Anillin Interacts with Microtubules to Define the Division Plane

Husni Haji Bik

A Thesis in the Department of Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science (Biology) at

Concordia University

Montreal, Quebec, Canada

April 2011

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

By: Husni Haji Bik

Entitled: Anillin Interacts with microtubules to define the division plan

and submitted in partial fulfillment of the requirements for the degree of

Master of Science (Biology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

Dr. Selvadurai Dayanadan Chair

Dr. Christopher Brett External examiner

Dr. William Zerges Examiner

Dr. Catherine Bachewich Examiner

Dr. Alisa Piekny Supervisor

Approved by Dr. Selvadurai Dayanadan
Chair of Department or Graduate Program director

Dean of Faculty

Date

Abstract

Anillin Interacts with Microtubules to Define the Division Plane

Husni Haji Bik

Cytokinesis is the process by which the cell divides into two daughter cells and is driven by the formation and ingression of an actin-myosin contractile ring, which is under the control of RhoA. The plane of division is dictated by the position of the mitotic spindle. In particular, central spindle-associated Ect2 (a GEF) generates active RhoA to form the contractile ring in the overlying cortex. We recently found that the conserved scaffold protein anillin interacts with Ect2 at the cortex, in addition to its many interactions with actin, myosin, septins and RhoA (and others). Anillin's interaction with Ect2 may anchor central spindle microtubules at the cortex to activate RhoA in a discrete zone, in a feedback manner. Consistent with this, depletion of anillin or Ect2 results in the loss of cortically-localized central spindle microtubules. Furthermore, disrupting the central spindle by inhibiting Aurora B or by MKLP1 depletion results in the broad localization of contractile ring proteins. In addition, depletion of both MKLP1 and anillin prevents the establishment of an equatorial plane as contractile ring components spread around the entire cell.

These studies suggest that there is a strong relationship between anillin and microtubules, which may help define the division plane. We found that there is a strong negative relationship between astral microtubules and anillin's cortical localization. Furthermore, we observed that anillin localization switches between the cortex and stable microtubules

depending on the cell cycle stage or condition (*i.e.* drug treatment). In the absence of Rho signaling (Ect2 or RhoA RNAi), anillin localizes to central spindle microtubules. Anillin also strongly localizes to taxol-stabilized microtubules. This suggests that RhoA activation coupled with microtubule instability switches anillin from the microtubules to the cortex. Disrupting central spindle microtubules by inhibiting Aurora B or by MKLP1 depletion in taxol-stabilized cells causes anillin to re-locate to the cortex, and differs between discrete and broad depending on the treatment. Using deletion constructs, we mapped the possible microtubule-localizing region on anillin to the C-terminus. We hypothesize that there could be multiple sites where one could be Ect2-dependent to anchor cortical central spindle microtubules and the other could be Ect2-independent. We are still determining if the second interaction is direct. Recently, *C. elegans* ANI-1/anillin was shown to localize to astral microtubules where it removes myosin from the cortex and polarizes the early embryo. In addition, multiple Rho effectors, including mDia2, relocate to central spindle microtubules in the absence of Rho signalling in human cells. We propose that interacting with microtubules could be a conserved mechanism to keep Rho effectors off the cortex to help define cortical domains.

Acknowledgements

I praise to God who gave me the strength and will to finish this study.

I would like to thank my supervisor, Dr. Alisa Piekny, for her patience, generosity guidance and support over the course of my studies. I would also like to thank my committee members, Drs. William Zerges and Catherine Bachewich for their guidance.

In addition, I would like to thank all the members of the Piekny's lab who have been great friends and gave me the advice and the help whenever I needed them.

I also owe my family a debt of gratitude for their unlimited support, both financially and emotionally throughout my degree.

Last but not least, I would like to thank Yaning Fan who has been a friend and family for me and supported me in every way when no one else could.

Table of Contents

List of Figures viii

List of Tables ix

1. Introduction 1

1.1 The Cell Cycle 1

1.2 Cytokinesis 4

1.3 Rho Pathway 8

1.4 Contractile Ring 11

1.5 Mitotic Spindle 13

1.6 Anillin 16

2. Material and methods 22

2.1 Cell Culture and Transfection 22

2.2 RNAi and Drug treatments 22

2.3 Constructs 23

2.4 Fixation and Immunoflourescence 24

2.5 Imaging 26

2.6 Quantitation of Images 26

2.7 Co-sedimentation and Western blot 28

3. Results 30

3.1 Anillin Localizes to Microtubules in Mitotic Cells 30

3.2 the discrete cortical localization of anillin is dependent on astral microtubules 31

3.3 The discrete cortical localization of anillin is dependent on central spindle microtubules 35

3.4 Anillin localizes to taxol-stabilized microtubules in mitotic cells 36

3.5 The localization of anillin to taxol-stabilized microtubules is central spindle- dependent 40

3.6 Anillin localizes to central spindle microtubules in the absence of active RhoA 42

3.7 The localization of anillin to either the cortex or to microtubules is Cdk-dependent 43

3.8 Identifying microtubule-targeting sites in anillin 45

4. Discussion 49

4.1 Anillin Localizes to Microtubules in Mitotic Cells 49

4.2 The discrete cortical localization of anillin is dependent on astral microtubules 50

4.3 The discrete cortical localization of anillin is dependent on central spindle microtubules 52

4.4 Anillin localizes to taxol-stabilized microtubules in mitotic cells 53

4.5 The localization of anillin to taxol-stabilized microtubules is central spindle- dependent 55

4.6 Anillin localizes to central spindle microtubules in the absence of active RhoA 55

4.7 The localization of anillin to either the cortex or to microtubules is Cdk-dependent 56

4.8 Identifying microtubule-targeting sites in anillin 57

5. References 60

List of Figures

Page

Figure 1.1 The Cell Cycle……3

Figure 1.2 The Rho Pathway……...11

Figure 1.3 PRC1……..15

Figure 1.4 Anillin…….20

Figure 3.1 Anillin Localizes to Microtubules in Mitotic Cells……...31

Figure 3.2 Astral microtubules restrict anillin localization……....33

Figure 3.3 Astral and central spindle microtubules restrict anillin localization…….34

Figure 3.4 The cortical localization of anillin depends on microtubule instability…38

Figure 3.5 Stable taxol-treated microtubules recruit central spindle proteins……...39

Figure 3.6 RhoA localization requires microtubule instability……..40

Figure 3.7 The central spindle is required for anillin's localization to microtubules in taxol-treated cells…….41

Figure 3.8 The cortical localization of anillin is Ect2-dependent…….43

Figure 3.9 The localization of anillin to the cortex or to microtubules depends on Cdk1 activity…….45

Figure 3.10 The microtubule-localizing region on anillin maps to the C-terminus..46

Figure 3.11 Anillin may co-sediment with microtubules..... 48

List of Tables

Table 2.1: The primer sequence and restriction enzyme sites……24

Table 3.1: The different treatments of HeLa cells and the resulted anillin's localization.....42

Table 3.2: The number of cells for each construct in the localization experiment…47

1. Introduction

Understanding the cell cycle is important to understand life, particularly development and diseases such as cancer. Scientists have been studying the mechanisms that regulate the cell cycle for decades. One of the areas of the cell cycle that is still not well understood is cytokinesis, which occurs at the end of mitosis to separate the cell into two daughter cells. Although the mitotic spindle is required to establish the division plane, the molecular signals that communicate the position of the mitotic spindle to the cortex are not known. In this study, I am proposing that the cortical scaffold protein anillin is one of these molecular signals.

1.1 The Cell Cycle

The cell is the building block of life. Organisms develop from a single cell and are able to fight illnesses and injuries, because of cell division. Depending on the environmental conditions, cells multiply to repair a tissue or to build the organism. The cell cycle is divided into several phases, two gap phases, a synthesis (S) phase and a mitotic (M) phase. During S phase, genetic information duplicates so that after the segregation of sister chromatids during M phase, two daughter cells form with identical genetic information. During the two gap phases (G1 and G2) before and after S phase, the cell grows in size and cytoplasmic organelles replicate preparing the cell to divide (Figure 1.1).[(1)]

The term ‘mitosis’ is taken from the Greek word for thread, which refers to the thread-like shape of the chromosomes during mitosis.[(2)] Mitosis is the last stage of the cell

cycle and its start, prophase, is marked by the condensation of chromosomes inside the nucleus. During prometaphase, the nuclear envelope breaks down, the mitotic spindle starts to form and associates with chromosomes. The chromosomes align in the equatorial plane during metaphase. The final stages of mitosis start with anaphase, when the duplicated DNA gets segregated by the mitotic spindle toward opposite spindle poles.[3] In addition to DNA segregation, a new pool of microtubules, called the central spindle, accumulates between segregating DNA, the cell elongates and an actin-myosin contractile ring forms at the overlying cortex in the equatorial plane.[4] Finally, during telophase, the actin-myosin ring ingresses to separate the two daughter cells, the segregated chromosomes decondense as the nuclear envelope reforms, and the central spindle microtubules (and remnants of the furrow) condense to form the midbody. The midbody is a platform for the delivery of vesicles from the golgi and endosomes, and is important for abscission of the daughter cells. We use the term 'cytokinesis' to represent the division of cytoplasmic content and physical separation of the daughter cells by abscission, from anaphase through telophase.[2, 3, 5]

The different cell cycle stages require specific kinase activities for their entry and progression. Cyclin-dependent kinase (Cdk)/cyclin complexes are formed and activated to drive entry into the different stages of the cell cycle. In metazoans, different complexes typically regulate progression through different stages. For example, in human cells Cdk4 or 6/Cyclin D regulate G1 entry, Cdk2/Cyclin E regulates S phase entry and progression, Cdk2/Cyclin A regulates S phase progression, Cdk1/CyclinA regulates G2/M transition, and Cdk1/Cyclin B regulates M phase entry. Despite the 'assigned' role of many different Cdk enzymes, knockout studies in mice illustrated that Cdk1 is the only essential Cdk.[1,2]

There are mechanisms to sense when errors have occurred and delay the cycle so the cell can repair errors before proceeding. For example, the checkpoint kinases 1 and 2 (Chk1 and 2) sense that DNA has been properly replicated before permitting the cell to cycle past S phase. Another important step in cell cycle progression is the inhibition of Cdk1/Cyclin B to trigger anaphase onset and mitotic exit. [2, 5]

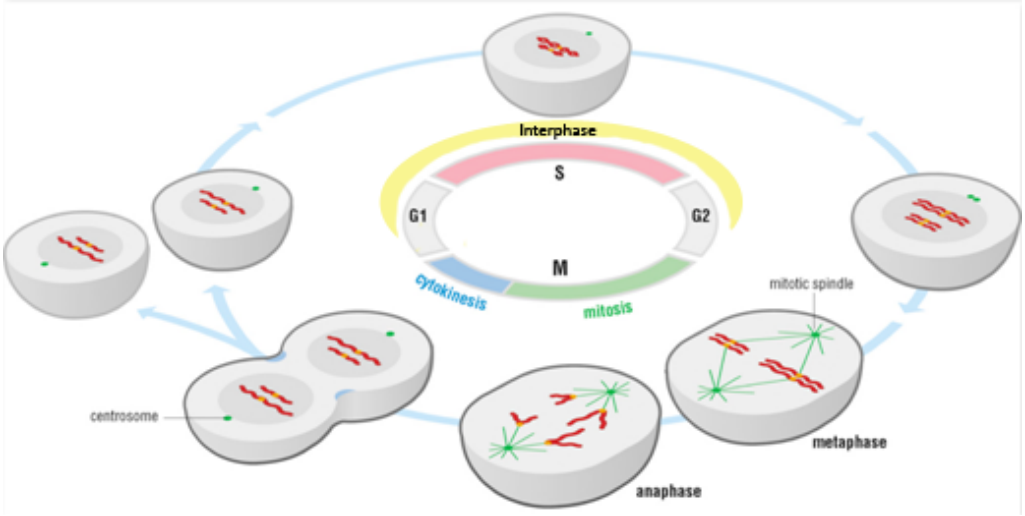

Figure1.1: A schematic of mammalian cells showing phases of cell cycle and the main stages of mitosis phase. Note: Figure is adapted from Morgan 2007. [41]

1.2 Cytokinesis

As mentioned earlier, cytokinesis is the process by which a single mother cell divides into two daughter cells during mitosis. It is a complicated process and many proteins play a role in its regulation, organization and progression. Cytokinesis can be divided into several stages depending on the structural changes of the cell during mitosis starting with specification of the division plane, then cleavage furrow formation and ingression, midbody formation and finally cell separation.[6]

Stage I. Positioning the Division Plane and Initiating Cytokinesis: It is very important to establish the cleavage furrow in the right location so that both daughter cells receive identical genetic material and requires both temporal and spatial control. [6]

Temporal control: In metazoans, cytokinesis is initiated by anaphase onset.[8] Studies have shown that inhibition of Cdk1 activity initiates cytokinesis, even in cells that have disrupted microtubules and are preventing form segregating their DNA. For example, cells treated with Cdk1 inhibitors enter 'cytokinesis' in the absence of microtubules by showing widespread and highly disorganized contractions for ~ 50 minutes. [6, 7] The disorganization of these contractions suggests the importance of microtubules in spatial control. Many of the temporal regulators that contribute to the timing of cytokinesis, such as the anaphase-promoting complex (APC)-mediated destruction of Cyclin B, control the down-regulation of Cdk1 activity.[5, 9] In general, studies have shown that cytokinesis occurs within a flexible ~50 minute window after anaphase onset.

Spatial control: The spatial control of division plane positioning mainly occurs through microtubules from the mitotic spindle. For example, moving the mitotic spindle to a new location within the cell results in a new cleavage furrow that bisects the newly positioned mitotic spindle.[6, 11] Although it is not well understood, different microtubule populations may have different roles in cleavage furrow positioning. Equatorial astral microtubules, which arise from centrosomes and extend to the division plane, and central spindle microtubules, generated between segregating chromosomes, deliver positive signals to the cortex. In contrast, astral microtubules that extend toward the polar cortex may deliver negative signals to promote cortical relaxation and prevent contractility away from the division plane.[10] However, the position of microtubules and their ability to deliver a positive cue or sequester positive cues could be more important vs. delivering negative cues.

The positive signal stimulated by microtubules to initiate cytokinesis is the RhoGEF Ect2, which activates the small GTPase RhoA. RhoA (described further in Section 1.3) is required for contractile ring formation and ingression. Ect2 is phosphorylated by Cdk1 and kept inactive until anaphase. After anaphase onset, dephosphorylated Ect2 is recruited to the central spindle (or stable equatorial-positioned astral microtubules), where it generates active RhoA in the equatorial plane of the cell.[10, 17, 30]

Stage II. Ingression of the Cleavage Furrow: RhoA activation leads to the recruitment and activation of other proteins to form and ingress the contractile ring.

