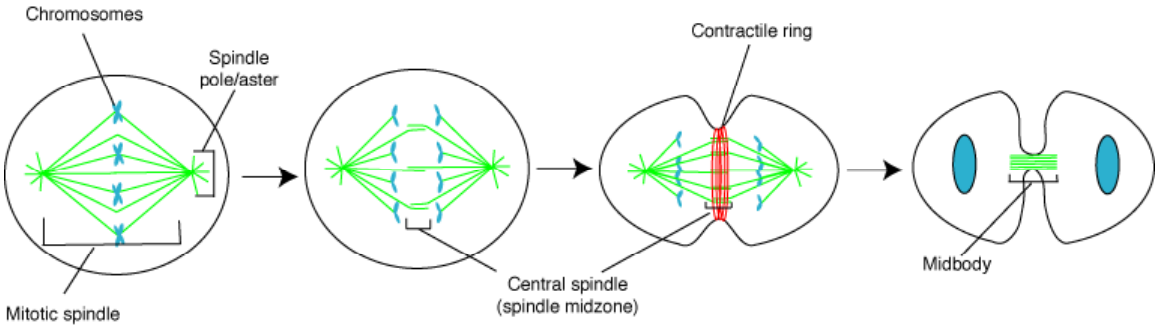

Figure 1.4. The central spindle and the midbody

The central spindle is a bundle of antiparallel microtubules that originates from the spindle poles but does not associate with the kinetochores of chromosomes. As chromosomes separate this bundle remains in the middle of the cell. Proteins that associate with this set of bundled microtubules recruits the proteins required for the contractile ring to form. This causes the contractile ring to form where the central spindle is located and the furrows begin to ingress at this location. After the completion of furrow ingression a remnant of the central spindle remains between the furrows, the midbody. The midbody is involved in the abscission of the two cells into two individual cells.

1.5.2 Protein Regulator of Cytokinesis 1 (PRC1)

PRC1 (protein regulator of cytokinesis 1) is a microtubule bundling protein and has been found to be an essential component for central spindle formation in many organisms (Jiang et al., 1998; Mollinari et al., 2002; Verbrugghe and White, 2004; Verni et al., 2004; Yamashita et al., 2005). PRC1 is localized to the nucleus during interphase but then moves to the spindle at the onset metaphase (Figure 1.5A). PRC1 then relocates to the spindle midzone where it organizes the central spindle (Figure 1.5B and C). During abscission PRC1 remains at the midbody in a structure called the flemming body (Figure 1.5D). When PRC1 levels are reduced via siRNA in cultured cells chromosomes are misaligned, do not segregate correctly and cytokinesis eventually fails (Mollinari et al., 2005; Zhu and Jiang, 2005). Furthermore, cells depleted of PRC1 have a number of

unexpected phenotypes. For example, chromosomes separate to a greater extent and furrows have an increased rate of ingression (Mollinari et al., 2005).

These phenotypes are related to the absence of a central spindle. When the central spindle is not formed, chromosomes can separate further because there is no restriction on how far the spindle poles can move. Moreover, microtubules destined to form the central spindle instead associate directly with the ingressing furrows (Mollinari et al., 2005). Central spindle proteins are still localized to the plus ends of the microtubules, but since PRC1 is not bundling the microtubules, these proteins instead are associated with the ingressing furrows (Mollinari et al., 2005). The association of these proteins with the membrane allows for the ingression of the furrows in PRC1 depleted cells but cytokinesis ultimately fails when furrows regress (Jiang et al., 1998; Mollinari et al., 2002; Verbrugghe and White, 2004; Verni et al., 2004; Mollinari et al., 2005; Yamashita et al., 2005). These data indicates that PRC1 is required to form the central spindle and that the central spindle is required for completion of cytokinesis.

Analysis of the role of PRC1 during cytokinesis is relatively recent, with PRC1 being identified in 1998 (Jiang et al., 1998). More recent research shows that the function of PRC1 is broader than just a microtubule bundling protein. PRC1 interacts with other central spindle proteins and regulate their activity during other phases of cell division (Ban et al., 2004). These studies suggest that the role of PRC1 is not fully understood. Previous studies in the worm have shown that PRC1 may not be absolutely required for cell division. When the PRC1 homologue, SPD-1, is mutated in the worm the central spindle does not form but the first few cell divisions occur correctly. One of the cells, the EMS cell, at the 4-cell stage consistently and often failed during cytokinesis (Verbrugghe and White, 2004). This suggests that PRC1 may have different roles in different cell types and more detailed analysis of its role during development is required.

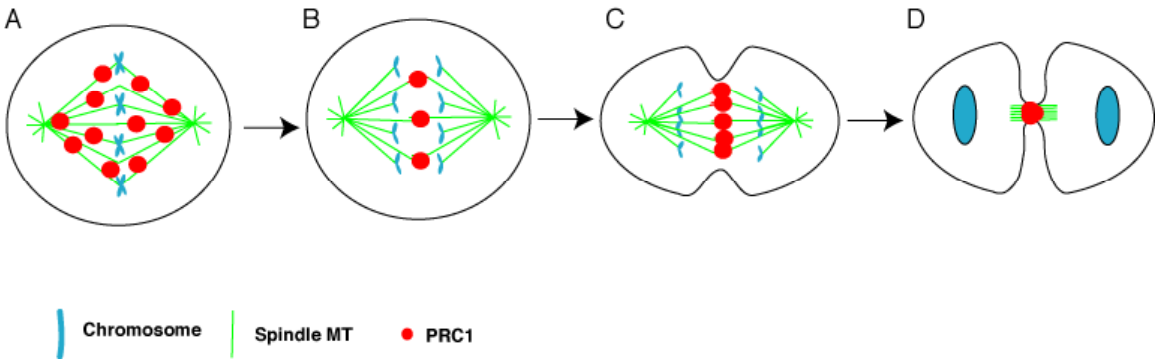

Figure 1.5. Localization of PRC1 through mitosis

(A) PRC1 is localized over the entire spindle during metaphase. (B and C) PRC1 then moves to the spindle midzone during anaphase (B) and telophase (C) where it is required for the bundling of the microtubules at the central spindle. (D) The area of PRC1 expression is then compressed into the flemming body of the midbody.

1.6 CILIA

Cilia and flagella are microtubule-based organelles that protrude from the surface of most vertebrate cells. There are two types of cilia, motile and stationary or primary cilia. Both these types of cilia have similar architectures; a microtubule based axoneme extending from the surface of the cell covered with a membrane that is continuous with the plasma membrane and a basal body at the base of the axoneme (Bossinger and Bachmann, 2004). Furthermore, both motile and stationary cilia have a basal foot and a rootlet, accessory structures to the basal body (Satir and Christensen, 2008). As evident by their distinction motile cilia move while stationary cilia remain in one position. Motile cilia are used in many tissues to move liquid over the tissue of interest (Sleigh et al., 1988). Most vertebrate cells have at least one primary cilia protruding from its

surface (Olsen, 2005). These primary cilia have been found to be hubs for particular signaling molecules and the ciliary structure is required for transduction of certain signaling pathways, such as the Hedgehog pathway (Corbit et al., 2005; Haycraft et al., 2005; May et al., 2005; Scholey and Anderson, 2006). Motile cilia can be singular such as on sperm (Sleigh and Barlow, 1982) or in a multi-ciliated cell as in lungs (Sleigh et al., 1988).

To facilitate the movement of motile cilia they have a different structure to the microtubules in their axoneme. Motile cilia have a 9+2 structure of tubules while primary cilia have a 9+0. Both types of cilia have 9 doublets of tubules in a polarized around the outside of the axoneme just under the membrane cortex in a circle (Figure 1.6A). Motile cilia also have a central pair of tubules, hence the +2. This central pair is missing in primary cilia (Figure 1.6B) (Satir and Christensen, 2008). Motile cilia are able to move by the presence of dyneins forming two arms, the inner dynein arm (IDA) and the outer dynein arm (ODA), extending from each of the outer doublets (Figure 1.6A, red lines). These arms allow the doublets to slide against one another to produce the effective stroke. The central pair interacts with the IDA and causes the cilia to beat in a directed fashion (Nicastro et al., 2006; Satir and Christensen, 2008).

Both types of cilia have a common structure at the base of the ciliary shaft, the basal body (Inglis et al., 2007). Basal bodies modified centrioles that are also microtubule based and have a similar structure to the ciliary axoneme but have nine microtubule triplets, instead of doublets, arranged in a similar manner to the axoneme and do not contain a central pair (Fliegauf et al., 2007). Basal bodies associate with vesicles and are trafficked to the apical surface where cilia are formed (Figure 1.6B) (Yang et al., 2005; Yang and Li, 2005; Park et al., 2008). As basal bodies are trafficked to the apical surface they associate with two main accessory structures, the basal foot and the rootlet

(Figure 1.6B and C). The basal foot is closely associated with the basal body and points in the direction of cilia beating (Boisvieux-Ulrich and Sandoz, 1991). The rootlet interacts with the actin cytoskeleton to stabilize the cilia (Hagiwara et al., 1997; Yang et al., 2002; Yang et al., 2005; Yang and Li, 2005). In multiciliated cells the rootlet and basal foot associated with each cilium all point in the same direction (Figure 1.6D). This allows all of the cilia to beat in the same direction for coordinated movement of liquid over the tissue (Park et al., 2005; Mitchell et al., 2007; Park et al., 2008; Vladar and Axelrod, 2008).

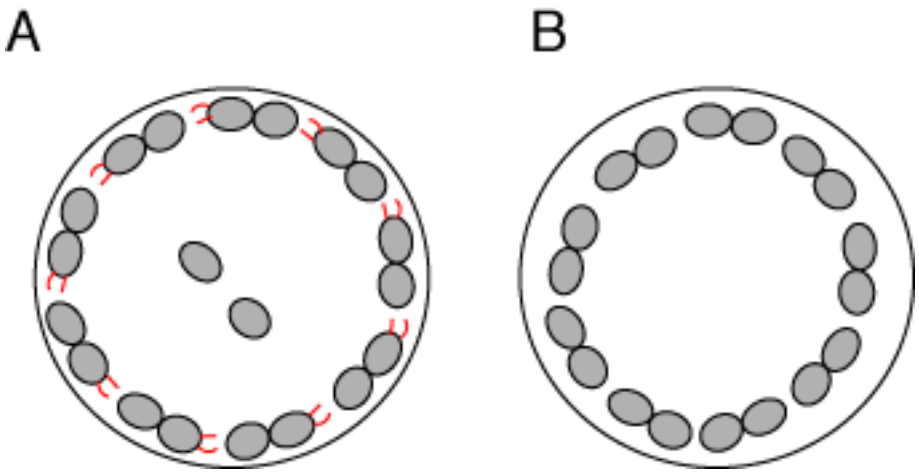

Figure 1.6. Structure of motile and stationary cilia

(A) Structure of motile cilia axoneme. Motile cilia have a 9+2 structure of microtubules. Around the outside of the axoneme are 9 doublets of microtubules with a central pair of single tubules. On each of the outer doublets are two arms of dynein (red), which facilitate the movement of the cilia. (B) Structure of stationary cilia. Stationary cilia have a 9+0 structure of microtubules in the axoneme. Around the outside of the axoneme are 9 doublets of microtubules. There is no central pair or dynein arms on the microtubules since they are not required to move.

1.6.1 Cilia and cell division

A large body of research concentrates on the connection between ciliogenesis and cell division. As mentioned above, most vertebrate cells have at least one primary cilia protruding from its surface (Olsen, 2005). The basal body of the primary cilia also acts as one of the centrioles of the centrosome and regulated break down of the cilia is required for proper progression of cell division (Rieder et al., 1979; Wheatley et al., 1996; Pan and Snell, 2007). The basal body is formed by the older centriole of the centrosome pair (Sorokin, 1962). This indicates that there is a communication between the cilia and cell division. In fact, there is an inverse relationship between cell proliferation and cilia formation, as cells differentiate they reduce their proliferation rate and form cilia (Fonte et al., 1971). Some cells that form cilia are completely differentiated and in the G0 stage of cell division (Tucker et al., 1979).

Recently it has been discovered that in sea urchins a ciliary kinesin subunit, KAP, must be translocated to the nucleus for the progression of mitosis. For this translocation to occur the cilia must be broken down and reabsorbed (Morris et al., 2004). Furthermore, diseases associated with defects in cilia formation, such as polycystic kidney disease, are now being linked to defects in the progression of the cell cycle (Mahjoub et al., 2002; Mahjoub et al., 2004). While the connection of cilia formation to the cell cycle is well studied the inverse is not as well studied. Proteins involved in cilia formation and maintenance are involved with cell cycle progression but what about the inverse: What cell divisions proteins are involved in ciliogenesis? Recently, more research has been conducted to help answer this questions. For example, Aurora-A, a kinase involved in spindle formation during cell division and centriole maturation, has been shown to be involved in ciliogenesis (Hannak and Heald, 2006; Pugacheva et al.,

2007). Further connecting cell division and the molecules involved in cell division to cell division requires more research.

1.6.2 Ciliogenesis

Ciliogenesis is the process by which a cilium is formed at the surface of a cell. As described, above the process of forming cilia comes when a basal body is formed near the Golgi and in multiciliated cells replicate in this area of the cell as well (Figure 1.7A). Basal bodies are then trafficked to the apical surface of the cell by use of vesicle transport (Figure 1.7B and C) (Yang et al., 2005; Park et al., 2008). The doublets of the ciliary axoneme then extend up to form the axoneme making the axoneme continuous with the basal body (Figure 1.6D) (Rosenbaum and Witman, 2002; Pazour and Witman, 2003). This extension of the axoneme relies on a process called intraflagellar transport (IFT). This process grows the cilia from the distal tip of the axoneme (Witman, 1975; Rosenbaum and Witman, 2002). Disruption of any of the many IFT molecules leads to defects in ciliogenesis (Rosenbaum and Witman, 2002; Pazour and Witman, 2003).

Recently it has been shown that defects in ciliogenesis leads to defects in neural tube closure (Huangfu et al., 2003; Huangfu and Anderson, 2005; Park et al., 2006). These defects are probably due to the inability of the neural plate to pattern properly. As mentioned above, without cilia Hedgehog signaling is unable to occur. Without Hedgehog signaling the neural plate may not be correctly differentiated and neural tube closure will fail (Huangfu and Anderson, 2005; Wallingford, 2006). These neural tube closure defects and defects in cilia formation have been linked to the PCP pathway (Park et al., 2006; Park et al., 2008; Vladar and Axelrod, 2008). When Dishevelled function is disrupted through use of dominant negatives or knocked down through use of morpholino anti-sense oligonucleotides ciliogenesis fails. Morpholinos to Dishevelled show defects

to docking of basal bodies to the apical surface whereas dominant negatives cause the polarity of basal bodies and thus ciliary beating to be disrupted (Park et al., 2005; Mitchell et al., 2007; Park et al., 2008; Vladar and Axelrod, 2008). These data is fairly recent but reveals that our understanding of ciliogenesis is not complete and some unexpected pathways may be involved in this process.

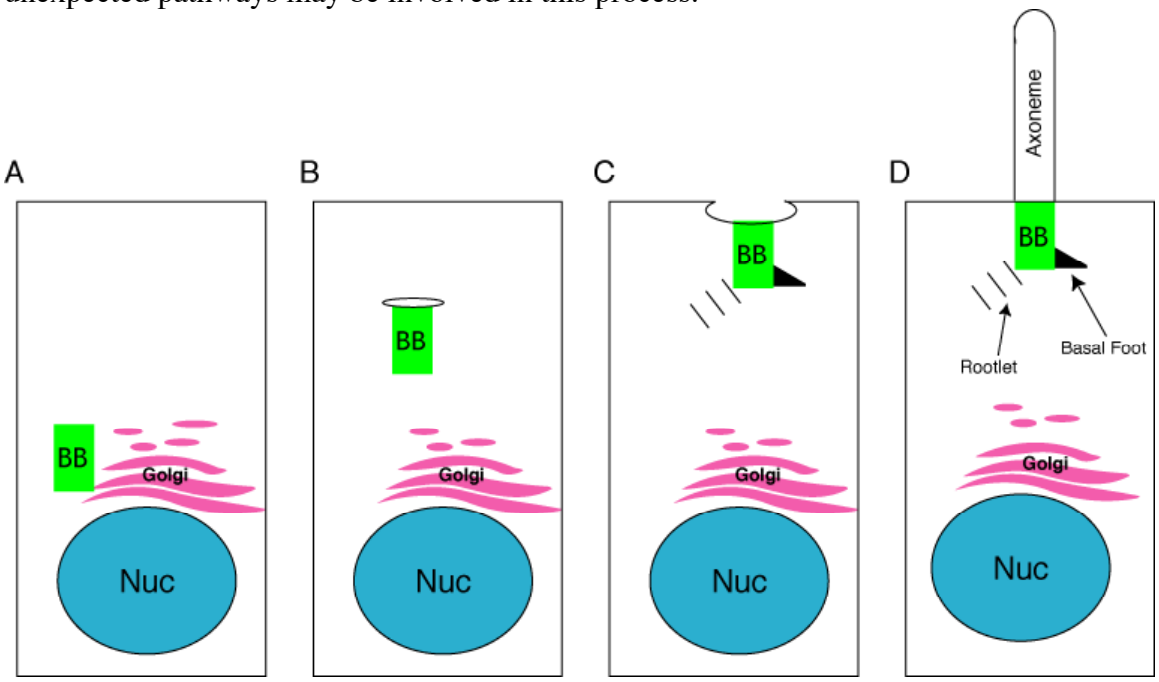

Figure 1.7. Ciliogenesis

(A) The centrioles of the centrosome separate near the Golgi. In the case of multiciliated cells the mother/older centriole replicates in this area to produce many basal bodies. (B) These basal bodies then associate with vesicles and are trafficked toward the apical surface of the cell. (C) As they are trafficked the basal bodies associate with two accessory structures, the basal foot (black triangle) and the rootlet (black lines). This completed basal body structure then docks to the apical membrane by the fusion of the vesicle with the plasma membrane. (D) The axoneme grows out from the basal body by the addition of axonemal units to the distal tip by intraflagellar transport.

1.6.2 Ciliogenesis and the exocyst

As mentioned above, to traffic the basal body to the surface of the cell a vesicle associates with the distal tip of the basal body (Figure 1.7B and C) (Sorokin, 1968; Park et al., 2008). Ciliogenesis has been found to require the exocyst complex and this complex is found at the base of cilia (Rogers, 2004; Zuo et al., 2009). The exocyst is a complex of eight proteins that is required for secretion from the cell through exocytosis (TerBush et al., 1996; Kee et al., 1997; Hsu et al., 2004). These data indicate this vesicle travels to the apical surface with the basal body and then exocytosis occurs to dock the basal body to the apical surface of the cell. The exocyst remains at the basal body most likely to maintain the ciliary sheath after if forms and to traffic membrane bound signaling molecules to the axoneme (Sorokin, 1968; Rogers et al., 2004).

1.6.4 Connecting ciliogenesis to cytokinesis

Centriolin has recently been discovered and found to be a major protein of centrosomes. Antibody staining has shown that Centriolin is localized to the basal body of primary cilia in cultured cells (Gromley et al., 2003; Gromley et al., 2005). Surprisingly, during cytokinesis centriolin relocalizes to the midbody and when knocked-down the final steps of cytokinesis fail (Gromley et al., 2003). Centriolin interacts with members of the exocyst complex and proteins associated with SNAREs (soluble N-ethylmaleimide-sensitive factor attachment receptor). More detailed analysis of the knock down phenotype showed that Centriolin is required for the recruitment of the exocyst to the midbody. When the exocyst is not localized to the midbody vesicles are shown to accumulate around the cytokinetic furrow indicating a failure of fusion between the vesicles and the plasma membrane. MKLP1 is required to localize centriolin to the midbody and thus shows a requirement for MKLP1 in recruiting the exocyst complex to

the cytokinetic furrow (Gromley et al., 2005). Previous findings in the Wallingford lab show that the process of ciliogenesis also requires vesicle fusion through the exocyst complex (Park et al., 2008). Based on the work of other members in the lab and the work of Gromley et al., we felt that the ciliogenesis and cytokinesis may share a number of the same molecules and the same molecular pathways. The central spindle proteins may be required at cilia, either for ciliogenesis or for maintenance of the cilium.

1.7 THE *XENOPUS LAEVIS* SYSTEM

Early embryos of the frog *Xenopus*, owing to their external development and large cell-size, provide un-paralleled access to cell behaviors associated with vertebrate development, including cytokinesis. Upon fertilization, *Xenopus* embryos undergo rapid, synchronous divisions until the mid-blastula transition (Newport and Kirschner, 1982). Soon thereafter, morphogenesis initiates with the onset of gastrulation, which is then followed by neural tube closure, which generates the hollow central nervous system (Keller, 1991; Wallingford, 2005). During this time, neural epithelial cells constitute the major population of dividing cells (Saka and Smith, 2001). The neural plate of *Xenopus* has two cell layers, a deep layer giving rise to the primary neurons and a superficial layer that only differentiates much later and continues to proliferate dramatically during general tube closure (Hartenstein, 1989; Chalmers et al., 2002). A second wave of cell proliferation occurs during the early tadpole stage, when epidermal epithelial cells proliferate extensively in the developing tail (Gibson et al., 2006). We chose to exploit these attributes to analyze both the mechanism of cell division and the orientation of cell divisions during development.

Chapter 2: Role of Cdc42 in spindle positioning and planar orientation of cell divisions during neural tube closure

2.1 INTRODUCTION

Oriented cell division is a process in which cells in a specific tissue divide in a specified alignment (Gong et al., 2004). Such alignment allows for axis formation in *C. elegans* (Gong et al., 2004; Gonczy and Rose, 2005), morphogenesis of tissues (Concha and Adams, 1998; Gong et al., 2004; Fischer et al., 2006; Saburi et al., 2008), segregation of differentiation factors and many other aspects important for development (Bellaiche et al., 2001a; Bellaiche et al., 2001b; Chalmers et al., 2003; David et al., 2005).

Oriented cell divisions occur prevalently in neural and/or neurogenic cells. In the closed neural tube, divisions that occur parallel to the plane of the neural epithelium are generally proliferative, with the daughter cells continuing to produce more cells, while divisions perpendicular to the apical membrane tend to be neurogenic, with one daughter cell remaining proliferative and the other exiting the cell cycle and differentiating (Haydar et al., 2003; Gotz and Huttner, 2005; Wilcock et al., 2007). Furthermore, proliferative divisions in the early neural plate may have oriented cell divisions with respect to a particular embryonic axis, such as the anterior/posterior or medio/lateral. This type of oriented cell division is seen in the neural plate of the chick and the zebrafish. In the chick, cells in the neural plate divide parallel to the long axis of the embryo (Sausedo et al., 1997). In the zebrafish, cells initially divide parallel to the long axis early during neurulation but then later switches to a perpendicular division (Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Gong et al., 2004).

Many of these oriented cell divisions are controlled by a similar set of proteins. The planar cell polarity (PCP) pathway have been found to be a large contributor to

setting up oriented cell divisions (Bellaiche et al., 2001a). In *Drosophila*, localization of determinants to different regions of the neuroblast PI cells allows for reorientation of the mitotic spindle (Bellaiche et al., 2001a; Bellaiche et al., 2001b; David et al., 2005). The PI cell divides in the plane of the epithelium to form the PIIa and PIIb cells. A spindle rotation occurs to ensure the proper division orientation of this division. These two cells under go a stereotypical set of oriented cell divisions, each of with is established by mitotic spindle rotations, to form five cells which then form the sensory organ (Roegiers et al., 2001). For the proper determination of these different neuroblasts Numb must be segregated correctly. Recently, Frizzled has been found to control the correct localization of Numb and partner of numb (Pon) and control spindle rotations associated with the correctly oriented cell divisions (Bellaiche et al., 2001a; Bellaiche et al., 2001b). In zebrafish, PCP signaling controls the oriented cell divisions seen early in neurulation (Gong et al., 2004). Cells in the future neural plate divided along the anterior to posterior axis during epiboly. These oriented cell divisions aid in the elongation of the embryo (Gong et al., 2004). It should be noted however, that this role for PCP signaling in controlling oriented cell division is not universal. For example, extension of the germband in *Drosophila* also relies on oriented cell divisions, but this orientation is independent of PCP signaling (da Silva and Vincent, 2007).

Another common player in developmental regulation of oriented cell divisions is the small GTPase, Cdc42. In *C. elegans*, the anterior of the embryo is determined by the localization of the Par complex (Par-3/Par-6/PKC-3) to the cortex of the one cell embryo and this localization is maintained by the small Rho-GTPase Cdc42 (Gotta et al., 2001; Kay and Hunter, 2001). In some cell lines, Cdc42 has been reported to control microtubule attachment to kinetochores (Yasuda et al., 2004), while in others it governs assembly of the cytokinetic septin cytoskeleton, as it does in yeast (Joberty et al., 2001;

Caviston et al., 2003; Garcia et al., 2006). Cdc42 controls the relocalization of the microtubule organizing center (MTOC) to the leading edge of cells in culture during migration (Etienne-Manneville and Hall, 2001). Cdc42 is also involved in bud site selection for yeast (Adams et al., 1990). Cdc42 localizes to the bud site in yeast, in the growing bud and appears to direct vesicle traffic to the bud (Ziman et al., 1993). These data indicate that Cdc42 has a clear role in controlling the polarity of cells through its subcellular localization. A clear role for Cdc42 in controlling oriented cell division in vertebrate somatic cells has not been reported as yet, but Cdc42 is essential for proper localization and organization of the meiotic spindle in mouse oocytes (Na and Zernicka-Goetz, 2006). Furthermore, Cdc42 is activated asymmetrically on meiotic spindles in *Xenopus* oocytes, where it links spindle position to the position of the cytokinetic ring (Ma et al., 2006). Very recently, Cdc42 has been shown to control the orientation of the spindle in HeLa and Caco2 cells (Jaffe et al., 2008; Mitsushima et al., 2009). HeLa cells divide parallel to the substrate to ensure that neither of the daughter cells loses contact with the substrate (Toyoshima and Nishida, 2007). Cdc42 controls the localization of the dynein/dynactin complex to the midcortex of the cell through PtdIns(3,4,5)P3 activation at this site. This dynactin localization modulates the interaction of the mitotic spindle with the cell cortex for proper orientation (Mitsushima et al., 2009). HeLa cells are not polarized and instead lay flat on the substrate. Caco2 cells, on the other hand, are polarized epithelial cells from the human intestine and have an apical/basal axis. These cells form cysts when grown in culture and cell division occurs perpendicular to the apical-basal axis (Jaffe et al., 2008). When Cdc42 is knocked down in Caco2 cells by siRNA the angle of division is randomized (Jaffe et al., 2008). The mechanism for this role of Cdc42 remains unclear but may also be related to positioning of dynactin at the lateral cortex.

Cdc42 has been shown to be involved in reorganizing cells in vertebrate culture in response to tension and forces. When endothelial cells are exposed to a shear force they move their MTOC, microtubule-organizing center, toward the direction of the force. Cdc42 is involved in the positioning of the MTOC in migrating cells (Tzima et al., 2003). Cells exposed to a shear force also reorganize their cytoskeleton and shape in response to shear force (Tzima, 2006). This reorganization is in response to the activation of the small Rho-GTPases. Cdc42 is activated in response to shear flow and becomes membrane localized to aid reorganization of the cytoskeleton (Li et al., 1999; Tzima et al., 2003). More recently it has been shown that Cdc42 regulates cell shape in epithelial cells by being locally activated at junctions (Otani et al., 2006). All of this data indicates that forces exerted on the cell affect the activation of Cdc42 and thus cell shape.

These differences in controlling the orientation of cell division and cell polarity indicate that further analysis of other organisms is required. The presence of oriented cell divisions in the neural tissue of the Zebrafish, chick and fly indicate that oriented cell divisions in neural tissue may be a common phenomenon in many organisms. Comparatively little research has be conducted on cell divisions in the neural plate of the *Xenopus laevis* embryo, making this organism a good candidate (Hartenstein, 1989; Saka and Smith, 2001; Wu et al., 2001). Combining data from cells in culture, *Xenopus* and other organisms we wish to formulate a more comprehensive model of the controlling factors of oriented cell division. Understanding how these and other systems differ in the control of the orientation of cell divisions should eventually lead to a model that will encompass all recent findings.

