Identifying Cues that Regulate the Position of the Contractile Ring during Cytokinesis

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Abstract

Identifying Cues that Regulate the Position of the Contractile Ring during Cytokinesis

Tara Kafiyeh Akhshi

Cytokinesis is the process where a mother cell cleaves into two daughter cells and is driven by the constriction of an actomyosin ring. Formation and ingression of the contractile ring is regulated by the mitotic spindle to couple cytokinesis with the segregation of sister chromatids. The central spindle forms in anaphase and recruits Ect2, a GEF that activates RhoA, for F-actin polymerization and nonmuscle myosin activation to assemble the ring in the equatorial plane. However, the molecular mechanism that regulates the localization of contractile proteins is poorly understood. For example, astral microtubules exclude the localization of contractile proteins at the polar cortex, but the molecular pathway is not known. Furthermore, other cues likely regulate the localization of contractile proteins at the polar cortex. In this study, I investigated the role of chromatin in mediating the polar exclusion of contractile proteins in Hela cells. First, I measured the minimum distance between chromatin and the boundary of accumulated contractile proteins during cytokinesis in control cells, and in cells treated with different conditions that affect the central spindle and/or astral microtubules. I found that chromatin position closely correlates with the localization of contractile proteins within 3-4 μm of the cortex. This suggests that chromatin has a diffusible cue that can regulate the organization of actomyosin. I also found that over-expression of active Ran targeted to the equatorial membrane alters anillin localization and causes asymmetric furrow ingression or oscillation. In addition, inactivating Ran-GTP results in the global localization of myosin and cytokinesis phenotypes. My data supports the idea that chromatin, likely via Ran-GTP, works in combination with the central spindle and astral

microtubules to ensure that the contractile ring forms at the correct time and location during cytokinesis.

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I love you and wish you were still among us. I can never forget everything you have taught me and all the great moments we had together. You were an amazing human being and an incredible friend.

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Table of Abbreviations

AHD – Anillin homology domain

ANI-1 – Anillin isoform 1 (*C. elegans*)

Cdk1 – Cyclin dependent kinase 1

CPC – Chromosome passenger complex

Cyk4 – Cytokinesis defect 4 (human MgcRacGAP; *Drosophila* RacGAP50C)

DAPI - 4, 6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DTT – Dithiothreiol

Ect2 – Epithelial cell transformer sequence 2

FBS – Fetal bovine serum

FH1/2- FH Afrikaner-1/2

GAP – GTPase activating protein

GEF – Guanine nucleotide exchange factor

GFP – Green fluorescent protein tagged vector

GTP – Guanosine triphosphate

HeLa – Henrietta Lacks cervical cancer cell line INCENP

– Inner centromere protein

KIF4 – Kinesin family member 4 (also referred to as KIF4A)

MAPs – Microtubule associated proteins

MBP – Maltose binding protein

MLC- myosin light chain

MKLP1 – Mitotic kinesin like protein 1

NDS – Normal donkey serum

Plk1 – Polo-like kinase 1

PIP 2- Phophatidylinositol 4, 5 phosphate

PRC1 – Protein regulator of cytokinesis 1

Ran- Ras-related nuclear protein

RCC1-Regulation of chromosome condensation 1

RhoA – Ras homolog gene family, member A

RNAi – RNA interference

ROCK – Rho dependent kinase

TBST – Tris buffer saline (Triton X)

TCA – Trichloroacetic acid

Chapter 1. Introduction

1.1 Cytokinesis

Cytokinesis is the last step of mitosis, when the cell undergoes a drastic change in shape to pinch into two daughter cells. It is driven by the formation and ingression of an actin-myosin ring that must be properly positioned to ensure the proper distribution of cytoplasm and chromosomes to each daughter cell. Actin and nonmuscle myosin II (hereafter myosin), are enriched in a well-defined zone at the equatorial cortex, where myosin binds and crosslinks actin filaments to exert tension required for actin constriction [Green *et al*., 2012; Ma *et al*., 2012]. Failure to form or stabilize the ring at the correct location can lead to failed cytokinesis and the formation of genomically instable cells that are prone to tumorigenesis [Piekny and Maddox, 2010]. Therefore, it is crucial to understand the mechanisms responsible for contractile ring formation and cytokinesis.

Active RhoA orchestrates assembly of the contractile ring, as it regulates the activity of several downstream effectors that mediate the formation of F-actin and myosin contractility. RhoA activates formins, which stimulate actin polymerization via the formin-homology FH1 and FH2 domains that bind to profilin and actin, respectively. In the absence of RhoA, formin activity is autoinhibited when the C-terminus of formin binds to its N-terminus, which prevents the FH1 and FH2 domains from promoting actin polymerization [Piekny *et al*., 2005]. Another RhoA effector, Rho kinase (ROCK), plays an important role in cytokinesis by regulating myosin light chain (MLC) phosphorylation, which is essential for myosin activity [Piekny *et al*., 2005].

Active RhoA also recruits anillin, which organizes the contractile ring [Bement *et al*., 2005; Piekny *et al*., 2005; Piekny and Glotzer, 2008]. Anillin is a scaffold protein, which stabilizes the division plane by crosslinking actin and myosin with the mitotic spindle and overlying membrane [Field and Alberts, 1995;

Straight *et al*., 2005; Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008; Piekny and Maddox, 2010]. Interestingly, anillin stabilizes RhoA localization, suggesting that there is a feedback mechanism between components of the ring and their upstream regulators [Piekny and Glotzer, 2008]. Part of this feedback system likely involves anillin's interaction with components of the central spindle (see section 1.2.1), which provides the signals to stimulate RhoA activation. For example, anillin has been shown to interact with Ect2 (RhoA GEF) in human cells and with RacGAP50C (Cyk-4 homologue that regulates central spindle assembly and activates Ect2) in *Drosophila* [D'Avino *et al*., 2008; Gregory *et al*., 2008; Frenette *et al*., 2012]. Both of these interactions may stabilize central spindle microtubules near the cortex, to reinforce the generation of active RhoA in a focused region of the equatorial cortex. Anillin also interacts with septin filaments, which may bridge anillin's localization to the overlying membrane [Oegema *et al*., 2000; Field *et al*., 2005; Maddox *et al*., 2005; Maddox *et al*., 2007]. Interestingly, septins also interact with myosin and microtubules, but their function in cytokinesis is not clear (Figure 1) [Joo *et al*., 2007; Spiliotis, 2010; Estey *et al*., 2011; Mostowy and Cossart, 2012].

1.2 Setting up the division plane

Microtubules determine the division plane by regulating the localization of RhoA at the equatorial cortex during cytokinesis. As described earlier, active RhoA regulates contractile ring assembly by stimulating the formation of active myosin filaments and long, unbranched F-actin [Piekny *et al*., 2005]. A classic experiment demonstrated that manipulating the position of the mitotic spindle in echinoderm embryos causes furrow regression and the formation of a new furrow at the site of the newly positioned spindle [Rappaport, 1986]. A follow-up study used a probe for active RhoA to show that the localization of active RhoA dynamically responds to changes in spindle position [Bement *et al*., 2005].

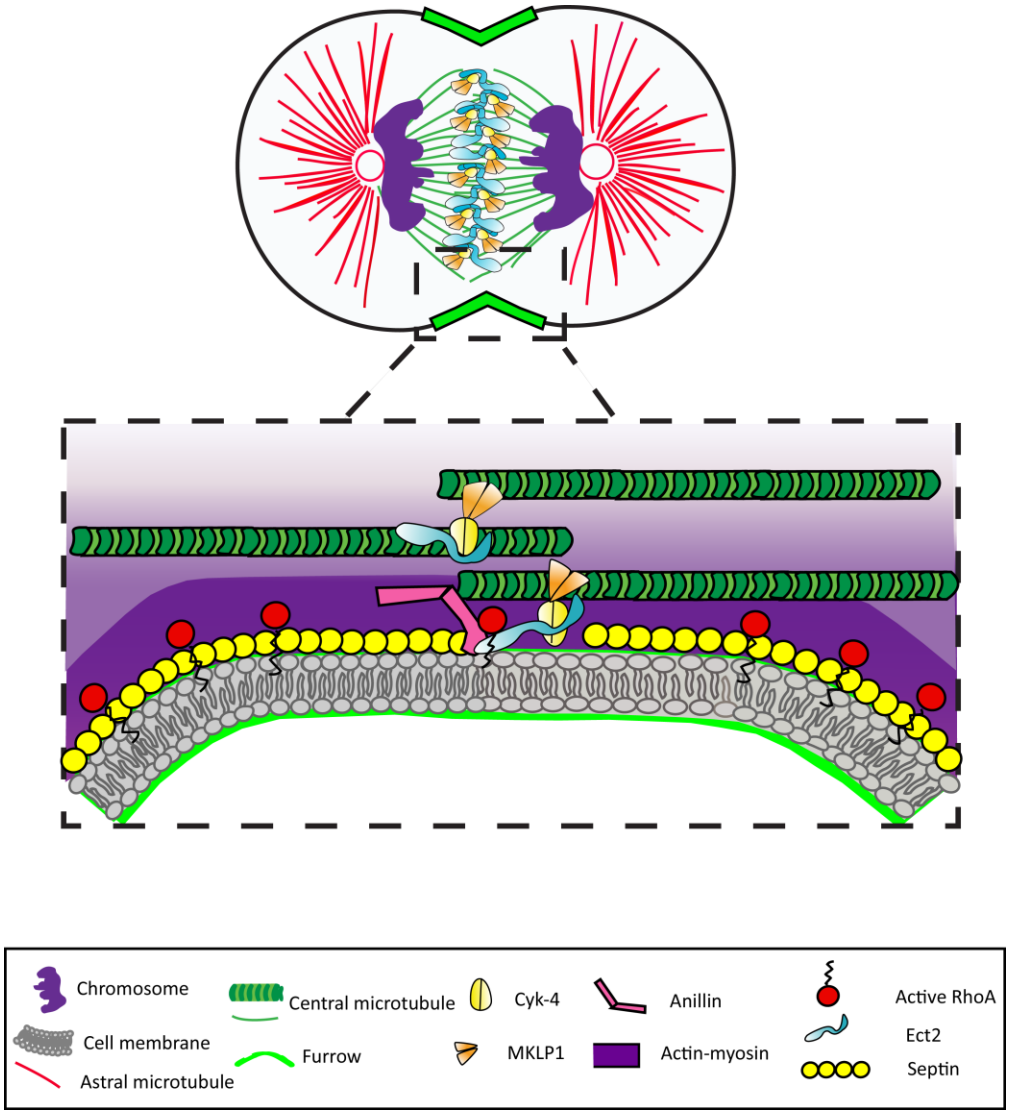


Figure 1. Cartoon schematic of a mammalian cell during furrow ingression. The central spindle delivers signals for contractile ring assembly at the equatorial cortex. Cyk4 and MKLP1, which form the centralspindlin complex, regulate central spindle assembly and recruit the RhoA GEF, Ect2, where it accumulates at the overlying cortex to activate RhoA. Active RhoA activates actin-myosin to form the contractile ring, and recruits other key components of the ring, including anillin. Anillin interacts with different proteins involved in cytokinesis including septins, actin and myosin. *Note, this figure is taken from Akhshi et al, 2013.*

The mitotic spindle provides cues that determine the division plane. The mitotic spindle is comprised of astral microtubules, which emanate from the centrosomes to the polar cortex, and the central spindle, composed of bundled, stable, antiparallel microtubules that form between the segregating chromosomes [Glotzer, 2009]. Both astral and central spindle microtubules provide signals to form and ingress the actin-myosin ring, and couples cytokinesis with chromosome segregation [Green *et al*., 2012]. In particular, polar astral microtubules exclude the localization of contractile proteins at the polar cortex and disrupting these microtubules using the drug Nocodazole causes contractile proteins to localize more broadly along the cortex [Murthy and Wadsworth, 2008]. The central spindle provides signals that direct the accumulation of contractile proteins at the equatorial cortex (Figure 1) [Fededa and Gerlich, 2012; Green *et al*., 2012; Akhshi *et al*., 2013]. There are also subsets of astral microtubules that emanate toward the equatorial cortex and are bundled by central spindle proteins [Canman *et al*., 2003; Inoue *et al*., 2004; Foe and von Dassow, 2008; Hu *et al*., 2008]. Disruption of the central spindle causes contractile proteins to localize to a broader zone, and co-disruption of both astral and central spindle microtubules causes contractile proteins to localize all around the cell cortex [Yüce *et al*., 2005; Lewellyn *et al*., 2010].