Actomyosin-based contractile ring: The contractile ring is composed mainly of actin filaments that overlap with nonmuscle myosin II filaments (described further in

Section 1.3). RhoA mediates actin polymerization through the activation of formin proteins such as mDia1 and stimulates myosin activity by activating kinases such as Rho-dependent Kinase (ROCK).[6, 10] Active myosin can pull actin filaments together to constrict the ring. In addition, disassembly of both actin and myosin is important to constantly re-model the ring through ingression. Other filament systems, including septins and anillin, are important for crosslinking the ring to maintain its position through ingression.

Membrane insertion during furrow ingression: Cytokinesis also requires the delivery of new membrane to increase the total membrane area to accommodate the formation of two new cells. Insertion of new membrane happens through the delivery of targeted vesicle insertion. Brefeldin A (BFA), an inhibitor of Golgi-derived vesicle transport, blocks this delivery and as a result, blocks cytokinesis.[46] The role of membrane delivery is not well understood, but likely there is cross-talk with the contractile ring to ensure coordinated growth with ingression and abscission.[6] Membrane composition is important and may act as a platform for RhoA signaling. For example, the furrow is enriched with $PI_{4,}5P_2$ phospholipids, and recent studies showed yeast Rho1 preferentially interacts with these lipids.[17, 42]

Stage III. Midbody Formation: At the end of furrow ingression, and presumably when active RhoA levels decrease, the contractile ring (and plasma membrane) forms a tight ring around the compacted central spindle microtubules to form a bridge that is 1-1.5 microns in diameter. Many proteins that were associated with the plus ends of central

spindle microtubules also condense in this region. Together these molecules form an electron dense structure called the mid-body.[6, 10]

Formation of the midbody is important for stabilizing the ingressed contractile ring and permitting the last stage of cytokinesis, abscission. The midbody also acts as a template for the delivery of membrane vesicles and localizes both the Fip3-Rab11 complex, as well as the exocyst, both of which are important for targeting endosomal-derived and golgi-derived vesicles, respectively. Failure to form a midbody prevents cell separation and causes the furrow to regress to form binucleate cells.

Stage IV. Cell Separation/Abscission: Abscission is the final event of cytokinesis. In this stage, the cytoplasmic bridge becomes as narrow as 0.2 microns in diameter and microtubule bundles start to disappear. Many proteins that are required for vesicle and membrane trafficking (as described above) and membrane fusion are essential for abscission.[6, 10]

Modulation of the plasma membrane: As described earlier, the composition of the plasma membrane changes during cytokinesis. For example, $PI_{4,5}P_2$ is enriched in the furrow and RhoA may preferentially associate with this phospholipid. During later stages of cytokinesis, another change that occurs is in the distribution of the membrane phospholipid phosphatidylethanolamine (PE). PE is usually asymmetrically distributed in the plasma membrane and is enriched in the inner leaflet compared with the outer leaflet. However, during cytokinesis, this lipid is localized in the outer leaflet of the plasma membrane in the cleavage furrow. The importance of PE is shown by experiments where its depletion causes a late block in cytokinesis. [6, 10, 15, 42]

1.3 Rho Pathway

As described earlier, the RhoA pathway is the main signaling pathway that regulates cytokinesis (Figure 1.2). RhoA is a GTPase that binds (and weakly hydrolyzes) GTP and GDP. Activation of RhoA is considered as the molecular switch that turns on the events required for cytokinesis. RhoA is inactive when bound to GDP, and active when GTP-bound [17, 47]. Ect2 is a guanine nucleotide exchange factors (GEF) that promotes GTP binding by exchanging GDP to GTP to activate RhoA for cytokinesis. The activity of RhoA is counterbalanced by GTPase activating proteins (GAPs), which stimulate GTP hydrolysis to GDP. Although excess GAP activity leads to the downregulation of RhoA, and probably occurs at the end of furrow ingression, some GAP activity is required to promote the cycling of RhoA during furrow ingression. It is not clear which GAP carries out this function, although Cyk-4 is a candidate. A conformational change happens when RhoA is activated by binding GTP, which increases its affinity for effectors including profilin, formin, anillin, Rho-dependent kinase and Citron kinase. When active Rho binds to one of these effectors, it can alter their activity or interactions with other proteins and lipids.[16, 17]

RhoA Localization: RhoA localizes to a discrete zone in the cleavage furrow area of dividing cells (Figure 1.2). This is the same zone where active RhoA regulates furrow formation and ingression, and it is likely that most of this RhoA is active. In support of this, a probe that specifically detects active RhoA localizes to the cleavage furrow, in the same place where endogenous RhoA localizes in fixed cells.[17]

Spatiotemporal Regulation of RhoA: As described earlier, Ect2 is the GEF protein that activates RhoA. Accordingly, Ect2 is required for the localization of RhoA to the

equatorial cortex and Ect2 depletion blocks cytokinesis and phenocopies RhoA. During metaphase, Ect2 is phosphorylated by Cdk1, which causes it to fold into an inactive conformation. After anaphase onset, Ect2 is dephosphorylated and binds directly to Cyk-4, which is part of the centralspindlin complex with the kinesin protein MKLP1. This binding recruits Ect2 to the central spindle and helps restrict the activation of RhoA to the equatorial plane of the cell. Cyk-4 is required for Ect2 activation, because depletion of Cyk-4 phenocopies Ect2 and blocks cytokinesis. MKLP1 depletion causes ectopic localization of active RhoA suggesting that only Cyk-4 is required for Ect2 activation and that the central spindle functions to restrict the localization of active Ect2.[17, 19, 30]

RhoA Effectors: After its activation, RhoA regulates numerous proteins that modify the cytoskeleton. In addition to mediating actin assembly, Rho effectors regulate the activation of nonmuscle myosin II to slide actin filaments. The key RhoA effectors involved in cytokinesis are profilins, formins, Anillin (will be discussed further in Section 1.6), Rho-dependent kinase (ROCK, also known as ROK) and Citron kinase (Citron K).[17, 48, 49]

Activation of RhoA is essential for contractile ring formation by directly activating the actin assembly factor formin.[19] After binding to active RhoA, formin becomes functional and promotes actin polymerization. The core structure of formin involves two formin-homology FH1 and FH2 domains [50]. These domains bind to profilin and actin, respectively, and mediate actin polymerization. This happens *in vivo* by mediating the association of two actin monomers and creating a binding site for a third monomer. The N-terminus has a DID (Diaphanous inhibitory domain) and the C-

terminus has a DAD (Diaphanous autoinhibitory domain), which binds to active RhoA. When active RhoA is absent, the C-terminus of formin binds to the DID to autoinhibit.[17]

Active RhoA also activates nonmuscle myosin II, another major component of the contractile ring. Myosin II is a hexamer containing pairs of heavy chains, essential light chains and regulatory light chains (RMLC) [51]. Upon binding to active RhoA, ROCK is activated and phosphorylates the serine at position 19 in RMLC, which allows myosin to assemble into filaments and activates the actin-dependent ATPase activity of the motor domain.[17] This slides the interacting actin filaments to close the ingressing contractile ring like a purse-string.

Active RhoA also activates Citron kinase, an essential kinase for cytokinesis in *Drosophila*, however, is not essential for cytokinesis in many mammalian cell types. Citron kinase phosphorylates RMLC at two positions, Thr18 and Ser19. Although the function of Citron kinase is not well understood, it might be required for midbody formation, by stabilizing the ingressed contractile ring. [17]

Inactivation of RhoA: RhoA must be inactivated at the end of cytokinesis. This may occur due to the removal of Ect2 from the central spindle, which becomes trapped in the newly formed nuclei. Downregulation of RhoA may also require a GAP protein. The GAP Cyk-4 is localized to the central spindle and becomes highly concentrated in the midbody, where it could act on RhoA. However, contradictory results have been obtained using GAP deletion mutants of Cyk-4 and it is not clear if its GAP domain is required for its function. Other GAP proteins that regulate RhoA have been found including RGA-3/-4 in *Caenorhabditis elegans*. [17, 18, 19, 52]

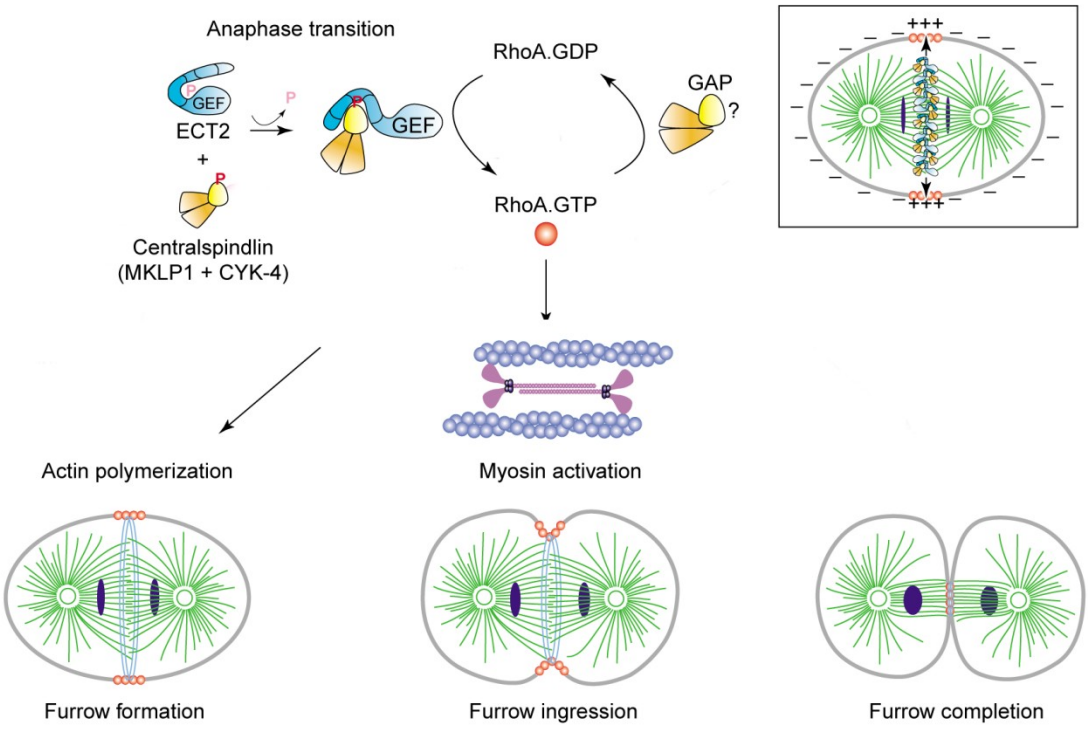

Figure1.2: A schematic of mammalian cells showing the Rho pathway and its main effectors. Note: Figure is adapted from Piekny et al, 2005. [17]

1.4 Contractile Ring

The contractile ring form is the main structure that ingresses separate the two daughter cells. However, the mechanism by which this contractile ring assembled, controlled and functioned is very complicated. The ring contains multiple filament systems that are conserved among organisms, particularly actin, myosin, septins and anillin. The progressive sliding of actin filaments by myosin likely provides the contractile force that causes furrow ingression. [19]

Contractile ring assembly: As described earlier, RhoA activation is the switch that triggers contractile ring assembly. In early anaphase, active RhoA directs assembly

of the contractile ring by activation of profilin, formin and nonmuscle myosin II. Anillin and septins are also recruited to stabilize the position of the contractile ring and will be discussed later (Section 1.6). Formin nucleates and directs the elongation of actin filaments around the equator of the cell and at the same time, nonmuscle myosin II concentrates in cortical spots. Nonmuscle myosin II within a spot captures actin filaments growing from adjacent locations and pulls actin filaments together.[20] The actin filaments have mixed polarities and are oriented in both directions around the ring. In animal cells, actin filaments are grouped into small bundles, but the lengths of these filaments are not known since it is difficult to track them for more than 0.25μm.

One of the big mysteries is how the contractile ring is anchored to the cortex through the overlying plasma membrane, and if it also has similar associations with the mitotic spindle. The contractile ring is linked to both the central spindle and membrane through Ect2's activation of RhoA (which is associated with the membrane through its C-terminal lipid tail). However, another key protein that mediates this function is anillin (will be discussed in Section 1.6), which contains binding sites not only for actin and myosin, but also for septins, the plasma membrane, RhoA and central spindle proteins (Ect2 and Cyk-4).

Mechanism of constriction and disassembly of the contractile ring: The proportion of actin and myosin filaments in the contractile ring remains constant through ingression [53]. This suggests that both actin and myosin filaments must depolymerize in proportion to the circumference of the ring as it closes. In mammalian cells, drugs that inhibit myosin activity, but don't block contractile ring assembly, result in an over-accumulation of myosin and actin filaments in the ring. In fission yeast, the intensity of

GFP tagged nonmuscle myosin II heavy chain was found to be constant during contraction of the ring.[20, 53] The rate of constriction also is proportional to the circumference of the ring and ranges from 0.5 nm s^{-1} for every 1μm in fission yeast to 3.5 nm s^{-1} per 1μm in nematode embryos. The presence of both antiparallel actin filaments and nonmuscle myosin II suggested that constriction uses a sliding-filament mechanism similar to muscle sarcomeres. However, contractile rings may use a more complex mechanisms since the filament arrangement is less-organized compared to sarcomeres and undergoes disassembly as the ring constricts.[18, 19, 20]

1.5 Mitotic Spindle

The microtubule cytoskeleton is reorganized during mitosis to form the bipolar mitotic spindle, which is important for segregating sister chromatids and dictating the position of the contractile ring for cytokinesis. The bipolar spindle contains microtubules from the centrosomes (minus ends at centrosomes and plus ends toward cortex or chromosomes) and from the kinetochores of chromosomes, which will incorporate into the spindle. Since the goal of the spindle is to segregate DNA, stable kinetochore attachments ensure the alignment and movement of sister chromatids to their respective poles. Many motor and other microtubule associated proteins have roles in assembling, modeling and generating forces for chromosome movement. Dynamic instability is an inherent property of microtubules that also is important for aligning and segregating chromatids.[54]

During anaphase, the microtubule-based central spindle forms between segregating chromatids. The central spindle contains antiparallel bundles of microtubules with the plus ends pointing toward the centre of the cell. These microtubules will elongate during anaphase, pushing the poles of the cell away from each other and causing cell elongation. As described earlier, the central spindle provides cues for contractile ring assembly and positions the division plane. Astral microtubules ('astral' refers to centrosome-derived microtubules that emanate toward the cortex in at the poles of the cell) may play a negative role in keeping contractile ring components away from the cell poles, and keeping them concentrated in the equatorial plane of the cell.[21]

Many proteins participate in central spindle assembly, stabilization and elongation and include mitotic kinases, kinesin motor proteins and microtubule-associated proteins (MAPs). The main proteins that will be discussed are PRC1 (Protein Regulator of Cytokinesis 1), which partners with the kinesin KIF4A, the centralspindlin complex (heterotetramer of Cyk-4 and the kinesin MKLP1) and the chromosome passenger complex (CPC; composed of Aurora B kinase, Survivin, INCENP and Borealin).[21]

PRC1 is a MT binding and bundling protein. PRC1 localizes to the mitotic spindle and concentrates in the spindle midzone during anaphase (Figure 1.3). Cdk1 phosphorylation inhibits PRC1's interaction with KIF4A, which ensures that the complex only localizes to microtubules in anaphase. PRC1 is the most upstream protein that functions in central spindle assembly. Depletion of PRC1 or its partner, KIF4A blocks central spindle formation altogether and blocks the recruitment of other central spindle proteins. Despite this strong phenotype, PRC1 is dispensable for division in many organisms, such as *C. elegans* and mammalian cells.[27]

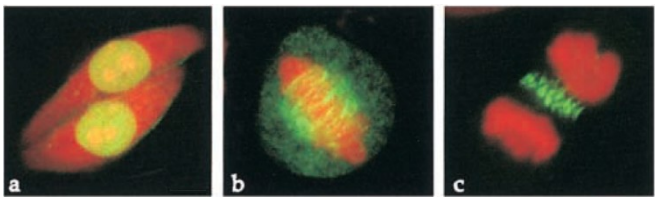

Figure1.3: Dividing mammalian cells showing PRC1 stained in green and DNA stained in red. PRC1 is located inside the nucleus during interphase and localizes to the central spindle during mitosis. Note: Figure is adapted from Mollinari et al, 2002. [27]

Centralspindlin also regulates central spindle assembly, but is not required for the localization of some central spindle proteins including PRC1. The centralspindlin complex is prevented from forming in metaphase by Cdk1 phosphorylation of MKLP1. In anaphase, MKLP1 dephosphorylation increases its ability to bind to Cyk-4 and its affinity for microtubules. Blocking phosphorylation by Cdk1 causes increased MKLP1 localization on metaphase spindle microtubules and leads to defects in chromosomes segregation.[28] One of the main functions of the centralspindlin complex is to recruit Ect2, the activator of RhoA (discussed in Section 1.3). [29] In order to bind to Ect2, CYK-4 must be phosphorylated by Polo-like kinase 1(Plk1) (figure 1.2).[30] The ability of Plk1 to regulate the binding and activation of Ect2 adds another layer of temporal control to cytokinesis.