Using *in vivo* 4-D analysis of the *Xenopus laevis* neural plate, we have found that cell divisions in the early neural ectoderm exhibit a unique set of oriented cell divisions. The majority of the cells in the early neural plate of the *Xenopus* divide in a medio/lateral

orientation but they are unique in the fact that they are not perfectly medio/lateral but instead divide at an oblique angle with respect to the midline. The anterior most daughter cell is oriented away from or toward the midline, in approximately equal proportion, at an angle of about 60°. Rapid mitotic spindle rotations during anaphase established this division polarity and place the division angle parallel with the long axis of cells. Surprisingly, the PCP pathway does not control the polarity of cell division in Xenopus as it is in the zebrafish and fly but instead is regulated by Cdc42 function. When Cdc42 function is perturbed, cells divide with more cells oriented obliquely away from the midline of the embryo. Furthermore we found that cells with altered Cdc42 function had over rotations of mitotic spindles as well as instable metaphase spindle. We also find that these effects are specific to the early neural plate; when Cdc42 function is altered in the epidermis of the tail bud embryo we do not see alterations to the stability of the mitotic spindle. These data reveal a role of Cdc42 in controlling the interactions between the mitotic spindle and the cell cortex during development.

2.2 RESULTS

2.2.1 Cdc42 is required to stably position metaphase spindles in neural plate cells.

As previously mentioned (Chapter 1) *Xenopus laevis* is an excellent model for analysis of cell divisions. Cdc42 has been shown to control very early embryonic divisions through positioning the mitotic spindle in *C. elegans*, *Xenopus* oocyte meiotic spindle and the mouse oocyte meiotic spindle (Gotta et al., 2001; Bement et al., 2005; Ma et al., 2006; Na and Zernicka-Goetz, 2006). We wished to examine if this was conserved through development. By use of expression of two different dominant negatives against Cdc42 in clones in *Xenopus* we were able to answer this question.

Cdc42 is in the small Rho-GTPase family, which also includes Rac and RhoA. We used Cdc42-N17 as a dominant negative to block Cdc42 function. Cdc42-N17 preferentially binds GDP and keeps the protein inactive. This construct may sequester GEFs (GTP-exchange factors) that are not specific for Cdc42 but instead Rac or Rho and may lead to Cdc42 non-specific effects (Zhang et al., 1995; Karnoub et al., 2004). For this reason we also used the Cdc42-F37A dominant negative (Zhang et al., 1995; Lamarche et al., 1996; Richman et al., 2002), which is more specific to Cdc42 function, blocking the interaction of Cdc42 and its downstream effector proteins (Lamarche et al., 1996).

Embryos were injected at the 4-cell stage with mRNA encoding histone-2B-GFP into both dorsal cells of the embryo. Embryos were then grown to the 8-cell stage and co-injected again with mRNA encoding membrane-RFP and either Cdc42-N17 or Cdc42-F37A into one animal dorsal embryo. This method labeled chromosomes along its entire neural plate, and half the neural plate also had labeled membranes and Cdc42 function perturbed. Wild-type clones and experimental clones can then be compared directly within a single embryo.

In control cells chromosomes condensed at the metaphase plate at one position in the cell and remained at approximately that position until anaphase onset (Figure 2.1A and a'). When Cdc42 function was altered we saw that chromosomes condensed at the metaphase plate and did not stably remain at that location (Figure 2.1B and b'). Often we saw chromosomes condense and then move from one side of the cell to the other before anaphase onset. In each of these perturbations of Cdc42 we saw the same alteration to spindle stability.

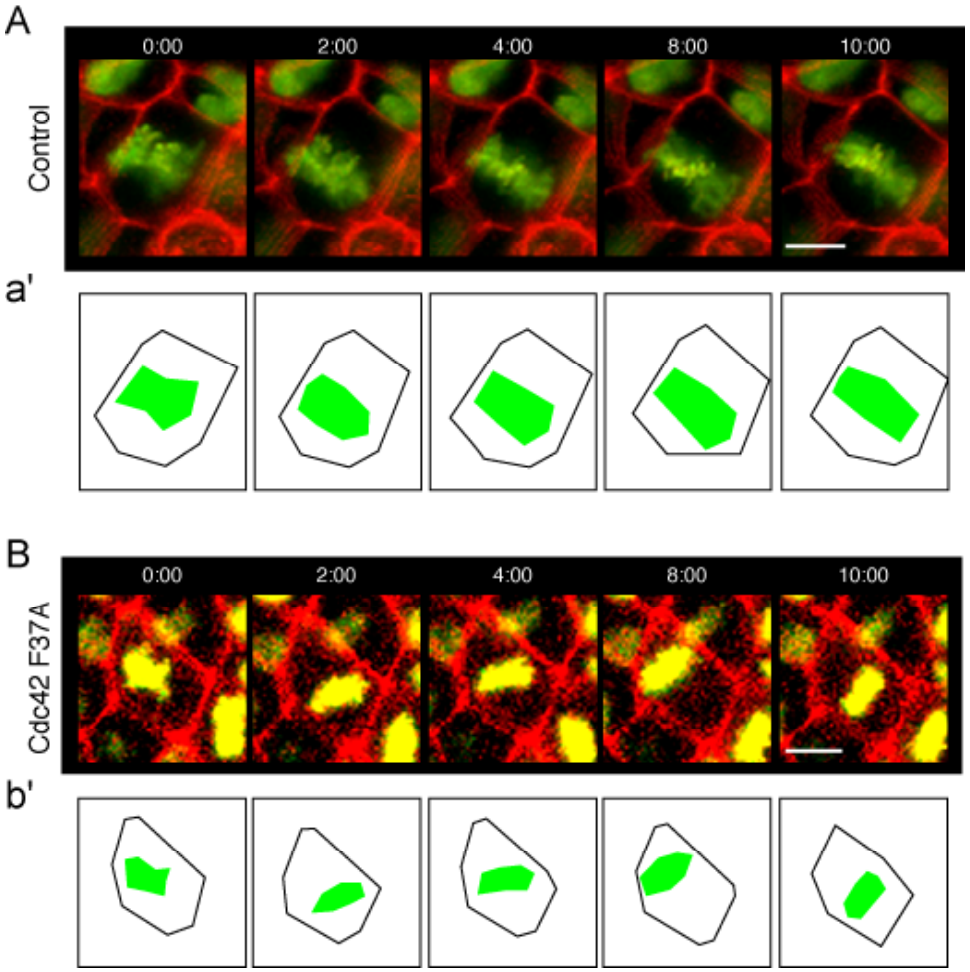

Figure 2.1. Mitotic chromosomes undergo drastic movements in neural cells expressing dominant negatives to Cdc42

(A) Still frames from a movie showing a control cell. The chromosomes can be seen to condense and remain at that location throughout metaphase. Scale bar = 10mm. (a') Cartoons of the cell of interest from above. Cell outlines are shown in black and nuclei are shown in green. (B) Still frames from a movie showing a cell expressing Cdc42-F37A dominant during metaphase. The chromosomes can be seen to move quickly from the upper left hand side of the cell to the lower left and back again. Scale bar = 10mm. (b') Cartoons of the cell of interest from above. Cell outlines are shown in black and nuclei are shown in green. Chromosomes move dramatically through the cell before aligning at the approximate middle of the cell and anaphase proceeds.

2.2.1.1 Cdc42 does not control spindle positioning in epithelial cells of the developing **Xenopus** *epidermis*

We next sought to quantify the excessive spindle movement following manipulation of Cdc42. Because of the overall movement of the tissue of the neural plate we needed to develop a method to correctly assess how much chromosomes move during metaphase. To do this we determined how much chromosomes moved with relation to the size of the cell, which should remain stable. Cell size (d1) and the distance between one side of the cell and the middle of the chromosomes (d2) were determined every minute for the 14 minutes before the onset of anaphase (Figure 2.2A). The ratio of these two numbers at each time point was taken and subtracted from the ratio of the next time point. The absolute value of this value divided by the time difference between the two time points was determined to be the spindle instability index (SII) (Figure 2.2A). The average SII for control cell chromosomes was about 5% while for Cdc42-N17 and Cdc42-F37A had an average of about 8% (Figure 2.2B). Furthermore, control cell chromosomes rarely moved more than 20% of the cell length at one time (Figure 2.2C) whereas Cdc42-N17 and Cdc42-F37A expressing cells frequently had chromosomal movement well above 20% with the maximum for both manipulations was well over 46% (Figure 2.2D and E). This difference in the average distance moved between control and experimental groups was extremely significant ($P<0.001$, Kuskal-Wallis multiple comparisons test).

Given that core cytokinesis mechanisms differ surprisingly between neural epithelial cells and epidermal epithelial cells examined *in vivo* (Kieserman et al., 2008), we next asked if epidermal cell division was affected by disruption of Cdc42. To examine the developmental role of Cdc42 in stabilizing spindles we examined the epidermis of the tail of an early tadpole embryo. Embryos were injected as described above but instead on

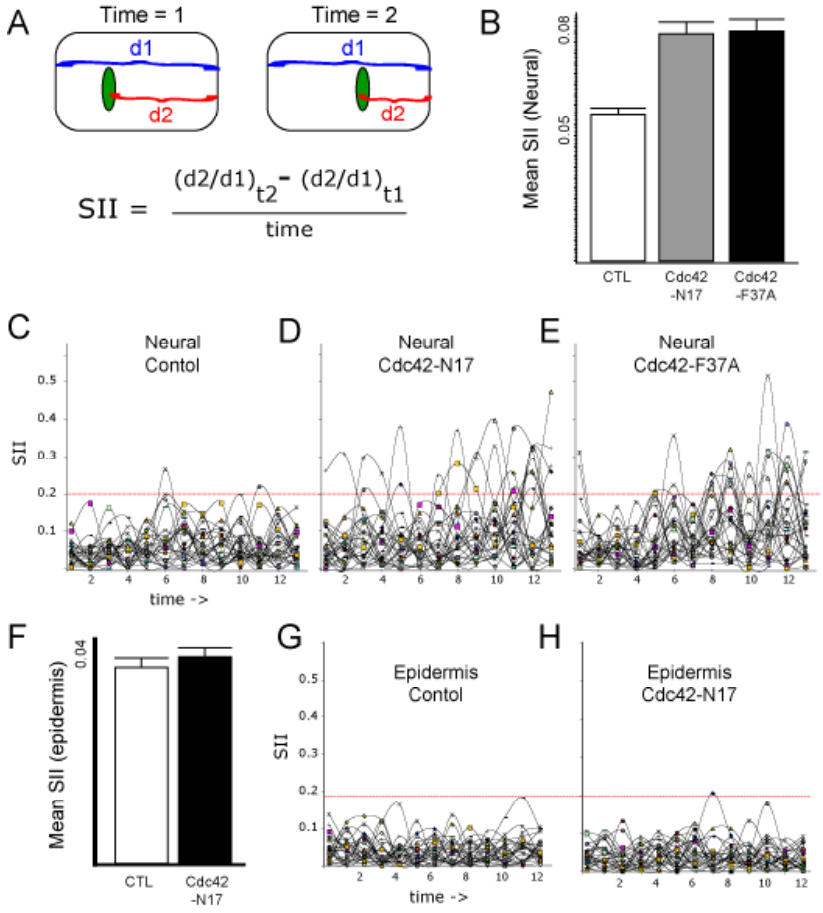

Figure 2.2. Cdc42 is required to stabilize metaphase plates in the early neural plate but not in the tail epidermis

(A) Diagram and equation of how chromosome stability was accessed. The Spindle Instability Index (SII) was assessed using the equation shown. (B) Graph showing the mean SII value for control cells (white bar), cells expressing Cdc42-N17 (gray bar) and cells expressing Cdc42-F37A (black bar). Error bars = SEM. (C) Graph showing instantaneous SII for each time point of all control cells in. The maximum instantaneous SII for control cells is 20% and is represented by the red line. (D) Graph showing instantaneous SII for each time point of all of Cdc42-N17 cells. (E) Graph showing instantaneous SII for each time point of all Cdc42-F37A cells. (F) Graph showing the mean SII value for control cells (white bar, 3 embryos, 26 cells) and cells expressing Cdc42-F37A (black bar, 2 embryos, 26 cells). Error bars = SEM. (G) Graph showing instantaneous SII for each time point of all of the 26 cells in control epidermal cells. The maximum instantaneous SII seen for control neural cells is 20% and is represented by the red line. (H) Graph showing instantaneous SII for each time point of all of the 26 cells in Cdc42-N17 expressing epidermal cells.

the ventral side of the embryo. We saw no change in the SII in control cells as compared to Cdc42-N17 or Cdc42-F37A (Figure 2.2F, G and H). This revealed a neural specific role for Cdc42 in controlling spindle stability.

2.2.2 Planar orientation of cell divisions in the closing neural tube of *Xenopus*

Since oriented cell divisions are often seen in neural cells (Concha and Adams, 1998; Haydar et al., 2003; Gong et al., 2004; Gotz and Huttner, 2005; Wilcock et al., 2007) we sought to ask if these types of division were present in the early neural plate of *Xenopus*. 4-D *in vivo* time-lapse movies were made of the closing spinal cord labeled with membrane-RFP and histone-GFP. The angle of cell division of 330 cells in 6 embryos were assessed and plotted on a rose diagram. This diagram is from 0° to 360° in a circular form. A histogram of each division value, 10° increments or bins, is set up over this circle. The number of divisions that fall with in a set 10° bin is plotted. The more divisions with in a bin the larger the bar. This analysis showed that the majority of cell divisions were oriented perpendicular to the anterior-posterior axis (Figure 2.3 C). This was unexpected based on division orientation seen in the zebrafish, where divisions are parallel to the anterior-posterior axis. Furthermore, the range of division angles is more widely varying than that seen in the zebrafish. The majority of the divisions occur at angles that are oblique with respect to the midline (Figure 2.3C). Cells divide with their anterior most daughter cell oriented away from or toward the midline (Figure 2.3A). Often we found that adjacent cells divided simultaneously with opposite orientations, one obliquely away from the midline and one obliquely toward (Figure 2.3 B).

To quantify the angle of division, each division was assigned based upon the position of the anterior-most daughter cell relative to the midline. To simplify this

process, all division angles were converted to represent divisions on the right side of the embryo. A positive value indicates an orientation obliquely toward the midline (Figure 2.3, pink arrows) and a negative value indicates a division obliquely away from the midline (Figure 2.3, blue arrows). A cell dividing parallel to the medio-lateral axis is given an angle of 0° (Figure 2.3E). Representative division angles are provided in Figure 2.3a' and Figure 2.3B and correspond to a color-coded rose diagram (Figure 2.3C).

2.2.3 Rotations of the mitotic spindle establish division orientation.

Mitotic spindle rotations often accompany oriented divisions and establish the angle of division (Kaltschmidt et al., 2000; Bellaiche et al., 2001a; Geldmacher-Voss et al., 2003; Haydar et al., 2003). Typically spindle rotations in neural cells occur to a stereotypical extent, they set up in the same orientation and rotate in the same direction the same number of degrees each time (Bellaiche et al., 2001a). To determine if this was the case with oriented cell divisions seen here we made movies of the mitotic spindles by injection of mRNA encoding tau-GFP, a microtubule-stabilizing factor (Kwan and Kirschner, 2005) and membrane-RFP. Indeed, we found that mitotic spindles underwent rotations just prior to anaphase onset (Figure 2.4A and B). Surprisingly, we found that the extent of rotations in the *Xenopus* neural plate varied from cell to cell (Figure 2.4A and B). To fully assess these rotations we examined the angle at which the spindle formed (Figure 2.4A and B, white line) and the angle at which anaphase occurred at (Figure 2.4A and B, yellow line). We found that the angle of spindle setup was essentially random with respect to the midline (Figure 2.4C). The angle of the spindle at the onset of anaphase exhibited the same oblique polarity seen when chromosomes were examined

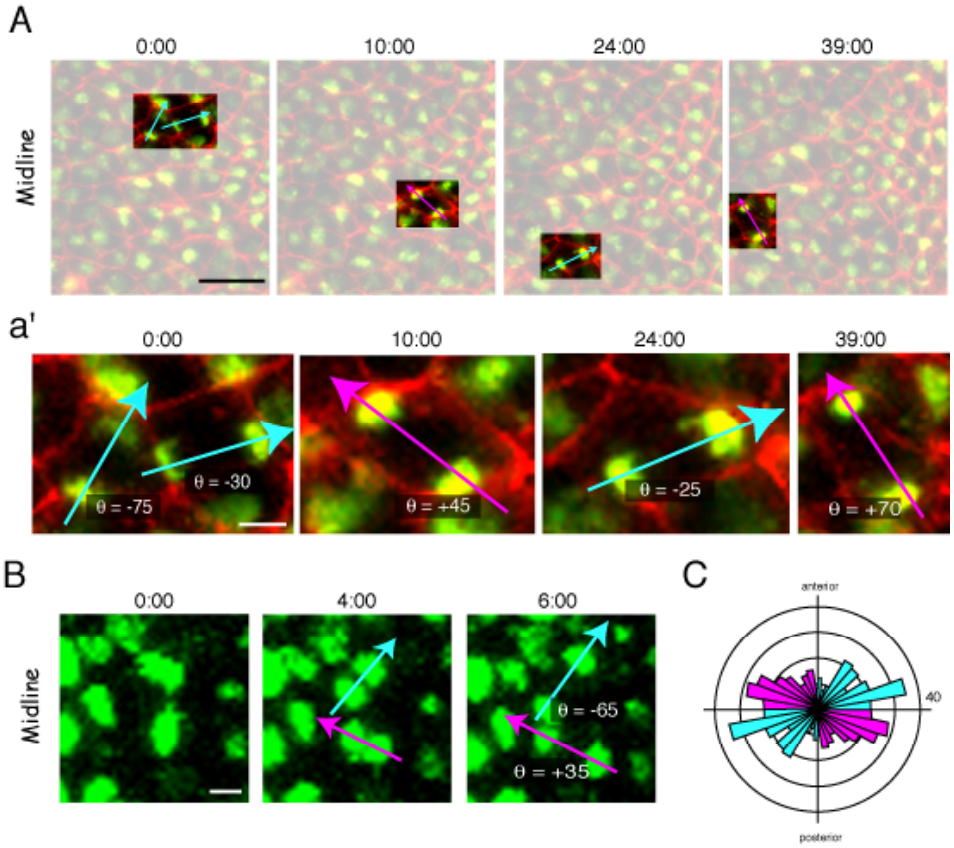

Figure 2.3. Cell divisions in the early neural plate are polarized at an oblique angle with respect to the midline

(A) Still frames from a movie of neural tube closure. Anterior is to the top and the midline is to the left. A few cells undergoing division are highlighted (non-transparent squares). Cells with their anterior daughter cell oriented away from the midline are denoted with a light blue arrow (T = 0 min and T = 24 min). Cells that divide with their anterior daughter cell toward the midline are dented with a pink arrow (T = 10 min and 39 min). Scale bar = 50μm. (a') Blow up of cells highlighted in figure 2.3A. The angle of division, with respect to the midline, is shown above the line for each cell. Scale bar = 10μm. (B) Still frames from a movie of cells dividing in the early neural plate. Cells adjacent in the anterior-posterior divide with opposite oblique polarities. The angles of these divisions are noted. Scale bar = 10μm. (C) Rose diagram of division orientation of 333 cells in 6 embryos. Division angles are binned from 0 to 360 in bins of 10 degrees. Pink bars represent cells dividing with their anterior daughter cell facing toward the midline. Light blue bars represent cells dividing with their anterior daughter cell facing toward the midline.

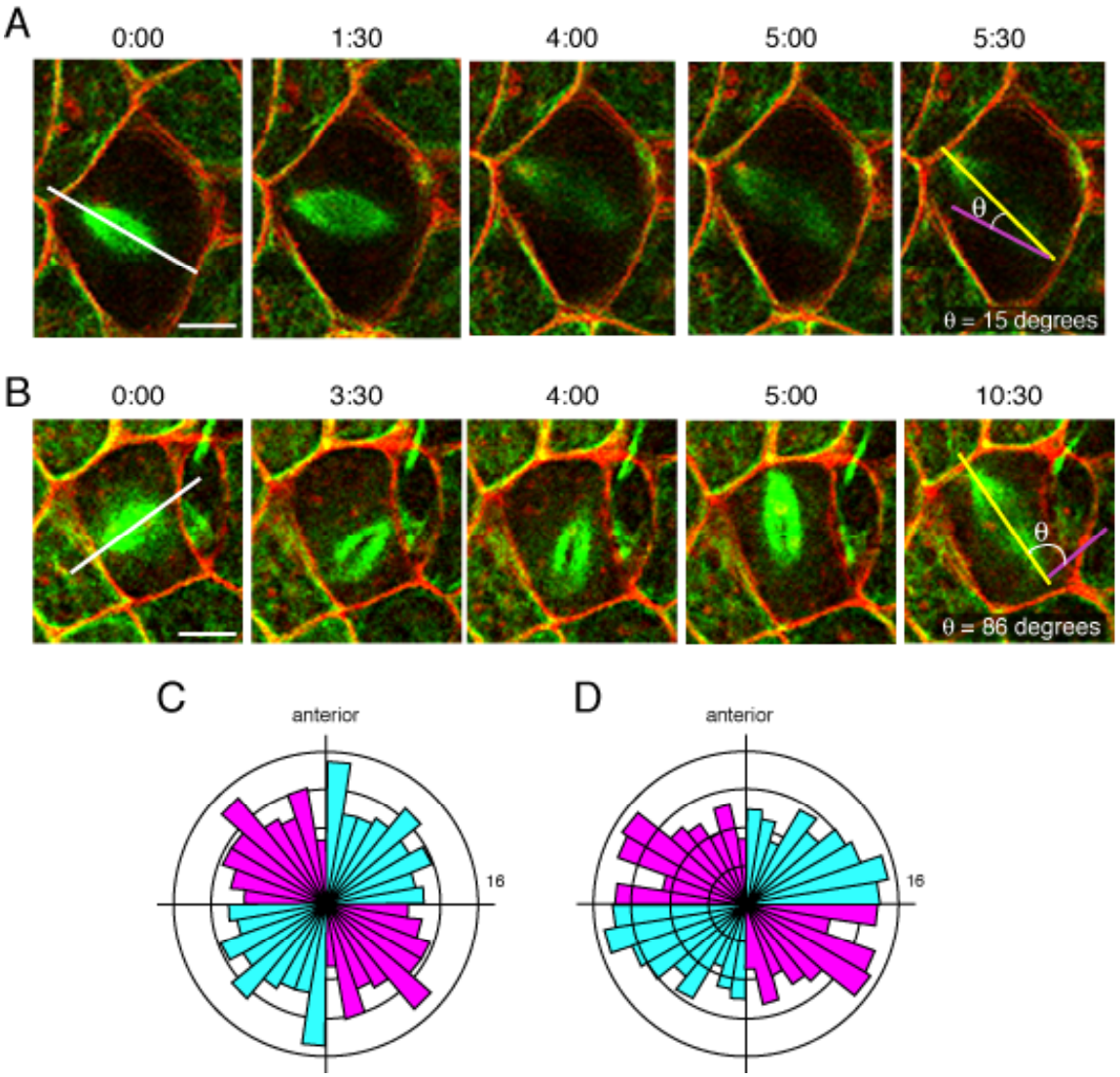

Figure 2.4. Mitotic spindle rotations establish division polarity

(A) Still frames from a movie of cells are expressing membrane-RFP and Tau-GFP. Mitotic spindles are easily seen and a rotation of 15º is seen before anaphase onset. White line represents orientation of spindle set up and corresponds to the purple line. Yellow line represents the orientation of spindle pole separation. Scale bar = 10μm. (B) Still frames from a different sector of the same movie as in the A panel. This spindle rotates a total of 86º before anaphase onset. White line represents orientation of spindle set up and corresponds to the purple line. Yellow line represents the orientation of spindle pole separation. Scale bar = 10μm. (C) Rose diagram of the angle of 144 cells in 6 embryos each expressing membrane-RFP and Tau-GFP. Angles of spindle set up are shown from 0º to 360º in bins of 10º.

(Figure 2.4D). This result indicates that the spindle rotations in these neural cells indeed establish the division polarity.

2.2.4 Disruption of Cdc42 function results in over-rotation of mitotic spindles

Because of the role of Cdc42 in controlling spindle stability, we asked if it also controlled spindle rotations. Indeed, we found that when Cdc42 function was disrupted caused dramatic over rotations of the mitotic spindle (Figure 2.5C compare to A). Because of toxicity effects by tau-GFP injection with either Cdc42 dominant negative we examined rotations of the chromosomes. We found that control cells had an average rotation of 45° with the majority of the rotations occurring between 0° and 60° and very few rotations exceeding 120° (Figure 2.5B and E). In contrast, cells expressing Cdc42-F37A had an average spindle rotation of 78° and often rotations were seen to exceed 90° with a number exceeding 200° (Figure 2.5D and E).

2.2.5. Cdc42 controls division orientation of cell division in early neural plate

We next examined if this alteration of the extent of spindle rotation caused an alteration to the orientation of cell division seen. Embryos were injected as above allowing for an internal control. The angle of division of control cells is easily comparable to manipulated cells. Following manipulation of Cdc42 by injection of either Cdc42-N17 or Cdc42-F37A the majority of the cell divisions occurred with the anterior-most daughter cell oriented away from the midline at an expense of the cells dividing toward the midline (Figure 2.6B and C, compare to Figure 2.6A). This caused a 12° shift of the division angle on average. While this shift is moderate, it is consistent with

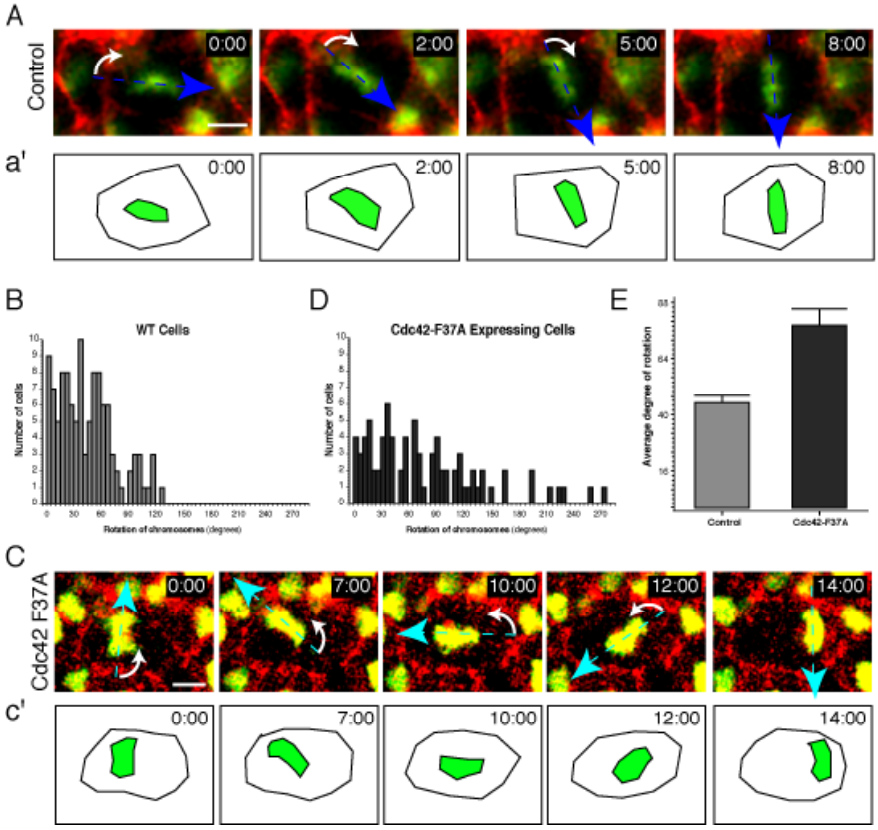

Figure 2.5. Alteration of Cdc42 function causes over rotations of mitotic spindles

(A) Still frames from a movie of a control embryo expressing histone-2B-GFP and membrane RFP. The blue arrow represents the major axis of the chromosomes during the rotation and the white arrow represents the direction of rotation of the chromosomes. Scale bar = 10μm. (a') Cartoons of the cell of interest from control nuclear rotation movie. (B) Histogram representing the rotation of 114 chromosomes from 4 control embryos. These values are shown in bins of 5º from 0º to 290º. (C) Still frames from a movie of cells expressing Cdc42-F37A, histone-2B-GFP and membrane-RFP. The light blue arrow represents the major axis of the chromosomes during the rotation and the white arrow represents the direction of the chromosomal rotation. Scale bar = 10μm. (c') Cartoons of the cell of interest from Cdc42-F37A nuclear rotation movie. (D) Histogram representing the rotation of 82 chromosomes from 4 embryos expressing Cdc42-F37A. These values are shown in bins of 5º from 0º to 290º. (E) Graph of the average rotation of chromosomes in control cells (light grey bar) and cells expressing Cdc42-F37A (dark grey bar). Error bars represent standard error for each (2.9 for control and 7.0 for Cdc42-F37A). A Mann-Whitney test, with a U-statistic of 3302.5 and a U' of 6045.5, was performed and showed a two-tailed P-value of 0.0005.