The molecular mechanism that directs contractile ring formation at the equatorial cortex via central spindle microtubules is well understood, but the mechanism by which astral microtubules regulate the localization of contractile proteins is unknown. In addition, cues that modulate the localization of contractile proteins could come from other components in the cell, such as chromatin, and it is not known how these systems work together.

1.2.1 The central spindle

During anaphase, several protein complexes bundle the anti-parallel microtubules that arise between segregating chromosomes to form the central

spindle. PRC1 selectively bundles the plus ends of anti-parallel microtubules and is negatively regulated by Cdk1 phosphorylation [Mishima *et al*., 2004; Glotzer, 2009; White and Glotzer, 2012]. PRC1 also interacts with kinesin KIF4, which regulates the length of the spindle midzone [Bieling *et al*., 2010]. Inhibition of KIF4 results in the formation of longer spindles and a broader zone of active RhoA during cytokinesis [Hu *et al*., 2011]. The chromosome passenger complex (CPC) and centralspindlin are two other complexes that play important roles in central spindle formation [Ruchaud *et al*., 2007; Carmena *et al*., 2009; Fededa and Gerlich, 2012]. Centralspindlin is a heterotetramer comprised of MKLP1 (a kinesin 6 motor protein) and MgcRacGAP (hereafter Cyk-4; a Rho family GTPase) [Mishima *et al*., 2002; Mishima *et al.*, 2004; Green *et al*., 2012]. Disrupting centralspindlin interferes with central spindle formation, and causes an increase in the breadth of RhoA localization [Yüce *et al*., 2005]. The CPC complex includes INCEP, Survivin and Aurora B kinase, and regulates the stable accumulation of centralspindlin at the midzone. For example, Aurora B phosphorylates MKLP1, which may release it from 14-3-3, a protein that impedes central spindle formation before anaphase [Douglas *et al*., 2010]. The CPC and centralspindlin may also function in separate pathways, since mutations in components of either complex in *C. elegans* embryos have milder phenotypes vs. when they are combined [Lewellyn *et al*., 2011]. Furthermore, the CPC may mediate crosstalk between microtubules and the actin-myosin cytoskeleton, particularly during the polarization of cells [Canman *et al*., 2003; Hu *et al*., 2008].

One of the main functions of centralspindlin is to regulate contractile ring formation via recruiting and activating Ect2, the GEF (guanine nucleotide exchange factor) for RhoA (Figure 1) [Yüce *et al*., 2005]. Ect2 is negatively regulated by Cdk1 phosphorylation, and in anaphase it binds to Cyk-4 via its N-terminus. Formation of the Ect2/Cyk-4 complex also requires Plk1 phosphorylation of Cyk-4 [Petronczki *et al*., 2007; Wolfe *et al*., 2009]. Ect2 is recruited to the central spindle through its interaction with Cyk-4, where it

stimulates RhoA activation at the overlying membrane [Satio *et al*., 2003; Somers and Saint, 2003; Yüce *et al*., 2005; Zhao and Fang, 2005]. Ect2 also interacts with the membrane, likely through domains in its C-terminus, which may provide Ect2 with access to RhoA [Chalamalasetty *et al*., 2006; Su *et al*., 2011; Frenette *et al*., 2012]. It is not clear how Ect2's interaction with Cyk-4 is coordinated with its membrane localization. Interestingly, the C1 domain of Cyk-4 also interacts with the membrane [Lekomtsev *et al*., 2012]. Cyk-4's ability to interact with the membrane may stabilize the Cyk-4/Ect2 complex to position Ect2 for RhoA activation. Furthermore, anillin also interacts with RacGAP50C (the *Drosophila* homologues of Cyk-4) in *Drosophila* or with Ect2 in human cells, and depleting anillin leads to a decrease in central spindle microtubules near the equatorial cortex [D'Avino *et al*., 2008; Gregory *et al*., 2008; Zhao and Fang 2005; Frenette *et al*., 2012]. Studies in *C. elegans* and in human cells have shown that Cyk-4 also may act as a GAP (GTPase activating protein) for Rac. Rac promotes the formation of short, branched F-actin by regulating the activity of effectors that modulate Arp2/3. Decreasing active Rac at the equatorial cortex, while permitting higher levels to accumulate at the cell poles, likely lead to the enrichment of different types of actin filaments that impart different cortical properties to the cell [Canman *et al*., 2008; Bastos *et al*., 2012]. However, some of the results from these studies could be explained by Cyk-4's role in promoting RhoA activation [Loria *et al*., 2012].

1.2.2 Astral Microtubules

Subsets of astral microtubules exclude the localization of contractile proteins at the polar cortex during cytokinesis, however, the molecular mechanism by which astral microtubules alter the organization of contractile proteins is not clear. Early experiments using echinoderm embryos and fused Ptk1 cells showed that furrows always form between astral arrays of microtubules [Rappaport, 1996; Oegema and Mitchison, 1997]. Studies using *C. elegans*

embryos demonstrated that contractile ring formation is first determined by signals coming from the astral microtubules, then from the central spindle [Dechant and Glotzer, 2003; Bringmann and Hyman, 2005]. When asters are too close together, furrow formation is blocked even in the presence of a spindle midzone, suggesting that astral microtubules inhibit the localization of contractile proteins [Dechant and Glotzer, 2003; Bringmann *et al*., 2005; Werner *et al*., 2007; Lewellyn *et al*., 2010]. As the centrosomes separate during anaphase, the furrow always forms at the region with the lowest density of astral microtubules [Dechant and Glotzer, 2003]. Also in *C. elegans* embryos, mutations that decrease astral microtubule length and shift the spindle toward one pole of the cell result in the formation of a second furrow [Werner *et al*., 2007, Tse *et al*., 2011].

In human cells, although contractile proteins spread along the cortex after disrupting the central spindle, they are restricted from the polar cortex, suggesting that astral microtubules also play an important role in restricting the localization of contractile proteins [Yüce *et al*., 2005]. Furthermore, disruption of astral microtubules using Nocodazole causes an increase in the breadth of RhoA localization [Murthy and Wadsworth, 2008].

Although the molecular mechanism by which astral microtubules regulate the localization of contractile proteins is not well understood, studies in *C. elegans* and human cells demonstrated that anillin may also be involved, since it localizes to astral microtubules [Tse *et al*., 2011; van Oostende, Haji Bik and Piekny, unpublished observations]. Anillin's cortical recruitment by active RhoA may compete with its localization to astral microtubules. This leads to the enrichment of anillin at the equatorial cortex, and its removal from the polar cortex, which would alter the organization of actin and myosin at the polar cortex [Tse *et al*., 2011; Haji Bik, Garcia Jaramillo and Piekny, unpublished data]. In support of a role for anillin in the 'astral pathway', while furrow forms and often ingress after disrupting the central spindle, co-depletion of anillin blocks furrowing altogether [van Oostende, Haji Bik and Piekny, unpublished observations].

A recent study using *C. elegans* embryos and human cells found the Rho GAP that likely counteracts Ect2's activation of RhoA for cytokinesis [Zanin *et al*., 2013]. This GAP, called MP-GAP, localizes around the entire cell cortex, where it globally downregulates RhoA activity [Zanin *et al*., 2013]. The localized enrichment of active Ect2 in the equatorial plane leads to the restricted generation of active RhoA in this plane. Interestingly, depletion of MP-GAP in human cells causes ectopic cortical contractility in the cell cortex, but cytokinesis occurs successfully and the zone of RhoA does not differ from control [Zanin *et al*., 2013]. Co-disruption of astral microtubules using Nocodazole causes RhoA to localize around the entire periphery of the cell, suggesting that the astral microtubules redundantly function with MP-GAP to exclude the localization of active RhoA [Zanin *et al.*, 2013]. Taken together, these studies lead to the conclusion that astral microtubules play a crucial role in restricting the localization of RhoA, but are not the only regulators of this pathway.

1.2.3 Chromatin/ Ran-GTP

Cues that restrict the localization of contractile proteins at the polar cortex also could come from chromatin. Ran is a GTPase that functions in nuclear transport, mitotic spindle assembly, and spindle positioning [Clarke and Zhang, 2008; Roscioli *et al*., 2010]. The levels of active Ran (Ran-GTP) are controlled by RCC1, the GEF for Ran, and RanGAP, which promotes GTP hydrolysis [Drivas *et al*., 1990; Ren *et al*., 1993]. In interphase cells, RCC1 levels are higher in the nucleus and RanGAP is higher in the cytosol to keep the levels of Ran-GTP high inside the nucleus. Ran mediates nuclear transport via its interaction with importins and exportins. In the cytosol, cargo proteins are imported into the nucleus by binding to importins, and are released when active Ran binds to importin beta causing the complex to dissociate [Clarke and Zhang, 2008; Roscioli *et al*., 2010]. Ran-GTP also interacts with exportins in the nucleus and facilities the removal of cargo out to the cytosol where RanGAP promotes inactivation of Ran and dissociation of the

complex [Clarke and Zhang, 2008; O'Connell *et al*., 2009]. RCC1 binds to histones H2A and H2B, which localizes active Ran to DNA and enhances its activity [O'Connell *et al*., 2009; Roscioli *et al*., 2010; Hutchins *et al*., 2011].