The CPC refers to a complex of Aurora-B kinase, INCENP, survivin and borealin, and localizes to kinetochores during metaphase and transfers to the central spindle during anaphase. Studies on cells that lack chromosomes show that the CPC localizes to the central spindle even without chromosomes.[21] The complex is redundant for central spindle formation, but is required for the stabilization of central spindle proteins

including the centralspindlin complex. Mutations in or depleting members of the complex cause defects in chromosome segregation and abscission.[31]

INCENP works as scaffold protein for the CPC and has two functional domains in its structure. The C-terminal domain binds and activates Aurora-B, and the N-terminal domain binds both Survivin and Borealin. Borealin directly interacts with both Survivin and INCENP, but not Aurora-B.[32]

The CPC also may communicate to the cortex. It is not clear what cortical component it interacts with, but the CPC also localizes to the cleavage furrow. Treating cells with drugs that inhibit polymerization of microtubules cause an enrichment of INCENP on the cortex. Studies have shown that it localizes to the tips of microtubules that reach the cortex suggesting that the CPC could 'deliver' cues for determining the cleavage plane.

As cytokinesis progresses, the central spindle becomes more compact and many of the central spindle-associated proteins also compact into the midbody. Although PRC1 and centralspindlin localize in the very centre of the midbody, the CPC spreads along the microtubules on either side of the midbody and it is not understood why they differ.

1.6 Anillin

Anillin is conserved across metazoans and functions as a scaffold based on its interaction with many proteins. Anillin contains an actin binding domain and myosin binding domain in its N-terminus and an Anillin Homology domain (AHD) and Pleckstrin Homology (PH) domain in its C-terminus. Anillin is required for cytokinesis in many organisms and localizes to the cleavage furrow with a pattern that strongly overlaps

with RhoA. It crosslinks different components of the cell; the plasma membrane, actin-myosin filaments, septin filaments and the mitotic spindle, and its depletion causes destabilization of the contractile ring (Figure 1.3).[33]

Actin: Anillin was first isolated as an F- actin-binding protein from *Drosophila* extracts. It also bundles F-actin and these functions are conserved in other organisms. Although they interact, anillin and F-actin localization to the cleavage furrow are independent from one other. In the absence of anillin, actin localizes outside the equatorial plane, suggesting anillin is required to stabilize the contractile ring.[33, 34] In addition to its interaction with actin, anillin binds to the formin mDia2.[33, 55, 61]

Myosin: Anillin directly interacts with active myosin (phosphorylated on Ser19) and indirectly through actin [56]. Similar to actin, anillin and myosin localize to the contractile ring independently of one another except in fission yeast *S. pombe*, where the anillin-like protein Mid1p localizes to the site of division in late G2 and is required to establish the division plane [62]. Also similar to actin, depletion of anillin disrupts the spatial stability of myosin at the cell equator during cytokinesis. Anillin constructs with deleted actin and myosin binding domains localize to the cleavage furrow suggesting that anillin binds to the cortical equatorial plane through its C-terminus domains while the N-terminus organizes the contractile ring.[33, 34, 56, 58, 60]

Septins: Septins are a group of GTP binding proteins that form heterooligomers and localize to the cleavage furrow during cytokinesis. Septins can directly bind to anillin and their localization to the furrow is dependent on anillin. Anillin binds to septins through the AH and PH domains in the C-terminus [60, 63]. Anillin localization to the

cleavage furrow depends on the septin protein (Peanut) and anillin constructs deleted for the PH domain cannot localize to the cortex.[33, 57, 58, 60, 61]

Rho: Anillin directly binds to human RhoA through its AH domain. As described earlier (Sections 1.1-1.4), RhoA activation is the molecular switch that starts cytokinesis. Anillin is as an effector for RhoA, by interacting with GTP-bound RhoA, and is dependent on RhoA for its localization to the cleavage furrow. Anillin may feedback to further stabilize active RhoA because RhoA localization is lost in anillin-depleted cells and over-expressing the AH domain restores RhoA localization.[33, 34] The mechanism for this feedback is not known, but could occur through anillin's other interactions (see below).[58, 64]

Ect2: Anillin binds to Ect2 through its AH domain. This interaction may be independent of RhoA binding. By binding to both Ect2 and RhoA, anillin could feedback to stabilize the localization of active RhoA in the furrow. As described earlier, Ect2 also binds to Cyk-4, which recruits it to the central spindle. This suggests that by binding to Ect2, Anillin also crosslinks the mitotic spindle to the contractile ring.[33]

Cyk-4: Anillin binds to RacGAP50C (Cyk-4 homologue in *Drosophila*) through its C-terminus [65, 66]. This interaction may have the same function as anillin binding to Ect2, to crosslink the mitotic spindle to the contractile ring. In both *Drosophila* and human cells, anillin depletion affects the ability of the central spindle to remain close to the cortex. It has not been shown in other organisms if anillin's interactions with Cyk-4 or Ect2 are conserved.[33, 59]

Microtubules: Drosophila anillin may interact with microtubules and studies have shown that anillin-rich structures co-localize with microtubules after specific drug

treatments. For example, Latrunculin A treatment of *Drosophila* S2 cells resulted in colocalization of anillin with the plus ends of microtubules [43]. This kind of association reflects a possible role for anillin in communicating the position of the mitotic spindle to the equatorial cortex.[21, 33]

Plasma membrane/lipids: The PH domain is known for its ability to interact with lipids and could allow anillin to interact with the plasma membrane (although this hasn't been shown). Deletion of the PH domain prevents anillin from localizing to the cortex [58, 60]. However, since anillin binds septins through its PH domain, which in turn can interact with the membrane, they could bridge the membrane interaction. In fission yeast, Mid1p directly localizes to the plasma membrane through a region in its C-terminus.[33, 60, 62]

Summary

Several important questions about cytokinesis remain unanswered. How is the division plane determined? It is clear that the mitotic spindle dictates the position, but how is this communicated to the cortex, and how is this communication maintained through furrow ingression? Anillin is a good candidate to carry out this function since it interacts with actin and myosin, RhoA, their upstream regulator, the central spindle and the plasma membrane and could coordinate all of the key components of the cell for contractile ring formation and ingression. Another question regards the end stages of cytokinesis. How is the midbody formed and how is the contractile ring stabilized at the midbody? Anillin is also a good candidate for this function since it interacts both with the contractile ring and the central spindle microtubules.

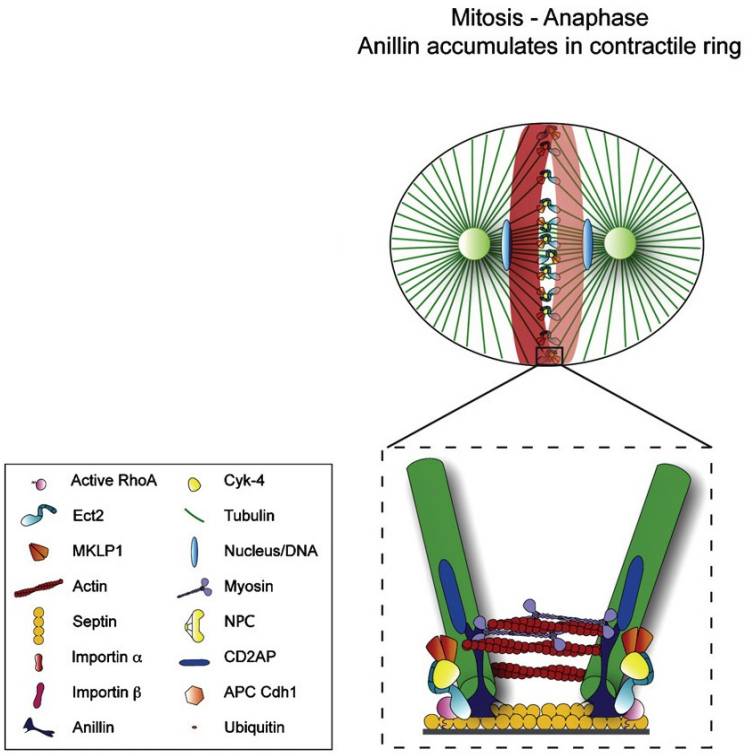

Figure1.4: A schematic of mammalian cells showing anillin accumulation in the contractile ring (shown in transparent red). A high-resolution view of Anillin's interactions with other cytokinesis proteins is shown in the hatched box. Note: Figure is adapted from Piekny & Maddox, 2010. [33]

Hypothesis

The mitotic spindle determines the division plane, presumably by communicating with the cortex. Anillin is a scaffold that interacts with components of the contractile ring as well as their upstream regulators. I hypothesize that anillin also interacts with microtubules to act as a molecular communicator between the mitotic spindle and the cortex to define the division plane.

2. Material and methods

2.1 Cell Culture and Transfection

HeLa cells were gown and maintained in Dulbecco's Modified Eagle Medium (DMEM; Wisent), which is a high glucose media supplemented with 10% fetal bovine serum (FBS; Wisent), 2 mM L-glutamine, 100 u penicillin and 0.1 mg/mL streptomycin (PS; Wisent) and kept in a humidified 5% CO_2 incubator at 37°C. For transfection purposes, HeLa cells were plated in media without antibiotics and transfected with short interference RNA (siRNAs; sequences in section 2.2) and/or co-transfected with DNA (0.5 – 2 ug; description of constructs in section 2.3) using Lipofectamine 2000 (Invitrogen) and Plus Reagent (Invitrogen) as per manufacturer's instructions. We used 5-fold less reagent than recommended by the manufacturer for best results and transfected cells between 50-70% confluency. Cells were transfected for approximately 30 hours for RNAi experiments (also when co-transfected with DNA), and 24 hours for DNA transfection.

2.2 RNAi and Drug treatments

As described above (section 2.1), we transfected Hela cells with siRNAs to knockdown the expression of endogenous genes. We used the following siRNAs in this study: Anillin siRNA CGAUGCCUCUUUGAAUAAA (Dharmacon), Ect2 siRNA GGCGGAAUGAACAGGAUUU (Dharmacon), MKLP1 siRNA

CGACAUAACUUACGACAAAUU (Dharmacon), and MCAK siRNA GCAGGCUAGCAGACAAAU (Dharmacon).

The following drugs were used in this study: 2.5 mM thymidine (blocks cells in S phase; Bioshop)[30], 10 μM taxol in DMSO (stabilizes microtubules; Bioshop)[36], 40 ng/mL nocodazole in DMSO (prevents microtubule polymerization; Sigma-Aldrich)[30], 100 nM hesperadin in 1 X PBS (inhibits aurora B kinase activity; Boehringer Mannheim)[37], 22.5 nM purvalanol A in DMSO (inhibits Cdk1 kinase activity; Sigma-Aldrich)[38] and 2 μM S-trityl-L-cysteine (STC; inhibits eg5 kinesin; Sigma-Aldrich)[30].

To synchronize HeLa cells in anaphase, they were treated with 2.5 mM thymidine for 12-16 hours (blocks cells in S phase; on occasion this was repeated), then released and after 7 hours were treated with 40 ng/mL nocodazole or 2 μM STC for 3 hours (both drugs block cells in metaphase), then released for 45 minutes (to permit entry into anaphase). To synchronize DNA-transfected cells, they were treated with 40 ng/mL nocodazole or 2 μM STC for ~6 hours, then released for 45 minutes prior to fixing. To generate monopolar cells, 7 hours after release from a thymidine block, they were treated with 2 μM STC for 3 hours, then treated with 22.5 nM purvalanol A (to stimulate mitotic exit) for 30 minutes.

2.3 Constructs

As described above (section 2.1), several DNA constructs were used in this study. For imaging experiments, we used the following GFP-tagged RNAi-resistant Anillin constructs: GFP:Anillin (1-1087; full-length), GFP:Anillin (608-1087; delta N-terminus), GFP:Anillin (250-1087; delta myosin binding domain), GFP:Anillin (340-1087; delta

myosin and actin binding domains), GFP:Anillin (1-460; 608-1087; delta middle), GFP:Anillin (100-460; 943-1087; delta Anillin Homology domain), and GFP:Anillin (1-943; delta PH domain)[39]. In addition, new constructs were generated for this study. GFP:Anillin (460-1087; delta actin and myosin binding domains and part of the middle), GFP:Anillin (340-460; delta actin bundling region and part of the middle) and GFP:Anillin (1-460; delta middle and C-terminus) all were generated by PCR using the primers in Table 2.1 and cloned into the pEGFPC1 vector (Clontech).

	Primers		Cut Site
Anillin 460-1087	Forward	**5'- GCTAAGCTTTGCAACTAGAAACCAAACAGGAA-3'**	AAGCTT Hind III
	Reverse	**5'- GAGGGATCCAGGCTTTCCAATAGGTTTGTA - 3'**	GGATCC BamHI
Anillin 340-460	Forward	**5'- GCTAAGCTTTGCAGACAGTTCCATCCAAGGGA -3'**	AAGCTT Hind III
	Reverse	**5'- GCGGGATCCTTGTTTGCTTTTTGAGTTTCC - 3'**	GGATCC BamHI
Anillin 1-460	Forward	**5'- CAGGAATTCTATGGATCCGTTTACGGAGAAA – 3'**	GAATTC EcoRI
	Reverse	**5'- CAGCCGCGGCTATTGTTTGCTTTTTGAGTTTCCGC - 3'**	CCGCGG SacII

Table 2.1: The primer sequence and restriction enzyme sites used to PCR and clone the indicated constructs.