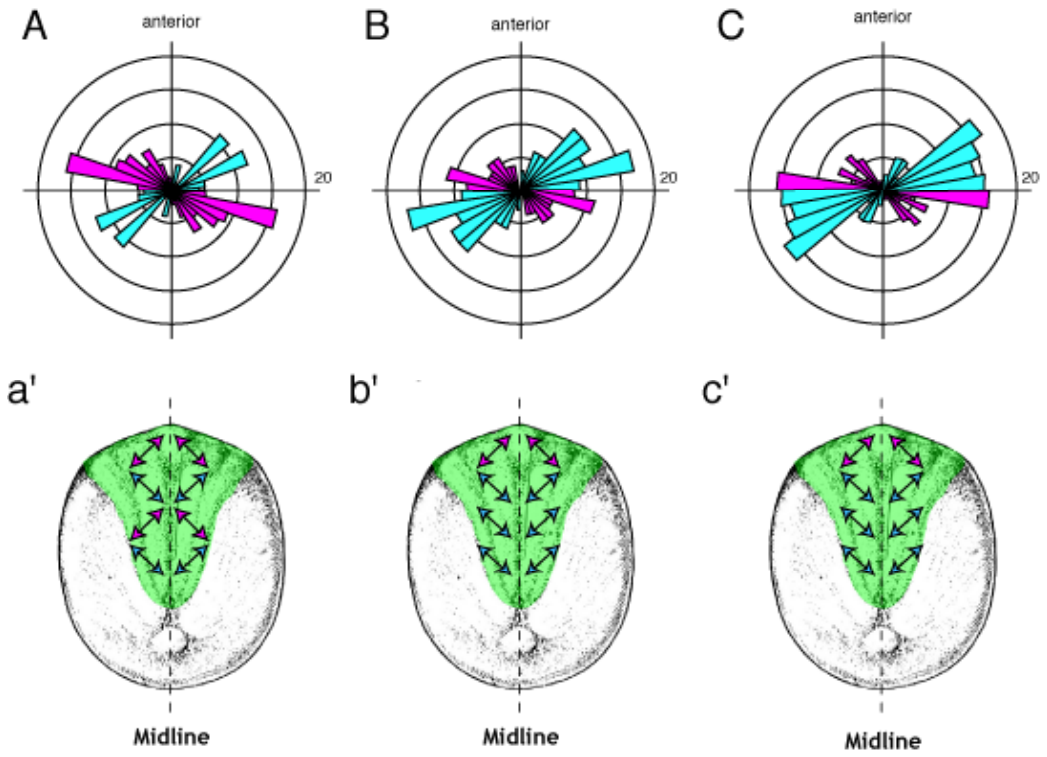

Figure 2.6 Cdc42 controls orientation of cell divisions in early neural plate

(A) Rose diagram of division polarity of a control clone. 99 cells from 5 embryos are binned from 0º to 360º in bins of 10º. (a') Diagram representing the polarity of division seen in control clones of Cdc42-N17 and Cdc42-F37A injected embryos. Double-headed pink arrows represent cells dividing in a medial manner and double-headed blue arrows represent cells dividing in a lateral manner. (B) Rose of the division polarity in a clone expressing a dominant negative to Cdc42, Cdc42-F37A. 73 cells from 3 embryos are binned from 0º to 360º in bins of 10º. (b') Diagram representing the polarity of division seen in Cdc42-F37A clones. Pink double-headed arrows represent cells dividing in a medial manner and blue double-headed arrows represent cells dividing in a lateral manner. (C) Rose diagram of the division polarity in a clone expressing a dominant negative to Cdc42, Cdc42-N17. 87 cells from 4 embryos are binned from –90º to +90º in bins of 10º. (c') Diagram representing the polarity of division seen in Cdc42-N17 clones. Pink double-headed arrows represent cells dividing in a medial manner and blue double-headed arrows represent cells dividing in a lateral manner.

previous results from the fly neuroblasts showing only small changes to the division angle in Ric8 mutants (David et al., 2005).

2.2.7 Spindle rotations align divisions with the cellular long axis, but cellular axis alignment is not affected by disruption of Cdc42.

Based on how instable spindles were and the over rotation of mitotic spindles indicate that the role of Cdc42 in controlling the orientation of cell divisions is through the interaction of the spindle with the cell cortex. Cells have been shown to preferentially divide along their long axis: "Hertwig's rule" (Hertwig, 1893; O'Connell and Wang, 2000; Thery and Bornens, 2006). We therefore determined if the neural cells in the *Xenopus* obeyed this rule. The cellular long axis was determined in dividing cells when the entered metaphase. We found that cells are initially polarized in the orientation of division and spindle rotations orient into this long axis (Figure 2.7A). The same analysis was conducted on cells expressing either Cdc42-N17 or Cdc42-F37A and no difference was found (Figure 2.7B and C, K-S test, $p=0.431$ and $p=0.083$ respectively). This indicates that Cdc42 does not determine the shape of cells and instead directly acts to orient and stabilize spindle rotations.

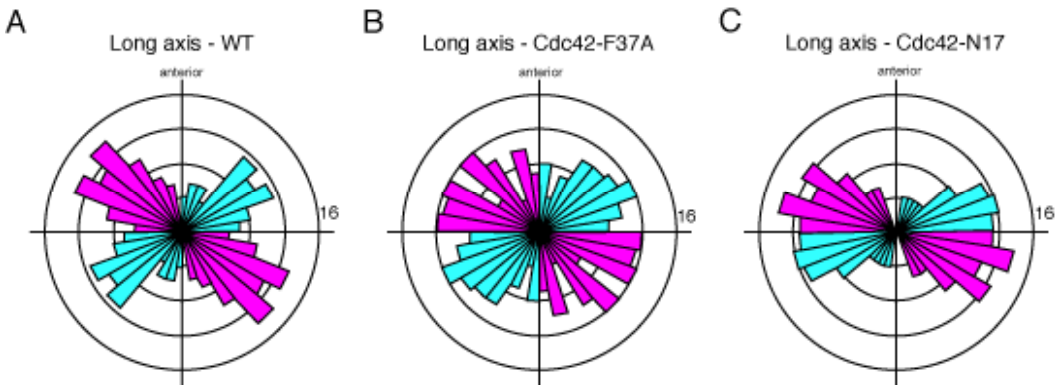

Figure 2.7. Long axis of cells is unaltered in cells expressing dominant negatives to Cdc42.

(A) Rose diagram representing the angle of the long axis of 84 dividing control neural cells in 6 embryos at initiation of metaphase. Angles of cellular long axis are shown from 0º to 360º in bins of 10º. Pink bars represent cells dividing with their anterior daughter cell facing toward the midline. Light blue bars represent cells dividing with their anterior daughter cell facing toward the midline. (B) Rose diagram representing the angle of the long axis of 99 dividing Cdc42-F37A expressing neural cells in 9 embryos at initiation of metaphase. Angles of cellular long axis are shown from 0º to 360º in bins of 10º. (C) Rose diagram representing the angle of the long axis of 79 dividing Cdc42-N17 neural cells in 8 embryos at initiation of metaphase. Angles of cellular long axis are shown from 0º to 360º in bins of 10º.

2.2.6 Cell division orientation is not controlled by PCP signaling and does not require normal neural tube morphogenesis.

The planer cell polarity (PCP) pathway has been shown to control oriented cell divisions in the fish and the fly (Gho and Schweisguth, 1998; Kaltschmidt et al., 2000; Bellaiche et al., 2001a; Gong et al., 2004). Therefore, we examined the role of these genes in the oriented cell divisions in *Xenopus* by use of dominant negatives to molecules in the PCP pathway. Use of Xdd1, a dominant negative to Dishevelled, did not disrupt oriented cell divisions in the superficial neural plate (Figure 2.8A). A Kolmogorov-Smirnov (K-S) test was not found to be significant when conducted on histograms of Xdd1 and control clones (p=0.892). To confirm these data we used a dominant negative

to Frizzled-8 function, Nfz-8 (Deardorff et al., 1998). We again found no difference in the orientation of cell divisions in the neural plate (Figure 2.8B, K-S test, p=0.296). Modification of the PCP signaling pathway by these dominant negatives has been shown to control neural tube closure in the *Xenopus* (Wallingford et al., 2000; Gong et al., 2004; Park et al., 2008).

These data suggested that the orientation of cell division did not depend on the closure of the neural tube. Injection of either Xdd1 or Nfz-8 mRNA into the entire neural plate gave significant neural tube closure defects indicating they were functioning correctly (not shown). To confirm the independence of cell division orientation from neural tube closure we used another dominant negative known to disrupt neural tube closure, Shroom3-ASD1 (Haigo et al., 2003). Expression of this dominant negative disrupted neural tube closure but did not cause alteration to the division polarity (Figure 2.8D K-S test, p=0.127).

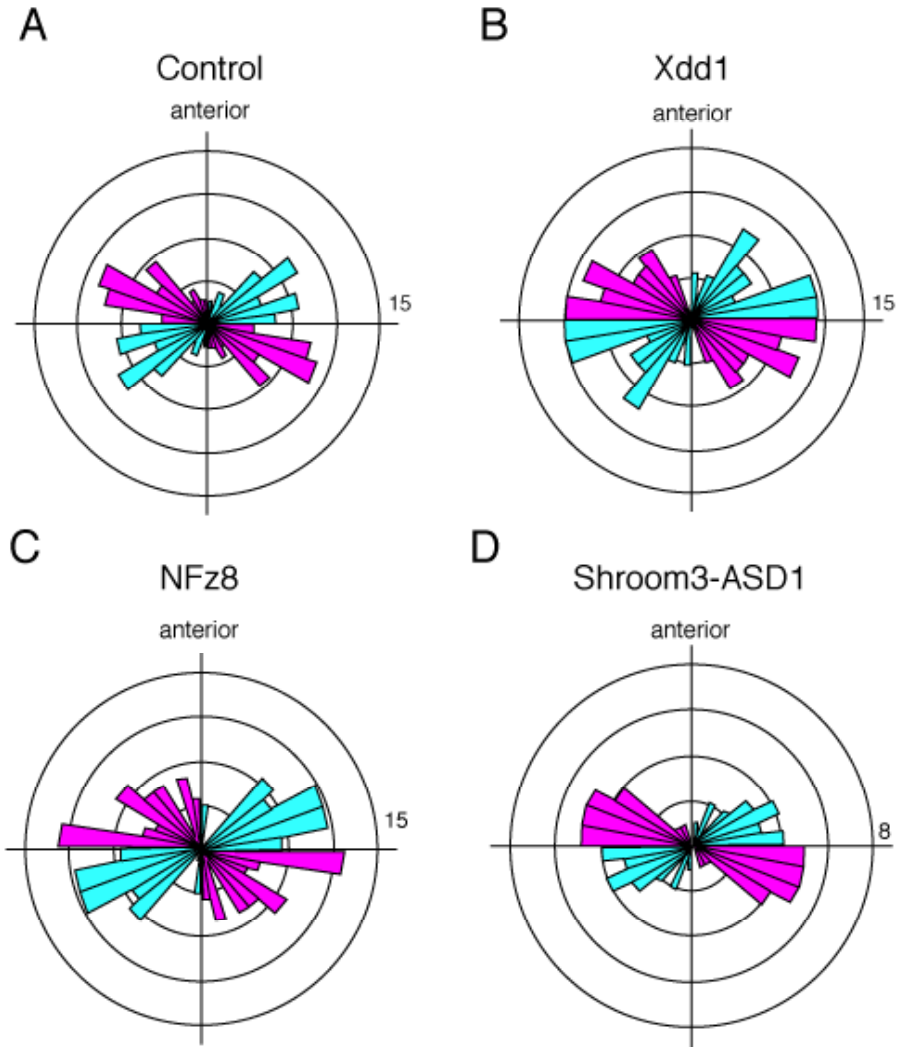

Figure 2.8. PCP signaling does not control cell division polarity

(A) Representative rose diagram of the division polarity in a control clone. 85 cells from 3 embryos are binned from 0º to 360º in bins of 10º. (B) Rose diagram of the division polarity in clones expressing Xdd1, a dominant negative to Dishevelled. 110 cells from 4 embryos are binned from 0º to 360º in bins of 10º. (C) Rose diagram of the division polarity in clones expressing a dominant negative to Frizzled Xfz8N. 100 cells from 5 embryos are binned from 0º to 360º in bins of 10º. (D) Rose diagram of the division polarity in clones expressing a dominant negative to Shroom-3, the ASD1 domain. 41 cells from 2 embryos are binned from 0º to 360º in bins of 10º.

2.3 DISCUSSION

Oriented cell divisions are important for normal development and a small number of conserved mechanisms appear to be associated with setting up the correct division plane. Previous studies have shown that cells tend to divide in one orientation (i.e. anterior-posterior or medio-lateral) within a given tissue and that spindles reliably rotate to a precisely defined degree (Concha and Adams, 1998; Bellaiche et al., 2001a; Bellaiche et al., 2001b; Gong et al., 2004). By contrast, we find that here these themes are not universal, and we describe a novel system of medio-lateral cell division in an intact vertebrate embryonic epithelium. Cells in the early neural plate of *Xenopus* divided in a medio-lateral fashion. These divisions are novel in the fact that the majority of divisions are not oriented perfectly in a medio-lateral orientation but the majority of the cells divide obliquely with relation to the midline in a mirror symmetric manner. We are able to define the division orientation of these cells by the anterior-most daughter cell. Cells in the *Xenopus* neural plate divide with the anterior most daughter cells facing obliquely toward or away from the midline in approximately equal proportion at a degree of about 60^{o} (Figure 2.3). Like other oriented cell divisions, rotations of the mitotic spindle establish the orientation of division in these cells. But unlike previously examined spindle rotations the extent of spindle rotation in the *Xenopus* neural plate was highly variable from cell to cell (Figure 2.4). We also found that while the extent of spindle rotation varied from cell to cell, all spindles rotated to align parallel to the cellular long axis (Figure 2.7).

Previous studies have also found that PCP signaling plays a major role in controlling orientated cell divisions and associated spindle rotations in vertebrate animals, but we find no role for PCP signaling in this case (Figure 2.8). Moreover, we find that in *Xenopus* the orientation of cell division is independent from the morphogenic

movements of the neural plate. When dominant negatives to PCP genes or to Shroom3, another gene required for neural tube closure, we see dramatic neural tube closure defects (Figure 2.7). This phenotype serves as a useful indicator for the efficacy of our reagents.

Surprisingly, we find that Cdc42 plays a key role in establishing division orientation in the closing *Xenopus* neural tube (Figure 2.6). Cells expressing two different dominant negatives to Cdc42, Cdc42-N17 or Cdc42-F37A, divided with more cells dividing obliquely away from the midline at the expense of the cells oriented toward it. Alteration of Cdc42 function causes neural tube closure defects (Choi and Han, 2002). This result may be important, as the neural plate is subject to defined mechanical strains, and such strains are likely to influence cell shape and cell division in epithelial sheets (Brodland and Veldhuis, 2002; Thery and Bornens, 2006; Benko and Brodland, 2007). To investigate the dependence of cell shape on neural tube closure we examined the long axis of cells as they divide. We find that cells divide along their long axis in control cells, thus obeying Hertwig's rule (Figure 2.8). Moreover the orientation of the cellular long axis is unaltered in perturbations of Cdc42 or PCP genes (Figure 2.8 and not shown), showing an independence of cell shape and neural plate morphogenesis.

Since cell shape was not altered in Cdc42 dominant negative cells we examined spindle dynamics in these cells and found a dramatic effect. Spindles were highly unstable and mitotic chromosomes were often seen to move from one side of the cell to the other. This was not seen in control cells where chromosomes remained stably localized at the metaphase plate (Figure 2.1 and 2.2). Quantification of these data showed that the chromosomes in cells expressing either Cdc42-N17 or Cdc-F37A had a significantly higher average SII (Figure 2.2). Moreover, such hyperactive nuclear movement is linked to spindle positioning in *C. elegans* (Couwenbergs et al., 2004). Cdc42 has been shown to control the reorientation of the MTOC in migrating cells by

governing association of the microtubules with the cell cortex (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001). Our results suggest a similar mechanism may function to position the spindle in *Xenopus* neural plate cells.

Previously we have found that cell divisions in the neural plate are highly specialized (Chapter 3). To determine if Cdc42 played a role in this specialization, we altered Cdc42 function in the epidermis of the tail. We found no change to the stabilization of the mitotic spindle in either Cdc42-N17 or Cdc42-F37A expressing cells (Figure 2.2). This implies that Cdc42 may play a role in coordinating the interaction of the spindle with the cell cortex in the early neural plate. To confirm this, we examined spindle rotations in cells with altered Cdc42 function. Control cells have a limited range of rotation, with very few spindles rotating more than 120°, cells with altered Cdc42 function had spindles that rotated in excess of 200° (Figure 2.5). These data suggest a model for the oblique angle of cell divisions in the neural plate. We propose that spindle rotations are essentially random, and that molecular cues on the cell cortex define "catch-points" that stop rotation prior to anaphase in accordance with a cell's long axis (Figure 2.9A and B). One such catch point is responsible for stopping spindle rotation such that the final division angle is oblique to the midline with the anterior-most daughter cell lateral to the more-posterior daughter cell. This catch-point requires Cdc42 function (Figure 2.9A, blue), while Cdc42-independent catch-point accounts for cells dividing obliquely toward the midline (Figure 2.9A and B, red). In this model, if the Cdc42-dependent catch-point is disrupted, spindles rotate excessively, and eventually are able to stop by responding to the other, Cdc42-independent catch (Figure 2.9B).

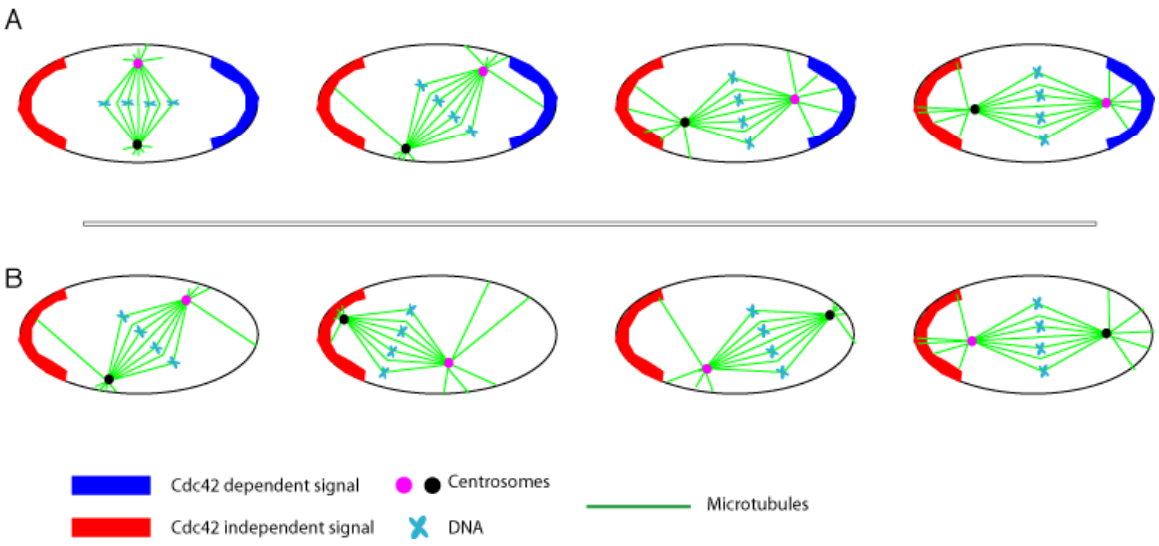

Figure 2.9. Model of Cdc42 control of spindle stability in early neural plate cells

(A) In a wildtype neural plate cells there are two sets of molecules on the cortex of the long axis of the cells. One is Cdc42 dependent (blue) and one is Cdc42 independent. A spindle forms and interacts with these molecules, via the astral microtubules, has a limited range of rotation (pink and black dots) and remains stable in the middle of the cell. (B) When Cdc42 function is altered the Cdc42 dependent orientation signal is disrupted (note lack of blue). When a spindle forms it is unable to remain stably in the middle of the cell because of the lack of interaction with the astral microtubules with the Cdc42 signal. Also the spindle over rotates (pink and black dots).

This general mechanism, by which multiple inputs "compete" to control spindle orientation, has been demonstrated in *Drosophila* neuroblasts (Bellaiche et al., 2001a; Bellaiche et al., 2001b; David et al., 2005). Further examination of the localization of Cdc42 and active Cdc42 in early neural plate cells will be required to aid in the understanding of these "catch-points"

These data point to a new level of diversity in the mechanisms of oriented cell division and suggest that additional *in vivo* studies will be important. Finally, this study provide a unique glimpse into the process of cell division in an intact epithelial cell sheet

that is simultaneously engaged in massive proliferation and a massive morphogenetic event.

Chapter 3: Developmental regulation of central spindle assembly and cytokinesis during vertebrate embryogenesis.

3.1 INTRODUCTION

Proper completion of cell division is critical to ensure segregation of genetic material and also contributes to morphogenesis in embryos (Rappaport, 1961; Gont et al., 1996; Glotzer, 2001; Chalmers et al., 2003). To correctly segregate DNA, accurate temporal progression and completion of the phases of the mitotic cell cycle: interphase, synthesis, metaphase, anaphase and telophase/cytokinesis, is required. Cells have an elaborate system of checkpoints to ensure that these phases are correctly completed. A number of these checkpoints make certain that the architecture of mitotic structures is correct. Furrow ingression is called cytokinesis and is an important step to ensuring the fidelity of cell division (Glotzer, 2001). A number of structures are necessary for the completion of this process, one of which is the central spindle (Severson and Bowerman, 2002; Glotzer, 2009). The central spindle is a microtubule-based structure containing overlapping anti-parallel non-kinetochore associated polar microtubules that dictate the site of cytokinetic furrow ingression (Glotzer, 2001; Glotzer, 2005). The central spindle determines the site of furrow ingression by locally recruiting and activating the small Rho GTPase RhoA. This local activation of RhoA is required for the formation of the contractile ring. RhoA, in its active form, activates actin assembly factors and regulates the activation state of myosin light chain (Werner and Glotzer, 2008).

While the central spindle structure is required for the localization and activation of RhoA at the site of cleavage its presence is not the sole factor. Many proteins are known to localize to this region of microtubule overlap and their localization leads to cleavage furrow initiation. Many of these proteins require the cooperation of other

central spindle proteins their proper localization. For example, Plk1 (polo-like kinase 1), a kinase required for the eventual activation of RhoA, requires the microtubule bundling protein PRC1 (protein regulator of cytokinesis). PRC1 has been shown to be required for proper completion of cytokinesis in many organisms (Jiang et al., 1998; Mollinari et al., 2002; Verbrugghe and White, 2004; Verni et al., 2004). When the expression of PRC1 is reduced cytokinetic furrows ingress but then regress after a period of time. (Jiang et al., 1998; Mollinari et al., 2002). This failure of abscission is because of the disorganization of the central spindle. Central spindle proteins do not localize correctly when the central spindle is not organized correctly (Jiang et al., 1998; Mollinari et al., 2002; Mollinari et al., 2005).

A number of unexpected phenotypes are seen when PRC1 is ablated in cultured cells. In control cells, with PRC1 present, chromosomes separate to about the geometric center of the new daughter cells before cytokinesis initiates (Figure 3.1A, anaphase). The central spindle is well organized between the chromosome masses and PRC1 is present at the central spindle through anaphase and telophase (Figure 3.1A, anaphase and telophase). In PRC1 depleted cells chromosomes separate much farther than in control cells (Figure 3.1B, telophase). Moreover, instead of a well-organized central spindle astral microtubules associate directly with the membrane of the cleavage furrow. This causes these PRC1 depleted cells to have a low density of microtubules in the spindle midzone (Figure 3.1B, anaphase and telophase). The final surprising phenotype that is observed in these cell (Mollinari et al., 2005) is that the cytokinetic furrows move inward at a faster rate as compared to the controls (Figure 3.1B, telophase) (Mollinari et al., 2005).

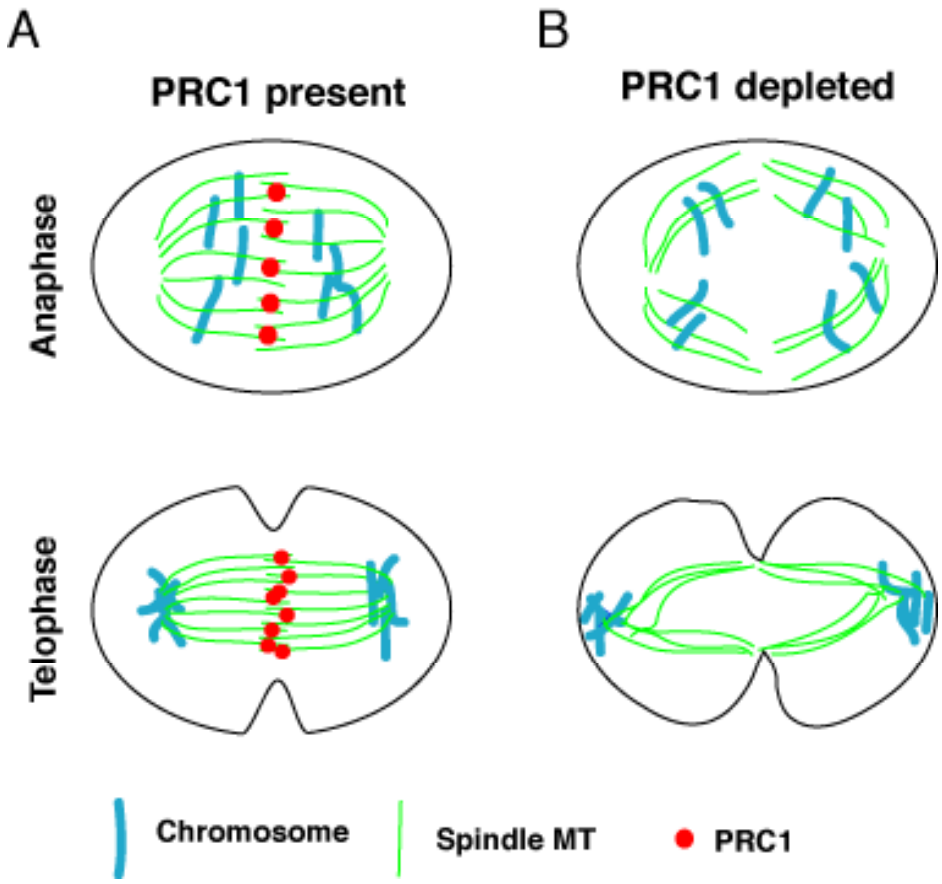

Figure 3.1. Depletion of PRC1 causes unexpected phenotypes in cultured cells

(A) In cultured cells with PRC1 present anaphase and telophase occur as expected. The chromosomes separate to the middle of the new daughter cells before cytokinesis initiates (anaphase). A properly bundled central spindle is observed and PRC1 is localized to the central spindle (anaphase and telophase). (B) In cultured cells with PRC1 depleted a number of unexpected phenotypes are seen. The central spindle is disorganized and there is a low density of microtubules in the spindle midzone (anaphase). The mitotic spindle microtubules now instead associate directly with the ingressing furrows (telophase) The chromosomes separate much farther than expected and closely appose the cell cortex before the onset of telophase (telophase). Finally, the rate of furrow ingression is accelerated as compared to control cells (telophase).