During mitosis, RCC1 remains associated with chromatin, where it generates high levels of Ran-GTP and promotes the dissociation of regulators of mitotic spindle assembly from importins. These microtubule motors and MAPs (microtubule associated proteins) regulate microtubule dynamics and organization to form the bipolar spindle [Carazo-Salas *et al*., 1999; Kalab *et al*., 1999; Ohba *et al*., 1999; Wilde *et al*., 1999; Zhang *et al*., 1999]. Using fluorescence resonance energy transfer (FRET)-based studies with *Xenopus* egg extracts, elevated levels of Ran-GTP were shown to persist in the vicinity of chromosomes during metaphase. The high levels of chromatin-associated Ran-GTP caused the release of importin-beta from spindle assembly factors such as NuMA and TPX2, to facilitate spindle assembly [Kaláb *et al*., 2002; Kaláb *et al*., 2006; Roscioli *et al*., 2010]. Experiments that lowered the levels of Ran-GTP resulted in a lower density of microtubules and a disorganized spindle. Conversely, increasing the levels of active Ran or using Ran mutants that are defective in hydrolysis, caused an increase in the polymerization of microtubules, even in the absence of centrosomes or chromosomes [Khodjakov *et al*.,2000; Kaláb *et al*., 2002; Clarke and Zhang, 2008; O'Connell *et al*, 2009; Roscioli *et al*., 2010]. Furthermore, these asters contained centrosome-associated proteins such as gamma-tubulin and NuMA [Wilde *et al*., 1999; Zhang *et al*., 1999; Clarke and Zhang, 2008]. Therefore, Ran-GTP promotes spindle assembly via releasing spindle assembly factors from importins. However, the over-expression of importin-beta in somatic cells did not block mitosis, but only postponed prometaphase, and increased the frequency of monopolar spindles, suggesting that Ran may be partially redundant with other mechanisms for mitosis [Kaláb *et al*., 2006; Clarke and Zhang, 2008].

Ran also regulates spindle orientation during metaphase [Kiyamitsu and Cheeseman, 2012]. Correct spindle position involves the cortically-associated

microtubule tethering complex, Galphai/LGN/NuMA, which interacts with the minus end motor protein complex, dynein-dynactin, to exert pulling forces on astral microtubules. Ran positions the mitotic spindle by blocking accumulation of the NuMA/LGN complex at the lateral cortex in the vicinity of chromosomes. This ensures that the microtubule-tethering complex accumulates at the polar cortex where it generates asymmetric pulling forces to re-position the spindle [Kiyamitsu and Cheeseman, 2012]. During anaphase, NuMA forms a different complex with 4.1G/R, which may continue to position the mitotic spindle through the latter stages of mitosis [Kiyamitsu and Cheeseman, 2013]. However, the role of Ran-GTP in regulating spindle position may differ among eukaryotes. For example, *Drosophila* Ran-GTP positively regulates localization of the LGN complex during asymmetric cell division [Wee *et al*., 2011].

Ran also may restrict the localization of contractile proteins at the polar cortex to determine the division plane during cytokinesis. Experiments showed that anillin localization decreased at the cell cortex that was in closest proximity to chromatin [Kiyomitsu and Cheeseman, 2013]. If the spindle shifts asymmetrically toward one pole, this system could recover a central division plane by shifting the localization of contractile proteins away from one pole, causing the 'depleted' cortex to expand outward. This mechanism of 'asymmetric membrane elongation' may help maintain the division plane, and coordinate it with chromatin segregation [Kiyomitsu and Cheeseman, 2013]. Although this data strongly supports a role for Ran as a negative regulator of contractile protein localization during cytokinesis, a study using *Drosophila* embryos proposed that Ran-GTP positively regulates celullarization in the syncytial *Drosophila* embryo, a process that shares some similarities with cytokinesis. Importin alpha and beta can bind to anillin, and this site overlaps with the septin (Peanut)-binding site. Therefore, high levels of Ran-GTP likely promote interactions between septin and anillin by dissociating importin alpha and beta from anillin [Silverman-Gavrila *et*

al., 2008]. Showing that Ran-GTP affects the localization of contractile proteins could alter our interpretation on the role of astral microtubules in cytokinesis.

Previous studies also support a role for Ran in regulating actin cap formation. In mouse oocytes, formation of an actin cap precedes polar body formation to ensure the minimum loss of cytoplasm [Deng *et al*., 2007]. First, Ran could promote the activation of Rac and/or Cdc42, required for polymerization of Arp2/3-mediated branched F-actin [Deng *et al*., 2007; Dehapiot *et al*., 2013]. Second, Ran may exclude ERM (Ezrin/Radixin/Moesin) family of actin-membrane crosslinkers from the cell cortex where the actin cap is formed and thus, regulates cortical actin dynamics [Dehapiot and Halet, 2013]. However, none of these studies addressed the mechanism by which Ran could regulate Rac, Cdc42 or ERM proteins in mediating actin cap formation.

Here, I investigate the role of chromatin in regulating the localization of contractile ring proteins during cytokinesis in human Hela cells. My results provide evidence to support the role of Ran-GTP in negative regulation of localization of contractile proteins. I show that the boundary of accumulated anillin at the equatorial cortex correlates with a minimum chromatin distance. When the central spindle and/or astral microtubules are disrupted, the minimum distance between the boundary of anillin and chromatin decreases, yet anillin is excluded from the polar cortex, suggesting that multiple pathways coordinate to regulate its localization. Removal of both the central spindle and astral microtubules in bipolar or “non-polar” systems show that chromatin is sufficient to alter the localization of anillin, predominantly within 3-4 µm of the cortex. I demonstrate that furrow-targeted over-expression of constitutively active Ran (Q69L) changes the boundary of anillin localization and causes asymmetric furrow ingression or oscillation. Interestingly, inactivating endogenous Ran using a tsRCC1 mutant leads to the global localization of myosin in anaphase and alters furrowing. This evidence in particular, supports a role for Ran-GTP, as well as, highlights the fact that different pathways work together to precisely position the contractile ring.

Chapter 2. Materials and Methods

2.1 Cell culture and transfection

Hela cells were plated and grown in Dulbecco's Modified Eagle Medium (DMEM; Wisent), supplemented with 10% fetal bovine serum (FBS; Thermo Scientific), 2 mM L-glutamine (Wisent), 100 U penicillin and 0.1 mg/mL streptomycin (Wisent). Cells were maintained in a humidified incubator with 5% CO_2 at 37°C. For transfection, cells were plated the day before at a confluency between %50-60 (>60% for mCherry:Neuromodulin Ran) on 25 mm coverslips [No. 1.5 (Harvard Apparatus); washed with 0.1 M HCl, then with isopropanol and air dried] or in dishes, in DMEM media without antibiotics (PS). DNA transfection was performed using Lipofectamine (Invitrogen) according to manufacturer's protocol, except that 3 µL of Lipofectamine was used per 2 mL of media for optimal transfection and to limit lethality, and the amount of DNA varied for each construct (e.g. 0.5 µg for H2B:GFP and H2B:mRuby, 1.5 µg for GFP:MLC and 2 µg for the Ran constructs). The cells were imaged 24-26 hours after starting the transfection. Co-transfection of DNA and siRNAs (3 µL of 2.0 nM siRNAs) was performed using Lipofectamine as outlined above, except that cells were imaged after 27-30 hours.

All of the conditions explained above were used for the temperature sensitive baby hamster kidney cells (tsBN2) expressing ts mutant RCC1, except that they were maintained at 33°C.

2.2 RNAi and drug treatments

The following short interference double strand RNA (siRNA) was used: MKLP1, 5' CGACAUAACUUACGACAAAUU 3' (Dharmacon).

The following drugs were used and dissolved in DMSO as 1000 X stocks: S-trityl-L-cysteine (STC; Sigma-Aldrich), Purvalanol A (Sigma-Aldrich) and Nocodazole (Sigma-Aldrich). To generate monopolar cells, 2 µM of STC was added

for 4-5 hours to accumulate cells in prometaphase. Subsequently, 22.5 μM Purvalanol A was added to the STC-arrested cells and imaged after 15 minutes. For Nocodazole treatments, 10 or 100 nM was used for 'low' or 'strong' doses, respectively. Low doses of Nocodazole were added to the cells during live imaging, ~3 minutes after cells exited metaphase and chromosomes had begun segregating. Cells were treated with strong doses of Nocodazole for 3 hours before imaging, and Purvalanol A was added during live imaging to promote exit from mitosis. For immunofluorescence, cells were fixed after 20-25 minutes of treatment with Purvalanol A.

2.3 Constructs

The following constructs and cell lines were previously made and used in this study: 1) a line of Hela cells stably expressing GFP:nonmuscle myosin light chain (MLC; active; T18E S19E) (Piekny and Glotzer, 2008; made by C. van Oostende), 2) H2B:GFP (Histone; Kanda *et al*., 1998) generously provided by Dr G. Hickson (Montreal, Canada), 3) a line of Hela cells stably expressing Anilin:GFP kindly provided by I. Posner (Dresden, Germany) and E. Zanin (Munich, Germany), and 4) tsBN2 cells with a ts mutation in RCC1, generously provided by Dr. I. Cheeseman (California, USA).

The following constructs were made for this study: 1) H2B:mRuby, which was generated by removing GFP from H2B:GFP using *BamHI* (New England Biolabs) and *XbaI* (New England Biolabs), and replacing it with mRuby from the pcDNA3-mRuby2 plasmid (kindly provided by Dr. C. Brett; Montreal, Canada). 2) mCherry:Neuromodulin (1-60) Ran (WT), which was generated by cloning WT Ran obtained via PCR using Ran cDNA (Cat#205957, True Clone) and a forward primer with an *XhoI* (New England Biolabs) cut site: 5' CCGCTCGAG ATGGCTGCGCAGGG 3' and a reverse primer with an *EcoRI* (New England Biolabs) cut site: 5' CGGAATTCTCACAGGTCATCATC 3', into the pTK24 plasmid (Addgene), which contains mCherry:Neuromodulin (1-60), using *XhoI* and *EcoRI* cut sites, and

verified by DNA sequencing at the Genome Quebec Innovation Centre, and 3) mCherry:Neuromodulin (1-60) Ran Q69L, which was generated from mCherry:Neurobmodulin (1-60) Ran WT via quickchange PCR using the forward primer 5' CAATGTATGGGACACAGCCGGCCTGGAGAAATTCGGTGGACTGAG 3' and reverse primer 5' CTCAGTCCACCGAATTTCTCCAGGCCGGCTGTGTCCCATACATTG 3', and verified by sequencing at the Genome Quebec Innovation Centre.