2.4 Fixation and Immunoflourescence

To determine the localization of GFP-tagged constructs or endogenous proteins in Hela cells, they were fixed and stained with primary and fluorescent secondary antibodies. Coverslips with cells (treated as described in sections 2.1-2.3), were washed with pre-warmed (37 ºC) 1 X cytoskeletal buffer (80mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA) then ice-cold methanol or freshly prepared 10% trichloroacetic acid (TCA) was

added to the cells for 20 minutes and kept at 4 ºC, and subsequently washed 3-4 times with 1 X TBST (0.5% Triton X-100, 0.15 M NaCl and 0.05 M Tris pH 7.4). Before immunostaining, the coverslips were placed on parafilm in a wet chamber (a dish with a lid and wet paper towels) and incubated with 5% NDS (Normal Donkey Serum) in 1 X TBST for 20 minutes to block unspecific binding of primary antibodies. To immunostain, cells were incubated with primary antisera diluted in 1 X TBST with 5% NDS for 2 hours at room temperature. The following primary antibodies and dilutions were used for immunofluorescence (IF), 1:100 rabbit polyclonal anti-GFP antibodies (gift from Dr. Glotzer, University of Chicago, IL), 1:50 mouse monoclonal anti-Plk1 antibodies (Santa Cruz), 1:100 rabbit polyclonal anti-anillin antibodies (Piekny and Glotzer, 2008), 1:100 mouse monoclonal anti-tubulin antibodes (DM1A; Sigma-Aldrich) and 1:2 media with mouse monoclonal anti-Myc antibodies (gift from Dr. Sacher, Concordia University, QC). After incubation with primary antibodies, cells were washed 3 times with 1 X TBST, then incubated with secondary antibodies with 5% NDS in 1 X TBST for 2 hours. The following secondary antibodies were used at 1:250 – 1:500 dilution, donkey or goat anti-mouse Alexa 488 (Invitrogen) and anti-rabbit Alexa 568 (Invitrogen), or anti-mouse Alexa 568 (Invitrogen), and anti-rabbit Alexa 488 (Invitrogen). After incubation with secondary antibodies, cells were washed 3 times with 1 X TBST, then 1:1000 1 mg/mL DAPI (Sigma-Aldrich) in 1 X TBST was added for 5 minutes, washed once with 1 X TBST, then washed with 0.1 M Tris pH 8.8. After removing the last wash, a drop of mounting media (5% n-propyl gallate (Alfa Aesar) in 50% glycerol 50 mM Tris pH9) was added to the coverslip, which was then placed onto a slide and sealed with nail polish.

2.5 Imaging

Images were captured on a Leica DMI6000B inverted microscope with the 40x/0.75 or 63x/1.4 NA objectives using an OrcaR2 (Hamamatsu) camera with Volocity acquisition software (PerkinElmer). Exposure times and gain settings were kept constant based on control slides. 0.5 μm Z-sections were collected using the piezo Z/ASI stage (Molecular Devices). All images were exported as TIFF's (16-bit images) and opened in Image J (NIH), where pixel intensities were adjusted based on control cells, then converted into 8-bit images for making figures in Adobe Photoshop and Illustrator. Also, Image J (NIH) was used to add colour (for overlay to show co-localization) and/or to generate maximum intensity Z-stack projections. All quantitations (see section 2.6) were performed in Image J (NIH), and values were exported into PowerPoint or Excel (Microsoft) for generating graphs.

2.6 Quantitation of Images

Two types of quantitations were done in this study. The first was to determine the breadth of cortical anillin localization in nocodazole treated cells compared to control cells (Figures 3.2 and 3.3). To do this, images were opened in Image J (NIH) and the distance of anillin distribution along both sides of the cortex were measured, and averaged together. We then measured the length of the cell and calculated the ratio of the length of anillin localization to the length of the cell. Cells that were selected for this quantitation were elongated with slightly ingressed furrows. The results were graphed using Excel and PowerPoint 2007 (Microsoft).

The second quantitation was to detect the change in cortical anillin localization and increase in microtubule localization in taxol treated cells compared to control cells. Images were opened in Image J (NIH), and five square points were measured on each side of the cell at the equatorial plane. The fluorescence intensity of cortical anillin was calculated as an average of all squares. Using the same process, we selected five points in the region of the central spindle and determined the average intensity of anillin in a more central plane of the cell. Finally, a ratio of anillin localization on the cortex to its localization in a more central plane (i.e. microtubules) was calculated. The results presented using Excel and PowerPoint 2007 (Microsoft).

For both quantitations, the standard deviation was found using the following equation:

$$S=\sqrt{\frac{\sum(X-M)^2}{n-1}}$$

Where S = standard deviation

Σ = Sum of

X = Individual score

M = Mean of all scores

N = Sample size (Number of scores)

Statistical Analysis

Graph data are presented as mean ± SD or SE of the means and were analyzed by the Student's *t* test. A *p* value of <0.05 is statistically significant. The following formula was used.

$$t = |\bar{x}_1 - \bar{x}_2| \div \sqrt{A * B}$$

Where,

$$A = (n_1 + n_2) \div n_1 n_2 ,$$

and

$$B = [(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2] \div [n_1 + n_2 - 2]$$

2.7 Co-sedimentation and Western blot

To determine if anillin interacts with microtubules, co-sedimentation experiments were performed. Hela cells expressing GFP:Anillin (250-1087) treated with taxol for 30 minutes or control (no taxol) were lysed with hypolysis buffer (10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.3 % Triton X-100, 1 mM DTT and protease inhibitors 1 mM PMSF, 10 µg/mL each leupeptin and pepstatin) and left at room temperature for 20 minutes, then centrifuged at 15,000 rpm for 20 minutes. The supernatant was removed and after collecting an aliquot (and adding 10 µL sample buffer; 90% 4 X SDS and 10% BME (2-Mercaptoethanol), the pellets were re-suspended in cytoskeletal buffer, and incubated on ice for 30 minutes with nocodazole. They were then centrifuged at 15,000 rpm for 20 minutes at 4 ºC and an aliquot was taken and resuspended in sample buffer. All samples were run by SDS-PAGE (BioRad) and then transferred to nitrocellulose

membrane (GE Healthcare) by wet-transfer (BioRad) for western blotting. The following primary antibodies were used for immunoblotting, 1:1000 mouse monoclonal anti-GFP antibodies (Roche), 1:2500 mouse monoclonal anti-tubulin antibodies (DM1A; Sigma-Aldrich). Anti-mouse Alexa 488 (Invitrogen) secondary antibodies were used at a 1:2500 dilution. Blots were scanned using the Typhoon Trio phosphoimager (GE) at 600 intensity. Image J was then used to open the 16-bit images and bands were quantitated based on selected regions of interest and pixel intensities. Figures were made by changing the images to 8-bit and cropping them in Adobe Photoshop, followed by their import into Adobe Illustrator.

3. Results

3.1 Anillin Localizes to Microtubules in Mitotic Cells

Previous studies showed that *Drosophila* anillin directly interacts with the central spindle protein Cyk-4, and our lab has found that human anillin can bind to Ect2, which is recruited to the central spindle by Cyk-4 [35]. Anillin depletion in *Drosophila* and human cells causes a decrease in the density of microtubules stabilized toward the equatorial cortex and causes the contractile ring to become laterally unstable, resulting in cytokinesis defects and the formation of binucleate cells [33]. These results suggest that anillin might be the key connector between the mitotic spindle and the contractile ring, a model that requires anillin interaction with microtubules. However, anillin has only been described as a cortical protein. To determine if anillin localizes to microtubules, we stained fixed Hela cells in different stages of mitosis for anillin. In metaphase cells, anillin was mostly cytosolic, with some protein visible at the cortex and some on astral microtubules (Metaphase/Ana-A; Figure 3.1). As shown before, anaphase cells displayed anillin localization at the equatorial cortex, but we also observed low levels of anillin on astral microtubules (Anaphase; Figure 3.1). Through late anaphase and early telophase, anillin predominantly localized to the equatorial cortex, although some anillin localized to central spindle microtubules (Early Telophase; Figure 3.1). During later stages of telophase, anillin localized to the midbody and central spindle microtubules (Telophase; Figure 3.1). These results show for the first time that anillin localizes to microtubules in mitotic cells, and its localization switches preferentially from astral microtubules to central spindle microtubules as cells progress through mitosis.

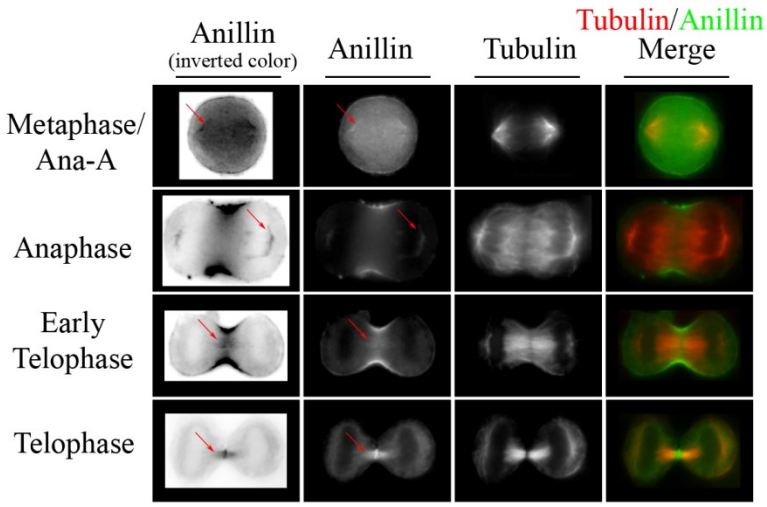

Figure 3.1 Anillin localizes to microtubules in mitotic Hela cells. Hela cells at varying mitotic stages were fixed and stained for anillin (green) and microtubules (red) as indicated. Inverted images of anillin are shown. The red arrows point to anillin localization on microtubules.

3.2 The discrete cortical localization of anillin is dependent on astral microtubules

We found that anillin localizes to astral microtubules just prior to and in early stages of cytokinesis. The polar astral microtubules might negatively regulate the distribution of contractile ring components in the poles, restricting them to the equator to establish the division plane [17]. In both *C. elegans* and human cells, anillin is thought to be one of the proteins that may communicate the position of astral microtubules, since its co-depletion with the central spindle completely blocks contractile ring formation. To further study the relationship between anillin and astral microtubules, we examined anillin localization in dividing Hela cells after disrupting astral microtubules with nocodazole. Nocodazole is an anti-cancer drug that prevents microtubule polymerization

and promotes cell cycle arrest if cells are exposed to low doses of the drug in metaphase. Hela cells in late anaphase/early telophase were treated with 40 ng/mL nocodazole for ten minutes, then were fixed and stained for anillin (green) and microtubules (red). As shown in Figure 3.2, the localization of anillin appeared broader in comparison to control cells and although the central spindle microtubules are clearly visible, the astral microtubules are greatly diminished. To quantitate the change in the localization of anillin, the breadth of anillin distribution on the cortex was measured and calculated as a ratio to the length of the cell (n = 17 for control cells, n = 17 for nocodazole-treated cells, dots represent individual cells; light grey shows standard deviation and the red line shows the mean; Figure 3.2). As shown in the graph, the mean ratio for nocodazole-treated cells was statistically different from control cells (0.6 vs. 0.42, respectively), supporting that anillin localization was broader in nocodazole-treated cells. Anillin did not localize to the polar regions of the cell, suggesting that the astral microtubules are not required to prevent polar accumulation of contractile ring proteins. However, they are required to maintain discrete localization of contractile ring components at the equator.

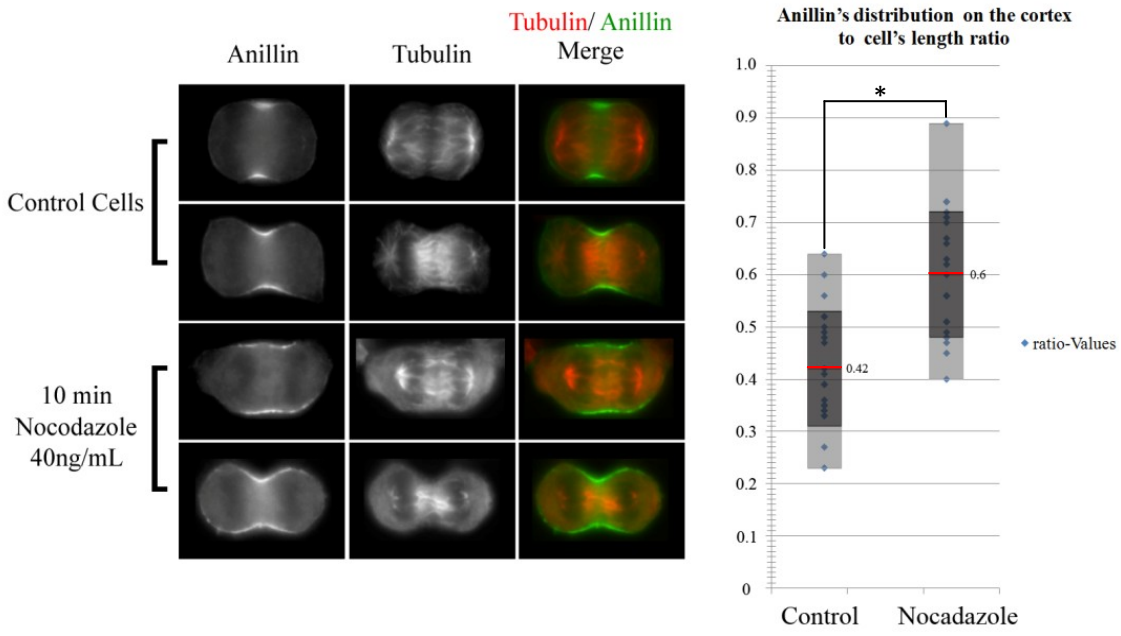

Figure 3.2 Astral microtubules restrict anillin localization. Dividing Hela cells treated with nocodazole were fixed and co-stained for anillin (green) and tubulin (red). The ratio of anillin distribution on the cortex vs. cell length is shown in the graph on the right. The dots represent individual cells, the red bar shows the average ratio and the dark grey area shows the standard deviation. $P < 0.005$ (independent sample student T-Test).

These results support a role for astral microtubules in restricting the localization of anillin, and possibly contractile ring proteins. However, we did not observe anillin localization in the poles and the mechanism by which astral microtubules regulate contractile ring proteins is not clear. If astral microtubules negatively regulate contractile ring proteins, then increasing the length of astral microtubules should decrease the breadth of their localization, causing a narrower division plane. Recently, it was shown that depletion of MCAK, a kinase required to depolymerize astral microtubules to model the bipolar spindle, caused an increase in the length of astral microtubules [(40)]. We performed MCAK RNAi in Hela cells to determine if lengthening the astral microtubules would decrease the localization of anillin (Figure 3.3). In MCAK RNAi cells, the breadth

of anillin localization was more restricted in comparison to control cells (0.23, n = 13 for MCAK RNAi cells vs. 0.3, n = 13 for control cells; Figure 3.3). Therefore, astral microtubules can impart a negative effect on the localization of contractile ring proteins, but other mechanisms or pathways prevent anillin from localizing to the poles of nocodazole-treated cells (perhaps something as simple as a limited gradient of a positive signal).