Most analysis of the central spindle and central spindle proteins has been conducted in cultured cells or in cleavage stage embryos (Roos and Camenzind, 1981; Salmon and Wolniak, 1990; Verbrugghe and White, 2004; Glotzer, 2005; Canman et al., 2008). The role of these proteins during embryonic development has not been well studied but regulation of the expression levels of these proteins throughout development may aid in the regulation of cell divisions at different stages. Analysis of SPD1, the PRC1 homologue, in the worm show that these central spindle proteins may play a developmental role in controlling cell divisions. When SPD-1, is mutated the central spindle does not form but the first few cell divisions occur correctly. One of the cells, the EMS cell, at the 4-cell stage consistently and often failed during cytokinesis while the other 3 cells are able to complete cell division properly (Verbrugghe and White, 2004). This suggests that SPD1 may have a different role in this EMS cell than it does in the other 3 cells. This also suggests a possible stage specific regulation of cell division where early during development SPD1/PRC1 may not be required for cell division but is later. We wished to compare cell divisions in different tissues during development, early and late, determine the differences and the causes for any differences seen.

Here we show that cell divisions in the early neural plate of the developing *Xenopus* embryo are highly modified as compared to cell divisions in the posterior region of the tail epidermis. Both of these tissues are derived from the ectoderm so these differences were quite surprising. Cells in the early neural plate early undergo exaggerated chromosome separation, delayed cytokinetic onset and rapid cytokinetic furrow ingression. In addition there is a low density of microtubules between separating chromosomes in the early neural plate, as compared to the late tail epidermis, instead microtubules associate with the ingressing cytokinetic furrows. These modifications

resemble phenotypes seen in cultured mammalian cells lacking PRC1 (Jiang et al., 1998; Mollinari et al., 2002; Kurasawa et al., 2004; Mollinari et al., 2005).

We show that the expression levels of PRC1 are developmentally regulated in these two ectodermally derived tissues. The expression level of PRC1 was found to be lower in the early neural plate than in the tail epidermis. It was found that the level of PRC1 mRNA and protein level were both reduced in the early neural plate as compared to the tail epidermis. Forced expression of PRC1 in early neural cells rescues both the exaggerated anaphase and low microtubule density. These data show that PRC1 is a developmental regulator of central spindle assembly and may be related to the specialization of the midbody in neural cells (Bancroft and Bellairs, 1975; Bellairs and Bancroft, 1975; Cohen et al., 1988; Dubreuil et al., 2007; Wilcock et al., 2007).

3.2 RESULTS

3.2.1 Novel modifications to mitotic mechanisms in early neural epithelial cells

Xenopus laevis embryos provide an exceptional model to study the mechanisms of cell division in an *in vivo* system. *Xenopus* an externally developing vertebrate and embryos have large easily visualized cells throughout development (Davidson and Wallingford, 2005). We chose to exploit these attributes in our study of cell divisions during development. To compare cell divisions we chose to study two ectodermally derived tissues, the early neural plate and the epidermis of the tail bud. Both tissues undergo abundant cell division and are situated on the surface of the embryo, making them easily accessible (Harris and Hartenstein, 1991; Saka and Smith, 2001; Wallingford, 2005; Gibson et al., 2006). Because of the conserved nature of cell divisions among

metazoans and the fact that these tissues are derived from the same germ layer we were surprised to find significant differences between them.

To assess cell divisions in these tissues we injected mRNAs encoding fluorescent fusion proteins to visualize particular structures in the cell. Membranes were labeled by injection of mRNA encoding myristylated red fluorescent protein (mem-RFP). The myristyl tag is a small peptide sequence that, when added to the N-terminus of a protein, targets it to the membrane (McIlhinney, 1998). To label nuclei/chromosomes we injected a histone-2B green fluorescent protein (H2B-GFP) encoding mRNA, which labels one of the subunits of the nucleosomes of condensed DNA. Analysis using mem-RFP and H2B-GFP revealed that cells in the early neural plate undergo exaggerated spindle elongation during anaphase B as compared to the tail epidermis. This movement caused chromosomes to closely approach the cell cortex before the onset of cytokinesis (Figure 3.2A, bracket and Figure 3.2C blue line). In the tail epidermis, chromosome movements were similar to those seen in cells in culture where chromosomes stopped in about the middle of the new daughter cell (Figure 3.2B, bracket and Figure 3.2C pink line). Exaggerated chromosome movement in the early neural plate was not accompanied by a significant difference in cell size. Associated with the exaggerated anaphase movements is a delay in the onset of cytokinesis. In the early neural plate, ingression of cytokinetic furrows began at approximately 6-7 minutes after the onset of anaphase while in the tail epidermis it began at 4-5 minutes (Figure 3.2A and B arrowheads). The completion of cytokinesis furrow ingression occurred for both tissues

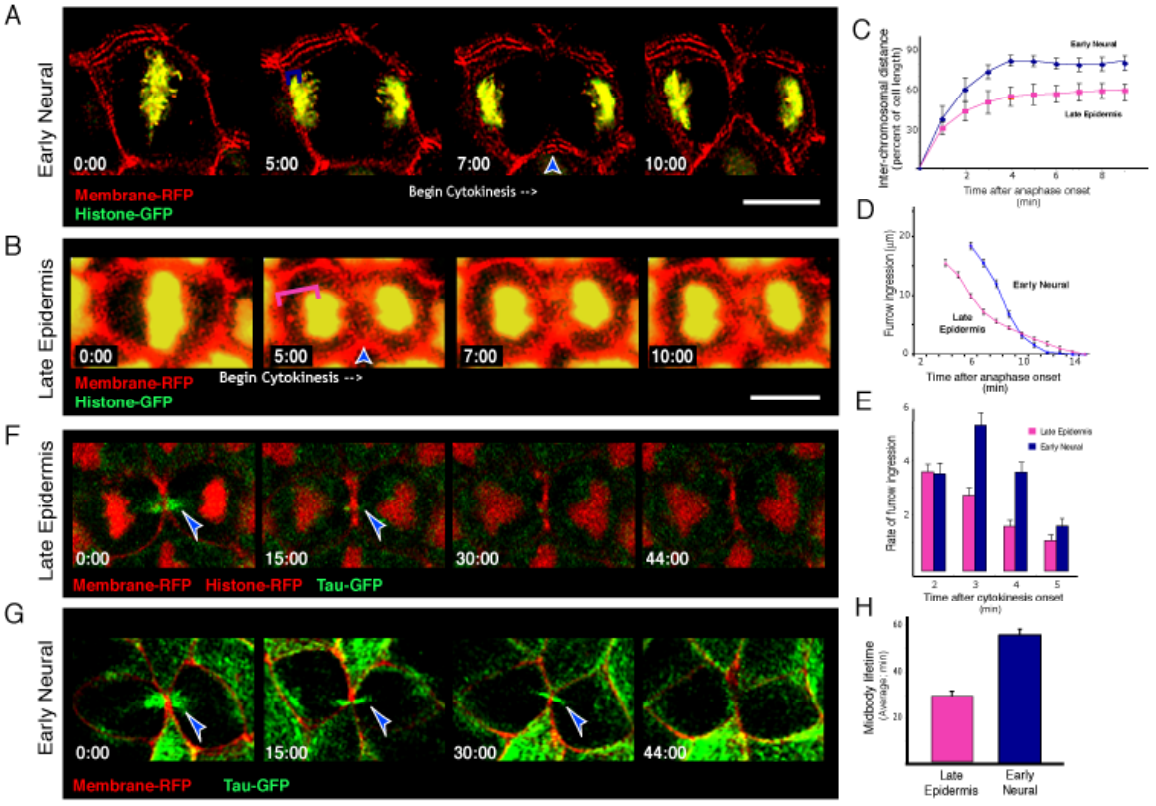

Figure 3.2 Modifications to cell division mechanism in early neural epidermis

(A and B) Still frames taken from movies of cell division in later tail epidermal cells (A) or in earlier neural epithelial cells. (B) Membranes are labeled with RFP and chromosomes are labeled with H2B-GFP. Bracket indicates distance between chromosomes and cell cortex; arrowhead indicates onset of cytokinesis. Scale bars = 10µm. (C) Graph illustrating chromosome separation during anaphase in early neural (blue) and later epidermal cells (red) expressed as percentage of cell length (N = 20 cells; 3 different embryos). (D) Graph showing progress of cytokinetic furrow ingression over time for early neural (red) and late epidermal (blue) cells. (N = 20 cells; 3 embryos) (E) Graph showing instantaneous rate of cytokinetic furrow ingression at time-points during mid-cytokinesis for early neural (red) and late epidermal (blue) cells (N = 20 cells; 3 embryos). (F and G) Still frames taken from movies showing the lifetime of the midbody in the late epidermis (G) and the early neural tissue (F). Microtubules are labeled with Tau-GFP and membranes with membrane-RFP. Arrowheads indicate midbodies. (H) Graph showing average midbody lifetime for late epidermal (red) and early neural (blue) cells (N = 14 cells; 3 embryos for neural; 11 cells; 2 embryos for epidermal).

at the same time, about 12 minutes after anaphase onset, indicating that the furrows may move at different rates in the two tissues. Furrow ingression rate was examined and it was found that for a short period of time during cytokinesis the furrows in the neural plate move at a faster rate (Figure 3.2D and E). Finally, the two tissues differed in the lifetime of the midbody. To examine microtubules during cell division mRNA encoding tau-green fluorescent protein (tau-GFP) was injected. Tau is a microtubule binding and stabilizing protein (Weingarten et al., 1975). Movies of tau-GFP revealed that the midbody persisted longer in the early neural plate than the tail epidermis, about 60 minutes and 30 minutes respectively (Figure 3.2F, G and H).

3.2.2 Decreased microtubule density in spindle midzone of early neural epithelia

The mitotic spindle and the central spindle are important for the control of the extent of sister chromosome separation (Glotzer, 2001). For this reason, we examined the spindle using movies of tau-GFP and α-tubulin immunostaining (Kwan and Kirschner, 2005). In the tail epidermis we saw a typical course of mitotic events. As anaphase and telophase progressed, a dense array of microtubules is present between the separating chromosomes. This array of microtubules is seen in both movies of tau-GFP and α-tubulin antibody staining and is the central spindle. In the tail epidermis, the midbody is formed by the compression of the central by the cytokinetic furrows (Figure 3.3A and C-F arrow). In the early neural plate this dense array of microtubules was not observed. Instead, there is a low density of microtubules between the chromosomes throughout anaphase and microtubules associate with the ingressing cytokinetic furrows during telophase (Figure 3.3B and G-J arrow and arrowhead).

To quantify the differences in microtubule density we measured the pixel intensity of microtubules between the spindle poles as indicated in Figure 3.5 (dashed

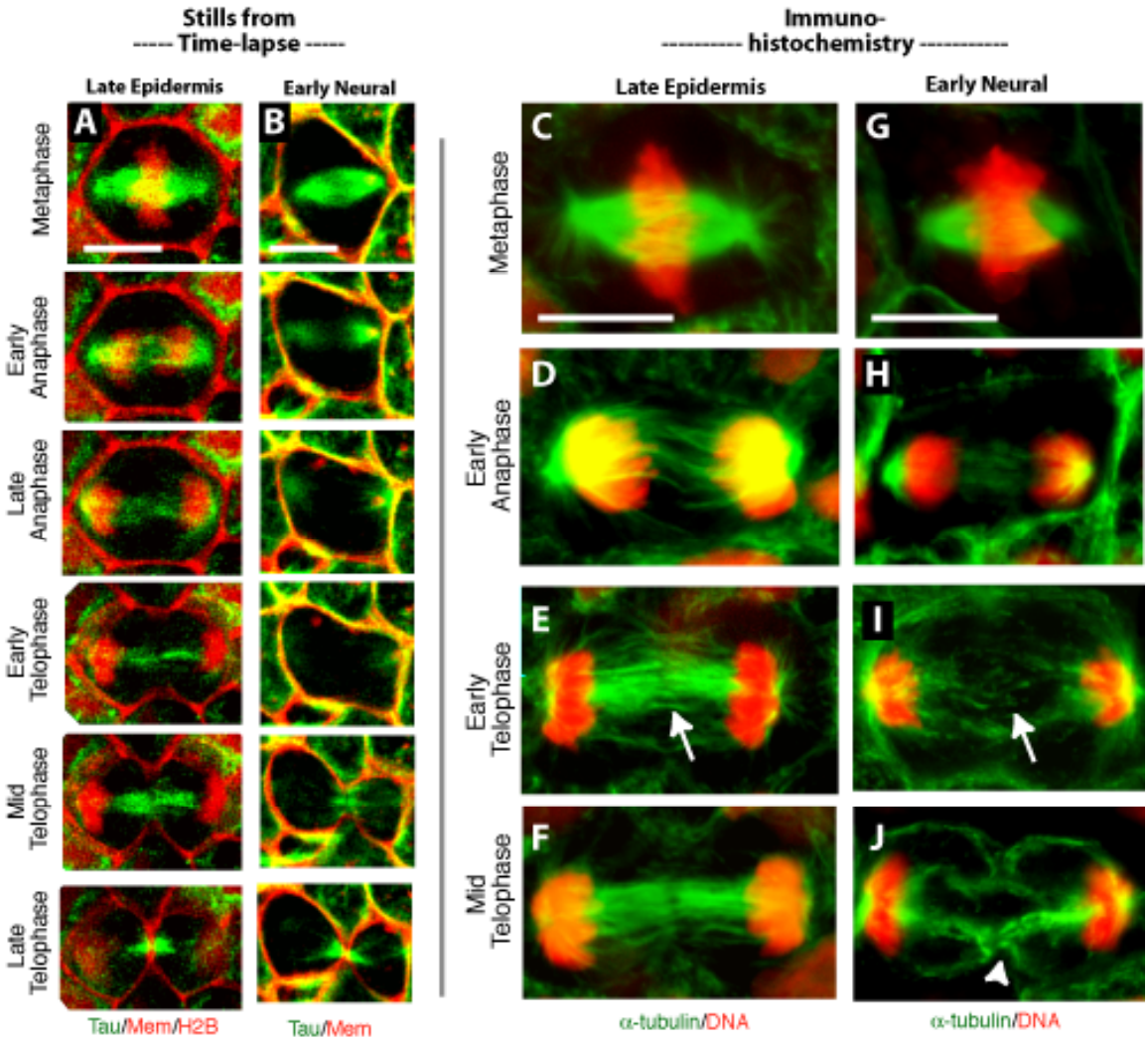

Figure 3.3 Decreased microtubule density in the spindle midzone of neural plate epithelial cells in vivo

(A and B) Still frames from movies of late epidermal epithelial cells (A) or early neural epithelial cells (B) showing the distribution of Tau-GFP-labeled microtubules during cytokinesis; membranes are labeled with membrane-RFP and chromosomes in the late epidermis are labeled with H2B-RFP. (C-J) Immunohistochemistry for α-tubulin in late epidermal (C-F) and early neural (G-J) cells confirm the microtubule distributions seen in time-lapse movies. All scale bars = 10μm.

lines in A-C). Cells at the same stage of the cell cycle, early telophase, were analyzed in the tail epidermis and the early neural plate. We consistently saw that the tail epidermis had a higher microtubule density between the spindle poles, and the average pixel intensity for several cells in the tail epidermis was higher than that in neural plate cells (Figure 3.5F compare dark blue line to the pink line).

3.2.3 PRC1 is dynamically expressed in the developing Xenopus embryo

Our data indicate that cells in the tail epidermis divide in a manner similar to mammalian cells in culture. In contrast, cells in the early neural plate divide with an exaggerated chromosome separation, rapid furrow ingression and low microtubule density between the separating chromosomes. These modifications to cell division are reminiscent of the phenotypes seen in cultured cells with depleted levels of the protein PRC1 (protein regulator of cytokinesis 1) (Jiang et al., 1998; Mollinari et al., 2002; Kurasawa et al., 2004; Verbrugghe and White, 2004; Verni et al., 2004; Mollinari et al., 2005). The similarity between these phenotypes suggested that PRC1 may control these differences in cell division in the developing *Xenopus laevis* embryo.

Using both *in situ* hybridization and immunostaining for PRC1 we observed decreased levels, but not a complete absence, of PRC1. *In situ* hybridization at the early neural stage showed low levels of PRC1 in the neural plate (Figure 3.4A, arrowheads indicate neural plate/epidermis boarder). At the early tadpole stage of development PRC1 was highly expressed in the tail bud, the kidney and the brain (Figure 3.4B). High levels of PRC1 expression in the tail bud correlate with the high level of cell division in this region. Antibody staining was consistent with the *in situ* data. Both stages had PRC1 protein expression but there was less overall in neural cells. To quantify the difference in PRC1 protein expression we imaged embryos at the two different stages

with identical staining and imaging parameters. Pixel intensity was then determined over a number of cell lengths including one midbody. PRC1 was localized to midbodies in both tissues and to this was measured to ensure that the level of PRC1 at the end of cytokinesis was the same. Indeed, we found that at the midbody the level of PRC1 was the identical (Figure 3.4C, D and E, arrow and arrowhead) but the level of PRC1 expression through the rest of the cells was reduced in the neural plate compared to the tail epidermis (Figure 3.4E compare blue line to pink line). Furthermore, the average PRC1 level over several embryos was lower in the early neural plate (Figure 3.4F).

PRC1 associated with the midbody indicating that a central spindle does eventually form. To determine when the central spindle formed in each of these tissues we examined when PRC1 associated with midzone microtubules by use of antibody staining. We found that PRC1 was often absent from midzone microtubules in the neural plate during early/mid telophase (Figure 3.4G and G'). At the same stages in the tail epidermis PRC1 localized to the spindle midzone microtubules at the expected mitotic phase, late anaphase/early telophase (Figure 3.4H and H'). At the late telophase/midbody stage of cell division PRC1 is localized to the midzone in cells in both tissues (Figure 3.4I-J').

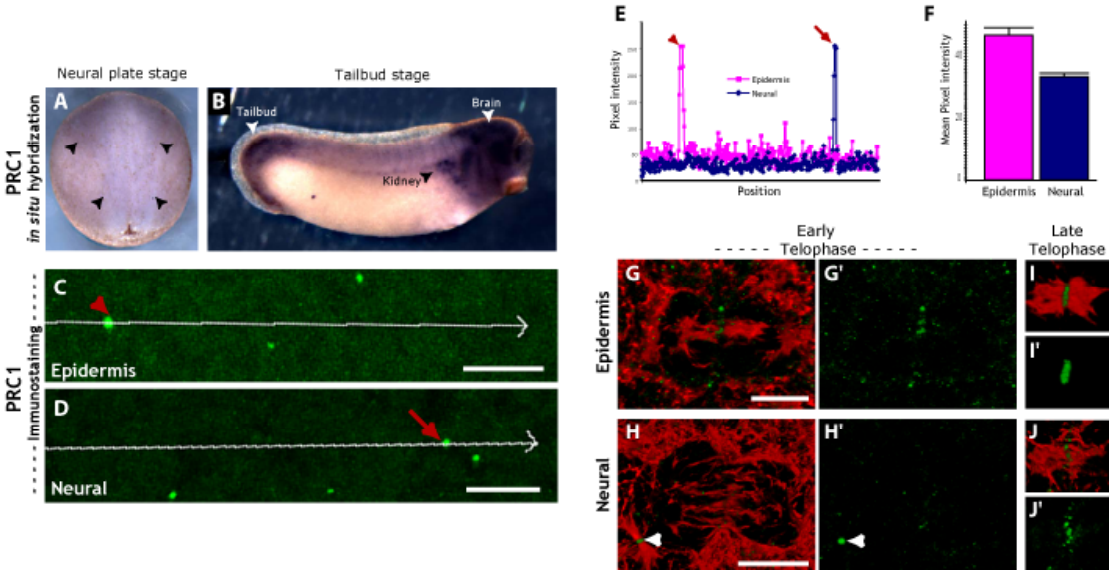

Figure 3.4 PRC1 is dynamically expressed in the developing Xenopus embryo

(A) *In situ* hybridization of PRC1 mRNA in an early neural plate stage embryo. Arrowheads outline the border of neural plate. (B) *In situ* hybridization of PRC1 mRNA in a late tail epidermis stage embryo. Arrowheads show areas of high expression of PRC1 mRNA, the brain, kidney and tail bud. (C) Immunostaining of PRC1 in the tail bud epidermis. White arrow indicates the line scan indicated by the pink line in panel E. Red arrowhead indicates a midbody stained by PRC1. (D) Immunostaining of PRC1 in the neural plate. White arrow indicates the line scan indicated by the blue line in panel E. Red arrow indicates a midbody stained by PRC1. Scale bar in C & D = 20μm. (E) Graph of line scans for PRC1 signal intensity in a representative tail bud epidermal cells (pink) and neural plate cells (blue). Arrow and arrowhead indicate the intense signal at midbodies in both cell types. (F) Graph mean pixel intensity of interphase, cytoplasmic PRC1 immunostaining signal for tail bud epidermis (pink; N = 12 embryos) and neural plate (blue bar. N = 7 embryos). (G) Immunostaining for PRC1 (green) and α-tubulin (red) in a tail bud epidermal cell at early telophase. PRC1 channel alone is shown in panel g'; PRC1 is concentrated around bundled microtubules in the midzone. (H) Immunostaining for PRC1 (green) and α-tubulin (red) in a neural plate cell at early telophase. PRC1 channel alone is shown in panel h'; despite cytokinesis onset, PRC is not concentrated in the midzone. Scale bar in G & H = 5μm. By late telophase, PRC localizes to the central spindle in both epidermal cells (I, i') and neural cells (J, j').

3.2.4 Excess PRC1 converts neural cell division mechanism to a more epidermal like mechanism

We next examined whether the low level of PRC1 in the neural plate could explain the modifications to cell division in this tissue. To test this idea, we used DNA injection of PRC1-GFP, in a mosaic manner, into the neural plate. Embryos were injected with histone-2B-RFP and membrane-RFP along the entire dorsal side at the 4-cell stage. At the 8-cell stage embryos are injected into one of the animal/dorsal cells with a plasmid in which a CMV promoter drives PRC1 expression. DNA was used because it avoids any early pleiotropic effects overexpression of PRC1 by delaying its expression until after the mid-blastula transition and the beginning of zygotic transcription (Vize et al., 1991). In neural cells expressing PRC1 at moderate levels, we saw increased density of midzone microtubules and a reduction in the extent of chromosome movement during anaphase-B to a similar degree as observed in the tail epidermis (Figure 3.5C, F, light blue line and G, light blue line).

3.2.5 MKLP1 onset to central spindle is delayed in early neural cells

In cultured cells depleted of PRC1 a mis-localization of a number of central spindle proteins was seen including MKLP1 (Mollinari et al., 2005). To examine if similar mis-localization events occur in the neural plate we investigated the onset of MKLP1 to the spindle midzone. MKLP1 is a kinesin like motor protein thought to be required for movement of vesicles to the cytokinetic furrows (Gromley et al., 2005). Antibody staining analysis of MKLP1 showed that it also had delayed onset to the central spindle. MKLP1 accumulated in the spindle midzone during early anaphase in epidermal cells, but not until late anaphase or early telophase in neural cells (Figure 3.6A and B**).** A similar delay was observed in time-lapse movies of embryos expressing MKLP1-GFP.

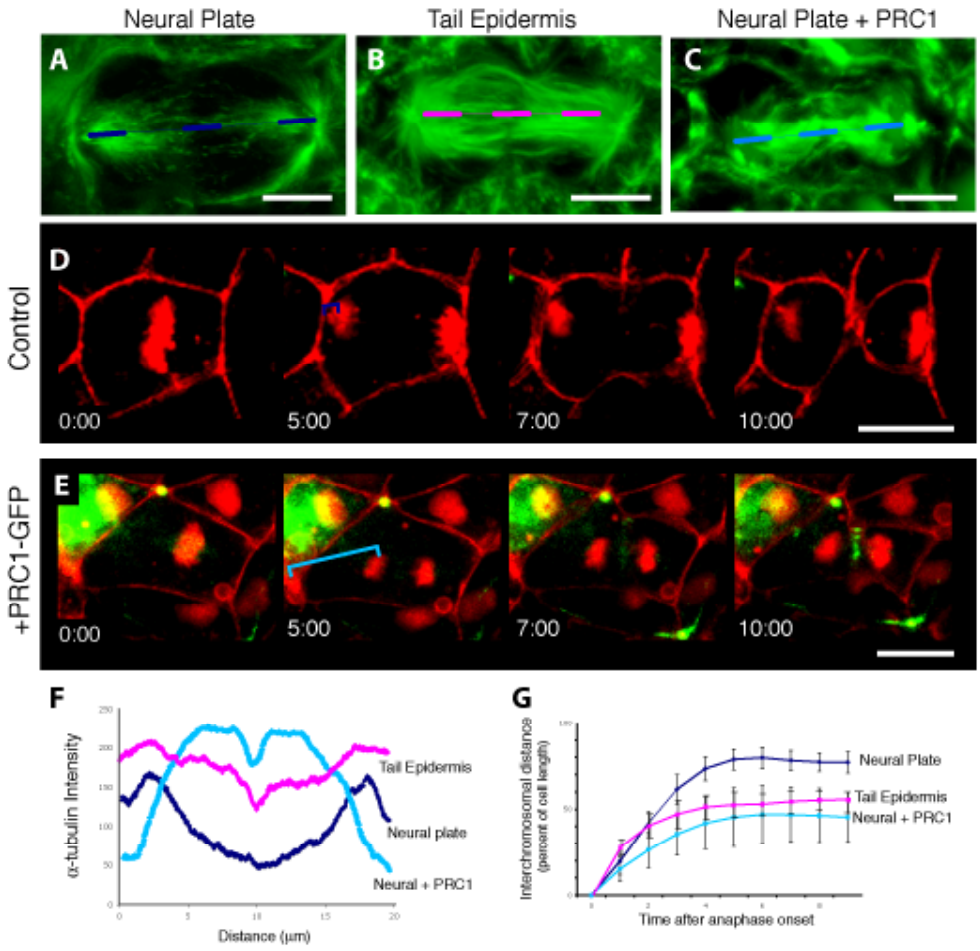

Figure 3.5 Excess PRC1 converts neural cell division mechanisms to a more epidermal-like mechanism.

(A) α-tubulin immunostaining of a neural plate cell during early telophase. Dark blue dashed line indicates position of line scanning for data shown in panel F. (B) α-tubulin antibody staining of a tail epidermis cell during early telophase. Pink dashed line indicates position of line scanning for data shown in panel F. (C) α-tubulin immunostaining of a neural plate cell overexpressing PRC1-GFP. Light blue dashed line position of line scanning for data shown in panel F. (D) Still frames from a movie of division in a GFP-negative early neural epithelial cell. (E) Still frames from a movie of division in a GFP-positive (PRC1-expressing) early neural epithelial cell. Brackets in panels B and C indicate the distance between chromosomes and the cell cortex. (F) Graph of α-tubulin intensity across the early telophase spindle of the early neural plate cells (dark blue line; N = 13), tail bud epidermis (pink line; N = 6) and early neural plate cells overexpressing PRC1-GFP (light blue line; N = 12). (G) Graph of anaphase

chromosome separation (expressed as percent of cell length) in neural plate cells (dark blue line; N = 12), tail bud epidermis (pink line; N = 20), and neural plate cells overexpressing PRC1 (light blue line; N = 12).