2.4 Fixation and immunofluorescence

Cells were fixed for immunofluorescence using trichloroacetic acid (TCA). 10% w/v TCA in water was prepared 30 minutes prior to fixation on ice using a freshly prepared 100% w/v stock, also made in water. Cells on coverslips were washed with pre-warmed (37˚C) cytoskeletal buffer (80 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA), then fixed with ice-cold 10% TCA for 16 minutes. After, cells were washed four times with 1 X TBST buffer (0.15 M NaCl, 0.05 M Tris, pH 7.4, 0.5% Triton X-100). Fixed cells were immunostained for microtubules and anillin using the following antibodies: 1:200 rabbit anti-anillin antibody (Piekny & Glotzer 2008), 1:200 mouse anti-tubulin antibody (DM1A, Sigma-Aldrich). The following secondary antibodies were used at 1:350 dilution: anti-rabbit Alexa 488 and anti-mouse Alexa 568 (Invitrogen). To perform staining, coverslips were placed on a piece of parafilm in a chamber with wet paper towels around it to keep the coverslips from drying during the procedure. Cells were 'blocked' using 150 µL of TBST with 5% NDS (Normal Donkey Serum) for 20 minutes. Then, 100 µL of primary antibodies diluted in TBST with 5% NDS were added to the cells, which were incubated for 2 hours at room temperature. Cells then were washed 3 times with TBST. After, 150 µL of secondary antibodies diluted in TBST with 5% NDS were added to the cells, which were incubated for another 2 hours at room temperature. Cells then were washed 2 times with TBST, after which 1:1000 DAPI (4, 6-diamidino-2-phenylindole; 1 mg/mL; Sigma-Aldrich) in TBST was added for 5 minutes. Cells then were washed once with TBST, and finally with 0.1 M Tris pH

8.8. Lastly, Tris was removed and a drop (~12 µL) of mounting media (4% n-propyl gallate in 50% glycerol, 0.1 M Tris pH 8.8) was added per coverslip. Then the coverslips were placed onto a glass slide, excess liquid was removed, and they were sealed with nail polish.

2.5 Imaging

Fixed cells were imaged using a Leica DMI6000B inverted microscope (Leica Microsystems) with the 63X/ 1.4 NA oil immersion objective and a CCD OcraR2 (Hamamatsu) camera with Volocity acquisition software (PerkinElmer). Images were acquired as Z-stacks (0.5 µm) using the Piezo Z stage (Mad City Labs). Exposures were set based on control slides (optimized levels between 3000 - 4000). Image files were exported as TIFFs, which were opened with Java-based software, Image J (NIH), and converted into maximum intensity Z-stack projections. Projections and merged colour images were then converted into 8-bit images and imported into Illustrator (Adobe) to make figures.

To perform live imaging, media was replaced with phenol red-free DMEM media. Cells were previously plated and transfected on round coverslips or dishes, depending on the experiment. For example, 25 mm round coverslips (No. 1.5) plated with cells were placed in a 35 mm Chamlide magnetic chamber (Quorum), or cells were plated in a 35 mm µ-Dish with 2 mL volume (ibidi), or in µ-Slide Angiogenesis tissue culture-treated 15-well dishes (ibidi). The latter was preferred when multiple treatments were being performed. Cells were kept at 37˚C (except for tsBN2 cells, which were kept at either 33˚C or 40 ˚C) and 5% CO_2 using the INU-TiZ-F1 stage series chamber. Live imaging was performed on an inverted Nikon Eclipse Ti microscope with a Livescan Swept Field confocal unit (Nikon), the 60X/1.4 NA oil immersion objective, a Piezo Z stage Nano-Z100 N (Mad City Labs), and with the iXON897 EMCCD camera (Andor). Images were collected using 200 ms exposures with 488 and 561 nm lasers (100 mW, Agilent) set between 20 - 60%, depending on the intensity of the signals (settings were kept constant for

related experiments), and 41 Z-stacks of 0.5 µm were taken every 1 minute per cell using Elements acquisition software (4.0 Nikon). To image tsBN2 cells in anaphase with the appropriate temperature, cells were kept at 33˚C, and cells in metaphase were selected. Just before anaphase (start of chromosome segregation), the temperature was upshifted to 40 ˚C to inactivate RCC1. Also, to determine the effect of downshifting the temperature (RCC1 activation) on tsBN2 cells, cells kept at 40 ˚C were imaged as the temperature was downshifted to 33˚C, and the time point that corresponded to 33˚C was noted.

2.6 Quantitation

To measure the breadth of accumulated contractile proteins, images were opened using ImageJ, and maximum intensity Z-projections were created. Then, line scans were performed along the cell cortex. The cytosolic levels were subtracted from the levels along the line scan, and the breadth was determined for different time points by measuring the GFP levels at half of the maximum intensity after cytosolic correction. To be consistent for timing among the cells, they were imaged from anaphase onset through furrow ingression and similar time points were selected for each treatment. The data was imported into an Excel (Microsoft) spreadsheet where graphs were generated from the data.

To measure the distance from chromatin to the boundary of accumulated contractile proteins, 4D movies were opened in Surpass (3D) Imaris ×64 7.6.0 software (Biplane). The boundary of accumulated contractile protein was calculated as described above, except that only 75% of the maximum levels of GFP were considered. Next, the minimum distance from chromatin to the boundary of contractile proteins was determined for 3-4 points per cell, and the average for each cell was calculated. Next, the average distance for multiple cells was calculated. Cells were imaged from anaphase through furrow ingression, and similar time points were chosen for mid anaphase and early telophase. To ensure accuracy in timing, other factors

including cell shape and the ratio of the distance between two chromosomes vs. cell length were considered. Data was imported into an Excel (Microsoft) spreadsheet where the average distance, standard deviation and student t test were calculated, and graphs were plotted.

In addition, kymographs of chromatin position during cytokinesis were performed using ImageJ (NIH) after Z stack projection, using a line drawn across the centre of the cell as shown in Figure 4B. All of the images and graphs were copied and pasted into Illustrator (Adobe) to make figures.

Chapter 3. Results

The main goal of my studies was to identify novel cue(s) that determine the division plane. Although the central spindle and astral microtubules have been shown to regulate the localization of contractile proteins, it was not known if other parts of the cell also affect their localization. I focused on the role of chromatin, and first determined if there was a correlation between chromosome position and the localization of contractile proteins during cytokinesis.

3.1 Characterization of the localization of contractile ring proteins during cytokinesis

In order to characterize contractile ring formation, I performed live imaging of GFP-tagged active myosin light chain (MLC E18 E19) or Anillin:GFP stably expressed in Hela cells from anaphase to early telophase. Both proteins showed similar patterns in their distribution (Figure 2A). In anaphase, they localized broadly along the cortex, but became more restricted to the equatorial plane as cells progressed through telophase (Figure 2A). The restriction of myosin and anillin correlated with the movement of chromosomes to their respective poles (Figure 2A). I quantitated changes in the breadth of myosin and anillin localization during mitosis. To do this, I normalized the levels of GFP along the cortex according to the cytosol, and measured the width of the GFP peak at half of the maximum GFP levels (Figure 2B). My data showed that myosin and anillin were very broad in early anaphase and became more restricted as cells progressed through anaphase. At 3 minutes after anaphase onset, the myosin zone was 14.1 +/- 0.4 μm, n=13 and the anillin zone was 15.5 +/- 0.48 μm, n=8, and after 5:20 minutes, 8.6 +/- 0.22 and 8.8 +/- 0.34 μm for myosin and anillin, respectively. Once the contractile ring began to ingress in early telophase (after 6:30 minutes), the breadth remained constant (8 +/- 0.3 and 8.5 +/- 0.24 μm, for myosin and anilin, respectively; Figure 2B). These data indicate that signals restrict the localization of

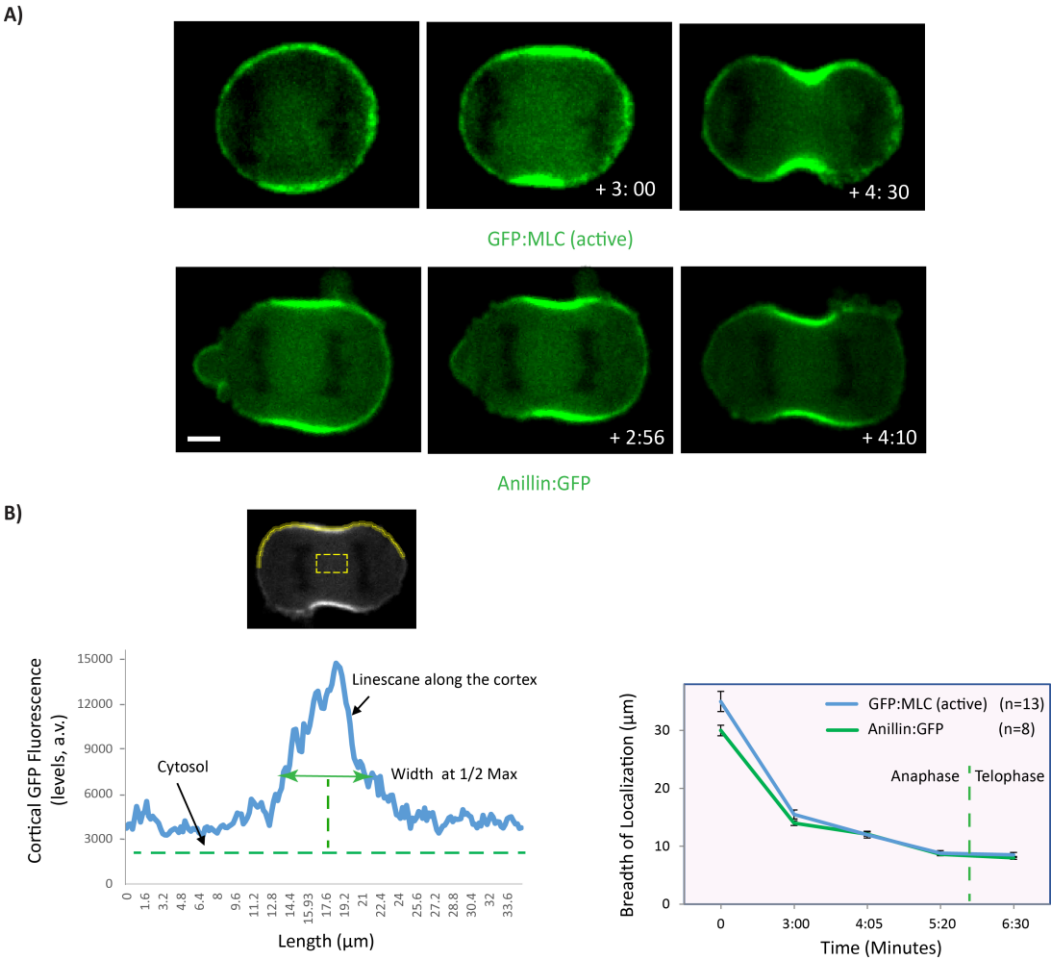


Figure 2. The breadth of contractile proteins decreases during cytokinesis. A) Time-lapse of Hela cells stably expressing GFP:MLC (active) or Anillin:GFP during anaphase and early telophase. Initially, MLC and Anillin localized more broadly along the cortex, but became more restricted as cells progressed through telophase. The scale bar represents 10 µm. **B)** The boundary of accumulated contractile proteins were determined by defining the number of pixels (width) at half of the maximum levels of GFP. Shown is a graph of a line scan drawn along the cell cortex (yellow line on cell image) with GFP fluorescence levels (Y axis) and corresponding length (in µm; X axis). GFP levels along the cortex were normalized according to the cytosol. Also shown is the corresponding graph with the breadth of GFP:MLC (active) or Anillin:GFP at various time points during cytokinesis. The Y axis shows the breadth (in µm) and the X axis shows the time. The error bars represent standard deviation.

contractile proteins from anaphase to early telophase. Since the timing of this restriction correlates with spindle elongation and the poleward movement of chromosomes, signals likely come from the mitotic spindle and chromatin to modify the localization of contractile proteins.