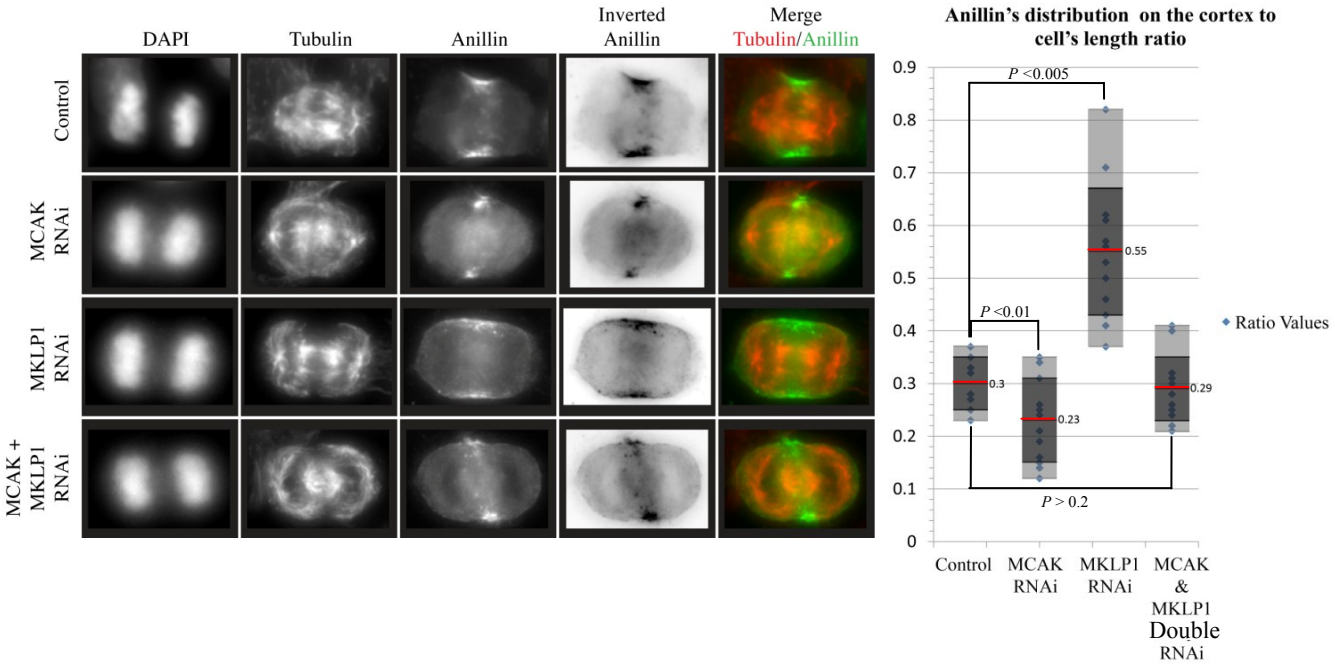

Figure 3.3: Astral and central spindle microtubules restrict anillin localization. Dividing Hela cells transfected with MCAK-RNAi, MKLP1-RNAi and co-depleted for both were fixed and co-stained for DAPI, tubulin (red) and anillin (green). Inverted images of anillin are shown. The breadth of anillin localization was calculated as a ratio of cell length. The dots represent individual cells, the red bar shows the average ratio and the dark grey shows standard deviation. *P* values are shown as indicated for the different means by the student T-test (independent samples student T-Test).

3.3 The discrete cortical localization of anillin is dependent on central spindle microtubules

The central spindle provides a stimulatory cue, RhoA activation, to form the contractile ring. It was previously shown that disruption of the central spindle causes active RhoA to spread along the cortex and is not restricted to a narrow equatorial plane. We performed MKLP1 RNAi to disrupt central spindle microtubules and determined the effect on anillin localization. Anillin localization significantly spread along the cortex, similar to what was previously observed for RhoA (0.55 for MKLP1 RNAi cells, n = 13 vs. 0.3 for control cells, n = 13; Figure 3.3). Since MCAK RNAi caused anillin to localize more narrowly, possibly due to the inhibitory role of astral microtubules, we co-depleted MKLP1 and MCAK to determine if combining them could restore a restricted zone of anillin. Indeed, in Hela cells treated with RNAi to both MKLP1 and MCAK RNAi simultaneously, anillin localization was more restricted in comparison to MKLP1 RNAi cells (0.29 for MKLP1 and MCAK RNAi cells, n = 13 vs. 0.3 for control cells, n = 13; Figure 3.3). Interestingly, anillin localization always followed the boundary of the astral microtubules, suggesting that their primary function is to delineate the localization boundary of anillin and other contractile ring proteins. Since anillin localizes to astral microtubules, one hypothesis is that anillin helps establish the division plane by 'reading' the position of these microtubules.

3.4 Anillin localizes to taxol-stabilized microtubules in mitotic cells

In addition to anillin's localization to astral microtubules, we observed anillin on central spindle microtubules, particularly in late anaphase and telophase cells. Central spindle proteins directly interact with anillin, suggesting that anillin could crosslink the central spindle to the cortex to maintain the division plane during furrowing. In furrowing cells, although active RhoA is initially high, it's activity decreases as furrow ingression completes. Since anillin is a RhoA effector, once RhoA activity diminishes, this could free anillin to localize to other proteins or locations, including its enrichment on the central spindle. To further investigate the relationship between anillin and central spindle microtubules, we manipulated the stability of microtubules and observed any effects on anillin localization. Taxol is an anticancer drug that stabilizes microtubules. Hela cells in anaphase were treated with 10μM taxol, then fixed and stained for anillin (red) and microtubules (green; Figure 3.4). Over time, anillin localization shifted from the cortex to microtubules in taxol-stabilized cells, and was predominantly at the cortex in control cells (Figure 3.4). To quantitate this change in localization, we measured the fluorescence intensity of anillin localization on the cortex in comparison to microtubules (n = 16 control cells vs. n = 20 cells after 5 min, 12 after 20 min, 12 after 30 min and 20 after 40 min taxol treatment, dots represent individual cells; dark grey shows standard deviation and the red line shows the mean; graph Figure 3.4). All of the taxol-treated cells had significantly lower cortical: microtubule ratios (mean of 1.39 after 5 min, 0.75 after 20 min, 0.69 after 30 min and 0.53 after 40 min taxol treatment) in comparison to control cells (mean of 1.93). Therefore, anillin's localization to microtubules occurs most predominantly after 20 minutes of taxol treatment. These results show that anillin can

localize to stable microtubules and that microtubule instability might be important for anillin's cortical localization.

The mitotic spindle undergoes morphological changes in anaphase cells treated with taxol; the spindle poles move apart and there is no obvious central spindle. We stained taxol-treated anaphase Hela cells with antibodies to Plk1 (a component of the central spindle) to determine how stabilizing microtubules affects the central spindle microtubules and/or proteins. Although there was no obvious central spindle structure based on tubulin staining, we observed Plk1 localization along the tips of stabilized microtubules (Figure 3.5). This result suggests that taxol-stabilized microtubules arise from the central spindle or that central spindle proteins are recruited onto stable astral microtubules. This also suggests that anillin preferentially localizes to stable microtubules labeled with central spindle proteins and one hypothesis is that anillin's localization to these microtubules could mimic its recruitment to the central spindle in telophase cells, after levels of active RhoA decrease.

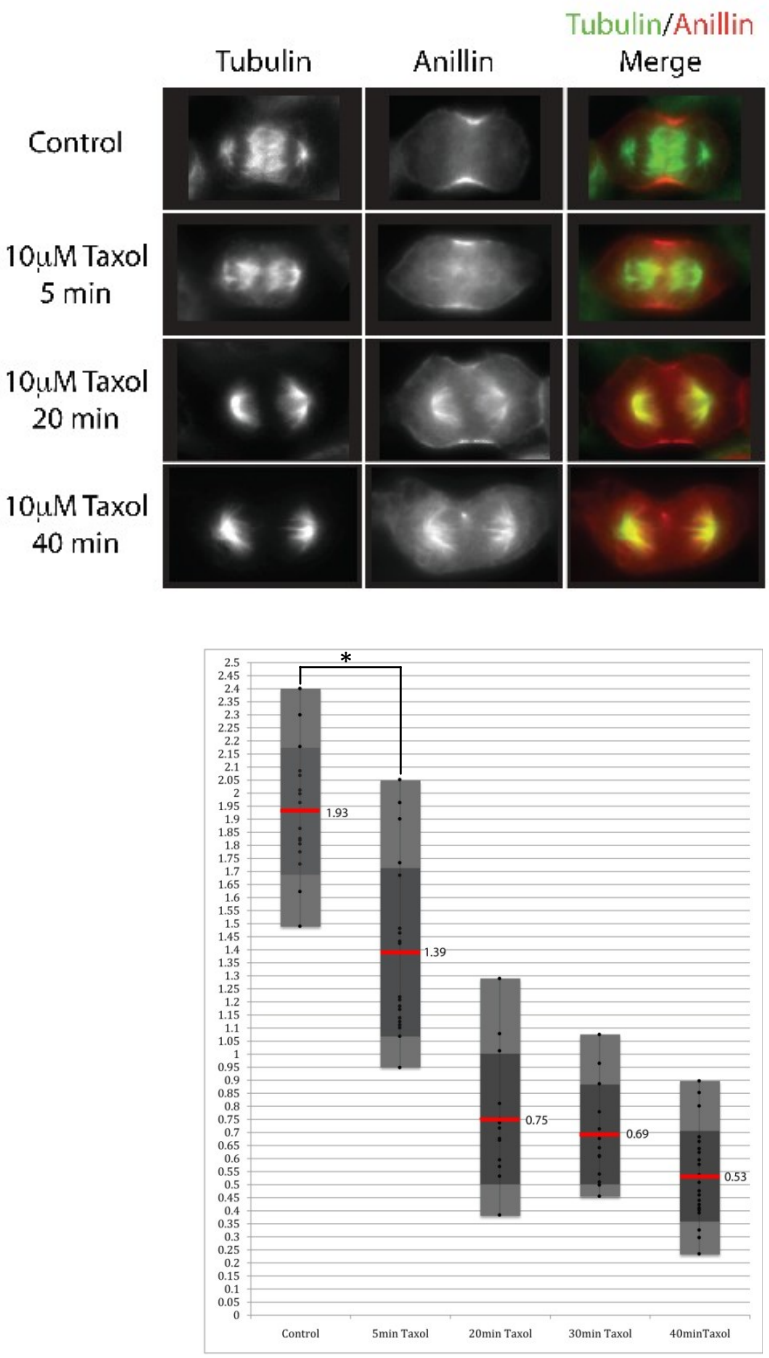

Figure 3.4: Anillin's cortical localization depends on microtubule instability. Hela cells were treated with taxol for varying periods of time as indicated, then were fixed and co-stained for tubulin (green) and anillin (red), and colocalization appears yellow. The ratio of fluorescence intensity of anillin at the cortex vs. on microtubules (Y-axis) was measured for control cells and cells treated with taxol for varying amounts of time as indicated. The dots represent

individual cells, the red bar shows the average ratio and the dark grey shows standard deviation. $P < 0.005$ (independent samples student T-Test).

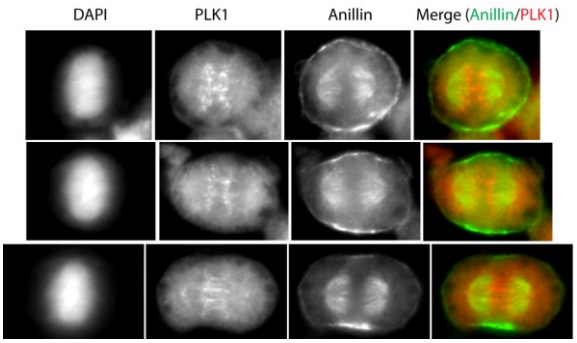

Figure 3.5: Stable taxol-treated microtubules recruit central spindle proteins. Hela cells were treated with taxol, then fixed and stained for Plk1 (a central spindle marker; red) and anillin (green).

Since we observed a strong decrease in cortical anillin localization in taxol-treated cells, we wanted to determine if other contractile ring proteins could be similarly affected. In particular, we wanted to examine RhoA localization, since anillin is a RhoA effector, and we hypothesize that active RhoA could compete with stable microtubules for anillin localization. As shown in Figure 3.6, RhoA was no longer discretely cortical after taxol-treatment. Furthermore, RhoA did not localize to microtubules, suggesting that anillin's ability to localize to microtubules is Rho-independent. Since anillin is a RhoA effector, the loss of RhoA from the cortex in taxol-treated cells could promote anillin's re-localization away from the cortex and on to microtubules. This could mimic anillin's enrichment onto central spindle microtubules in telophase cells, when RhoA activity decreases.

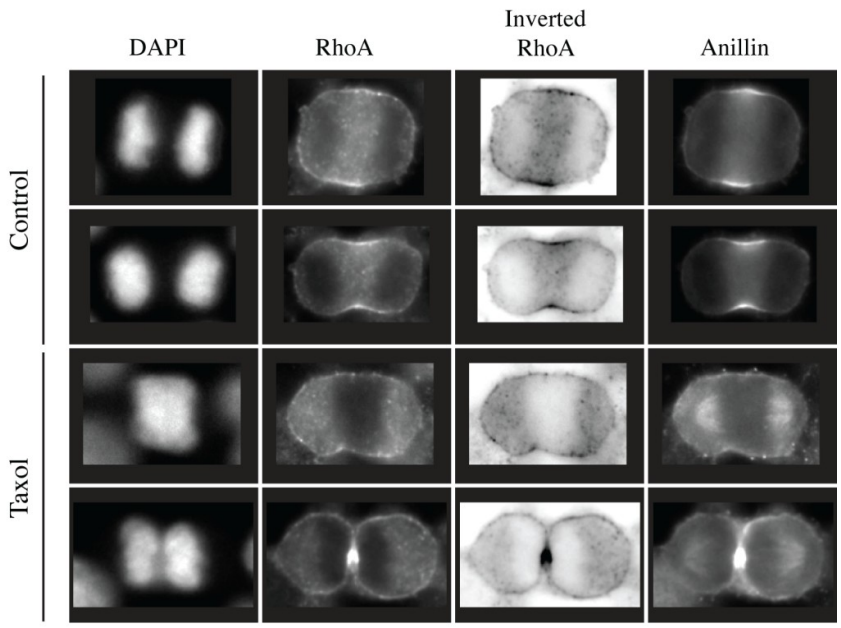

Figure 3.6: RhoA localization requires microtubule instability. Dividing Hela cells treated with taxol were co-stained for DAPI, RhoA and anillin. Inverted images for RhoA are shown.