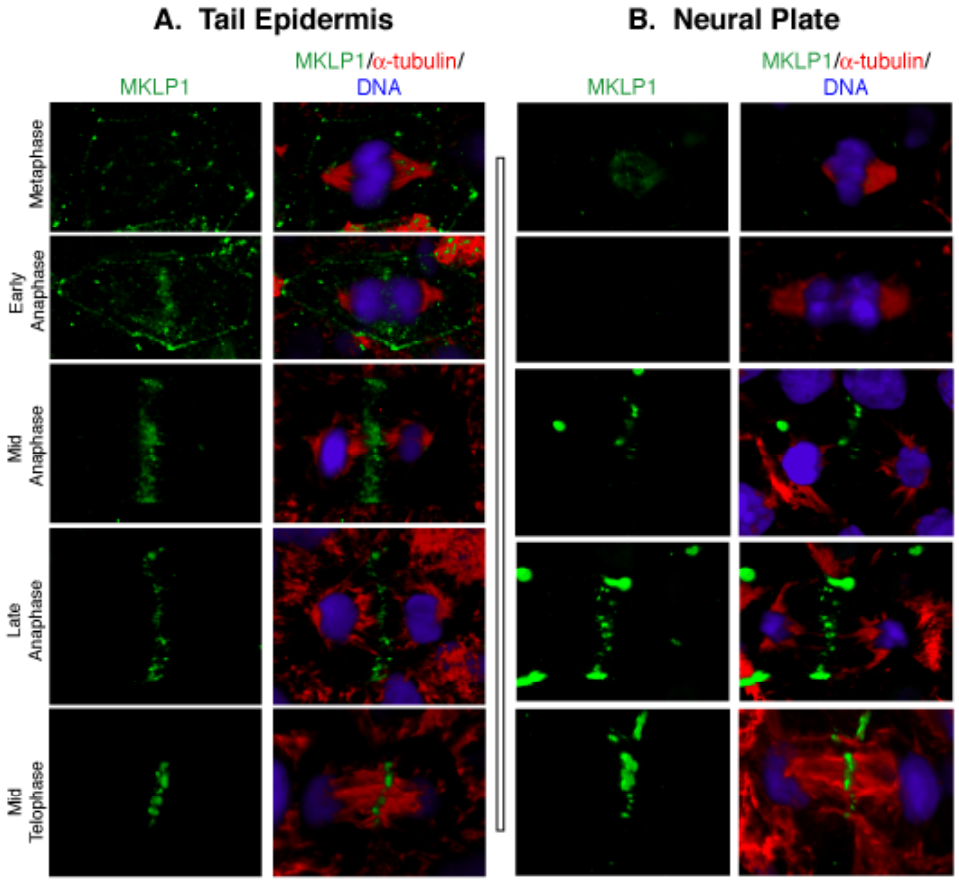

Figure 3.6 MKLP1 is recruited to central spindle later in the early neural plate

(A) Immunohistochemistry of MKLP1 localization during cell division in the tail epidermis. Distance between separating nuclei were assessed and compared to graph in Figure 3.1C. For telophase distance between ingressing furrows was measured and compared to graph in figure 3.1D. (B) Immunohistochemistry of MKLP1 localization during cell division in the early neural epithelial cells. Distance between separating nuclei were assessed and compared to graph in Figure 3.1C. For telophase distance between ingressing furrows was measured and compared to graph in figure 3.1D.

3.2.6 Midbodies in the neural plate are highly arched and release from the apical surface of cells

Our MKLP1-GFP movies also showed another striking phenomenon. After cell division was completed, puncta of MKLP1-GFP were released from the apical surface of the cells into the future lumen of the neural tube (Figure 3.7A). This indicated that the midbody is most likely released from the apical surface of these neural cells. This release was not observed in cells of the tail epidermis, suggesting that there might be a difference in the structure of the midbody. Both antibody staining and *in vivo* imaging showed that the midbody in neural plate cells had a highly arcuate structure (Figure 3.7B and C) and central spindle proteins and the midbody were closely localized to the apical surface of the cell. We did not observe this in the tail epidermis; instead the central spindle and the midbody spanned most of the apical/basal axis of the cell (Figure 3.7B and C). This indicates that the midbody in early neural cells may be specialized as compared to the tail epidermis.

3.2.7 High-level overexpression of PRC1 in neural cells causes ectopic microtubules and disrupts cytokinesis

When we examined the expression of PRC1 in the neural plate of DNA injected embryos, the cells had varying levels of the protein. This variability is due to mosaic expression of the gene of interest, with one cell with high expression being directly adjacent to a cell with low or no expression (Vize et al., 1991). When PRC1 is expressed at a low level or a moderate level we saw reduced chromosome separation and enhanced microtubule density in the midzone (see above). When PRC1 is expressed at very high levels, we encountered much different phenotypes. Cells had thick cables of PRC1-GFP positive microtubules (Figure 3.8A-A"). These cables were previously seen in

mammalian cells in culture with forced over-expression of PRC1. These cables broke down during the onset of metaphase, formed a mitotic spindle and underwent proper cytokinesis (Mollinari et al., 2002). Once the cells re-entered interphase the large bundles of microtubules reformed but the general morphology of the cells was normal. In cells of the *Xenopus* early neural plate, we saw that cells containing the large bundles contained multiple nuclei and were much larger than normal cells (Figure 3.8B). Movies of these cells revealed that they are still susceptible to mechanisms of PRC1 regulation previously seen (Jiang et al., 1998; Zhu and Jiang, 2005). Cables broke down during the onset of metaphase and formed a mitotic spindle. Anaphase began in these cells but ultimately failed and no cytokinetic onset was seen. This indicates that the cells in the *Xenopus* neural cells are very sensitive to changes in PRC1 levels.

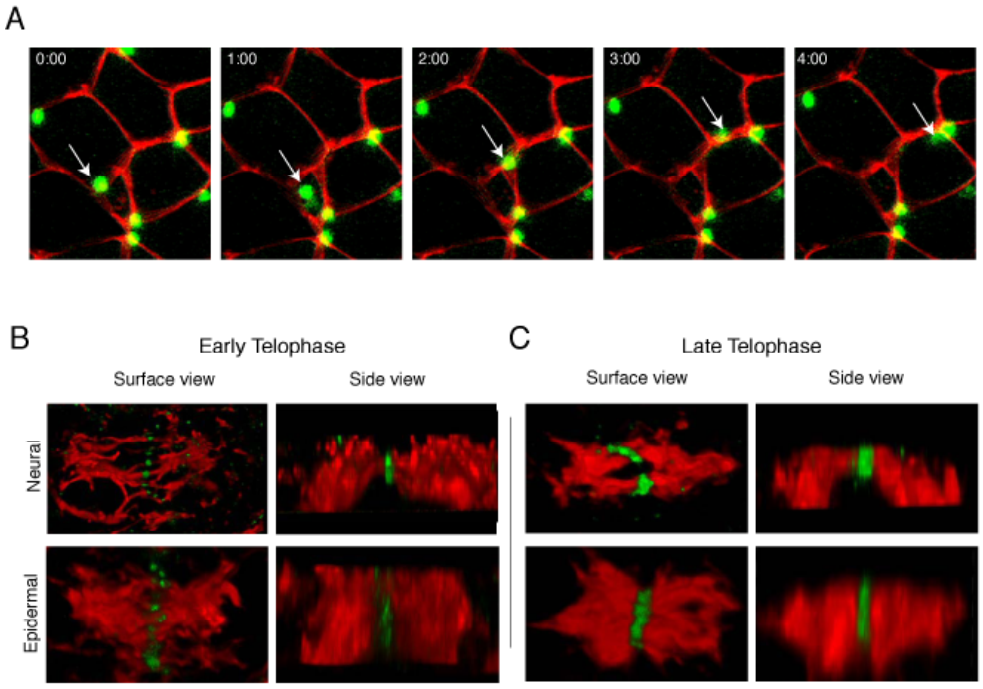

Figure 3.7 Midbody architecture differs in early neural plate and releases from the apical surface of cells

A) Still frames from a movie of MKLP1-GFP and membrane-RFP in the neural plate. Arrows point at a stably localized puncta of MKLP1, marking the midbody, that releases out of the apical surface of the tissue. B) Zoomed in view of the midbody at early telophase. The midbody is labeled with antibody staining of a-tubulin and PRC1 in both the neural plate (upper row) and the tail epidermis (lower row) at early telophase. Shown is an X-projection of a stack (left hand column) and a Z-projection of the same stack (right hand column). C) Zoomed in view of the midbody at late telophase. The midbody is labeled with antibody staining of a-tubulin and PRC1 in both the neural plate (upper row) and the tail epidermis (lower row) at early telophase. Shown is an X-projection of a stack (left hand column) and a Z-projection of the same stack (right hand column).

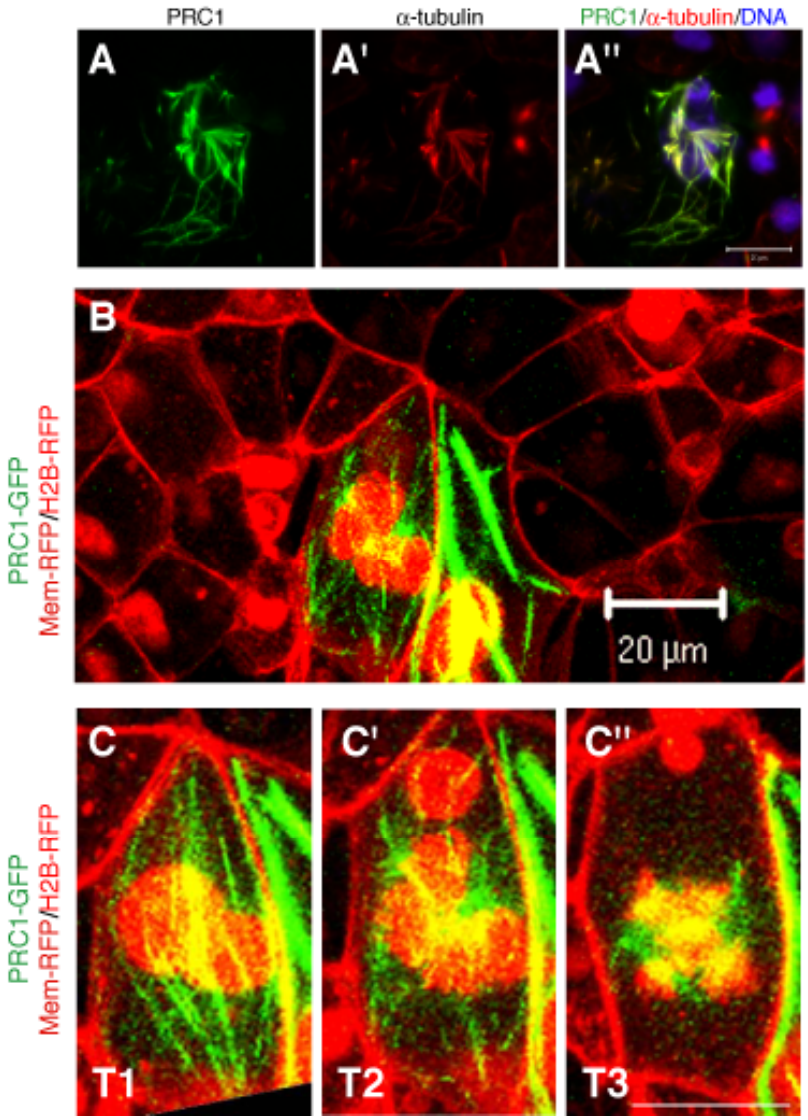

Figure 3.8 High-level overexpression of PRC1 in neural cells causes ectopic microtubules and disrupts cytokinesis

(A-A") Immunohistochemistry of α-tubulin in a neural cell expressing high levels of PRC1-eGFP. Showing colocalization of PRC1 and α-tubulin in large cables (A) Localization of PRC1-eGFP. (A') Localization of α-tubulin. (A") Merged image of PRC1, α-tubulin and DNA. (B) Still frames from a movie of PRC1-eGFP mosaics in the early neural plate. Cells expressing high levels of PRC1 are multi-nucleated and contain large cables of PRC1 associated microtubules. (C-C") Still frames over time from the same movie in figure 3.5B. Cables of microtubules break down at the onset of metaphase to form a multipolar spindle with more than one metaphase plate.

3.3 DISCUSSION

3.3.1 Central spindle assembly is developmentally regulated by PRC1 expression levels

Proper completion of cell division ensures the faithful segregation of genetic material and growth of organisms (Rappaport, 1961; Gont et al., 1996; Glotzer, 2001; Chalmers et al., 2003). Most of the previous analysis of the mechanisms of cell division has occurred in cultured mammalian cells and in early invertebrate embryos (Glotzer, 2001; Wang, 2001; Mazumdar and Mazumdar, 2002). Comparatively little data is available about the mechanisms of cell division during vertebrate development (Hartenstein, 1989; Concha and Adams, 1998; Saka and Smith, 2001; Geldmacher-Voss et al., 2003; Gong et al., 2004). Even less *in vivo* time-lapse data is available (Concha and Adams, 1998; Geldmacher-Voss et al., 2003; Gong et al., 2004). Here we present a method to analyze cell divisions during different stages of development in an intact, developing vertebrate.

To guarantee proper cell division the structures controlling this process must be correctly formed. One of the most important structures for cell division is the central spindle (Glotzer, 2001; Glotzer, 2005). The central spindle is a dense bundle of antiparallel microtubules shown to be required for cytokinesis (McCollum, 2004). Many proteins required for cytokinesis are recruited to this structure and if their localization is disrupted the structure of the central spindle is also disrupted and cytokinesis progresses but does not complete (Severson and Bowerman, 2002). Much of the previous work on central spindle proteins has been focused on the centralspindilin complex and passenger protein complex (Adams et al., 2001; Terada, 2001; Mishima et al., 2002; Mishima and Glotzer, 2003). Recent research has elucidated the role of the microtubule bundling

protein PRC1 in maintenance of the central spindle (Jiang et al., 1998; Mollinari et al., 2002; Verbrugghe and White, 2004; Verni et al., 2004).

Here we show that the structure of the central spindle is developmentally regulated in *Xenopus laevis*. We made *in vivo* 4-D movies of cell division at two different stages of development in two ectodermally derived tissues, the early neural plate and the tail bud epidermis. In the tail epidermis we found a typical central spindle structure throughout anaphase and telophase/cytokinesis (Figure 3.3A and C-F). In the early neural plate instead we found a dispersed central spindle during anaphase, which became more bundled and "normal" in appearance as telophase progressed (Figure 3.3B and G-J). We have found that regulation of the level of PRC1 expression in each of these tissues explains these differences. The neural plate has a low level of PRC1 expression compared to the tail epidermis (Figure 3.4). We propose that, due to this low level of expression, the microtubules of the central spindle are unable to be bundled until late in the cytokinetic process.

3.3.2 Differences in central spindle structure lead to changes to the mechanism of cell division

Interestingly, we find that alterations to the structure of the central spindle lead to changes in the mechanisms of cell division. In the tail epidermis cell division proceeds similar to cells in culture (Rappaport, 1961; Glotzer, 2001). During anaphase the chromosomes stop poleward movement when they reach the approximate geometric center of the new daughter cell. At this point, approximately 4 minutes after anaphase onset, telophase begins with the ingression of the cytokinetic furrows (Figure 3.2A). In contrast, early neural plate cells have chromosomes that travel much further than the center of the new daughter cell and instead closely approach the cell cortex (Figure 3.2B).

Furthermore, we find that cytokinesis onset is does not occur until the chromosomes stop pole ward motion, approximately 6 minutes after anaphase onset (Figure 3.2B).

Our data show that that modifications to cell division are caused by the differences in the structure of the central spindle because of a reduction of the level of PRC1 in the early neural plate. When we forced expression of PRC1 in the neural plate, the differences observed were relieved. When PRC1 is moderately over-expressed in the early neural plate a well-structured central spindle was formed and the chromosomes do not as closely appose the cell cortex (Figure 3.5). If when PRC1 was highly over-expressed cells were much larger, multinucleated and failed to divide properly (Figure 3.8). This indicates that cells in the early neural plate are highly sensitive to large changes in PRC1 expression and that in this tissue the altered central spindle structure is important for the high fidelity of cell division.

3.3.3 Differences in cytokinesis may be related to specialization of the midbody

As the cytokinetic furrows ingress they compress the central spindle down to a dense bundle called the midbody. The midbody is a microtubule-based structure that is thought to be required for the final abscission of dividing cells (Krishan and Buck, 1965; Saxton and McIntosh, 1987; Skop et al., 2004; Gromley et al., 2005). Different cells have been shown to discard their midbodies in different ways. For cultured cells to complete abscission, one of the daughter cells adopts the midbody (Figure 3.9A-C) (Mishima et al., 2002; Glotzer, 2005; von Dassow and Bement, 2005; Dhonukshe et al., 2007; Pohl and Jentsch, 2008).

In neural cells the midbody has been found to be a highly specialized (Bancroft and Bellairs, 1975; Bellairs and Bancroft, 1975; Dubreuil et al., 2007; Wilcock et al., 2007). The apical process in mature neural-epithelial cells is microtubule based and is

derived from the midbody (Wilcock et al., 2007). Furthermore, midbodies are released from the apical surface of neural cells to maintain the balance of differentiation factors (Figure 3.9D-G) (Dubreuil et al., 2007). Differentiation factors are primarily found at the apical membrane of cells (Bellaiche et al., 2001a; Bellaiche et al., 2001b; David et al., 2005; Dubreuil et al., 2007). Very small differences in segregation of these factors can lead to differentiation of one of the daughter cells (Kosodo et al., 2004). If these neural cells underwent abscission like cultured cells, one of the daughter cells would adopt more of the differentiation factors via the membrane around the midbody. Instead, these neural cells have modified their disposal of the midbody to release it from the apical membrane and out into the lumen of the neural tube. This allows for both of the cells to retain equal amounts of the midbody.

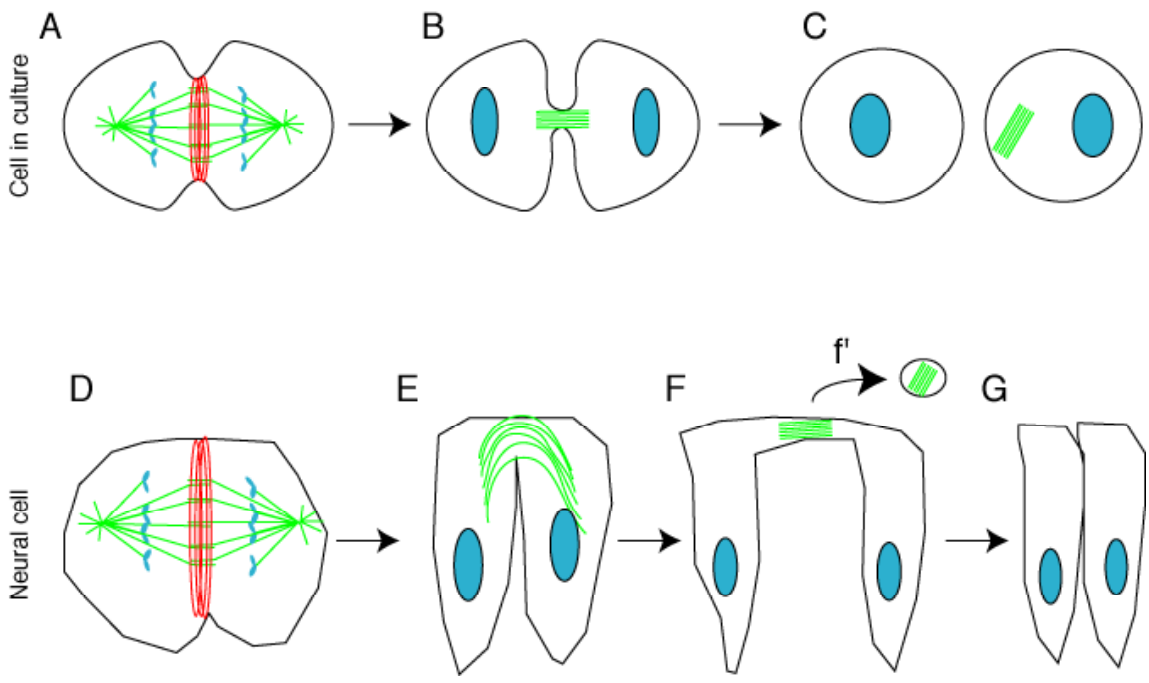

Figure 3.9. Neural cells discard their midbody differently than cells in culture

A) A cell in culture undergoing telophase. B) Cell at the late telophase/cytokinesis stage of the cell cycle. The midbody is seen as a bridge between the two new daughter cells. C) Vesicle traffic occurs from one of the daughter cells to the midbody. Vesicles fuse with the plasma membrane on one side of the midbody and allow one of the new daughter cells to adopt the midbody. D) A neural cell undergoing telophase. E) Neural cell at the mid/late telophase/cytokinesis stage. The midbody is seen as a highly arched bridge between the two cells. F) Neural cell at the late telophase/cytokinesis stage. Vesicle traffic occurs from both of the new daughter cells to the midbody. f') Vesicles fuse on both sides of the midbody with the plasma membrane leading to the release of the midbody off of the apical surface. G) Two new daughter neural cells with equal apical membranes.

During our time-lapse analysis we found that the midbody in *Xenopus* neural plate was also released from the apical surface of the cell. This release may be related to the control of the differentiation state of these neural cells. Most of the factors that control differentiation of cells lie at the apical surface. Symmetric cell divisions maintain the same level of these differentiation factors between each of the daughter cells keeping them proliferative. Asymmetric cell divisions cause one cell to gain more of the differentiation factor and this cell remains proliferative while the other differentiates (Bellaiche et al., 2001a; Bellaiche et al., 2001b; Dubreuil et al., 2007). This release of the midbody in neural cells is in contrast to the situation during Hela cell division where one of the daughter cells adopts the midbody to complete abscission (Mishima et al., 2002; Glotzer, 2005). We present the following model for the cell divisions in these two different cell types. Cells in the tail epidermis divide in the standard way. During anaphase chromosomes separate and stop at approximately the geometric center of the new daughter cell (Figure 3.10A, anaphase). During this period the central spindle forms by the bundling of the antiparallel microtubules by PRC1 (Figure 3.10, red dots). The cytokinetic furrows ingress at the central spindle (Figure 3.10A, early telophase). This central spindle is then compressed into the midbody by the cytokinetic furrows. In the tail epidermis this midbody spans the entire apical to basal axis (Figure 3.10A, mid/late telophase). In the early neural plate this process is different. Chromosomes separate to a further extent in the early neural plate (Figure 3.10B, anaphase). Moreover, the central spindle is not well organized until late in telophase. Astral microtubules instead associate with the ingressing cytokinetic furrows. These furrows also move at a faster rate than the furrows in the tail epidermis (Figure 3.10B, early telophase, compare to early telophase in 3.9A). At this stage the central spindle begins to form with PRC1 at bundles of antiparallel microtubules. This altered central spindle structure may lead to a highly

arched midbody (Figure 3.10B, mid telophase). This highly arched midbody most likely enables to the release of the midbody from the apical membrane (Figure 3.10B, late telophase). Also the requirement of vesicle trafficking to both sides of the midbody may lead to the midbody having a longer life time in these neural cells (Figure 3.2F-H).

Together these modifications lead to the maintenance of the state of these neural cells until differentiation is required. Cells in the tail epidermis may not need these modifications to the midbody because they are closer to their final fated state. The modifications to cell division seen in these neural cells; exaggerated anaphase, delayed cytokinesis, rapid furrow ingression and a long lasting midbody, may be a consequence of the differences in central spindle structure. They may also be another evolutionary role to these modifications. Cells in the early neural plate are under a large amount of tension by the movement of the tissues surrounding them. The process of neural tube closure in *Xenopus laevis* is one of large morphogenic movements. The embryo gets becomes thin and lengthens by a process called convergent extension. A tube of ectoderm, the future central nervous system, is formed and internalized by invagination of the neural tissue and the medial movement of the epidermis. Convergent extension is the process whereby cells intercalate between one another to create a longer thinner tissue (Davidson and Keller, 1999; Wallingford et al., 2002). The rolling of the neural tissue into a tube is caused by cell shape changes (Jacobson and Gordon, 1976). Medial movement of the epidermis on the neural tissue aids this rolling action (Alvarez and Schoenwolf, 1992). These motions apply mechanical tension on the cells in the neural tube. The modifications seen in these early neural plate cells may be a response to these motions as a way to avoid aneuploidy. The cells may have their chromosomes move as far apart as possible, have the cytokinetic furrows ingress as fast as possible and have the

midbody as long lasting as possible to avoid chromosome slippage back through the cytokinetic bridge. Further research on this is required to fully address this question.

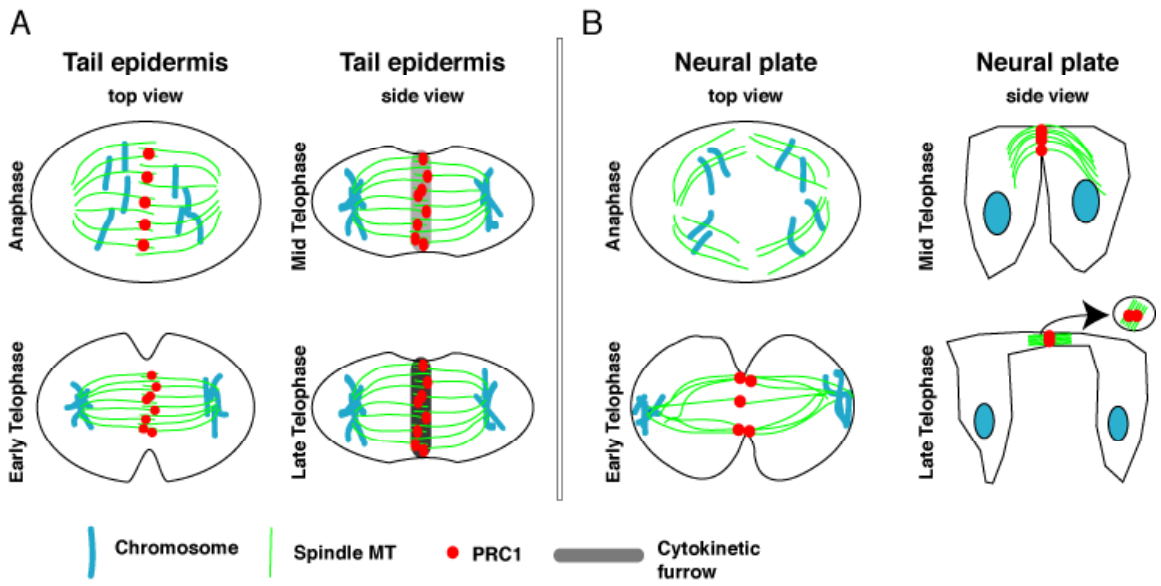

Figure 3.10. Model of final abscission in the tail epidermis and the early neural plate.

A) Model of division in the tail epidermis. First column represents a top view of a tail epidermal cell during anaphase and early telophase. A well bundled central spindle is observed and PRC1 localizes to the overlapping antiparallel microtubules. The second column represents a side view of the same cell during mid and late telophase. The black bar across the central spindle represents the ingressing cytokinetic furrows. The midbody is seen to span almost the entire apical to basal axis of the cell. B) Model of division in the early neural plate. First column represents a top view of a neural cell during anaphase and early telophase. A well bundled central spindle is not observed. PRC1 localizes to the overlapping antiparallel microtubules that are associating with the ingressing furrows and begins to form a central spindle later during the cell cycle. The second column represents a side view of the same cell during mid and late telophase. The midbody is highly arched and is close to the apical membrane of the cell. The midbody is eventually is released into the future lumen of the neural tube.

Chapter 4: Possible role of central spindle proteins in ciliogenesis

4.1 INTRODUCTION

Ciliogenesis is the process by which a cilium is formed at the surface of a cell. Cilia and flagella are microtubule based organelles that protrude from the surface of most vertebrate cells. There are two types of cilia, motile and stationary or primary cilia (Bossinger and Bachmann, 2004). Motile cilia move liquid over tissues such as the lung and kidney (Sleigh et al., 1988). Most vertebrate cells have at least one primary, or stationary, cilium protruding from its surface (Olsen, 2005). These primary cilia have been found to be hubs for particular signaling molecules and the ciliary structure is required for transduction of certain signaling pathways, such as the Hedgehog pathway (Scholey et al., 2004; Corbit et al., 2005; Haycraft et al., 2005; May et al., 2005; Scholey and Anderson, 2006). Cilia are formed at the surface of cells by a specific set of events called ciliogenesis. Ciliogenesis occurs when basal bodies are formed by the replication of centrioles near the golgi (Figure 4.1A). These basal bodies then associate with vesicles, possibly from the golgi as well, and are trafficked toward the apical surface of the cell (Figure 4.1B) (Yang and Li, 2005; Park et al., 2008). As the basal body is being trafficked apically it associates with two structures the basal foot and the rootlet (Figure 4.1C and D) (Boisvieux-Ulrich et al., 1990; Hagiwara et al., 1997; Yang et al., 2002; Yang and Li, 2005). The vesicle associated with the basal body then fuses with the apical membrane of the cell thus docking the basal body to the apical surface of the cell (Figure 4.1C). The cilia can now form by extension of the microtubules that make up the basal body (Rosenbaum and Witman, 2002; Pazour and Witman, 2003). This extension of the axoneme relies on a process called intraflagellar transport (IFT). This process grows the cilia from the distal tip of the axoneme (Witman, 1975; Rosenbaum and Witman, 2002).