Recently, Kiyomitsu and Cheeseman (2013) provided evidence supporting a role for chromatin in regulating the localization of anillin. Thus, I determined whether there is a correlation between the poleward movement of chromosomes and the equatorial accumulation of contractile proteins. I performed live imaging of Anillin:GFP using stable Hela cell lines, transfected with Histone:mRuby (H2B:mRuby) to visualize chromatin (Figure 3A). Then, I measured the distance of chromatin to the boundary of accumulated Anillin:GFP in anaphase and early telophase, just before ingression. First, I determined the boundary of Anillin:GFP as explained above (except that 75% of the maximum GFP levels were considered), then performed measurements from this boundary to chromatin in 3D using Imaris software (Figure 3B). The average distance in anaphase between the chromatin and anillin was 4.98 +/- 0.61 µm (n=10), and in early telophase was 5.07 +/- 0.45 µm (n=13). My measurements indicate that there is an average, measurable distance between chromosomes and the boundary of accumulated contractile proteins during anaphase and early telophase, and this distance remains constant after anaphase.

3.2 Is the distance between chromatin and the accumulation of contractile proteins at the equatorial cortex constant after manipulating chromosome position or the breadth of contractile proteins?

My studies suggested a correlation between chromosome position and the localization of contractile proteins. However, these studies were done in cells with intact mitotic spindles. As described earlier, the central spindle helps determine

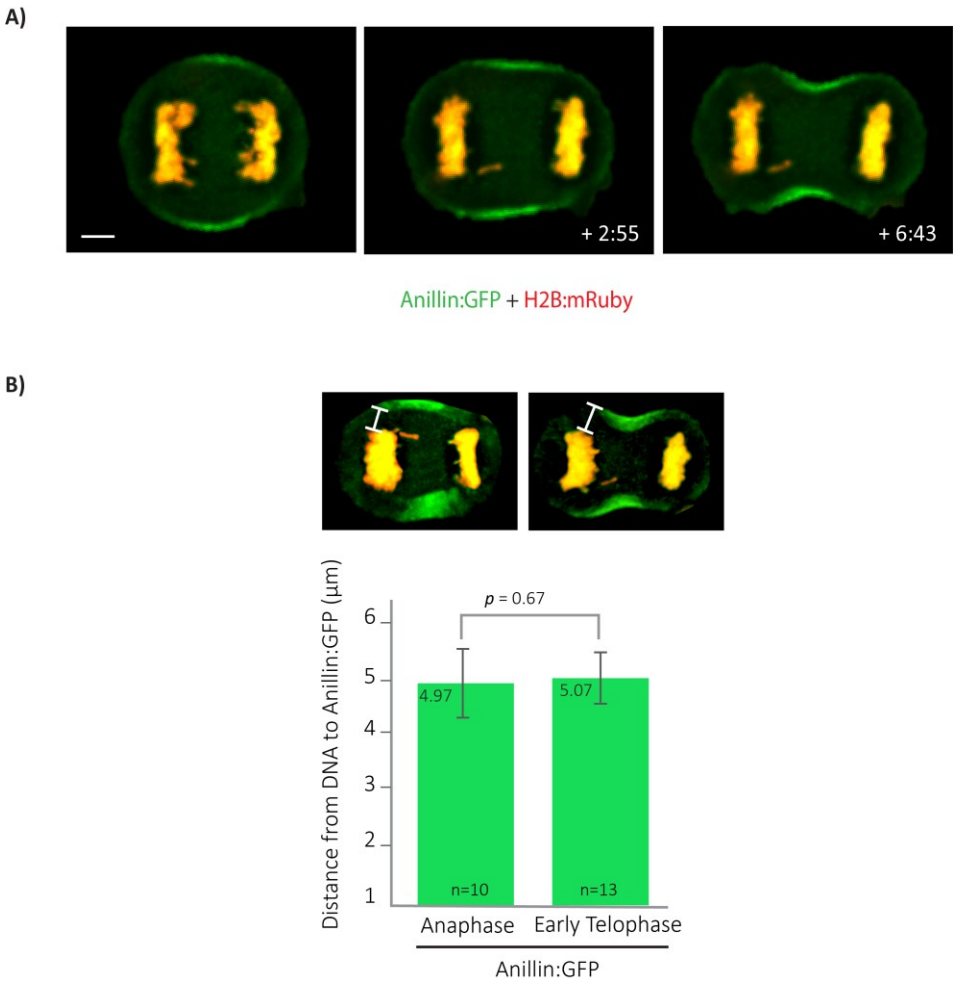
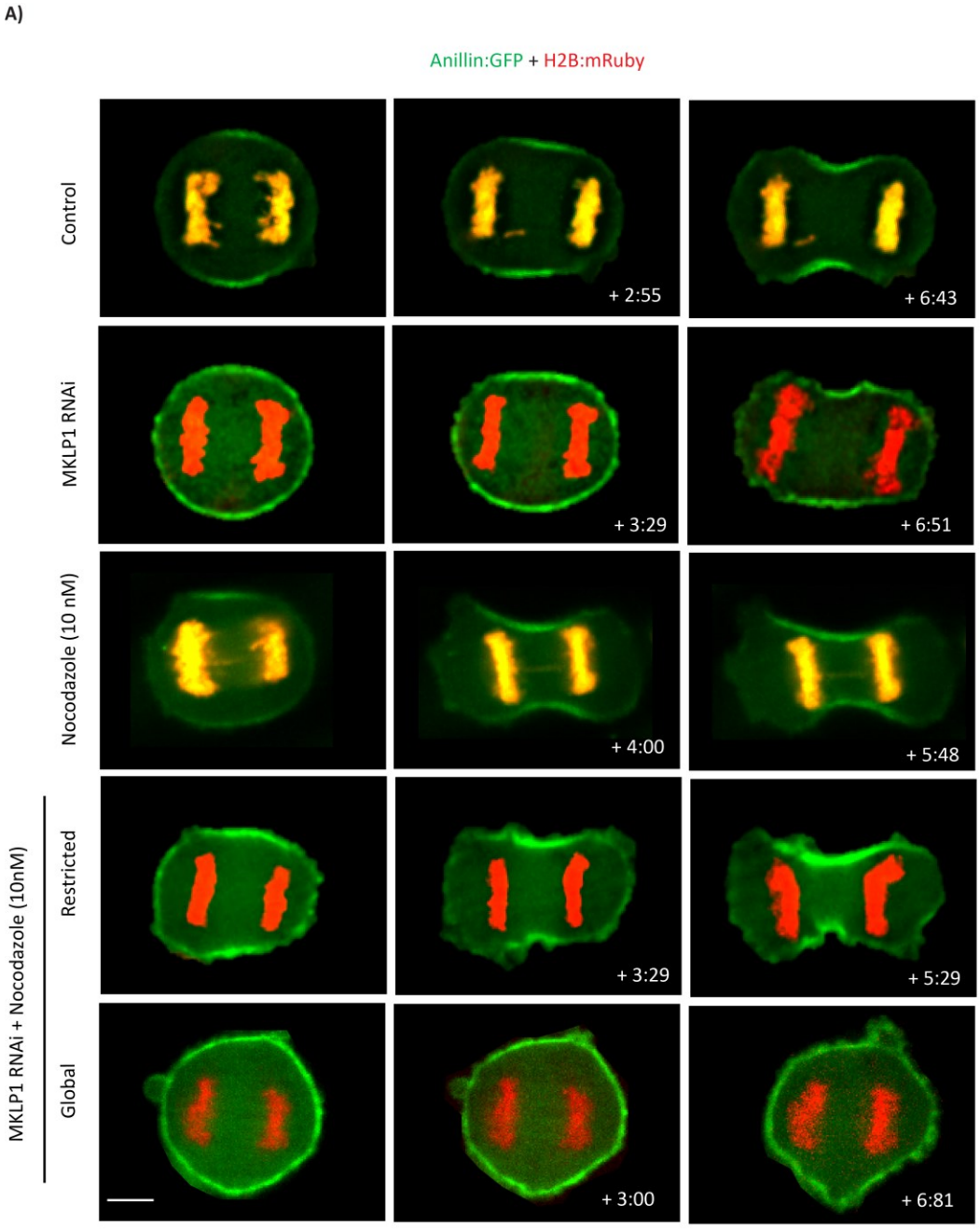


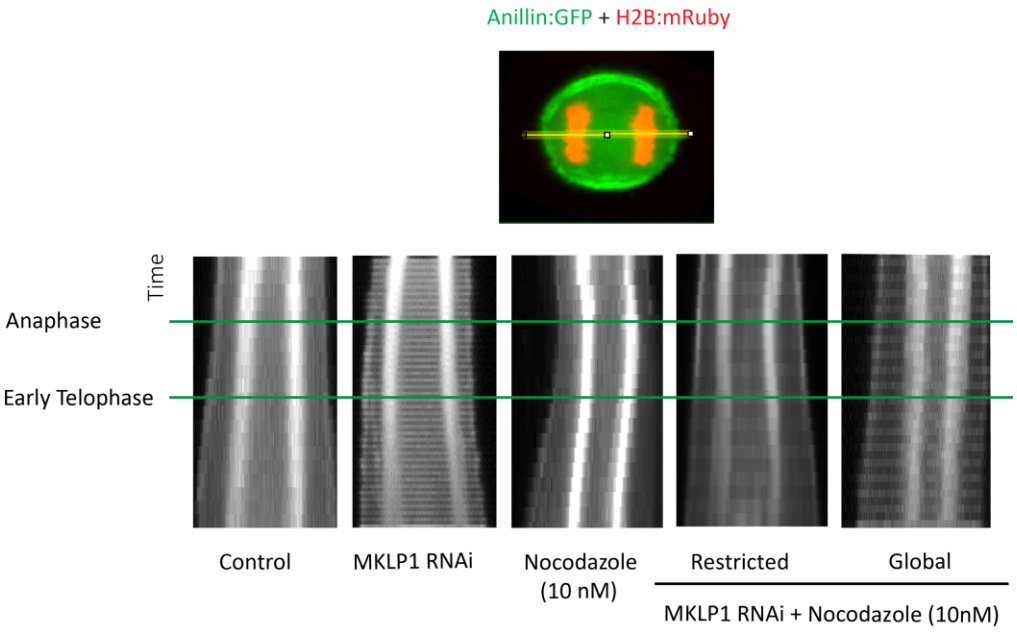
Figure 3. The position of chromatin correlates with the boundary of Anillin:GFP during anaphase and early telophase. A) Time lapse of Hela cells stably expressing Anillin:GFP and transfected with Histone:mRuby (H2B:mRuby), during anaphase and early telophase. Scale bar represents 10 µm. **B)** A graph shows the average distance from chromatin to the boundary of accumulated Anillin:GFP at the equatorial cortex in anaphase and early telophase cells. The Y axis shows the distance (in µm) and the X axis shows the cells in anaphase vs. telophase. An example of a measurement is shown in the picture above the graph. The average distance in anaphase is 4.98 +/- 0.61 µm (n=10), and in early telophase is 5.07 +/- 0.45 µm (n=13). The *p* values for student t tests is shown on the graph. The error bars represent standard deviation.