3.5 The localization of anillin to taxol-stabilized microtubules is central spindle-dependent

We showed that anillin localizes to the central spindle in telophase cells when RhoA activity decreases, and anillin localizes to taxol-stabilized microtubules that are decorated with central spindle proteins. To investigate the dependence of anillin's localization on the central spindle, we disrupted the central spindle by either MKLP1 RNAi or using 1μM hesperadin (inhibitor of Aurora B kinase) in combination with taxol treatments. As described earlier, MKLP1 and Aurora B contribute to central spindle formation and their removal disrupts the central spindle. As shown in Figure 3.7 and Table 3.1, anillin no longer localized to microtubules after disruption of the central spindle in taxol-treated cells and re-localized to the cortex in all experimental conditions

(except control cells treated with taxol alone). In addition, depending on the length and timing of the treatments (MKLP1 RNAi for 30 hours, or hesperadin for 30 or 40 minutes), anillin localized broadly over the entire cortex or in a discrete cortical location, respectively. There was one exception with the hesperadin treatment, when cells were treated with taxol and hesperadin simultaneously for 30 min and anillin also localized broadly at the cortex. This experiment suggests that as long as the central spindle has a chance to form and contact the cortex, this may cause anillin to localize discretely to the cortex after its disruption, vs. broadly when the central spindle may not have formed and contacted the cortex. Therefore, anillin's ability to localize to taxol-stabilized microtubules is central spindle-dependent. Furthermore, anillin's discrete cortical localization requires an intact central spindle that has contacted the cortex.

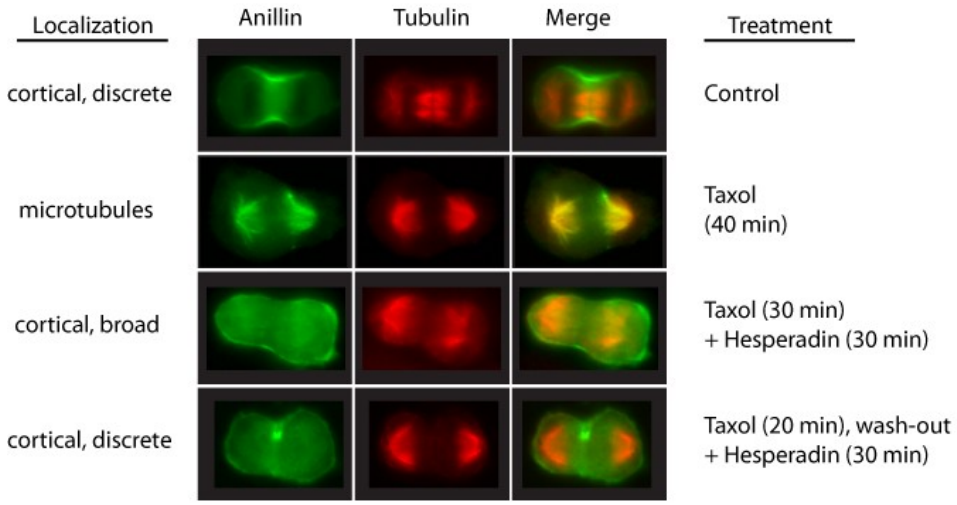

Figure 3.7: The central spindle is required for anillin's localization to microtubules in taxol-treated cells. Hela cells in late anaphase/early telophase treated with either MKLP1 RNAi (for 30 hours) or the Aurora B kinase inhibitor hesperadin (20-30 minutes) and taxol (20-30 minutes) were fixed and co-stained for anillin (green) and tubulin (red). Colocalization is yellow. Table 3.1 shows the different treatments that were used in this experiment.

Treatment	Localization
Control (no treatment)	Cortical, discrete
Tax (40min)	Microtubules
MKLP1 RNAi	Cortical, broad
MKLP1 RNAi + Tax (40min)	Cortical, broad
Tax (30min) + Hes (30min)	Cortical, broad
Tax (20min) wash-out + Hes (30min)	Cortical, discrete
Hes (30min) wash-out + Tax (20min)	Cortical, discrete
Hes (40min) + Tax (20min)	Cortical, discrete
Tax (40min) + Hes (20min)	Cortical, discrete

Table 3.1: The different treatments of HeLa cells showed that anillin's localization to taxol-stabilized microtubules is dependent on central spindle microtubules.

3.6 Anillin localizes to central spindle microtubules in the absence of active RhoA

Anillin is enriched on central spindle microtubules during late telophase, after RhoA activity decreases. In earlier stages of cytokinesis, anillin is enriched on the cortex in the equatorial plane. To assess the importance of RhoA in anillin's localization, we decreased the levels of active RhoA using RNAi to its GEF, Ect2. In Ect2-depleted cells, anillin localized more strongly to central spindle microtubules (Figure 3.8) in comparison to control cells where it localized cortically (Figure 3.8). Anillin's central spindle localization appeared more strongly in telophase cells in comparison to anaphase cells. The central spindle was still present after Ect2 depletion, although other studies in our lab have shown that cortically-associated pools of central spindle microtubules and proteins are decreased in Ect2 RNAi cells (unpublished observations). These results suggest that RhoA activation is required to enrich anillin localization on the cortex, and in its absence,

anillin localizes to central spindle microtubules. Furthermore, anillin's ability to localize to either the cortex or central spindle microtubules is cell cycle-dependent.

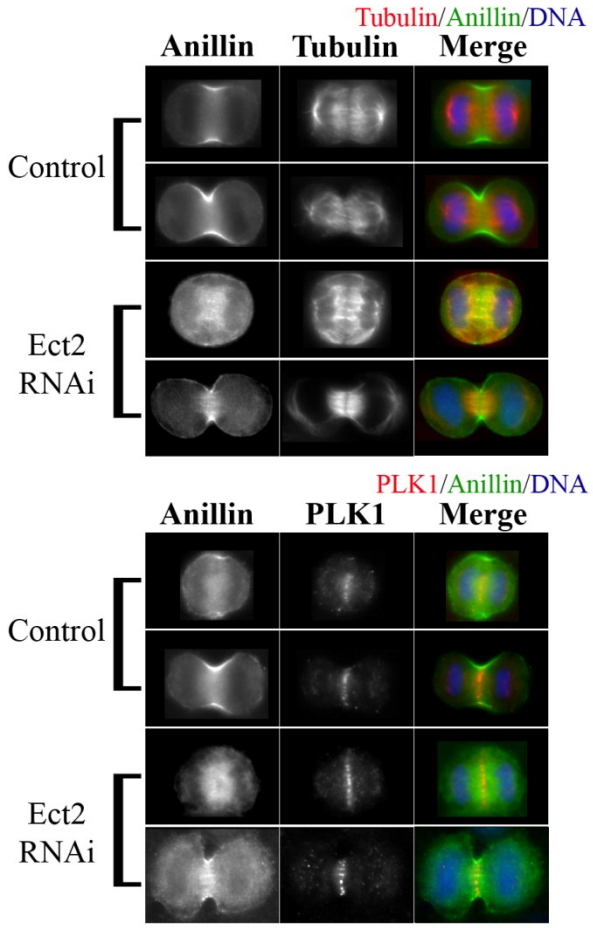

Figure 3.8: The cortical localization of anillin is Ect2-dependent. Hela cells were treated with Ect2 RNAi for 30 hours, fixed and co-stained for DNA (blue), anillin (green) and tubulin or Plk1 (red). For both control and Ect2 RNAi cells, an anaphase cell is shown in the top panel, and a telophase cell in the bottom panel.

3.7 The localization of anillin to either the cortex or to microtubules is Cdk-dependent

Active RhoA recruits anillin to the cortex, and we found a cell cycle timing in the enrichment of anillin either to the cortex or to central spindle microtubules in the absence of active RhoA. As previously described, RhoA activation is Cdk1-dependent. After entry into anaphase, Cdk1 activity drops and Ect2 is dephosphorylated, permitting its activation and recruitment to the central spindle through its interaction with Cyk-4. Therefore, the cortical recruitment of anillin requires Cdk1 inactivation. To determine if Cdk1 inactivation is also required for anillin's localization to taxol-stabilized microtubules, we treated cells with taxol for ~3 hours, which arrested cells at metaphase with fragmented centrosomes. We then added the Cdk1 inhibitor purvalanol A and observed the effect on anillin localization. As controls, we also treated cells with other drugs for about 3 hours (nocodazole – blocks cells in metaphase and STC – blocks cells in prometaphase) then with purvalanol A to observe anillin's recruitment to the cortex. As shown in Figure 3.9, while Cdk1 inhibition stimulated cortical recruitment of anillin in cells blocked with nocodazole or STC, Cdk1 inhibition stimulated anillin's recruitment to microtubules in taxol-blocked cells. This result suggests that anillin's ability to localize to microtubules also requires cell cycle exit, via Cdk1 inhibition, and anillin is only competent to interact with either the cortex or microtubules during later stages of mitosis.

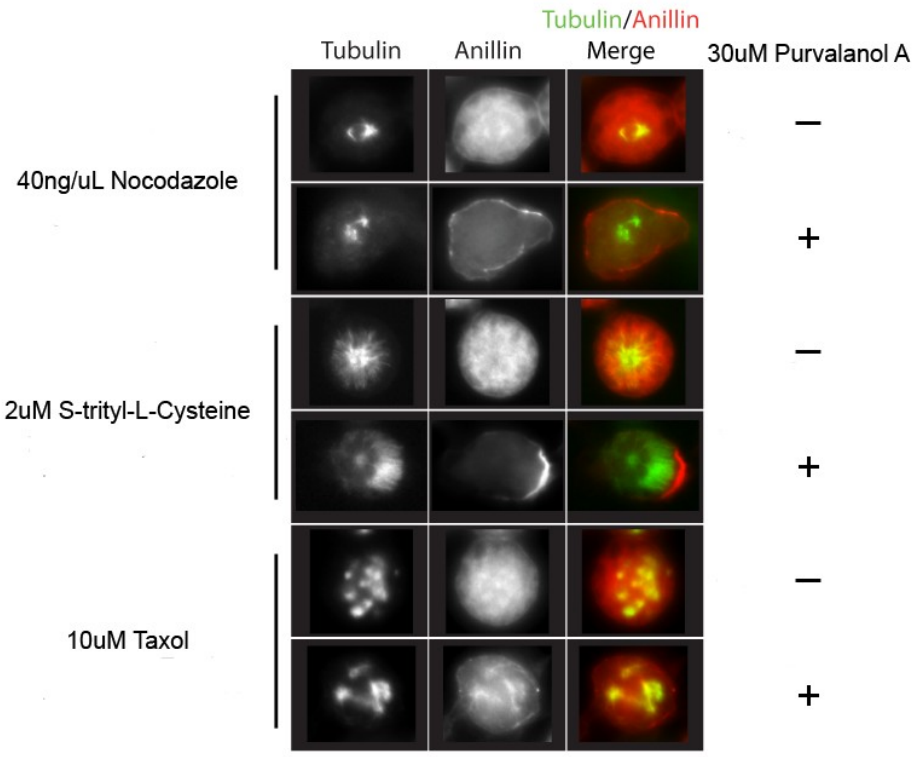

Figure 3.9: Anillin's localization to the cortex or to microtubules depends on Cdk1 activity. Hela cells were treated for three hours with nocodazole, STC or taxol as indicated by the star, which arrested the cells in either prometaphase or metaphase, then treated with the Cdk1 inhibitor purvalanol to stimulate mitotic exit. Cells were fixed and co-stained for tubulin (green) and anillin (red).

3.8 Identifying microtubule-targeting sites in anillin

Our results show that anillin localizes to microtubules, and this localization is enriched by Cdk1 inactivation, as well as by decreased RhoA activity. We also showed that anillin's enrichment to microtubules changes from astral microtubules to central spindle microtubules as the cell cycle progresses. Our next step was to identify a minimal region for mediating localization (and possibly binding) to microtubules in anillin. We co-transfected various GFP-tagged RNAi resistant anillin deletion constructs (missing various domains or regions of the protein; Figure 3.10) with anillin RNAi, then treated

cells with taxol to determine their localization to taxol-stabilized microtubules. In general, we observed strong localization, weak localization or no localization (examples of cells for each category are shown; graph shows percentage of elongated cells; Figure 3.10). All constructs containing C-terminal regions were able to localize to microtubules (although some were stronger vs. others). Therefore, the minimal targeting region maps to the C-terminus of anillin, although we cannot rule out the hypothesis that there may be multiple microtubule-targeting sites and we are currently testing this.

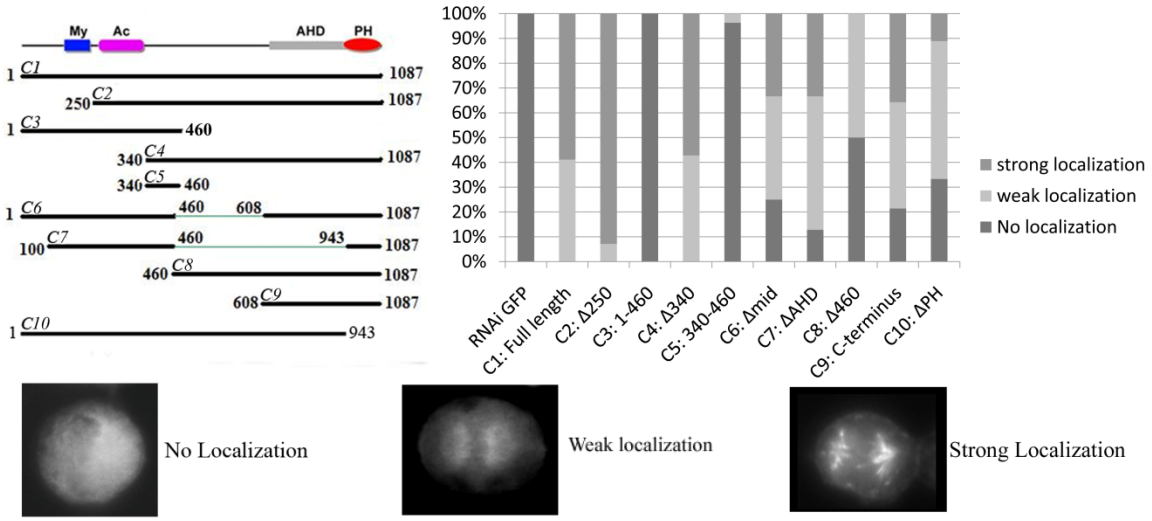

Figure 3.10: The microtubule-localizing region on anillin maps to the C-terminus. Different GFP-tagged RNAi-resistant anillin constructs were co-transfected with anillin RNAi and analyzed for their ability to localize to microtubules in taxol-treated cells (schematic of constructs in the elongated cells is shown at top left). Three predominant patterns were observed; no localization (cytosol), weak localization (cytosol and microtubules) and strong localization (microtubules). A graph of the proportion of localization patterns observed for each construct in elongated cells (graph for round cells is not shown) is shown at the upper right of the figure and examples of cells for each type of localization are shown below. Table 3.2 with the actual data is shown below.