Disruption of any of the many IFT molecules leads to defects in ciliogenesis (Rosenbaum and Witman, 2002; Pazour and Witman, 2003).

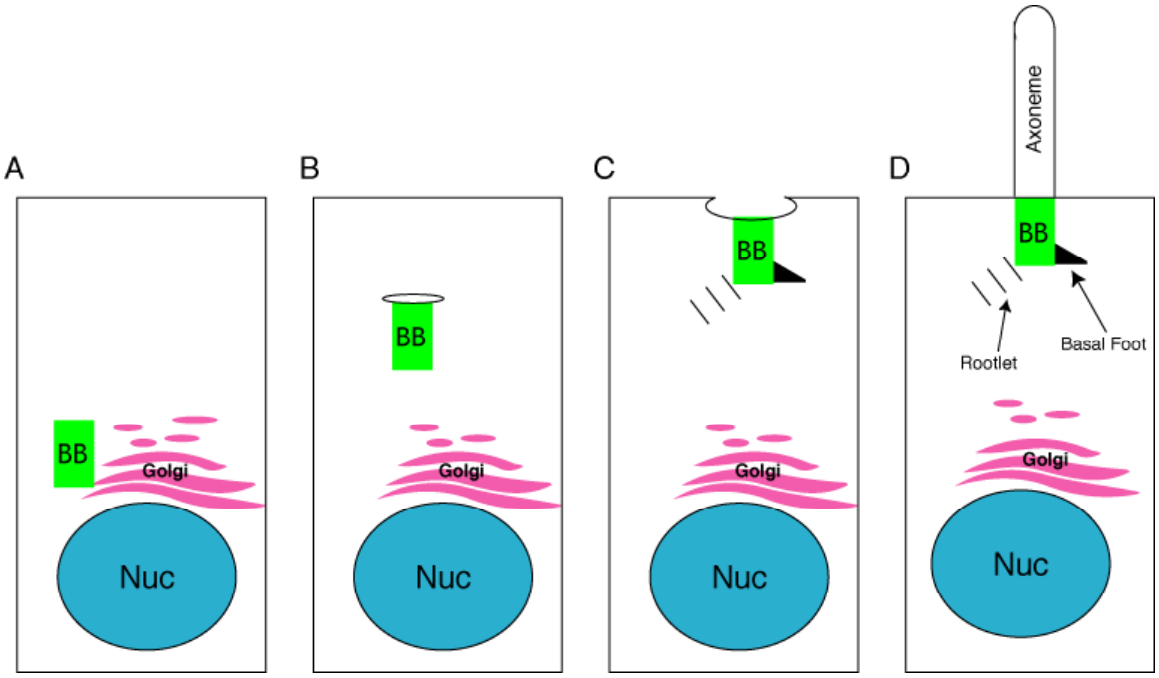

Figure 4.1. Ciliogenesis

(A) The centrioles of the centrosome separate near the golgi. In the case of multiciliated cells the mother/older centriole replicates in this area to produce many basal bodies. (B) These basal bodies then associate with vesicles and are trafficked toward the apical surface of the cell. (C) As they are trafficked the basal bodies associate with two accessory structures, the basal foot (black triangle) and the rootlet (black lines). This completed basal body structure then docks to the apical membrane by the fusion of the vesicle with the plasma membrane. (D) The axoneme grows out from the basal body by the addition of axonemal units to the distal tip by intraflagellar transport.

When ciliogenesis is disrupted during development a number of diseases, disorders and syndromes are seen, called ciliopathies. Along with other defects to development ciliopathies often present with defects to neural tube closure (Badano et al., 2006; Chen, 2008; Leitch et al., 2008; Tallila et al., 2008). These neural tube closure defects have been linked to defects in hedgehog signaling and patterning of the neural tissue (Huangfu et al., 2003; Huangfu and Anderson, 2005). The planar cell polarity (PCP) pathway also has been found to play a role in neural tube closure by aiding in convergent extension and cell shape changes that occur during this time (Wallingford and Harland, 2002; Ueno and Greene, 2003; Ciruna et al., 2006). As mentioned previously (Chapter 1), PCP signaling organizes cells within the plane of a tissue as opposed to the apical basal axis, which is perpendicular to the plane of the tissue (Nechiporuk and Vasioukhin, 2006). PCP signaling controls the outgrowth of lamellipodia on each side of the cell through the localized organization of actin filaments (Shih and Keller, 1992). Recently, PCP signaling has been shown to be involved in ciliogenesis by regulation of basal body docking and the polarity of basal bodies (Park et al., 2006; Park et al., 2008). When PCP signaling is disrupted, through morpholino knockdown, basal bodies are not trafficked to the apical surface of the cell. This may be because of disruption of the actin cytoskeleton at the apical surface (Park et al., 2006; Park et al., 2008). This data is fairly recent but reveals that our understanding of ciliogenesis is not complete and some unexpected pathways may be involved in this process.

The epidermis of the *Xenopus* tadpole is an excellent model system in which to study ciliogenesis. This tissue is a mucociliary epithelium that closely resembles that of the lining of the airway in vertebrates (Hayes et al., 2007). Both of these tissues contain goblet cells, that secrete mucus, and ciliated cells, with dozens of motile cilia that beat to move the mucus over the tissue (Steinman, 1968; Billett and Gould, 1971; Knowles and

Boucher, 2002; Rogers, 2003; Nokhbatolfoghahai et al., 2005). The vast number of cilia on these ciliated cells makes it easy to analyze defects by assessing the length and/or number of cilia present.

A large body of research concentrates on the connection between ciliogenesis and cell division. The basal body of the primary cilium also acts as one of the centrioles of the centrosome and regulated breakdown of the cilium is required for proper progression of cell division (Rieder et al., 1979; Wheatley et al., 1996; Pan and Snell, 2007). There is an inverse relationship between cell proliferation and cilia formation, as cells differentiate they reduce their proliferation rate and form cilia (Fonte et al., 1971).

The progression of cell division in sea urchins relies on the translocation of a ciliary kinesin subunit, KAP, to the nucleus. For this translocation to occur the cilia must be broken down allowing the release of KAP into the cytoplasm where it can be translocated to the nucleus (Morris et al., 2004). Furthermore, diseases associated with defects in cilia formation, such as polycystic kidney disease, are now being linked to defects in the progression of the cell cycle (Mahjoub et al., 2002; Mahjoub et al., 2004).

Recently there has been an increase in research connecting the molecular mechanisms of ciliogenesis and cytokinesis (Gromley et al., 2003; Gromley et al., 2005; Zhao et al., 2006; Spektor et al., 2007). Centriolin has recently been discovered and found to be a major protein of centrosomes. During cytokinesis Centriolin localizes to the midbody and when knocked-down the final steps of cytokinesis fail (Gromley et al., 2003). Centriolin interacts with members of the exocyst complex and proteins associated with SNAREs (soluble N-ethylmaleimide-sensitive factor attachment receptor). More detailed analysis of the knock down phenotype showed that centriolin is required for the recruitment of the exocyst to the midbody. When the exocyst is not localized to the midbody vesicles are shown to accumulate around the cytokinetic furrow indicating a

failure of fusion between the vesicles and the plasma membrane. MKLP1 (mitotic kinesin-like protein 1) is required to localize centriolin to the midbody and thus shows a requirement for MKLP1 in recruiting the exocyst complex to the cytokinetic furrow (Gromley et al., 2005). Previous findings in the Wallingford lab show that the process of ciliogenesis also localization of the exocyst to basal bodies (Park et al., 2008). This research shows a link between ciliogenesis and cytokinesis but the inverse relationship is poorly understood and further research is required. While the connection of cilia formation to the cell cycle is well studied the inverse is not as well studied.

Based on the work of other members in the lab and the work of Gromley et al., we sought to find out what cell divisions proteins are involved in ciliogenesis. Fully understanding the connection between ciliogenesis and cell division, cilia to cell division and cell division to cilia, will aid in understanding a number of ciliopathies. This will also lead to a better understanding of both these processes by gleaning information from each other. Specifically, vesicle trafficking appears to be involved in each of these processes (Gromley et al., 2005; Park et al., 2008). The specifics of vesicle trafficking in either cell division or ciliogenesis are not well known and connection of the two may aid in understanding this.

To this end we examined the localization of centriolin and central spindle proteins in the epidermis. Here we show that Centriolin, MKLP1, INCENP (inner centromere protein), PRC1 and CENP-E (centromere protein-E) all localized in a punctate pattern at the apical surface of multiciliated cells. To elucidate the exact localization of these proteins we did co-localization analysis with proteins known to localize to the basal body, γ-tubulin, or the rootlet, a basal body accessory structure, CLAMP. We found that centriolin was localized to the basal body while MKLP1, INCENP, PRC1 and CENP-E all appear to localize to the rootlet. Confirming this, MKLP1 and INCENP were both

shown to co-localize with CLAMP (Calponin-homology and microtubule associated protein), a protein associated with the rootlet. We hypothesize that since both processes involve vesicle trafficking these central spindle proteins may regulate the docking of the basal body to the apical surface of the cell or regulate traffic in to the ciliary axoneme. Future collaboration with other lab members will further elucidate the role of these central spindle proteins in the formation of cilia.

4.2 RESULTS

4.2.1 Select central spindle proteins localize in puncta in multiciliated cells

Based on the work of Gromley and other members in the Wallingford lab, we examined the localization of central spindle proteins in the multi-ciliated cells of the epidermis of the *Xenopus* tadpole. Antibody staining was conducted on proteins known to localize to the central spindle and α-tubulin to mark the microtubules of the cilium. PRC1, MKLP1, CENP-E and INCENP were all found to localize in a punctate pattern at the apical surface of ciliated cells just below the ciliary axonemes (Figure 4.2A, E, B, F, C, G, D and H). These central spindle proteins also localized correctly in cytokinetic cells (Figure 4.2a', b', c' and d', arrow for each) indicating that in addition to their known localization in dividing cells, these proteins had an additional localization in ciliated cells. Based on the pattern of localization in the ciliated cells, we thought these proteins are associated with the basal body or its associated structures, the basal foot or the rootlet.

4.2.1.1 Not all central spindle proteins show consistent localization in ciliated cells

While the majority of central spindle proteins examined showed possible basal body localization there were some inconsistencies. INCENP has been found to be in a

complex with Aurora-B, Survivin and Borealin at the central spindle (Nakajima et al., 2009). Antibody staining of Aurora-B showed proper localization in cytokinetic cells (Figure 4.3A and a', arrow), but in multi-ciliated cells there was no punctate localization under the ciliary axonemes (Figure 4.2C). Antibody staining of Survivin showed the punctate possible basal body staining in multi-ciliated cells (Figure 4.2B and D). This antibody did not show correct localization in dividing cells (Figure 4.2B'). Because of these discrepancies, we did not pursue analysis of Aurora-B or Survivin.

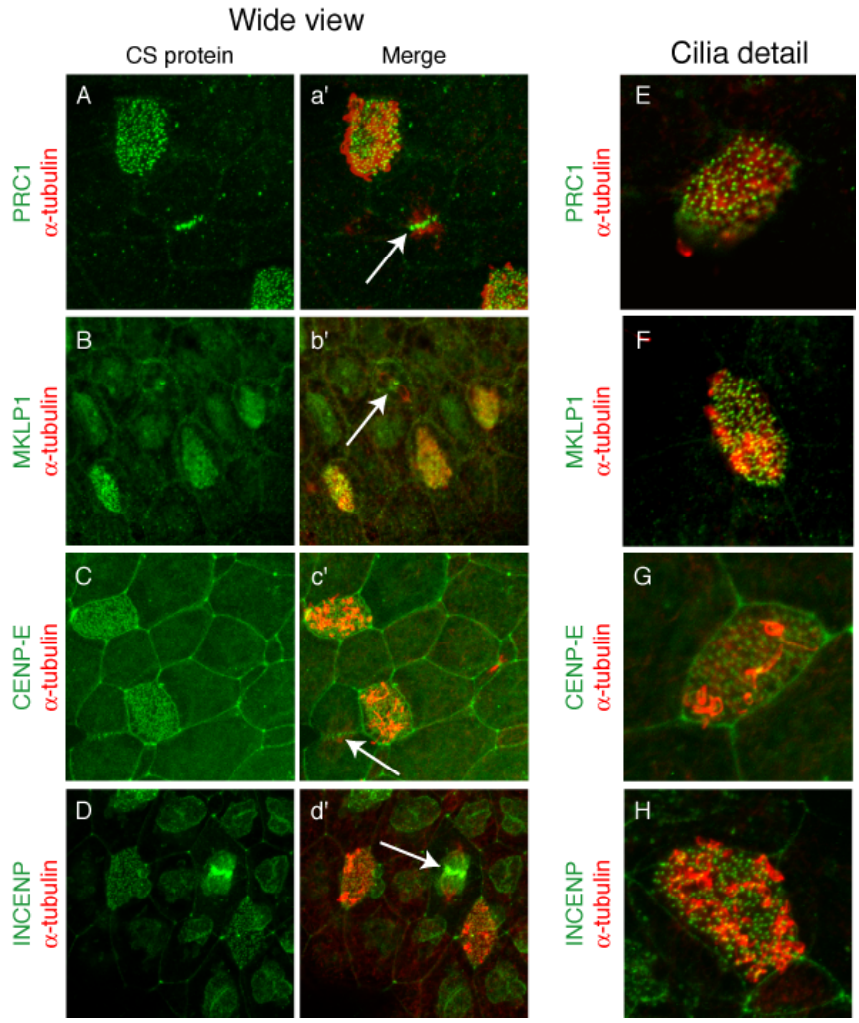

Figure 4.2. Select central spindle proteins localize in puncta at apical surface of multiciliated cells

(A) Immunostaining of PRC1 in the mucociliary epidermis of the *Xenopus*. (a') Merged image of PRC1 and α-tubulin immunostaining. Arrow indicates dividing cell with central spindle localization of PRC1. (B) Immunostaining of MKLP1 in the mucociliary epidermis of the *Xenopus*. (b') Merged image of MKLP1 and α-tubulin immunostaining. Arrow indicates dividing cell with central spindle localization of MKLP1. (C) Immunostaining of CENP-E in the mucociliary epidermis of the *Xenopus*. (c') Merged image of CENP-E and α-tubulin immunostaining. Arrow indicates dividing cell with central spindle localization of CENP-E. (D) Immunostaining of INCENP in the mucociliary epidermis of the *Xenopus*. (d') Merged image of INCENP and α-tubulin immunostaining. Arrow indicates dividing cell with central spindle localization of INCENP. (E) Close up image of PRC1 puncta and α-tubulin in a ciliated cell. (F) Close

up image of MKLP1 puncta and α-tubulin in a ciliated cell. (G) Close up image of CENP-E puncta and α-tubulin in a ciliated cell. (H) Close up image of INCENP puncta and α-tubulin in a ciliated cell.

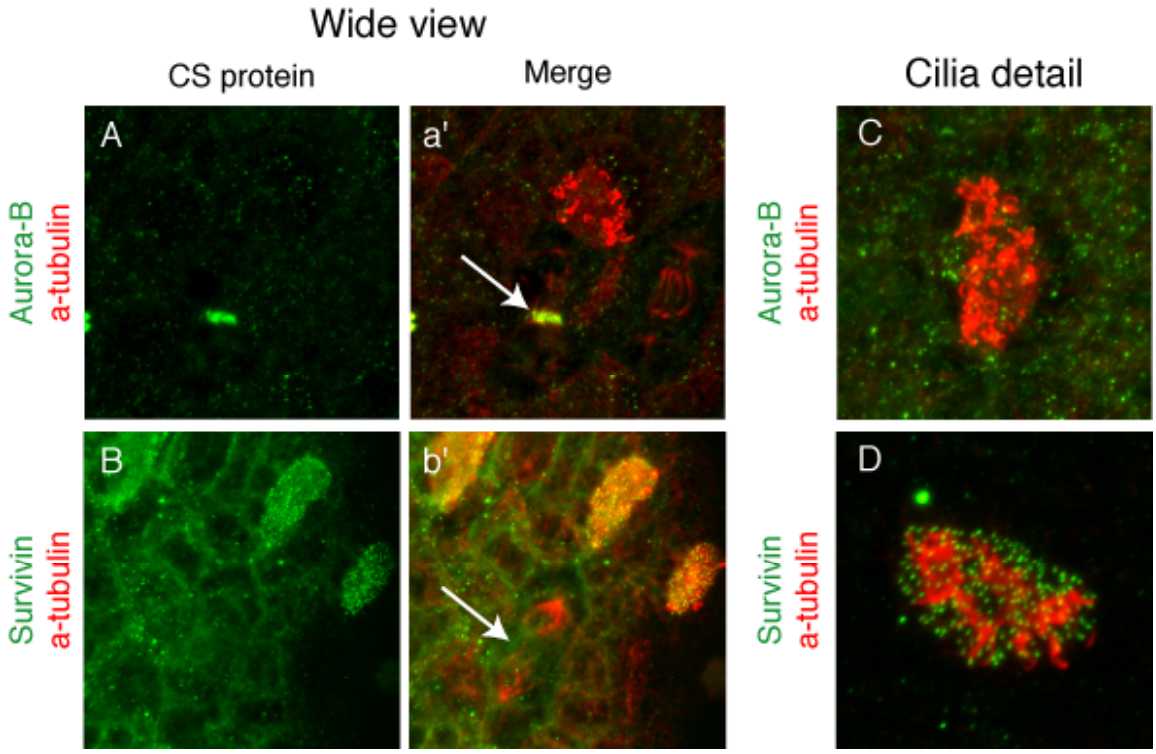

Figure 4.3. Not all central spindle proteins show consistent localization

(A) Immunostaining of Aurora-B in the mucociliary epidermis of the *Xenopus*. (a') Merged image of Aurora-B and α-tubulin immunostaining. Arrow indicates dividing cell with central spindle localization of Aurora-B. (B) Immunostaining of Survivin in the mucociliary epidermis of the *Xenopus*. (b') Merged image of Survivin and α-tubulin immunostaining. Arrow indicates dividing cell with no central spindle localization of Survivin. (C) Close up image of α-tubulin in a ciliated cell showing no puncta of Aurora-B. (D) Close up image of Survivin puncta and α-tubulin in a ciliated cell.

4.2.2 Central spindle proteins do not localize to the basal body but centriolin does

Based on the localization of some central spindle proteins seen in multi-ciliated cells, we wished to examine their localization in more detail. A number of proteins are known to localize to specific structures of the basal body complex. Since the basal body is a modified centriole γ-tubulin labels the basal body proper (Beisson and Wright, 2003). We used antibody staining of both γ-tubulin and the central spindle proteins of interest to determine co-localization. PRC1, MKLP1 and INCENP do not co-localize with γ-tubulin (Figure 4.4A-a"', B-b"' and C-c"'). Instead, they are localized adjacently to γ-tubulin puncta, in an anterior and dorsal manner with respect to the γ-tubulin (Figure 4.3c'", b'" and c'"). This could indicate that these proteins are localized to either the basal foot or the rootlet and will be assessed in section 4.2.3. Because of the weak antibody staining of CENP-E we were not able to assess its localization with respect to γ-tubulin.

Gromley and colleagues found that centriolin was a major component of centrioles. They found that centriolin localized to the centrosomes throughout the cell but also localized to the midbody ring during cytokinesis (Gromley et al., 2003; Gromley et al., 2005). Furthermore, they found that centriolin localized to the basal body of primary cilium of COS-7 cells (Gromley et al., 2003). We wished to examine if this localization was also seen in *Xenopus* multi-ciliated cells. Indeed, we did find centriolin localized to the basal body region of multi-ciliated cells (Figure 4.3D). Moreover, we find that centriolin co-localizes with γ-tubulin (Figure 4.3d'-d'"), confirming that centriolin is localized to the basal body.

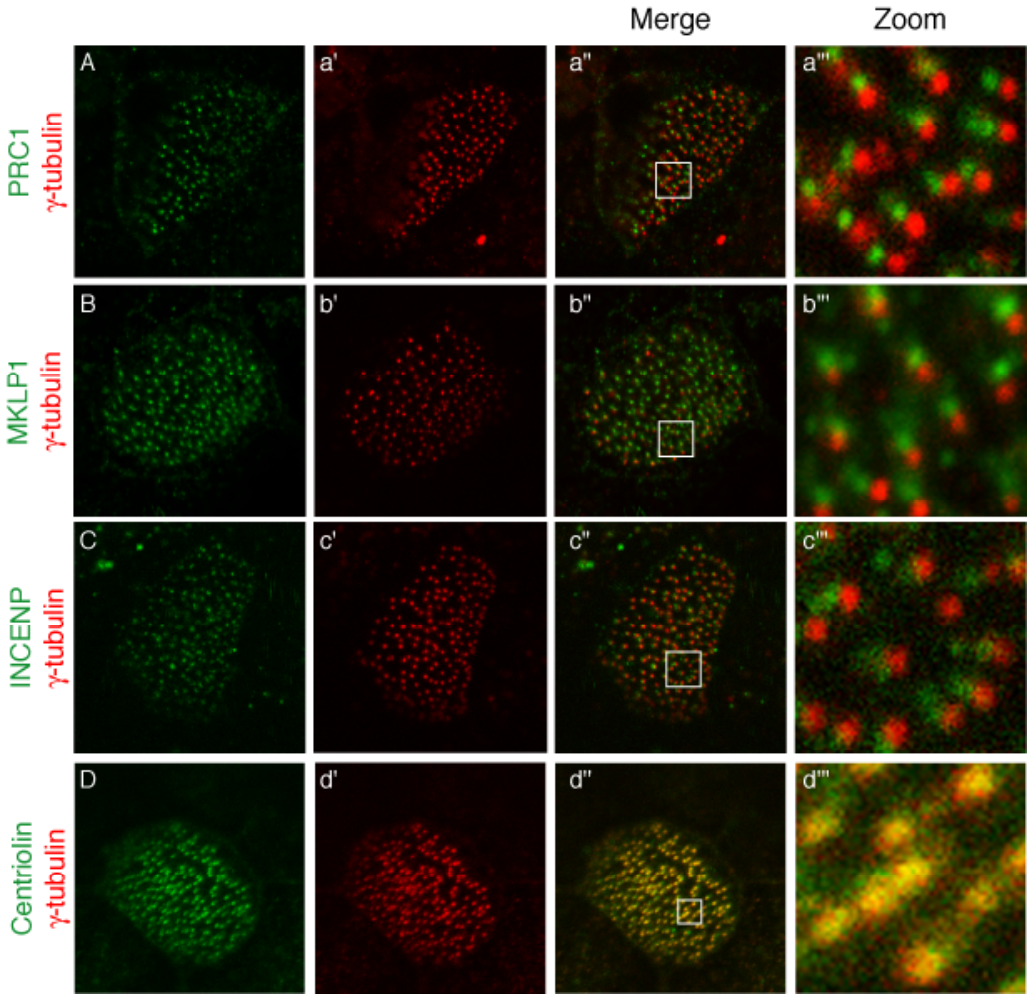

Figure 4.4. Central spindle proteins do not localize to basal body but centriolin does

(A) Immunostaining of PRC1 in a multiciliated cell. (a') Immunostaining of γ-tubulin in the same cell. (a") Merged image of PRC1 and γ-tubulin immunostaining showing that they do not co-localize. (a'") Blow up of white square in a". (B) Immunostaining of MKLP1 in a multiciliated cell. (b') Immunostaining of γ-tubulin in the same cell. (b") Merged image of MKLP1 and γ-tubulin immunostaining showing that they do not co-localize. (b'") Blow up of white square in b". (C) Immunostaining of INCENP in a multiciliated cell. (c') Immunostaining of γ-tubulin in the same cell. (c") Merged image of INCENP and γ-tubulin showing that they do not co-localize. (c'") Blow up of white square in c". (D) Immunostaining of centriolin in a multiciliated cell. (d') Immunostaining of γ-tubulin in the same cell. (d")Merged image of centriolin and γ-tubulin showing that they do co-localize. (D'") Blow up of white square in d".

4.2.3 Central spindle proteins localize to the rootlet.

The striated rootlet is an accessory structure associated with the basal body of cilia that extends away from the basal body. This structure is known to be required for maintaining stability of cilia and has been found to also be a docking station for vesicle transport into the cilia (Fariss et al., 1997; Yang et al., 2005; Yang and Li, 2005; Park et al., 2008). The rootlet can be visualized by marking rootletin or CLAMP, the two major proteins of the structure (Yang et al., 2002; Park et al., 2008).

For motile cilia, the basal foot points in the direction that cilia beat, while the rootlet is localized to the other side, presumably for stability (Yang et al., 2005). *Xenopus* cilia, in the mucociliary epidermis, beat in a posterior/ventral manner (Konig and Hausen, 1993; Mitchell et al., 2007) meaning that the rootlet is localized to the anterior and dorsal to the basal body. Based on the localization of the central spindle proteins with relation to the basal body, anterior and dorsal, we hypothesized that they were localized to the rootlet. To test this we used a CLAMP-RFP fusion protein. We injected embryos with CLAMP-RFP mRNA and conducted immunostaining of the central spindle proteins. The rootlet is pointed at its distal tip (Lundin, 1997) and CLAMP-RFP shows this morphology well (Figure 4.5b' and c'). We noticed that the central spindle proteins also had a pointed appearance in our previous antibody staining (Figure 4.1 and 4.3). We found that MKLP1 and INCENP both colocalized with CLAMP-RFP but their localizations were not the same. MKLP1 had a very pronounced pointed appearance but in the opposite direction and CLAMP (Figure 4.4A-a" and B-b"). INCENP, on the other hand, consistently localizes to the center of the CLAMP-RFP region (Figure 4.4C-c" and D-d').