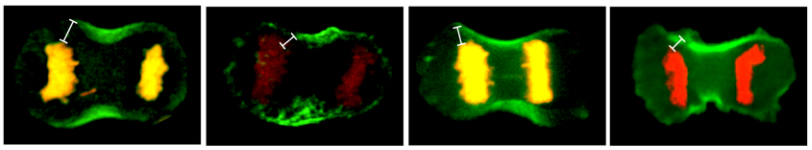
the division plane as it promotes RhoA activation at the equatorial cortex by recruiting Ect2. Central spindle disruption leads to ectopic RhoA activation and causes the global localization of contractile proteins in anaphase, and an increase in the breadth of contractile proteins in early telophase [Green *et al*., 2012; Akhshi *et al*., 2013; van Oostende, Haji Bik and Piekny, unpublished observations]. Astral microtubules also play a role in positioning contractile ring components, since disruption of astral microtubules results in an increase in the breadth of contractile proteins [Green *et al*., 2012]. I wanted to know how the distance between chromatin and the boundary of Anillin:GFP would change if the mitotic spindle, and/or position of the chromosomes were altered. These experiments included disrupting the central spindle, disrupting astral microtubules, co-disrupting both the central spindle and astral microtubules, creating monopolar cells, and removing all microtubules.

3.2.1 Central spindle disruption using MKLP1 RNAi

I wanted to determine how the distance between chromatin and the boundary of accumulated contractile proteins changes in cells with a disrupted central spindle. Hela cells stably expressing Anillin:GFP, co-transfected with H2B:mRuby, and treated with MKLP1 RNAi were imaged through anaphase and early telophase. Similar to previous studies, Anillin:GFP localized around the entire cortex of anaphase MKLP1-depleted cells, but was excluded from the polar cortex by early telophase (Figure 4A). Kymographs show changes in the position of chromosomes over time and showed that in cells lacking a functional central spindle, chromosomes moved closer to the polar cortex in comparison to control cells (Figure 4B). I measured the distance between chromatin and the boundary of accumulated Anillin:GFP in early telophase cells, and it was smaller in MKLP1-depleted cells in comparison to control cells (3.46 +/- 0.41 µm, n=12 vs. 5.07 µm for the control; Figure 4C). These data suggest that the central spindle helps restrict the localization of contractile proteins during anaphase and early


A)
Anillin:GFP + H2B:mRuby
Control
MKLP1 RNAi
Nocodazole (10 nM)
MKLP1 RNAi + Nocodazole (10nM)
Restricted
Global
+ 2:55
+ 6:43
+ 3:29
+ 6:51
+ 4:00
+ 5:48
+ 3:29
+ 5:29
+ 3:00
+ 6:81


Anillin:GFP + H2B:mRuby
Time
Anaphase
Early Telophase
Control
MKLP1 RNAi
Nocodazole (10 nM)
Restricted
Global
MKLP1 RNAi + Nocodazole (10nM)



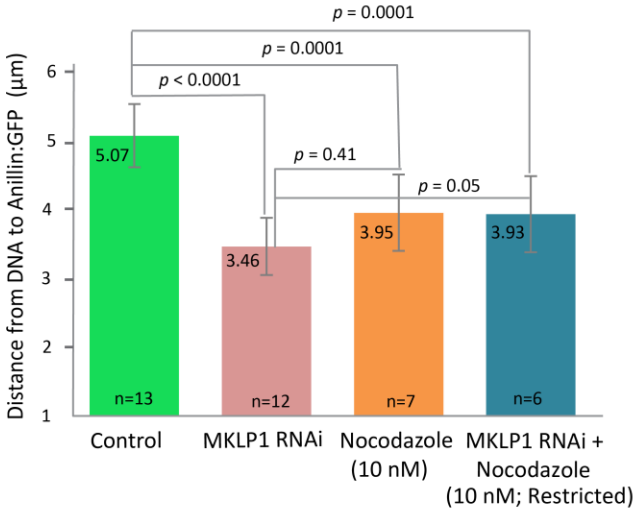

p = 0.0001
p = 0.0001
p < 0.0001
p = 0.41
p = 0.05
Distance from DNA to Anillin:GFP (µm)
6
5
4
3
2
1
5.07
3.46
3.95
3.93
n=13
n=12
n=7
n=6
Control
MKLP1 RNAi
Nocodazole (10 nM)
MKLP1 RNAi + Nocodazole (10 nM; Restricted)

Figure 4. Various treatments reveal the minimum distance that chromatin may be able to influence the localization of contractile proteins. A) Time lapse of Hela cells stably expressing Anillin:GFP and transfected with Histone:mRuby (H2B:mRuby), during anaphase and early telophase after various treatments that disrupt the central spindle (MKLP1 RNAi), astral microtubules (10 nM Nocodazole), or both. Scale bar represents 10 µm**. B)** Kymographs show chromatin position as cells progress from anaphase to telophase in control and treated cells. Timepoints corresponding to mid-anaphase and early telophase are marked on the kymographs by the lines. The picture of the cell above shows the corresponding region used for the kymographs. **C)** A graph shows the average distance from chromatin to the region of accumulated Anillin:GFP in the different treatments. The Y axis shows the average distance from chromatin to the boundary of Anillin:GFP (in µm), and the X axis shows the different sets of cells [control 5.07 +/-0.45 µm, n=13, MKLP1 RNAi 3.46 +/- 0.41 µm, n=12, Nocodazole (10 nM) 3.95 +/- 0.55 µm, n=7, and MKLP1 RNAi and Nocodazole (10 nM; restricted) 3.93 +/- 0.55 µm, n=6]. The *p* values for student t tests to compare data between the treatments and control cells, as well as between the treatments, are shown on the graph. The error bars represent standard deviation.

telophase. However, during early telophase, other signals exclude the localization of contractile proteins from the polar cortex.

3.2.2 Astral microtubule disruption using Nocodazole

I wanted to determine how the removal of astral microtubules would influence the distance between chromatin and the boundary of contractile proteins. Therefore, Hela cells stably expressing Anillin:GFP transfected with H2B:mRuby were treated with a low dose of Nocodazole (10 nM), which preferentially depolymerizes astral microtubules, but leaves central spindle microtubules intact [Murthy and Wadsworth, 2008; Zanin *et al*., 2013]. I added the drug about 3 minutes after anaphase onset when the central spindle had formed and chromosomes started to move apart. Consistent with previous studies, disrupting astral microtubules caused an increase in the breadth of

Anillin:GFP, however, anillin was still excluded from the polar cortex both in anaphase and in early telophase cells (Figure 4A). Unlike the MKLP1-depleted cells, kymographs showed that the chromosomes remained more centrally positioned (Figure 4B). I measured the distance from chromatin to the boundary of accumulated Anillin:GFP in telophase cells. Chromatin was closer to anillin in comparison to control cells (3.95 +/- 0.55 μm, n=7, vs. 5.07 μm for control cells; Figure 4C). These data show that astral microtubules, similar to the central spindle, also restrict the localization of contractile proteins during cytokinesis. Since contractile ring proteins are excluded from the polar cortex after disruption of astral microtubules, other pathways likely restrict their localization.

3.2.3 Central spindle and astral microtubule disruption using MKLP1 RNAi and Nocodazole

I wanted to determine if chromatin is sufficient to regulate the localization of contractile proteins. To do this, I removed both the central spindle and astral microtubules, and measured distances between chromatin and the boundary of accumulated contractile proteins. Cells stably expressing Anillin:GFP transfected with H2B:mRuby and MKP1 siRNAs were treated with Nocodazole (10 nM) after cells were in anaphase and chromosomes had moved apart. Once Nocodazole was added, in 57.1% (n=14) of the cells, chromosomes did not move further apart and Anillin:GFP localized around the entire cortex (global; Figure 4A). In contrast, in 42.9% (n=14) of the cells, the chromosomes traveled toward the poles, and Anillin:GFP was cleared from the polar cortex (restricted; Figure 4A). The kymographs showed that the chromosomes moved toward the poles in cells displaying the restricted phenotype, but were more centrally positioned in cells displaying the global phenotype (Figure 4B). The distance between chromatin and the boundary of accumulated anillin in cells with the restricted phenotype was 3.93 +/- 0.55 μm (n=6; Figure 4C). In contrast, when chromosomes was more centrally positioned, it was an average of 5.5 +/- 0.6 μm (n=8) from the cortex

where Anillin:GFP was enriched. Therefore, although the central spindle and astral microtubules modulate the localization of contractile proteins during cytokinesis, there is a correlation between the proximity of chromatin to the cortex, and the localization of contractile proteins.

3.2.4 Monopolar Cells

I also wanted to determine the correlation between chromatin and the accumulation of contractile proteins in cells with different geometries. Previous studies showed that cells can be induced to polarize in a single axis. These monopolar cells are generated using S-Trityl-L-Cysteine (STC) to prevent the separation of centrosomes, followed by the addition of Purvalanol, a Cdk1 inhibitor, to force cells to exit mitosis [Canman *et al*., 2003; Hu *et al*., 2008]. An extensive array of bundled microtubules forms on one side of the chromosomes, and contractile proteins accumulate at the cortex at the end of these microtubules. I generated monopolar cells using Hela cells stably expressing Anillin:GFP and transfected with H2B:GFP. As chromosomes shifted to one side, Anillin:GFP delocalized from the cortex in the vicinity of chromatin and accumulated at the opposite cortex (Figure 5A). Measuring the distance from chromatin to the boundary of Anillin:GFP showed an increase in the minimum distance in comparison to bipolar control cells (6.61 +/- 0.65µm, n=15 vs. 5.07+/- 0.45 µm for control cells; Figure 5B). This difference in distance likely arises from changes in cell geometry, which could be due to the nature of the signal and how it reaches the cortex.

3.2.5 Microtubule disruption with Nocodazole

I wanted to determine if chromatin is sufficient to influence the localization of contractile proteins in cells that have more random geometry and polarity. To do this, cells were treated with Nocodazole (100 nM) to remove all microtubules, then treated with a Cdk1 inhibitor, Purvalanol, to force mitotic exit.