Quantitative Analysis		Transfected	No Localization	Weak Localization	Strong Localization
RNAi GFP	Round Cells	19	19	0	0
	Elongated Cells	5	5	0	0
Δ 250	Round Cells	16	8	4	4
	Elongated Cells	14	0	1	13
Δ 340	Round Cells	53	39	12	2
	Elongated Cells	7	0	3	4
340-460	Round Cells	20	20	0	0
	Elongated Cells	7	6	1	0
1-460	Round Cells	12	12	0	0
	Elongated Cells	8	8	0	0
Δ 460	Round Cells	12	11	1	0
	Elongated Cells	6	3	3	0
Δ mid	Round Cells	41	34	6	1
	Elongated Cells	11	3	5	4
Δ AHD	Round Cells	97	59	38	0
	Elongated Cells	39	5	21	13
Δ PH	Round Cells	44	41	3	0
	Elongated Cells	9	3	5	1
C-terminus	Round Cells	37	27	8	2
	Elongated Cells	14	3	6	5
Full Length	Round Cells	29	9	20	0
	Elongated Cells	17	0	7	10

Table 3.2: The number of cells showing no localization, weak localization or strong localization to microtubules for both round cells (likely in metaphase or early anaphase) and elongated cells (late anaphase or telophase). (Nona Chamankhah helped to quantitate the number of cells for the different constructs)

To determine if anillin binds to microtubules, we transfected cells with a GFP-tagged construct (Δ250), which localizes strongly to taxol-stabilized microtubules, and assessed its ability to co-sediment with either taxol-stabilized microtubules or non-treated microtubules from cell lysates. We also determined if anillin can be released from microtubules after their depolymerization with nocodazole (and cold) treatment. A western blot co-stained for GFP (to detect anillin) and tubulin is shown in Figure 3.11.

Fractions were collected from the supernatant of cell lysates (shown on the left) either with or without taxol treatment. Fractions collected from pellets treated with nocodazole and cold are shown on the right. The ratios of tubulin and anillin released from the pellet (by cold temperature and nocodazole) vs. the supernatant were calculated based on fluorescent intensity and show that proportionally more anillin was released from the taxol-pellet vs. the control pellet (Figure 3.11). Our data suggests that anillin preferentially co-sediments with taxol-stabilized microtubules. We are carrying out additional co-sedimentation experiments using purified microtubules and purified anillin protein, or lysates with over-expressed anillin to further analyze anillin's ability to bind microtubules.

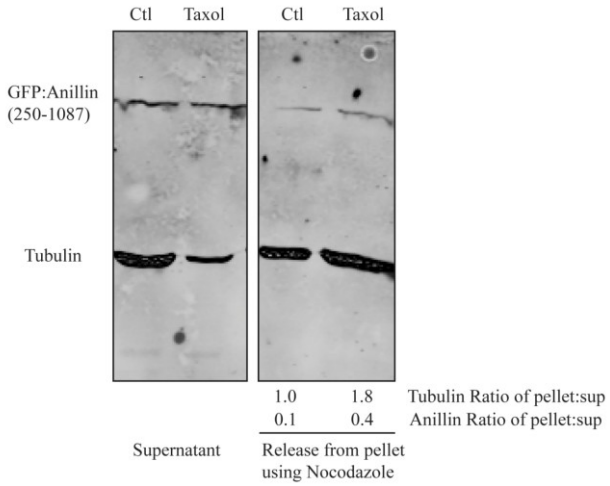

Figure 3.11: Anillin may co-sediment with microtubules. Lysates from cells transfected with GFP:anillin (250-1087) treated with taxol or no drug (control) were pelleted. Supernatants are shown in the western blot on the left, co-stained for GFP (to detect anillin) and tubulin. Pellets were resuspended and treated with cold and nocodazole, and re-pelleted. Supernatants are shown in the western blot on the right, co-stained for GFP and tubulin. The ratio of tubulin or anillin in the pellet vs. the supernatant were calculated based on fluorescence intensity and are shown below.

4. Discussion

Our results show that anillin may interact with microtubules during cytokinesis. We propose that this interaction communicates the position of the mitotic spindle to the cortex to define cortical domains. During early cytokinesis, anillin is on astral microtubules, which may deliver anillin to the central region of the cortex, but also negatively regulates its localization in the polar cortex to help define the division plane. During later stages of cytokinesis, anillin is on central spindle microtubules, which are required to maintain the division plane and form a stable midbody.

4.1 Anillin Localizes to Microtubules in Mitotic Cells

Anillin is an essential scaffold protein that is known to bind to various proteins at the cortex such as actin, myosin and RhoA [33]. Anillin was also found to bind to central spindle proteins such as Cyk4 and Ect2, which are required to activate RhoA in the equatorial plane of the cell [35]. Anillin depletion leads to the loss of centrally positioned microtubules toward the cortex and destabilization of the contractile ring at the division plane [33, 35]. Also, anillin is required for the localization of contractile ring proteins in cells with disrupted central spindles, suggesting it may function as part of the 'astral' pathway, which helps to determine the division plane. These results suggest that anillin might communicate the position of the mitotic spindle to the cortex to help establish and maintain the division plane.

Our results show that anillin localizes to microtubules during cytokinesis (Figure 3.1). Anillin localization shifted from astral microtubules in the early stages of

cytokinesis to central spindle microtubules and the midbody in late stages of cytokinesis. In the early stages of cytokinesis, anillin localization to astral microtubules is consistent with it being a member of the 'astral' pathway. This interaction could deliver anillin to the cortex in the central region, but could also remove or inhibit anillin in the polar regions, to promote assembly of contractile ring proteins in the centre of the cell. Anillin localization on central spindle microtubules could play a role in helping to anchor or position these microtubules at the cortex. Also, anillin's localization to the central spindle and midbody increases when active RhoA levels decrease after furrow ingression, where anillin could play a role in stabilizing the midbody. In general, this is the first time anillin has been shown to localize to microtubules in mitotic cells and supports our hypothesis that it might have an important role in communication between the mitotic spindle and the cortex. Since all of our data was collected using fixed cells, it will be important to show that the localization of anillin on microtubules also occurs in live cells (and is not an artefact of fixation) and to study the dynamics of this localization.

4.2 The discrete cortical localization of anillin is dependent on astral microtubules

Previous studies suggested that astral microtubules control the distribution of contractile ring components at the cortex. Since anillin localizes to astral microtubules in early cytokinesis, and is a contractile ring component, we determined how disruption of the astral microtubules would affect anillin localization. We used nocodazole, an anticancer drug that prevents the polymerization of microtubules. It is effective in blocking astral microtubules since they are more dynamic compared to central spindle

microtubules, which are more stable and allow us to observe the effect of losing the astral microtubules. Nocodazole-treated cells showed a broader distribution of anillin along the cortex (Figure 3.2), although we only measured cells in mid-late anaphase, and our results could differ for cells in other stages of cytokinesis. Anillin did not localize to the poles of the cell, which contradicts the hypothesis that astral microtubules have an inhibitory role in the distribution of contractile ring components and that it does this through anillin. However, there could be a limited amount of positive signal from the central spindle microtubules, and without further stimulation this also restricts the signal and the distance it can spread. The broadening of the cortical pool of anillin suggests that the astral microtubules do play some type of inhibitory or restrictive role to limit the position of the contractile ring. Since we only examined cells in late anaphase, we were unable to determine if astral microtubules are also required to deliver anillin to the cortex (in addition to negatively regulating its localization in the poles). I am currently investigating this by looking at nocodazole-treated cells in earlier stages of cytokinesis.

To further study the role of astral microtubules in positioning the division plane, we stabilized astral microtubules and determined the effect on anillin localization. MCAK is required to model the mitotic spindle and its depletion prevents depolymerization and causes longer astral microtubules to form in cells. If astral microtubules negatively control anillin cortical localization then longer microtubules should cause a more discrete localization of anillin. As shown in (Figure 3.3), MCAK-RNAi cells had more discrete anillin localization compared to that of the control cells. This supports our hypothesis that there is a negative relationship between the astral microtubules and anillin localization. However, it is not clear how the astral microtubules

are able to negatively regulate anillin and other contractile ring proteins. One hypothesis is that anillin localization to microtubules, brings it off the cortex, which may prevent the organization of contractile ring proteins. We did not notice if there was more anillin localized to microtubules, but this must be properly quantitated. Also, anillin localization on microtubules may be transient and the best way to study this may be to use live imaging.

4.3 The discrete cortical localization of anillin is dependent on central spindle microtubules

The central spindle has a stimulatory effect on contractile ring assembly and ingression, by recruiting and activating the RhoGEF Ect2 to activate RhoA in the central plane of the cell. However, it is not clear if the central spindle actually contributes to positioning the division plane, or if it is just required as a template to activate RhoA, which can diffuse to the overlying cortex. We studied the effect of central spindle disruption on anillin localization, by depleting MKLP1, which is part of the centralspindlin complex. Figure 3.3 shows that anillin localization is significantly broader in MKLP1-depleted cells compared to the control cells. This result is consistent with previous studies showing that RhoA localizes more broadly in MKLP1-depleted cells, and supports the hypothesis that the central spindle plays a role in determining the division plane.

Our results show that astral and central spindle microtubules have different, yet complementary, functions and that the disruption of any of these microtubules results in a

broader localization of cortical anillin, and potentially other contractile ring components (Figures 3.2 & 3.3). To assess how these microtubule sets work together, we combined MCAK and MKLP1 RNAi and determined the effect on anillin localization. MCAK co-depletion restored anillin localization patterns closer to control cells and interestingly, anillin always localized to the boundary of the long astral microtubules (Figure 3.3). Our results suggest that even when we disrupt the central spindle, enhancing the inhibitory effect of the astral microtubules will compensate for central spindle disruption. Therefore, the main function of the astral microtubules may be to restrict the localization of contractile ring components.

4.4 Anillin localizes to taxol-stabilized microtubules in mitotic cells

We observed anillin localization on both astral microtubules and central spindle microtubules in non-treated mitotic Hela cells. The localization to central spindle microtubules is enriched as cells progress through cytokinesis, and coincides with decreased levels of active RhoA. Since the central spindle microtubules are very stable, we determined what would happen to anillin localization if we further stabilized microtubules using taxol. Taxol is an anticancer drug that works by preventing the disassembly of the microtubule polymers, which stabilizes microtubules. Figure 3.4 shows that with increasing times of treatment, anillin localization shifted from the cortex to microtubules. Since these studies were performed with fixed cells, it will be important to replicate them using live cells, to ensure anillin's localization to microtubules is not an artefact of fixation. These results suggest that microtubule dynamic instability is

important for anillin's cortical localization and anillin's localization to stable microtubules competes with its cortical localization. One hypothesis is that taxol-treated cells mimic what happens during late stages of cytokinesis, when active RhoA levels drop. Since RhoA is the effector for recruiting anillin to the cortex, lowering its levels allows the stable central spindle microtubules to better compete for anillin. Since the population of cells used for these studies were asynchronous, it will be interesting to determine how the timing and strength of anillin's microtubule localization compares with its stage in cytokinesis. Furthermore, the striking overlapping localization pattern between anillin and microtubules suggests that anillin might be able to directly interact with microtubules.

The central spindle appeared to morphologically change after taxol treatment. We stained cells with Plk1, a central spindle marker, to help mark the central spindle microtubules. Figure 3.5 shows that central spindle proteins localized to the tips of stabilized microtubules, even though they were no longer antiparallel and overlapping. Therefore, the stabilized microtubules that anillin localized to have central spindle identity. This supports our hypothesis that anillin preferentially localizes to stable, central spindle microtubules. However, we also proposed that active RhoA competes for anillin's microtubule localization, since RhoA activity drops at the end of cytokinesis when anillin localizes more strongly to the central spindle. We examined RhoA localization in taxol treated cells, and as shown in Figure 3.6, RhoA lost its localization to the furrow in comparison to non-treated cells. Therefore, taxol-treatment may resemble the end of cytokinesis, when RhoA activity drops and anillin transfers to the central spindle microtubules.

4.5 The localization of anillin to taxol-stabilized microtubules is central spindle-dependent

Anillin localizes to taxol-stabilized microtubules marked with central spindle proteins. To determine if anillin's localization to taxol-stabilized microtubules is central spindle-dependent, we disrupted the central spindle by depleting MKLP1 or inhibiting Aurora B kinase with the inhibitor hesperadin. We also altered the timing and order of treatments. Figure 3.7 and Table 3.1 show that long-term disruption of the central spindle caused anillin localization to shift to the cortex, even in the presence of taxol. Also, if the central spindle was disrupted for shorter times, then anillin localized discretely to the cortex. We hypothesize that the central spindle must contact the cortex to cause the discrete localization of anillin that is observed with shorter hesperadin treatments. However, our studies were done using fixed cells and it will be important to replicate these experiments using live cells to better address this hypothesis. Therefore, The localization of anillin to microtubules stabilized with taxol is dependent on the central spindle and supports our hypothesis that anillin favors localization to central spindle microtubules.

4.6 Anillin localizes to central spindle microtubules in the absence of active RhoA

Our data supports the hypothesis that anillin localization is regulated by RhoA activity levels (Figures 3.1 & 3.6). When RhoA levels are high, anillin is predominantly on the cortex, but as RhoA levels decrease toward the end of cytokinesis, anillin localizes

to central spindle microtubules. To investigate the effect of RhoA activity on anillin localization, we co-transfected Hela cells with Ect2. Ect2 is the upstream activator of RhoA for cytokinesis and its depletion results in low levels of active RhoA. Figure 3.8 shows that in the absence of Ect2, anillin's localization to the central spindle was enhanced in comparison to control cells. This supports our hypothesis that the localization of anillin to the cortex is dependent on active RhoA, and that active RhoA competes with microtubules for anillin localization. These results also show that the localization of anillin to microtubules occurs independently of active RhoA (and Ect2) and anillin either directly interacts with microtubules, or indirectly interacts with microtubules through other proteins.

4.7 The localization of anillin to either the cortex or to microtubules is Cdk-dependent

Ect2, which regulates RhoA activity, requires decreased Cdk1 activity for its activation. Previous studies showed that Cdk1 inhibition is sufficient to promote the activation of RhoA and the recruitment of contractile ring proteins. We also show in (Figure 3.9), that Cdk1 inhibition in either nocodazole or STC-blocked cells caused the recruitment of anillin to the cortex. We also found that the localization of anillin to microtubules was Cdk1-dependent. Therefore, our results suggest that the localization of anillin to the cortex or to microtubules requires low Cdk1 activity. The dependence on Cdk1 suggests that anillin could be regulated by phosphorylation, which could alter its conformation for direct association with microtubules, or interaction with other proteins.

It will be interesting to determine if other cell cycle kinases (or phosphatases) regulate the localization of anillin.