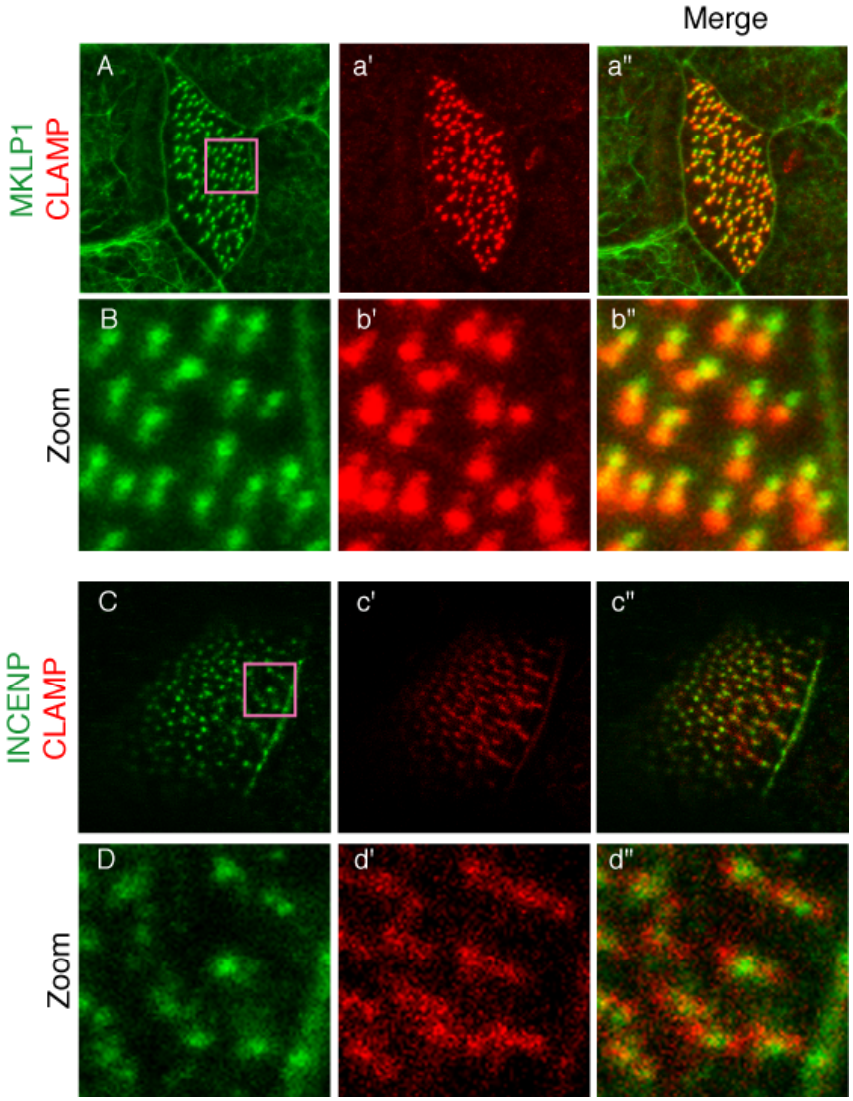

Figure 4.5. Central spindle proteins appear to localize to the rootlet

(A) Immunostaining of MKLP1 in a multiciliated cell. Pink box represents area used in B-b". (a') Image of CLAMP-RFP in a multiciliated cell. (a") Merged image of MKLP1 and CLAMP-RFP showing co-localization. (B) Hi-mag image of a small area of image in figure 4.4A. (b') Hi-mag image of a small area of image in figure 4.4a'. (b") Hi-mag image of a small area of image in figure 4.4a" showing the co-localization of MKLP1 and CLAMP. (C) Immunostaining of INCENP in a multiciliated cell. Pink box represents area in D-d". (c') Image of CLAMP-RFP in a multiciliated cell. (c") Merged image of INCENP and CLAMP-RFP showing co-localization. (D) Hi-mag image of a small area of image in figure 4.4A. d') Hi-mag image of a small area of image in figure 4.4a'. (d")

Hi-mag image of a small area of image in figure 4.4a" showing the co-localization of INCENP and CLAMP

4.3 DISCUSSION

The localization of central spindle proteins to the basal body region of cilia was surprising but not completely unexpected. At the central spindle, these proteins are known to be required for completion of cytokinesis (Bowerman, 2001; Terada, 2001; Severson and Bowerman, 2002). When these proteins are absent from the central spindle, cytokinetic furrows will progress but cytokinesis will not complete and furrows will eventually regress (Glotzer, 2001; Mollinari et al., 2002; Glotzer, 2005; Mollinari et al., 2005). Recently it has been found the cause of this defect is vesicle trafficking to and vesicle fusion at the cleavage furrow (Bowerman and Severson, 1999; Straight and Field, 2000; Gromley et al., 2005).

Centriolin has recently been discovered and found to be a major protein of centrosomes. Centriolin also plays a surprising role during cytokinesis. During cytokinesis centriolin localizes to the midbody) (Gromley et al., 2003; Gromley et al., 2005). Centriolin then recruits components of the exocyst complex and other components required for vesicle/plasma membrane fusion (Figure 4.6B) (Gromley et al., 2003; Gromley et al., 2005). The localization of MKLP1 at the midbody is required for Centriolin's localization there (Figure 4.6B) (Gromley et al., 2005; von Dassow and Bement, 2005). For proper localization of MKLP1 at the midbody other central spindle proteins are required. PRC1 aids in the localization of Aurora-B and Survivin (Mollinari et al., 2005). Aurora-B and Survivin exist in a complex with INCENP at the central spindle and flemming body (Adams et al., 2000; Uren et al., 2000; Adams et al., 2001) and INCENP aids in the localization of MKLP1 (Kaitna et al., 2000; Zhu et al., 2005).

These data show that the localization of the exocyst to the spindle midzone requires complex interactions of central spindle proteins.

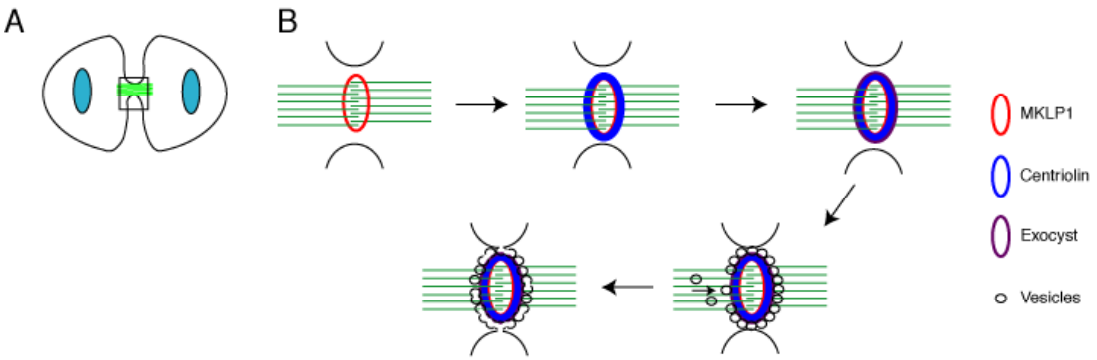

Figure 4.6. The role of Centriolin and vesicle trafficking during cytokinesis

(A) Cell at the late telophase/cytokinesis stage of cell division. Black box indicates region blown up in Figure 4.6B. (B) MKLP1 (red circle) is recruited to the spindle midzone (green lines). MKLP1 recruits Centriolin (blue circle) to the midzone, which then recruits the Exocyst and SNAREs. Vesicles (black circles) are then transported to the spindle midzone by traveling along the microtubules of the midbody. By the aid of the exocyst and the presence of SNAREs at the spindle midzone these vesicles fuse together and with the plasma membrane, completing abscission.

Another process known to use the exocyst complex is the process of ciliogenesis. (Zuo et al., 2009). The process of apical docking of the basal bodies requires the exocyst (Sorokin, 1968; Cohen et al., 1988; Park et al., 2008). The exocyst complex also remains associated with the basal bodies after docking has occurred, in *Xenopus* epidermis (Park et al., 2008). This localization may indicate that continued fusion of vesicles at the base of cilia is required for ciliary maintenance or transport of molecules in and out of the cilium.

The localization of central spindle proteins to the rootlet may be required for the initial docking of the basal body to the apical surface. Another possibility is that these

proteins are required at the rootlet for continued transport of vesicles to the cilium. The process of intraflagellar transport (IFT) has been found to be required for cilia formation and maintenance (Kozminski et al., 1995; Sloboda, 2002). IFT transport moves ciliary cargo along the microtubule doublets to the tip of the cilium where the cargo is released. IFT particles in ciliary axonemes are not associated with membranous vesicles (Sloboda, 2002; Bisgrove and Yost, 2006). Cargo directly interacts with IFT particles. IFT particles consist of two complexes, IFT complex A and IFT complex B (Figure 4.7). These two complexes also interact with microtubule motors, kinesin and dynein (Bisgrove and Yost, 2006). This allows for anterograde and retrograde transport through the cilium (Figure 4.7). While vesicles are not involved in transport in the axoneme, vesicles from the golgi transport the IFT proteins to the base of the cilium (Follit et al., 2006).

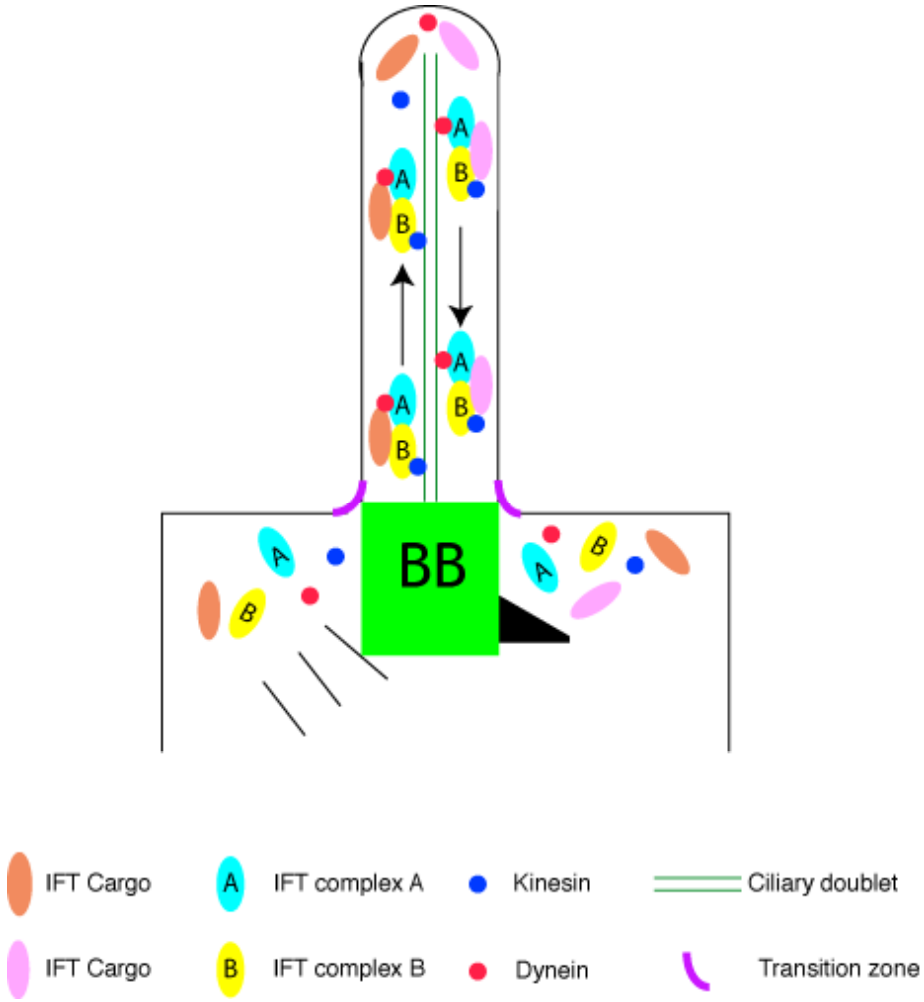

Figure 4.7. Model of intraflagellar transport

Intraflagellar transport (IFT) controls the transport of cargo in and out of cilia as well as maintains the ciliary structure. IFT particles consist of two complexes, IFT complex A (light blue oval) and IFT complex B (yellow oval). IFT transport occurs along the microtubule doublets (green lines, here simplified to show one doublet of nine) Kinesin (blue circle) controls anterograde transport of cargo (orange and pink ovals), while dynein (red circle) controls retrograde transport. Exchange of cargo and motors occurs at the tip of the cilium. At the base of the cilium there is a specialized area of membrane called the transition zone that restricts traffic of cargo in to and out of the ciliary axoneme.

We believe that the ciliary rootlet may have a secondary function in directing vesicle transport to the basal body. Vesicles from the Golgi may transport to the rootlet, possibly through the attachments of the rootlet with the cytoskeleton, and the cargo in the vesicles can then be transported in the cilium through IFT (Figure 4.8). This transfer from vesicle to IFT may occur by fusion of these vesicles with the membrane at the base of the cilium, which has been found to be very specialized (Figure 4.8) (Reiter and Mostov, 2006; Vieira et al., 2006). The localization of the central spindle proteins to the rootlet may aid in the fusion of vesicles to this specialized membrane (Figure 4.8). In the fly, specialized membrane rafts have been found to be required for completion of cytokinesis and central spindle formation (Szafer-Glusman et al., 2008). Since both processes require specialized membranes and similar set of proteins we feel that further research is required to fully elucidate the role of central spindle proteins in ciliogenesis.

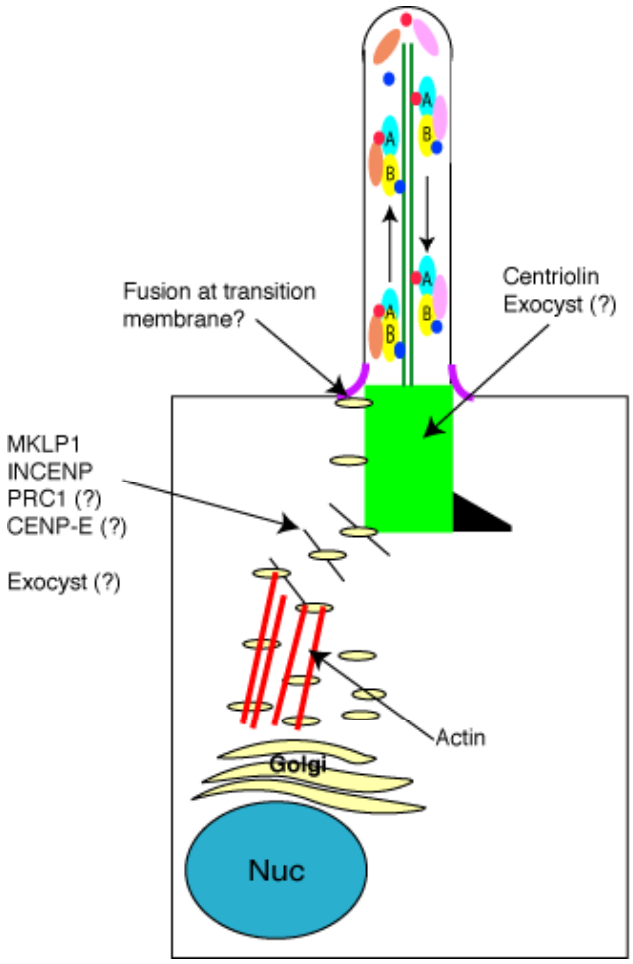

Figure 4.8. Model of role of central spindle proteins in cilia maintenance

Vesicles with cargo destined for the cilia originate from the golgi. These vesicles travel by the actin cytoskeleton to the rootlet of the cilium. At the rootlet are the central spindle proteins, MKLP1, INCENP, PRC1 and CENP-E. The exocyst may also lie at the rootlet and aid in the fusion of the vesicles with the membrane at the base of the cilia or may aid in the exchange of cargo within the vesicles with the IFT complex. Alternatively, the exocyst may be associated with the basal body proper and have a similar function. This may be more likely because of the presence of Centriolin at the basal body.

Chapter 5: Conclusions and Future Directions

In this work, I identified and characterized developmentally regulated aspects to cell division in the *Xenopus laevis*. I found that cells in the early neural plate divide in an oriented manner. This orientation is established by Cdc42 controlled maintenance of stable interactions between the spindle and the cell cortex. This role of Cdc42 is developmentally regulated and cells dividing later in a related tissue, the tail epidermis, are not under this control. Moreover, we find that the cell divisions in the early neural plate are further specialized in their mechanisms of cell division. Cells in the early neural plate exhibit exaggerated anaphase-B movements, a delayed onset of cytokinesis and a rapid cytokinetic furrow ingression as compared to the late tail epidermis, another ectodermally derived tissue. These modifications to the mechanism of cell division appear to be because of a reduced level of PRC1, a microtubule bundling protein, and thus modifications to the central spindle structure. Finally, we find that cytokinetic mechanisms may be functionally related to the process of ciliogenesis. We find proteins known to localize to the central spindle localized to the rootlet of the basal body of cilia in multiciliated cells of the mucociliary epidermis. This localization may be related to vesicle transport during both these processes.

5.1 Oriented cell divisions in the early neural plate

Oriented cell division is the process where cells in a tissue all divide in a particular orientation thereby contributing to the elongation or general shape of that tissue (Strutt, 2005). These types of cell divisions are seen in many organisms including the fly, fish and the worm (Concha and Adams, 1998; Gotta et al., 2001; Geldmacher-Voss et al., 2003; Gong et al., 2004; Baena-Lopez et al., 2005; Gonczy and Rose, 2005). We find

that there are oriented cell divisions in the neural tube of *Xenopus laevis*. Cell divisions are oriented medio-laterally. The orientation of cell division in this tissue has another variable that has not previously been assessed; the relation of the most anterior daughter cell to the midline. We find that the anterior most daughter cell is obliquely oriented with relation to the midline instead of perpendicular to it. Cells are oriented obliquely away from or toward the midline in equal proportion. Moreover, we find that rotations of the mitotic spindle establish this orientation of division and that cells divide along their long axis.

5.1.1 Cdc42 controls division orientation

Many oriented cell divisions have a few controlling factors in common. In particular, the planar cell polarity (PCP) pathway and Cdc42 have been found to be major components of establishing oriented cell division (Bellaiche et al., 2001b; Gotta et al., 2001; Kozminski et al., 2003; Gong et al., 2004; David et al., 2005; Gonczy and Rose, 2005; Ma et al., 2006; Na and Zernicka-Goetz, 2006; Saburi et al., 2008). We found that modifications to PCP signaling did not alter the orientation of cell division. Instead when two different dominant negatives to Cdc42 are expressed we find that more cells are oriented obliquely away from the midline at the expense of the cells that were oriented toward the midline. We find that this appears to be because of an alteration of the mitotic spindle with the cell cortex since alteration does not cause changes to the cell shape. Cells with altered Cdc42 function are still elongated in the same manner as control cells.

5.1.2 Cdc42 controls oriented cell division through spindle cortex interactions

During our examination of cells with altered Cdc42 function, we found that spindles were highly instable. Chromosomes were seen to "bounce" from one side of the cell to the other during metaphase. This indicates that Cdc42 might have a role in controlling spindle-cell cortex interactions. Confirming this we find that spindles in Cdc42 altered cells over rotate before anaphase onset. This role of Cdc42 is developmentally regulated. When we assessed these roles in the late tail epidermis we found that there was no difference in the stability of chromosomes during metaphase. This shows that Cdc42 has a neural specific role in controlling interactions between the mitotic spindle and the cell cortex leading to oriented cell divisions in this tissue.

We propose that spindle rotations are essentially random, and that molecular cues on the cell cortex define "catch-points" that stop rotation prior to anaphase in accordance with a cell's long axis (Figure 5.1A and B). One such catch point is responsible for stopping spindle rotation such that the final division angle is oblique to the midline with the anterior-most daughter cell lateral to the more-posterior daughter cell. This catch-point requires Cdc42 function (Figure 5.1A, blue), while Cdc42-independent catch-point accounts for cells dividing obliquely toward the midline (Figure 5.1A and B, red). In this model, if the Cdc42-dependent catch-point is disrupted, spindles rotate excessively, and eventually are able to stop by responding to the other, Cdc42-independent catch (Figure 5.1B).

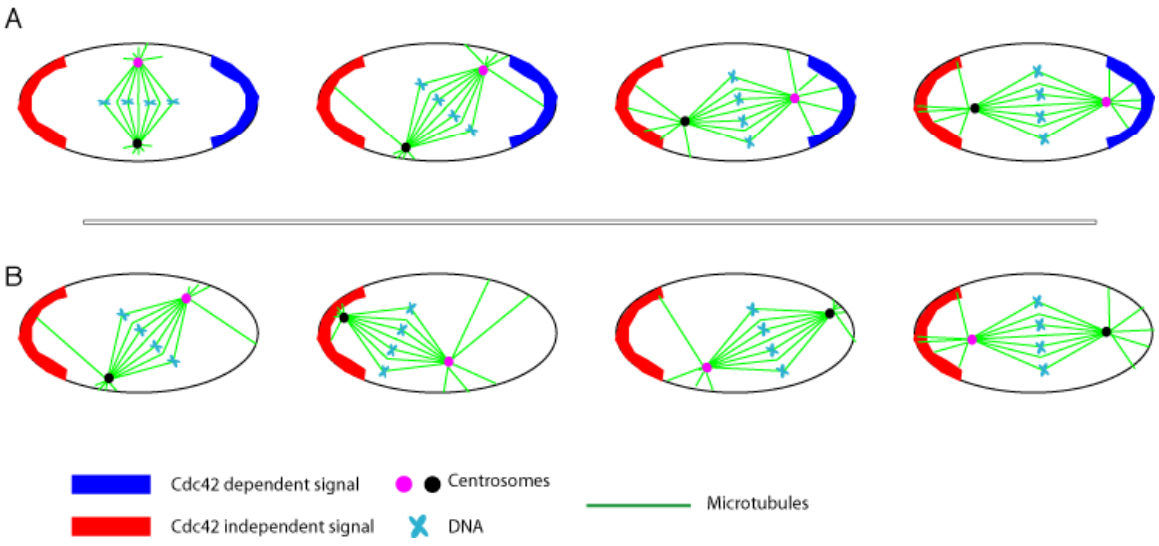

Figure 5.1. Model of Cdc42 control of spindle stability in early neural plate cells

(A) In a wildtype neural plate cells there are two sets of molecules on the cortex of the long axis of the cells. One is Cdc42 dependent (blue) and one is Cdc42 independent. A spindle forms and interacts with these molecules, via the astral microtubules, has a limited range of rotation (pink and black dots) and remains stable in the middle of the cell. (B) When Cdc42 function is altered the Cdc42 dependent orientation signal is disrupted (note lack of blue). When a spindle forms it is unable to remain stably in the middle of the cell because of the lack of interaction with the astral microtubules with the Cdc42 signal. Also the spindle over rotates (pink and black dots).

5.2 THE MECHANISM OF CELL DIVISION IN THE NEURAL PLATE IS MODIFIED AS COMPARED TO THE TAIL EPIDERMIS

Further examination of cell division in the neural plate showed that these divisions are highly specialized. Proper completion of cell division is critical to ensure segregation of genetic material and also contributes to morphogenesis in embryos (Rappaport, 1961; Gont et al., 1996; Glotzer, 2001; Chalmers et al., 2003). To correctly segregate DNA accurate completion of the phases of the mitotic cell cycle: interphase, synthesis, prophase, metaphase, anaphase and telophase/cytokinesis, is required.

Modifications can occur at many of these steps that can cause divisions in different organisms to look different. Most of the analysis of the mechanisms of cell has been conducted on cells in culture. While these studies are fundamental to understanding the process of cell division they do not address how these mechanisms are regulated during development. Basic mechanisms of cell division remain relatively ill-defined in early vertebrate embryos.

In vivo time-lapse analysis of cell division at two separate stages of development revealed that the mechanisms differed. Chromosomes in the early neural plate exhibit exaggerated anaphase-B movements, delayed onset of cytokinesis, rapid cytokinetic furrow ingression and a low density of midzone microtubules as compared to the late tail epidermis, another ectodermally derived tissue.

5.2.1 Modifications to cell division is caused by reduced levels of PRC1 expression

These modifications to the mechanism of cell division appear to be because of a reduced level of PRC1, a microtubule bundling protein, in the early neural plate. We found that this lack of PRC1 caused alterations to the structure of the central spindle. The central spindle is a set of bundled anti-parallel microtubules that concentrates several proteins and protein complexes required for cytokinesis (Glotzer, 2005). The position of the central spindle dictates the position of the contractile ring and thus the cleavage furrows (Adams et al., 1998; Gatti et al., 2000; Kimura et al., 2000; Dechant and Glotzer, 2003). In cultured cells depleted of PRC1 these modifications to cell division are seen (Jiang et al., 1998; Mollinari et al., 2002; Kurasawa et al., 2004; Verbrugghe and White, 2004; Verni et al., 2004; Mollinari et al., 2005). *In situ* hybridization and western blot showed reduced levels of PRC1 in the early neural plate as compared to the late tail

epidermis. Overexpression of PRC1 abrogated the modifications to cell division seen in the neural plate confirming that its absence is the cause of these modifications.

I present the following model (also described in Chapter 3) to describe the differences in cell division between the cells in the neural plate and the tail epidermis. During anaphase, in the tail epidermis, chromosomes separate during this period the central spindle forms by the bundling of the antiparallel microtubules by PRC1 (Figure 5.2, red dots). Bundling of microtubules stops the separating chromosomes by reducing the movements of anaphase B (the movement of the spindle poles from one another). The cytokinetic furrows ingress at the central spindle (Figure 5.2A, early telophase). This central spindle is then compressed into the midbody by the cytokinetic furrows. In the tail epidermis this midbody spans the entire apical to basal axis (Figure 5.2A, mid/late telophase side view).

In the early neural plate this process is different. The low level of PRC1 in these cells delays the formation of the central spindle until later on in the cell cycle. Because there is little to no hindrance on the movement of the chromosomes during anaphase B the chromosomes are able to separate to a further extent (Figure 5.2B, anaphase). Because there is no properly bundled central spindle these microtubules instead associate with the ingressing cytokinetic furrows. These furrows also move at a faster rate than the furrows in the tail epidermis (Figure 5.2B, early telophase, compare to early telophase in 3.10A). At this stage the central spindle begins to form with PRC1 at bundles of antiparallel microtubules. This altered central spindle structure may lead to a highly arched midbody (Figure 5.2B, mid telophase). This highly arched midbody most likely enables to the release of the midbody from the apical membrane (Figure 5.2B, late telophase). Also the requirement of vesicle trafficking to both sides of the midbody may lead to the midbody having a longer life time in these neural cells (Figure 3.2F-H).

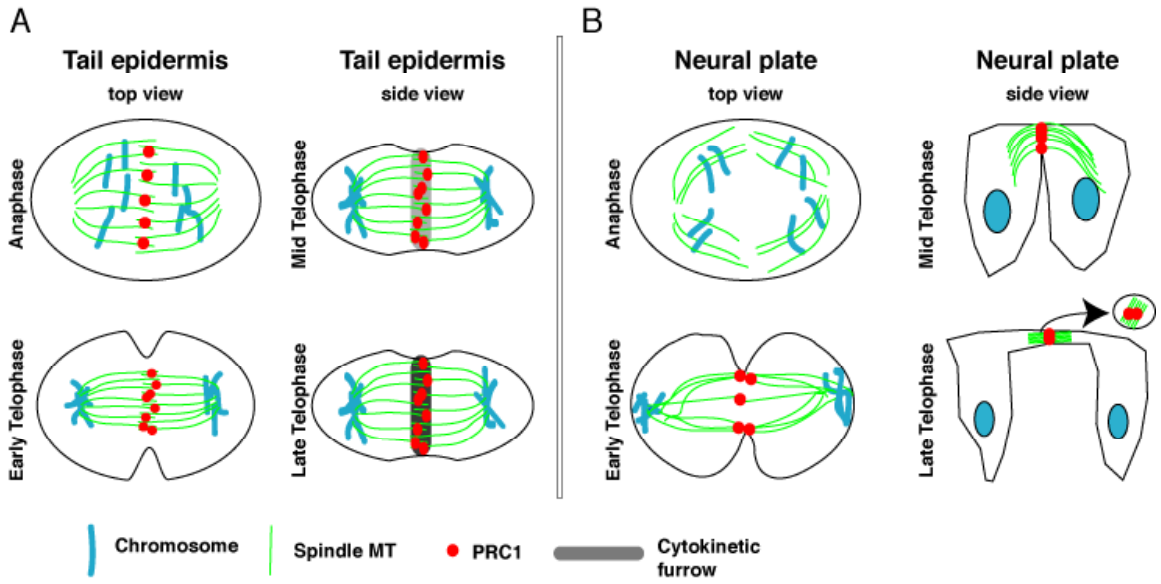

Figure 5.2.. Model of division and abscission in the tail epidermis and the early neural plate.

(A) Model of division in the tail epidermis. First column represents a top view of a tail epidermal cell during anaphase and early telophase. A well bundled central spindle is observed and PRC1 localizes to the overlapping antiparallel microtubules. The second column represents a side view of the same cell during mid and late telophase. The black bar across the central spindle represents the ingressing cytokinetic furrows. The midbody is seen to span almost the entire apical to basal axis of the cell. (B) Model of division in the early neural plate. First column represents a top view of a neural cell during anaphase and early telophase. A well bundled central spindle is not observed. PRC1 localizes to the overlapping antiparallel microtubules that are associating with the ingressing furrows and begins to form a central spindle later during the cell cycle. The second column represents a side view of the same cell during mid and late telophase. The midbody is highly arched and is close to the apical membrane of the cell. The midbody is eventually is released into the future lumen of the neural tube.