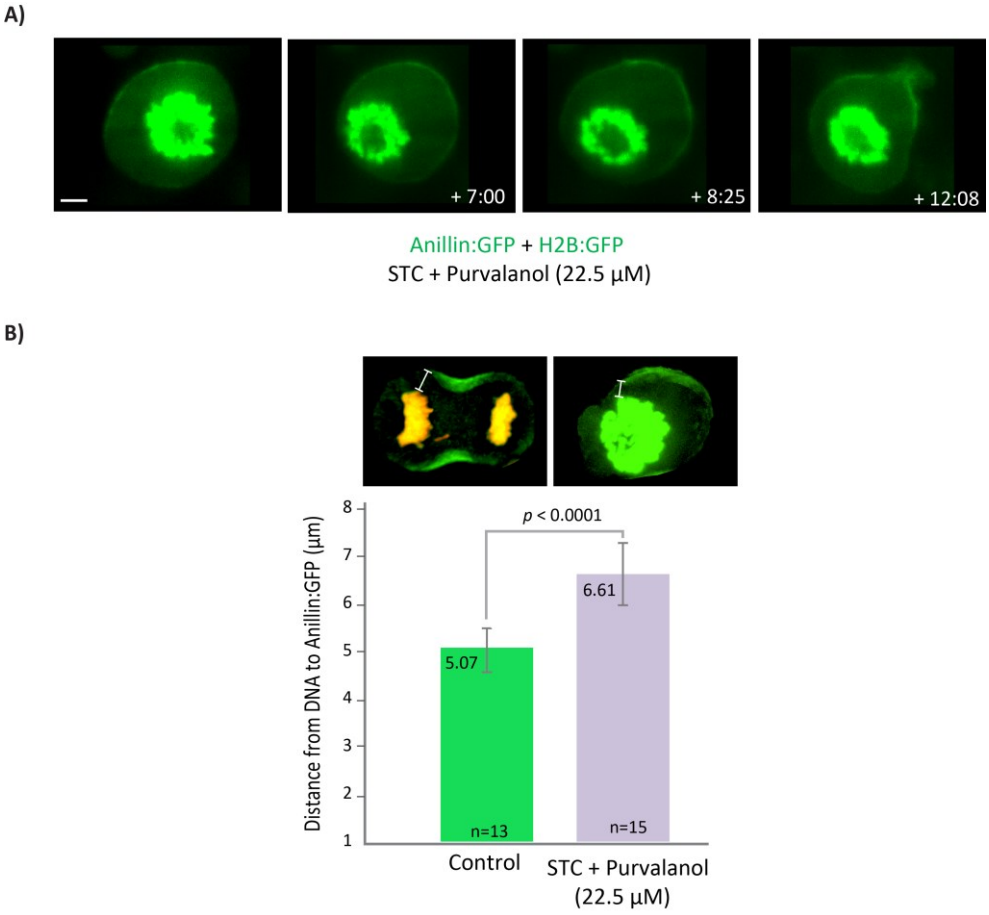


Figure 5. Chromatin distance to accumulated contractile proteins varies in cells with different geometries. A) Time lapse of Hela cells stably expressing Anillin:GFP and transfected with Histone:GFP (H2B:GFP), treated with S-Trityl-L-Cysteine (STC) for 4-5 hours and Purvalanol A (22.5 µM), for 20-25 minutes. After purvalonol (22.5 µM) was added to the cells, chromatin shifted to one side of the cell and Anillin:GFP accumulated at the opposite side. The scale bar represents 10 µm. **B)** A graph shows the average distance from chromatin to the boundary of accumulated Anillin:GFP. The Y axis shows the distance from chromatin to the boundary of contractile proteins (µm), and the X axis shows the different sets of cells. The distance for monopolar cells is 6.61 +/- 0.65 µm, n= 13. The *p* values for student t is shown on the graph. The error bars represent standard deviation.

First, I performed immunofluorescence on fixed Hela cells to ensure that no/few polymerized microtubules remained after Nocodazole treatment (Figure 6A). Next, I performed live imaging using cells stably expressing Anillin:GFP, transfected with H2B:mRuby and treated with Nocodazole for 3 hours, then treated with Purvalanol. Two phenotypes were observed based on chromatin position. In one group, chromatin remained in the middle of the cell, and Anillin:GFP localized globally (38.9%, n=18). In the second group, chromatin remained close to one part of the cortex, and Anillin:GFP accumulated at other regions of the cortex away from this site (61.1%, n=18; Figure 6B). I measured the distance from chromatin to the boundary of accumulated Anillin:GFP, which was similar to control 'bipolar' cells (5.04 +/- 2.09 µm, n=11; Figure 6C). However, in cells with disrupted microtubules, the standard variation was much higher than for control 'bipolar' cells (2.09 vs. 0.45 for the control). In microtubule-disrupted cells with more centrally positioned chromatin, and globally localized anillin, chromatin was an average of 8.76 +/- 2.04 µm, n=7, from the cortex. These data suggest that chromatin is sufficient to restrict the localization of contractile proteins, but must be positioned near the cortex. The high variability in the data shows that the geometry of the cell is also important in helping to restrict the localization of contractile proteins from chromatin.

Taken together these data indicate that chromatin is sufficient to restrict the localization of contractile proteins, however, it must be positioned near the cell cortex, likely within 3-4 µm. Together with the central spindle and astral microtubules, chromatin may help to precisely position the contractile ring, and may become essential when these other pathways are perturbed.

3.3 The role of Ran in regulating the localization of contractile proteins

Next, I wanted to determine the molecular nature of the cue coming from chromatin that may regulate the localization of contractile proteins. One of the main signals associated with chromatin is Ran-GTP, which is known to regulate

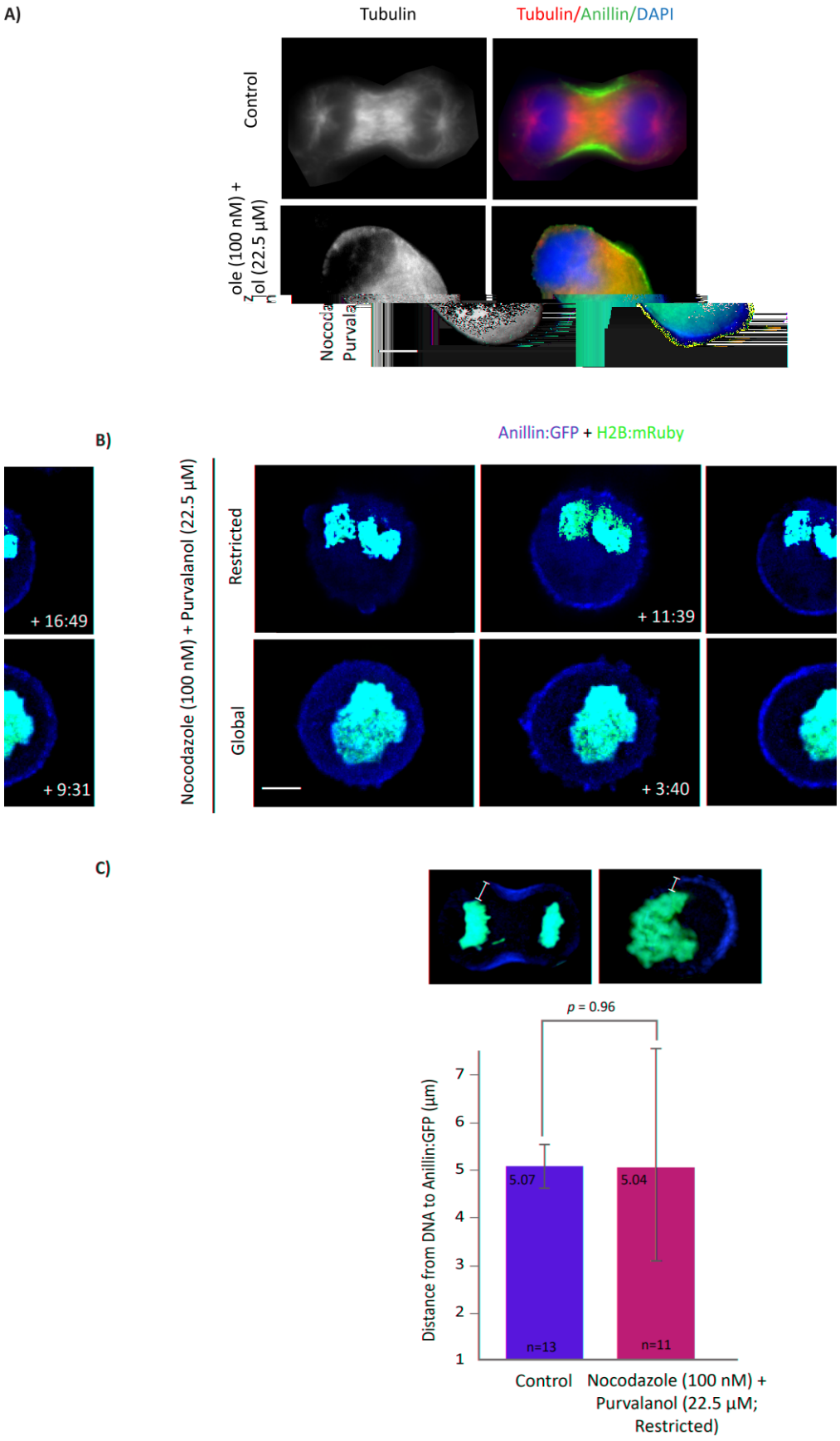

A)
Tubulin
Tubulin/Anillin/DAPI
Control
ole (100 nM) +
ol (22.5 µM)
B)
Anillin:GFP + H2B:mRuby
Nocodazole (100 nM) + Purvalanol (22.5 µM)
Restricted
Global
+ 16:49
+ 9:31
+ 11:39
+ 3:40
C)
p = 0.96
Distance from DNA to Anillin:GFP (µm)
5.07
5.04
n=13
n=11
Control
Nocodazole (100 nM) + Purvalanol (22.5 µM; Restricted)

Figure 6. Disrupting microtubules using Nocodazole (100 nM) reveals the minimum distance that chromatin can influence the localization of contractile proteins A) TCA fixed Hela cells immunostained for anillin (green) and tubulin (red), and stained for DAPI (to show chromatin), show the difference in microtubules in non-treated cells (top) in comparison to Nocodazole and Purvalanol-treated cells (bottom). **B)** Time lapse of Hela cells stably expressing Anillin:GFP and transfected with Histone:mRuby (H2B:mRuby), treated with Nocodazole (100 nM) and Purvalanol A (22.5 μM). The top cell shows one of the observed phenotypes, when chromatin remains close to one part of the cortex, and Anillin:GFP accumulates at the opposite cortex (61.11%, n=18). The bottom cell shows the second observed phenotype, when chromatin remains in the middle of the cell and Anillin:GFP localizes distance from chromatin to the boundary of accumulated Anillin:GFP. The Y axis shows the distance from chromatin to the boundary of contractile proteins (μm), and the X axis shows the different sets of cells. The distance for Nocodazole-treated cells (restricted), is 5.04 +/- 2.09 μm, n=11. The *p* value was calculated by the student t test and is shown on the graph. The error bars represent standard deviation.

mitotic spindle assembly [Clarke and Zhang, 2008]. Recently, Ran-GTP was indirectly shown to regulate the localization of anillin [Kiyomitsu and Cheeseman, 2013]. Anillin localized to the cortex near chromatin in Cdk1-inhibited cells with disrupted microtubules and inactive RCC1 (RanGEF) [Kiyomitsu and Cheeseman, 2013]. Since these cells had random geometry and polarity, I further investigated the role of Ran in regulating the localization of contractile proteins in bipolar cells.