4.8 Identifying microtubule-targeting sites in anillin

Our data shows that anillin localizes to microtubules; to astral microtubules in early anaphase cells, to central spindle microtubules in anaphase/telophase cells and to taxol-stabilized microtubules. This data supports an interaction between anillin and microtubules. To identify the minimal microtubule-binding region on anillin we used different anillin deletion constructs to determine their localization to taxol-stabilized microtubules. We also initiated co-sedimentation studies to biochemically study the region of anillin that mediates the interaction between anillin and microtubules. As shown in (Figure 3.10), most of the constructs localized to microtubules except for C3 (1-460) and C5 (340-460). C8 (460-1087) and C10 (1-943) showed low levels of microtubule-localization, but it is difficult to derive a conclusion based on low sample size. In general, the ability of most constructs to localize suggests that there are multiple microtubule-binding sites and these site(s) lie in the C-terminus of the protein, since all constructs except for C3 and C5 contained at least one domain from the C-terminus. We also obtained preliminary data showing that C2 co-sediments with microtubules, and may do so more strongly (not statistically analyzed) with taxol-stabilized microtubules. We are currently testing additional constructs containing only N-terminal domains, as well as more biochemical studies with C2 and in vitro purified C-term anillin protein, to test these hypotheses. Anillin could have different ways of interacting with astral vs. central spindle microtubules, and these could be mediated by different sites. We only assessed

localization in taxol-treated cells, and may only be studying the domain that mediates anillin binding to central spindle-labeled microtubules. A follow-up study could be to also identify regions that regulate anillin's interaction with astral microtubules.

Previous studies have shown that anillin can interact with central spindle binding proteins Cyk-4 and/or Ect2 depending on the organism. This interaction could bridge one of the microtubule binding sites. However, we detected anillin on microtubules in the absence of Ect2 suggesting that the ability of anillin to interact with microtubules is Ect2-independent, or anillin has multiple sites, where one is Cyk-4/Ect2-dependent and the other is independent.

In summary, our results suggest that after Cdk1 inactivation, RhoA is activated by Ect2 at the central spindle, which recruits anillin to the cortex. Anillin also localizes to astral microtubules, which may help establish the division plane by initially delivering anillin to the central cortex, but also by negatively regulating anillin and other cortical proteins. Once RhoA is strongly enriched at the cortex, anillin localization switches from astral microtubules to the cortex where it stabilizes the contractile ring. As cytokinesis progresses, anillin localizes predominantly to the cortex, but also localizes to the central spindle. It may help anchor central spindle microtubules at the cortex, to help maintain the division plane. In addition, anillin localizes more strongly to the central spindle in telophase, after RhoA activity levels have dropped. At this stage, anillin could participate to help form the midbody. Interestingly, other Rho effectors, including mDia2 (formin) and citron kinase [44, 45], also relocate to the central spindle in later stages of cytokinesis and in the absence of active RhoA suggesting that this could be a general theme of Rho effectors in cytokinesis. In general, we are describing, for the first time, interactions

between anillin and microtubules, which may perform multiple functions including communicating the position of the mitotic spindle to the cortex to help define the division plane, and for midbody transition in late stages of cytokinesis.

5. References

1. Y. Budirahardja and P. Gönczy (2009). **Coupling the cell cycle to development**. Development 136, 2861-2872
2. T. J. Mitchison and E. D. Salmon (2001). **Mitosis: a history of division**. Nature Cell Biology 3, 17-21
3. J. Pines and C. L. Rieder (2001). **Re-staging mitosis: a contemporary view of mitotic progression**. Nature Cell Biology 3, 3-6
4. M. Glotzer (2009). **The 3Ms of central spindle assembly: microtubules, motors and MAPs**. Nature reviews: Molecular cell biology 10, 9–20
5. D. N. Robinson and J. A, Spudich (2004). **Mechanics and regulation of cytokinesis**. Current Opinion in Cell Biology 16, 182–188
6. M. Glotzer (2001). **Animal cell cytokinesis**. Annu. Rev. Cell Dev. Biol. 17, 351–86
7. J. C.Canman, D. B, Hoffman and E, D, Salmon (2000). **The role of pre- and post-anaphase microtubules in the cytokinesis phase of the cell cycle**. Current Biology 10, 611–614
8. C. B. Shuster and D. R. Burgess (2002). **Transitions regulating the timing of cytokinesis in embryonic cells**. Current Biology 12, 854–858
9. A. Echard and P. H. O'Farrell (2003). **The degradation of two mitotic cyclins contributes to the timing of cytokinesis**. Current Biology 13, 373–383
10. G. Normand and R. W. King (2010). **Advances in experimental medicine and biology**. Landes Bioscience and Springer Science+Business Media, LLC. 676 , 27 – 55

11. R. Rappaport (1997). **Cleavage furrow establishment by the moving mitotic apparatus**. Develop. Growth Differ. 39, 221 – 226

12. S. P. Wheatley and Y. L. Wang (1996). **Midzone microtubule bundles are continuously required for cytokinesis in cultured epithelial cells**. The Journal of Cell Biology 135, 981-989

13. C. A. Cooke, M. M. S. Heck, and W. C. Earnshaw (1987), **The inner centromere protein (INCENP) antigens: movement from inner centromere to midbody during mitosis**. The Journal of Cell Biology 105, 2053-2067

14. S. P. Wheatley, A. Carvalho, P. Vagnarelli and W. C. Earnshaw (2001). **INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis**. Current Biology 11, 886–890

15. K. Emoto and M. Umeda (2000). **An essential role for a membrane lipid in cytokinesis: regulation of contractile ring disassembly by redistribution of phosphatidylethanolamine**. The Journal of Cell Biology 149, 1215–1224

16. M. J. Berridge (2009). **Cell signalling pathways. Cell Signalling Biology**. Portland Press Limited 2, 1 - 118

17. A. Piekny, M. Werner and M. Glotzer (2005). **Cytokinesis: welcome to the Rho zone**. TRENDS in Cell Biology 15, 651- 658

18. M. Glotzer (2005). **The Molecular requirements for cytokinesis**. Science 307, 1735 – 1739

19. M. Werner and M. Glotzer (2008). **Control of cortical contractility during cytokinesis**. Biochemical Society Transactions 36, 371–377

20. T. D. Pollard (2010). **Mechanics of cytokinesis in eukaryotes**. Current Opinion in Cell Biology, 22, 50 – 56

21. M. Glotzer (2004). **Cleavage furrow positioning**. The Journal of Cell Biology 164, 347–351

22. B. Vaart, A. Akhmanova and A. Straube (2009). **Regulation of microtubule dynamic instability**. Biochemical Society Transactions 37, 1007–1013

23. D. J. Sharp, G. C. Rogers and J. M. Scholey (2000). **Microtubule motors in mitosis**. Nature Review 407, 41 – 47

24. A. L. Manning and D. A. Compton (2008). **SnapShot: nonmotor proteins in spindle assembly**. Cell 134, 694- 695

25. M. Gatti, M. G. Giansanti, and S. Bonaccorsi (2000). **Relationships between the central spindle and the contractile ring during cytokinesis in animal cells**. Microscopy Research and Technique 49, 202–208

26. J. C. Gatlin and K. Bloom (2010). **Microtubule motors in eukaryotic spindle assembly and maintenance**. Seminars in Cell & Developmental Biology 21, 248–254

27. C. Mollinari, J. P. Kleman, W. Jiang, G. Schoehn, T. Hunter, and R. L. Margolis (2002). **PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone**. The Journal of Cell Biology 157, 1175–1186

28. M. Mishima, V. Pavicic, U. neberg, E. A. Nigg and M. Glotzer (2004). **Cell cycle regulation of central spindle assembly**. Nature 430, 908 – 913

29. Y. Nishimura and S. Yonemura (2005). **Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis**. Journal of Cell Science 119, 104-114

30. O. Yüce, A. Piekny, and M. Glotzer (2005). **An ECT2–centralspindlin complex regulates the localization and function of RhoA**. The Journal of Cell Biology 170, 571–582

31. U. Gruneberg, R. Neef, R. Honda, E. A. Nigg, and F. A. Barr (2004). **Relocation of aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2**. The Journal of Cell Biology 166, 167–172

32. A. A. Jeyaprakash, U. R. Klein, D. Lindner, J. Ebert, E. A. Nigg, and E. Conti (2007). **Structure of a survivin–borealin–INCENP core complex reveals how chromosomal passengers travel together**. Cell 131, 271–285

33. A. J. Piekny, A. S. Maddox (2010). **The myriad roles of anillin during cytokinesis**. Seminars in Cell & Developmental Biology 21, 881–891

34. L. Zhang and A. S. Maddox (2010). **Anillin**. Current Biology 20. 135 – 136

35. P. Frenette, E. Haines, M. Kinal, P. Pakarian, and A. Piekny (2011). **Anillin promotes the centralspindlin-directed activation of RhoA during cytokinesis**, Paper in progress

36. K. B. Shannon, J. C. Canman, C. B. Moree, J. S. Tirnauer, and E. D. Salmon (2005). **Taxol-stabilized microtubules can position the cytokinetic furrow in mammalian cells**. Molecular Biology of the Cell 16, 4423–4436

37. C. B. O ’ Connell, J. Lonˇ c arek, P. Hergert, A. Kourtidis, D. S. Conklin, and A. Khodjakov (2008). **The spindle assembly checkpoint is satisfied in the absence of**

interkinetochore tension during mitosis with unreplicated genomes. Journal of Cell Biology 183, 29–36

38. B. A. Wolfe, T. Takaki, M. Petronczki, M. Glotzer (2009). **Polo-like kinase 1 directs assembly of the HsCyk-4 RhoGAP/Ect2 RhoGEF complex to initiate cleavage furrow formation**. PLoS Biology 7, 1 – 15
39. A. J. Piekny and M. Glotzer (2008). **Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis**. Current Biology 18, 30–36
40. G. J. Gorbsky (2004). **Mitosis: MCAK under the Aura of Aurora B**. Current Biology 14, 346–348
41. D. O Morgan (2007). **The cell cycle: principles of control**. New Science Press
42. K. Emoto, H. Inadome, Y. Kanaho, S. Narumiya, and M. Umeda (2005). **Local change in phospholipid composition at the cleavage furrow is essential for completion of cytokinesis**. The Journal of Biological Chemistry 280, 37901–37907
43. G. R. X. Hickson and P. H. O' Farrell (2008). **Rho-dependent control of anillin behavior during cytokinesis**. The Journal of Cell Biology 180, 285–294
44. U. Gruneberg, R. Neef, X. Li, E. H. Y. Chan, R. B. Chalamalasetty, E. A. Nigg, and F. A. Barr (2006). **KIF14 and citron kinase act together to promote efficient cytokinesis**. The Journal of Cell Biology 172, 363–372
45. S. Watanabe, K. Okawa, T. Miki, S. Sakamoto, T. Morinaga, K. Segawa, T. Arakawa, M. Kinoshita, T. Ishizaki, and S. Narumiya (2010). **Rho and anillin-dependent control of mdia2 localization and function in cytokinesis**. Molecular Biology of the Cell 21, 3193–3204

46. J. C. Sisson, C. Field, R. Ventura, A. Royou, and W. Sullivan (2000). **Lava Lamp, a novel peripheral golgi protein, is required for *drosophila melanogaster* cellularization**. The Journal of Cell Biology 151, 905–917

47. L. C. Haeusler, L. Blumenstein, P. Stege, R. Dvorsky, M. R. Ahmadian (2003). **Comparative functional analysis of the Rac GTPases**. Federation of European Biochemical Societies 555, 556 – 560

48. H. Kosako, T. Yoshida, F. Matsumura, T. Ishizaki, S. Narumiya and M. Inagaki (2000). **Rho-kinase/ROCK is involved in cytokinesis through the phosphorylationof myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow**. Oncogene 19, 6059 – 6064

49. F. Di Cunto, S. Imarisio, E. Hirsch, V. Broccoli, A. Bulfone, A Migheli, C. Atzori, E. Turco, R. Triolo, G. P. Dotto, L. Silengo, and F. Altruda (2000). **Defective neurogenesis in citron kinase knockout mice by altered cytokinesis and massive apoptosis**. Neuron 28, 115–127

50. A. F. Severson, D. L. Baillie, and B. Bowerman (2002). **A formin homology protein and a profilin are required for cytokinesis and arp2/3-independent assembly of cortical microfilaments in *C. elegans***. Current Biology, Vol. 12, 2066–2075

51. M. Amano, M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi (1996). **Phosphorylation and Activation of Myosin by Rho-associated Kinase (Rho-kinase)**. The Journal of Biological Chemistry 271, 20246–20249

52. T. Tatsumoto, X. Xie, R. Blumenthal, I. Okamoto, and T. Miki (1999). **Human ECT2 is an exchange factor for Rho GTPases, phosphorylated in G2/M phases, and involved in cytokinesis**. The Journal of Cell Biolog 147, 921–927

53. A. Carvalho, A. Desai, and K. Oegema (2009). **Structural memory in the contractile ring makes the duration of cytokinesis independent of cell size**. Cell 137, 926–937

54. W. M. Saxton and J. R. Mclntosh (1987). **Interzone microtubule behavior in late anaphase and telophase spindles**. The Journal of Cell Biology 105, 875-886

55. M. G. Giansanti, S. Bonaccorsi and M. Gatti (1999). **The role of anillin in meiotic cytokinesis of Drosophila males**. Journal of Cell Science 112, 2323-2334

56. A. F. Straight, C. M. Field, and T. J. Mitchison (2005). **Anillin binds nonmuscle myosin ii and regulates the contractile ring**. Molecular Biology of the Cell 16, 193–201

57. M. Versele and J. Thorner (2005). **Some assembly required: yeast septins provide the instruction manual**. Trends Cell Biology 15, 414–424

58. A. J. Piekny and M. Glotzer (2008). **Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis**. Current Biology 18, 30–36

59. S. L. Gregory, S. Ebrahimi, J. Milverton, W. M. Jones, A. Bejsovec, and R. Saint (2008). **Cell division requires a direct link between microtubule-bound RacGAP and anillin in the contractile ring**. Current Biology 18, 25–29

60. K. Oegema, M. S. Savoian, T. J. Mitchison, and C. M. Field (2000). **Functional analysis of a human homologue of the *Drosophila* actin binding protein anillin suggests a role in cytokinesis**. The Journal of Cell Biology 150, 539–551

61. C. M. Field and B. M. Alberts (1995). **Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex**. The Journal of Cell Biology 131, 165-178

62. S. Celton-Morizur, N. Bordes, V. Fraisier, P. T. Tran, and A. Paoletti (2004). **C-terminal anchoring of mid1p to membranes stabilizes cytokinetic ring position in early mitosis in fission yeast**. Molecular and Cellular Biology 24, 10621–10635

63. M. Kinoshita, C. M. Field, M. L. Coughlin, A. F. Straight, and T. J. Mitchison (2002). **Self- and actin-templated assembly of mammalian septins.** Developmental Cell 3, 791–802

64. W. Zhao and G. Fang (2005). **MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis**. PNAS 102, 13158–13163

65. P. P. D'Avino, T. Takeda, L. Capalbo, W. Zhang, K. S. Lilley, E. D. Laue and D. M. Glover (2008). **Interaction between anillin and RacGAP50C connects the actomyosin contractile ring with spindle microtubules at the cell division site**. Journal of Cell Science 121, 1151-1158

66. S. L. Gregory, S. Ebrahimi, J. Milverton, W. M. Jones, A. Bejsovec, and R. Saint (2008). **Cell division requires a direct link between microtubule-bound racgap and anillin in the contractile ring**. Current Biology 18, 25–29