5.3 THE POTENTIAL ROLE OF CENTRAL SPINDLE PROTEINS IN CILIOGENESIS OR CILIA MAINTENANCE

Ciliogenesis is the process by which cilia are formed at the surface of a cell. Most vertebrate cells have at least one primary cilia protruding from its surface (Olsen, 2005).

These primary cilia have been found to be hubs for particular signaling molecules, such as the Hedgehog pathway (Corbit et al., 2005; Haycraft et al., 2005; May et al., 2005; Scholey and Anderson, 2006). Cilia can also be mobile, for the movement of liquid over a tissue. Both types of cilia have a common structure at the base of the ciliary shaft, the basal body (Inglis et al., 2007). Basal bodies are modified centrioles that associate with vesicles and are trafficked to the apical surface and associate with two main accessory structures, the basal foot and the rootlet. (Yang et al., 2005; Park et al., 2008). The basal foot is closely associated with the basal body and points in the direction of cilia beating (Boisvieux-Ulrich and Sandoz, 1991). The rootlet is on the opposite side of the basal body and interacts with the actin cytoskeleton to stabilize cilia (Hagiwara et al., 1997; Yang et al., 2002; Yang et al., 2005). Recently it has been found that centriolin, a major component of centrioles localizes to the midbody and controls vesicle traffic to this region (Gromley et al., 2003; Gromley et al., 2005). We find that centriolin localizes to the basal body. Gromley and colleagues also found that the localization of centriolin to the midbody relied on MKLP1, a central spindle protein required for cytokinesis. This data implied that there might be a connection between centriolin localization at the basal body and central spindle proteins. Indeed, we find MKLP1, INCENP, PRC1 and CENP-E localized to the basal body region. Further analysis revealed that these proteins are localized to the rootlet.

We believe that the ciliary rootlet may have a secondary function in directing vesicle transport to the basal body. Vesicles from the Golgi may transport to the rootlet, possibly through the attachments of the rootlet with the cytoskeleton, and the cargo in the vesicles can then be transported in the cilium through IFT (Figure 5.3). This transfer from vesicle to IFT may occur by fusion of these vesicles with the membrane at the base of the cilium, which has been found to be very specialized (Figure 5.3) (Reiter and

Mostov, 2006; Vieira et al., 2006). The localization of the central spindle proteins to the rootlet may aid in the fusion of vesicles to this specialized membrane (Figure 5.3). In the fly, specialized membrane rafts have been found to be required for completion of cytokinesis and central spindle formation (Szafer-Glusman et al., 2008). Since both processes require specialized membranes and similar set of proteins we feel that further research is required to fully elucidate the role of central spindle proteins in ciliogenesis.

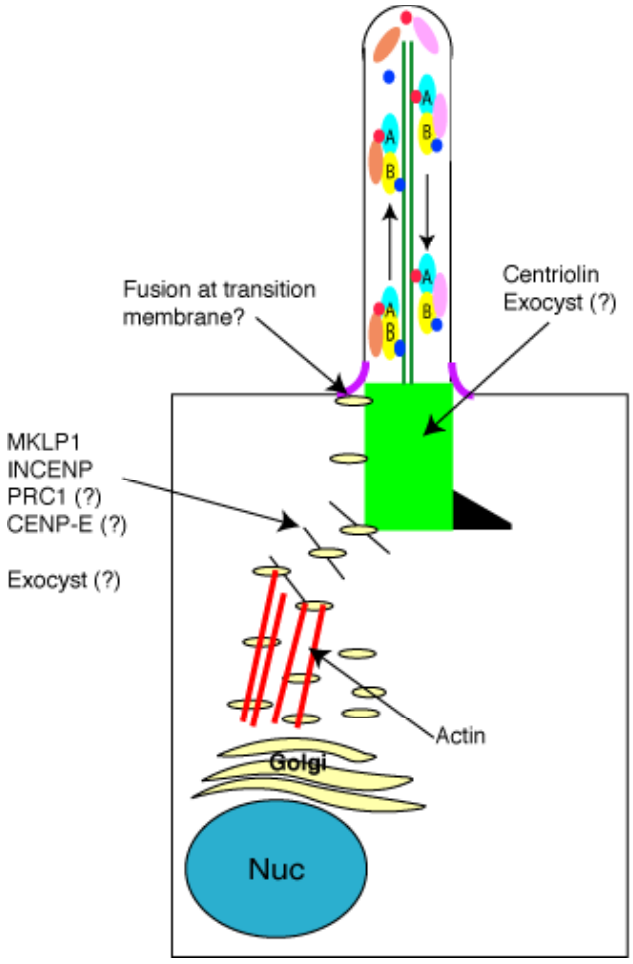

Figure 5.3. Model of role of central spindle proteins in cilia maintenance

Vesicles with cargo destined for the cilia originate from the golgi. These vesicles travel by the actin cytoskeleton to the rootlet of the cilium. At the rootlet are the central spindle proteins, MKLP1, INCENP, PRC1 and CENP-E. The exocyst may also lie at the rootlet and aid in the fusion of the vesicles with the membrane at the base of the cilia or may aid in the exchange of cargo within the vesicles with the IFT complex. Alternatively, the exocyst may be associated with the basal body proper and have a similar function. This may be more likely because of the presence of Centriolin at the basal body.

5.4 FURTHER DIRECTIONS

5.4.1 Oriented cell divisions

Here we showed that Cdc42 mediated the interaction of the mitotic spindle and the cell cortex to stabilize the spindle and restrict mitotic spindle rotations in early neural plate cells, leading to an alteration of the orientation of cell division. The mechanism behind how Cdc42 controls this interaction is unknown and further research concentrating on this mechanism is required. Sensors have been created to show the localization of GTP-bound or active form of the small Rho-GTPase (Bement et al., 2005; Bement et al., 2006). These sensors may be useful to show the localization of active Cdc42. This analysis of the localization may shed light on the function of Cdc42 in spindle dynamics. Furthermore, analysis of the localization of Cdc42 downstream effector proteins may aid in this understanding.

Analysis of the actin cytoskeleton in these early neural cells will help in understanding how actin interacts with the cell cortex. Cdc42 is known to reorganize the actin cytoskeleton in a number of cells in response to a signal (Glotzer and Hyman, 1995; Stowers et al., 1995). While cortical actin may not differ from control cells and Cdc42 altered cells more detailed actin structures may not be correctly formed. In conjunction with this the localization of dynein–dynactin complex should be examined. This complex is known to coordinate the interaction between microtubules and actin and is a prime candidate for mislocalization by these dominant negatives to Cdc42 (Glotzer and Hyman, 1995).

5.4.2 Role of PRC1 in modifications to cell division

Here we have described cell divisions only at two stages of development. Further examination of cell divisions during development is required to fully understand developmental regulation of cell division. We show that varying PRC1 is required for the modifications seen in these two stages but if this is the case in other stages is unknown. Bundles of microtubules are localized under the cytokinetic furrows of cleavage stage embryos (Danilchik et al., 2003). We have found that over expression of PRC1 at cleavage stages cause drastic defects to cell division. Cleavage furrows are seen or they form and then retract (data not shown). Cells appear to attempt to divide, as noted by waves of compression over the entire surface of the embryo. This may possibly be because of an over bundling of microtubules in these cells. If PRC1 bundles microtubules along the entire surface of the cells there is no one specific region of ingression and instead the entire membrane tries to ingress. Analysis of the level of microtubule bundling in PRC1 over expressing cells will answer this question. Also analysis of other stages with rapid cell division will further elucidate the role of PRC1 in controlling the mechanisms of cell division.

5.4.3 Role of central spindle proteins in ciliogenesis

Other members of the Wallingford lab have undertaken this project. Dissecting the role of central spindle proteins in cytokinesis from their role in ciliogenesis in the epidermis of the frog embryo has been difficult. Use of temperature sensitive mutants of these proteins in *C. elegans* seems to be able to aid in answering this question. *C. elegans* have two sets of 12 neurons, which aid in chemosensory or thermosensory movements. Each of these 12 neurons contains a cilia specific for the neuron. Proteins localize to the ciliary axoneme and transduce a response in the worm when they bind to a

chemosensory cue. When ciliogenesis is disrupted the worms are unable to respond to chemotactic cues (Scholey et al., 2004; Bargmann, 2006). Full knock out of central spindle proteins are embryonic lethal because of their requirement for full cytokinetic completion (Raich et al., 1998; Kaitna et al., 2000; Verbrugghe and White, 2004). Temperature sensitive mutations avoid these early defects by allowing the protein to function early in development at the permissive temperature. Shifting the worms to the restrictive temperature will disrupt protein function. If this is done before cilia form, simple chemotaxis assays will show if these genes are indeed involved in ciliogenesis.

Appendices

Appendix A: Materials and Methods

Materials and Methods:

Collection and manipulation of embryos

Female *Xenopus laevis* were injected with 700ccs of human chorionic gonadotropin hormone (HCG) and stored at 18ºC overnight. The next day eggs were isolated and fertilized. Embryos were de-jellied using a 3% cystine solution in 1/3x MMR.

Time-lapse analysis of cell divisions in the neural plate and tail epidermis

Embryos were injected dorsally, at the 4-cell stage, with 200pg histone-eGFP, 200pg membrane-RFP, 50pg tau-eGFP, 200pg histone-RFP or any combination of these to examine cell divisions in the neural plate. To examine the epidermis, all cells were injected at the 4-cell stage. To examine the neural plate, embryos were grown to stage 13 and then placed upside down in a culture dish. This dish contained 2% agarose with wells made from a hair comb. To image the tail epidermis embryos were grown to stage 30 and covered in agarose in a culture dish. Both of these sets of embryos were imaged on a Zeiss Pascal LSM5, at 10x (na - 0.3), 20x (na – 0.5), 40x (oil, na – 1.3) or 63x (oil, na – 1.4) and cell divisions were analyzed using Image-Pro Plus.

Immunohistochemistry for microtubules

For MT immunostaining, embryos were grown to appropriate stages and fixed in 1X MEMFA at 4°C overnight. Embryos were completely dehydrated in 100% methanol and stored overnight at –20°C in methanol. Embryos were then bleached in a light box in 60% methanol/10% H2O2. Rehydration was done by 5 minute washes in methanol/TBS or TBST mixtures: 50% methanol in TBS, 25% methanol in TBST and finally 100% TBST. Embryos were then blocked for 1 hour in 300ul of TBS + 10% FBS + 5% DMSO. Primary antibody, 1:250 dilution for mouse-anti-a-tubulin (Sigma), was added to this mixture and embryos were incubated overnight at 4°C. Embryos were washed 5x 1 hour in TBST, blocked again as described above, and secondary antibody was added, 1:200 dilution for anti-mouse-alexa-488, and embryos were incubated again overnight at 4°C. Secondary antibody solution was discarded and embryos were washed 5x 1 hour in

TBST. In some cases, during the last wash a dilution of 1:2000 of propidium iodide was added to label nuclei.

Immunohistochemistry for central spindle proteins

For immunostaining of central spindle and passenger proteins, for both chapter 3 and 4, embryos were grown to appropriate stages and fixed in DENTs fix (80% methanol / 20% DMSO) overnight at 4°C. Embryos were fully dehydrated in methanol and stored in –20°C overnight. Embryos were slowly rehydrated by 5-minute washes in 75% methanol in H2O, 50% methanol in PTW, 25% methanol in PTW and finally PTW. Embryos were bleached in a light box in a solution of 10% H2O2, 0.5% formamide and 2.5% 2X SCC. Embryos were then washed 3 times in TBST, 5 minutes each. Embryos were blocked in 300ul of TBS + 10% FBS + 5% DMSO. Primary antibody was added; rabbit-anti-PRC1 1:5 (BioLegend), rabbit-anti-MKLP1 1:200, rabbit-anti-INCENP 1:800, Aurora-B 1:50, and embryos were incubated overnight at 4°C. Embryos were washed 2x 1 hour in TBST. TBST was removed and 300ul of mouse-anti-a-tubulin antibody + blocking solution was added (1:250) and embryos were incubated for 1 hour at RT. Embryos were washed again for 5x 1 hour washes in TBST. In some cases, during the last wash a dilution of 1:2000 of DAPI was added to label nuclei.

Analysis of immunostaining images:

For measuring midzone MT density, pixel intensity measurements (8 bit, 1-255) were made from projections of stacks. All measurements were made from maximum intensity projections of 10μm stacks made by collecting 11 overlapped 4.5μm optical sections (Z-interval of 1μm) using a 40x objective and 4.5x zoom. All slices are 8-bit and have not been modified post-acquisition.

For measurements comparing PRC1 levels, pixel intensity measurements were made from stacks. All measurements were made from maximum intensity projections of 26.8μm stacks made by collecting 13 overlapped 4.5 μm optical sections (Z-interval of 2.23 μm) using the 40x objective and 1x zoom. All images were 8-bit, and no post-acquisition modifications were performed. The images contain very small saturated regions corresponding to intense PRC1 signal at midbodies; the saturated regions at midbodies were removed for the population level quantification.

For MT density, all images were collected at 63x using comparable settings. Stacks of varying sizes were made to incorporate all visible spindle microtubules in each cell.

All quantified images were taken from embryos stained in Dent's fixative. Much *Xenopus* autofluorescence results from byproducts of aldhehyde fixation, so these embryos have extremely low background signal. Nonetheless, we imaged unstained embryos and found that neural plate cells had slightly higher background signal than did

epidermal cells. Thus, the differences in α-tubulin signal and PRC1 signal presented here may very slightly greater than presented.

Determination of division polarity

Stacks were projected and exported from the Zeiss LSM5 Pascal program and analyzed using Image-Pro Plus. The polarity of cell division was assessed at the onset of telophase for all cells. Lines were drawn bisecting the center of each set of daughter chromosomes in a posterior-to-anterior manner. The features of these lines were then exported to MS Excel. The angles of divisions were converted placed into a –90° to +90° range and histograms were made using Delta-Graph. Kolmogorov-Smrinov tests were performed using an on-line tool available at http://www.physics.csbsju.edu/stats/KS-test.html.

Effect of molecular effectors on cell division polarity

To examine the effect of PCP signaling and Cdc42 function embryos were first injected with 200pg of histone-eGFP along the entire dorsal side at the 4-cell stage. These embryos were grown to the 8-cell stage and 200pg of membrane-RFP along with either 500pg Xdd1, 500pg Xfz8N, 1ng Shroom3-ASD1, 750pg Cdc42-F37A or 750pg Cdc42-N17 was injected into one of the dorsal animal blastomeres (Figure 1C). To examine the effect of Cdc42 on the tail epidermis embryos are injected into one of the ventral cells with 150pg of histone-eGFP, 150pg of membrane-RFP and 750pg of Cdc42-N17 or 750pg of Cdc42-F37A. Embryos were then imaged and analyzed as above.

Quantifying spindle rotations

This longer axis of the metaphase spindle was assessed upon initial formation, and again one minute before anaphase onset. The difference of these angles were determined and plotted in Delta-Graph. The Mann-Whitney comparison test was conducted on the two sets of data using GraphPad Instat software.

Analysis of cell divisions in neural plates over-expressing PRC1

Embryos were injected as before with mRNAs for fluorescent fusion proteins. These mRNAs were injected into the entire dorsal side of the embryo at the 4-cell stage. These injected embryos were grown to the 8-cell stage and one of the animal dorsal blastomeres was injected with 100pg of human PRC1-GFP DNA.

Analysis of the effect of over-expression of PRC1 in cleavage stage embryos

Embryos were injected with 25pg of xPRC1-RNA was injected with a 1/10 dilution of fluorescein-dextran into one cell at the 2-cell stage or all 4 cells at the 4-cell stage. Embryos were placed in a culture dish lined with modeling clay and imaged using Image Pro-Plus and the Leica stereoscope. These embryos and their clutch mates were lysed (see western blot method) and used for western blot analysis of PRC1 expression levels.

In situ hybridization of xPRC1

Embryos were grown to appropriate stages and fixed overnight in 1X MEMFA at 4ºC. Embryo were completely dehydrated in menthol and stored overnight at –20ºC in menthol. Embryos were then rehydrated slowly and in situ hybridization was conducted as described in Sive et al. with digoxygenin-labeled, antisense full-length probe to *Xenopus* PRC1.

Western Blot of xPRC1

25 Embryos were grown to correct appropriate stages and lysed; 50mM Tris (pH 8.0), 150mM NaCl, 1mM EGTA, 0.5%NP40, 0.5% Triton-X 100. Lysates were spun twice at 4ºC for 20 minutes each and the supernatant was saved each time. Sample buffer was added to each protein lysate at a concentration of 1X (25 mM Tris-Cl pH 6.8, 1% SDS, 0.002% bromophenol blue, 0.002% phenol red, 2mM EGTA, 5% glycerol and 1%bME). Lysates were stored at –20ºC until use and boiled at 100ºC for 10 minutes prior to each use. Proteins were run on 12% poly acrylamide gel with a 5% stacking gel and blotted onto a nitrocellulose membrane. Membranes were blocked in a PBS and 5% milk solution for two hours and then incubated with 1/2000 dilution of rabbit-anti-gamma-tubulin (Abcam) or a 1/500 dilution of rabbit-anti-PRC1 (BioLegend). Secondary goat-anti-rabbit HRP antibody was added after washing at a 1/5000 dilution (Pierce).

Expression of Cdc42-N17 and Cdc42-F37A in neural tube

Embryos were injected as before with mRNAs for fluorescent fusion proteins. These mRNAs were injected into the entire dorsal side of the embryo at the 4-cell stage. These injected embryos were grown to the 8-cell stage and one of the animal dorsal blastomeres was injected with 750pg of Cdc42-N17 or Cdc42-F37A mRNA.

Quantifying spindle rotations in embryos expressing Cdc42-N17 or F37A

This longer axis of the metaphase chromosome mass was assessed upon initial condesation, and again one minute before anaphase onset. This was done for control cells expressing Cdc42-F37A and Cdc42-N17, in both the tail epidermis and the neural plate and cells. The difference of these angles were determined and plotted in Delta-Graph. The Mann-Whitney comparison test was conducted on the two sets of data using GraphPad Instat software.

Analysis of colocalization of central spindle and basal body components

Embryos were grown to the 4-cell stage and injected with CLAMP-GFP or CLAMP-RFP mRNA into both of the ventral cells. Embryos were grown to stage 30 and fixed in DENT's fix and immunostained as above in "Immunohistochemistry for central spindle proteins".

To assess the colocalization of central spindle proteins with γ-tubulin uninjected embryos were grown to stage 30 and fixed in DENT's fix. Emrbyos were then co-stained with the central spindle protein of interest (rabbit antibodies) and mouse-anti-γ-tubulin at a 1:250 dilution. The central spindle antibodies were identified by goat-anti-rabbit-Alexa-488 and the gamma tubulin antibody was identified by goat-anti-mouse-Alexa-555. Other embryos were injected with γ-tubulin-RFP at the 4-cell stage into both of the ventral cells and immunostained as above.

Appendix B: Miscellaneous Experiments

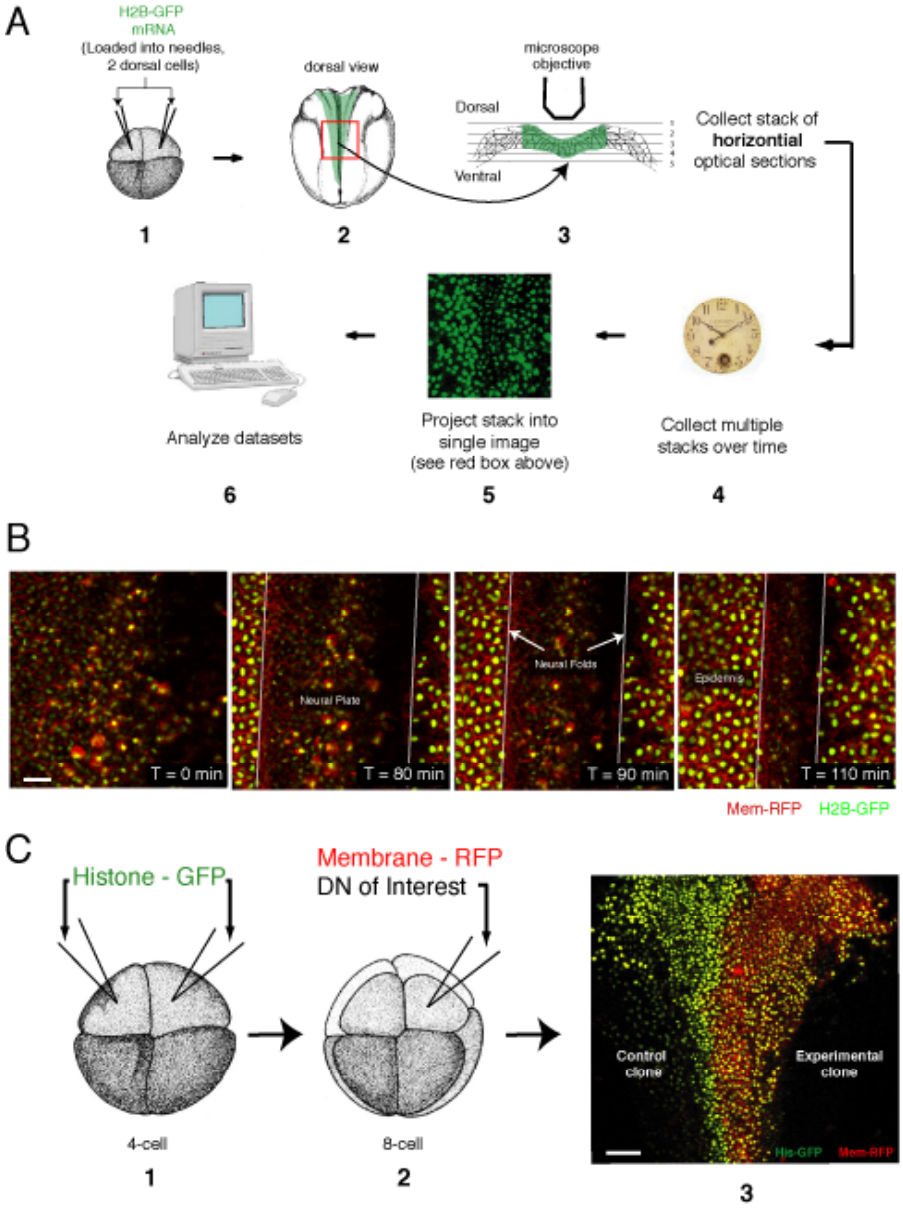

Figure D.1: Method for analysis of cell divisions during neural tube closure

(A) Method to analyze cell divisions during neural tube closure in *Xenopus laevis*. Eggs are collected and undergo in vitro fertilization as described in methods section. Solutions of mRNA, previously made by an in vitro transcription kit (Sigma) are made to label appropriate portions of cells (i.e. Chromosomes via H2B-GFP or membranes via mem-

RFP). Mixes of mRNA are put in small glass needles and are injected directly in to the embryo at the four-cell stage by use of a picospritzer (**1**). For analysis for the early neural plate the two dorsal cells are injected and for analysis of the late tail epidermis all four cells are injected. Embryos are then grown to an appropriate stage, 13 for early neural plate and 30 for late tail epidermis (**2**). Embryos are then placed on a microscope and imaged. Images of the early neural plate are obtained from horizontal confocal slices of the dorsal surface of the spinal chord region prior to neural tube closure. Images of the late tail epidermis are obtained from sagittal confocal slices of the most posterior surface of the flank of the embryo (**3, see red boxes**). Stacks are obtained every 30 seconds or 1 minute for 150 iterations (**4**). Movies are then projected into an X confocal projection in the Pascal program (**5**). Confocal projections are then exported to tiff files and analyzed by use of ImagePro Plus (**6**). (B) Use of this method allows for easy analysis of morphogenesis of the neural plate. Movies are started at early neural plate stage (T = 0 min). As time progresses neural folds come into view (T = 80 min). These neural folds continue to move laterally until the neural plate is no longer well seen and the future epidermis of the embryo is easily seen (T = 90 min and T = 110 min). Scale bar = 50μm. (C) Method to assess division polarity effectors. Embryos are injected to have different clones for analysis of polarity effectors. Embryos are injected on their entire dorsal side with a histone-2B-GFP marker at the four-cell stage (1). Embryos are grown up one stage, to the eight-cell stage, and injected with a membrane-RFP marker and an mRNA of interest (2). Embryos are grown to the early neurula stage and cells injected with the mRNA of interest can be distinguished by the expression of GFP and RFP while control cells only express the GFP marker (3). Scale bar = 100μm.

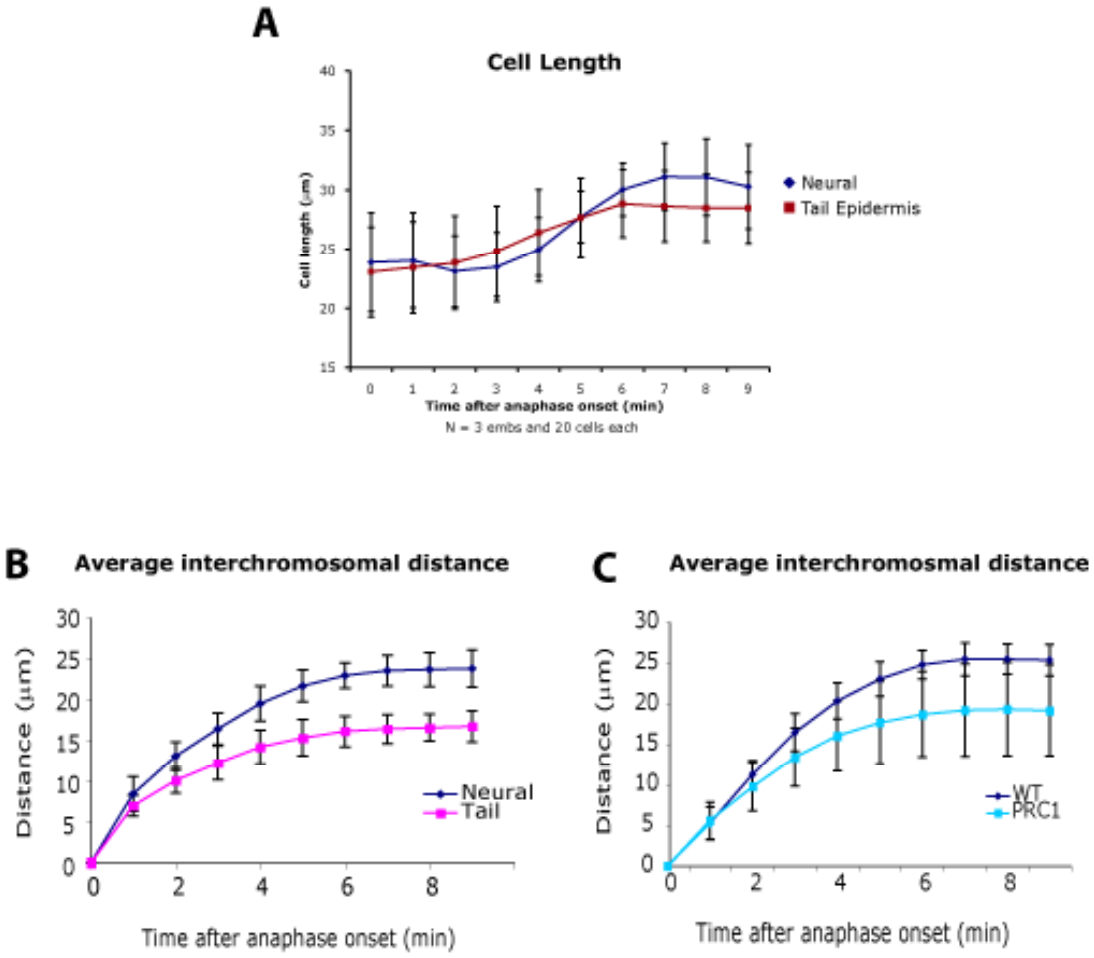

Figure D.2 Neural tube and tail epidermal cells are similar in size.

(A) Graph of cell size during the course of anaphase in cells within the neural tube (blue line) and the tail epidermis (red line). (B) Graph of chromosome separation expressed in microns over time for neural (blue line) and epidermal (pink line) cells. (C) Graph of chromosome separation expressed in microns over time for normal neural plate cells (dark blue line) or PRC1-GFP expressing cells (light blue line) in mosaic embryos..

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Vita

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