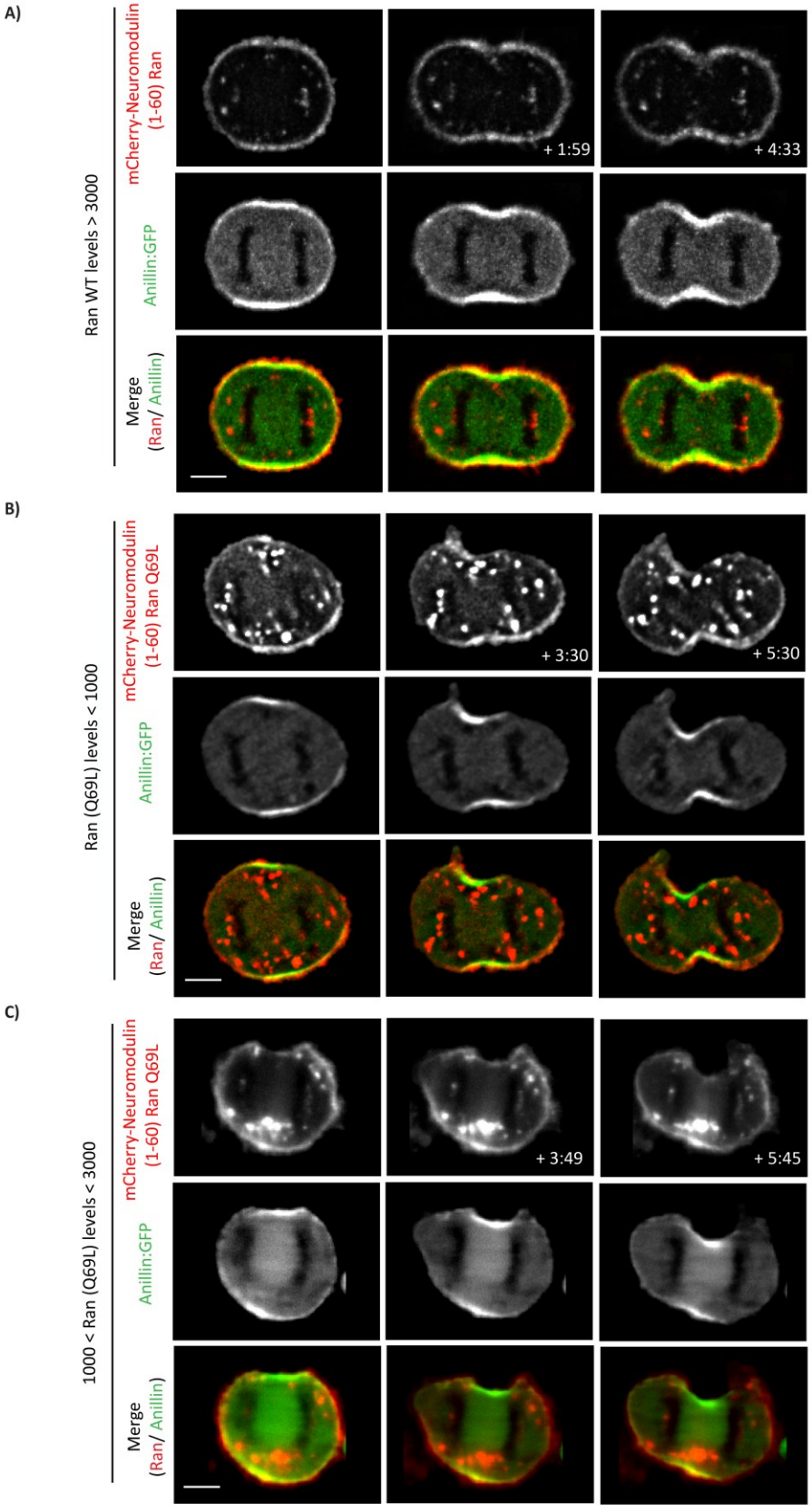
3.3.1 Active Ran influences Anillin localization and causes cytokinesis phenotypes

First, I determined the role of Ran-GTP in regulating the localization of contractile proteins in bipolar cells. I designed two mCherry-tagged constructs to artificially target either wild-type (WT) or constitutively active Ran (Q69L) to the equatorial membrane, by fusing each to a domain from Neuromodulin (1-60 aa) that specifically binds to phosphatidylinositol 4, 5-bisphosphate ($PI_{4,5}P_2$), which is enriched in the furrow during cytokinesis [Clarke and Zhang, 2008; Liu *et al*., 2012].

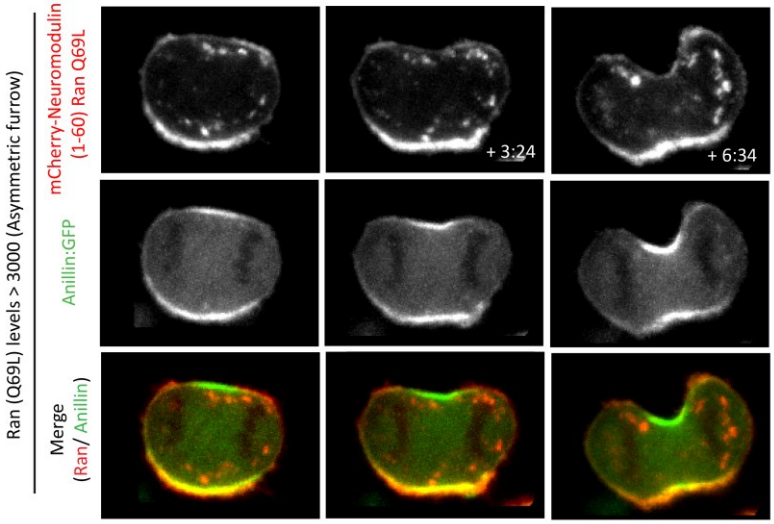
These constructs were transfected into Hela cells stably expressing Anillin:GFP, and imaged during cytokinesis. While over-expression of Ran WT did not dramatically alter the localization of anillin or cause cytokinesis phenotypes (levels of Ran above 3000; Figure 7A), over-expression of active Ran caused an increase in the breadth of anillin localization and cytokinesis phenotypes in a level-dependent manner (Figure 7B, C and, D). Based on the expression levels of active Ran, I sorted the cells into three groups. In the first group, the expression levels of active Ran were less than 1000, and anillin localization was similar to control cells (n=12) (Figure 7B). However, 41.6% of these cells formed slightly asymmetric furrows, although this was not quantitated (Figure 7B). In the second group, the expression levels of active Ran were between 1000 and 3000, and 66.6% (n=15) of the cells appeared to have broader anillin localization and displayed asymmetric furrow ingression (Figure 7C). Lastly, when the levels of active Ran were more than 3000, 62.5 % (n=8) of the cells showed asymmetric furrow ingression similar to the second group, and an increase in the breadth of Anillin:GFP localization. In addition, 37.5% (n=8) of the cells from the third group displayed oscillation phenotypes similar to anillin RNAi cells (Figure 7D). These data suggest that as the levels of Ran-GTP in the furrow increase, anillin moves away from this region and spreads along the cortex toward the poles. In cells with the highest levels of Ran-GTP in the furrow, this may cause enough of a change in anillin to cause a phenotype that resembles anillin depletion. This data suggests that Ran-GTP may be the signal associated with chromatin that affects the localization of contractile proteins during cytokinesis.

3.3.2 Loss of active Ran causes global accumulation of active MLC:GFP and asymmetric furrow formation

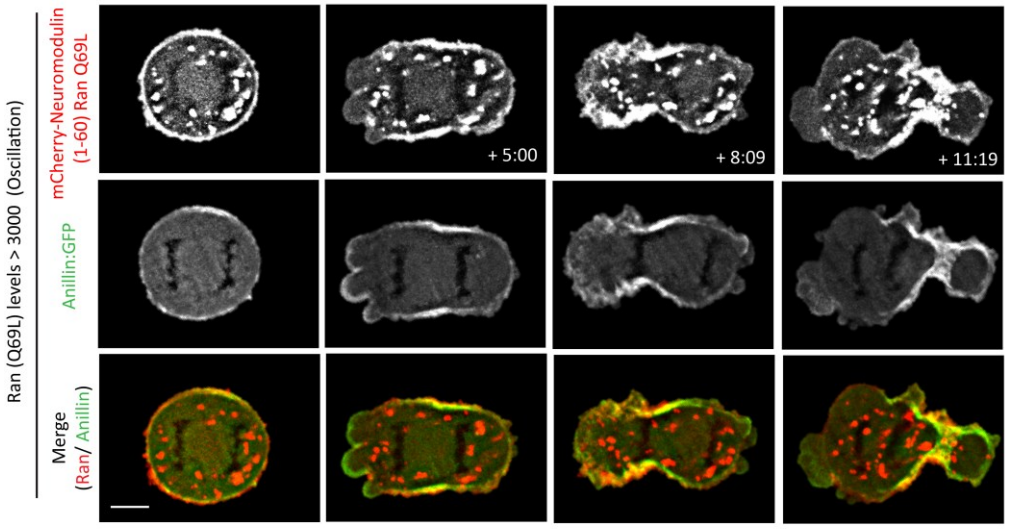
Next, I determined how the absence of endogenous Ran-GTP could affect the localization of contractile proteins in bipolar cells. Accordingly, I examined


A)
Ran WT levels > 3000
mCherry-Neuromodulin (1-60) Ran
Anillin:GFP
Merge (Ran/ Anillin)
+ 1:59
+ 4:33
B)
Ran (Q69L) levels < 1000
mCherry-Neuromodulin (1-60) Ran Q69L
Anillin:GFP
Merge (Ran/ Anillin)
+ 3:30
+ 5:30
C)
1000 < Ran (Q69L) levels < 3000
mCherry-Neuromodulin (1-60) Ran Q69L
Anillin:GFP
Merge (Ran/ Anillin)
+ 3:49
+ 5:45

D)



62.5% (n=8)



37.5% (n=8)

Figure 7. The levels of active Ran influences Anillin localization and causes cytokinesis phenotypes. A) Time lapse of Hela cells stably expressing Anillin:GFP (green) and transfected with mCherry-tagged Neuromodulin (1-60 aa) Ran WT (red). The expression levels of the cell shown were above 3000. **B)** Time lapse of Hela cells stably expressing Anillin:GFP (green) and transfected with mCherry-tagged Neuromodulin (1-60aa) Ran (Q69L; red). The cell shown had less than 1000 levels of active Ran. **C)** Time lapse of Hela cells stably expressing Anillin:GFP (green) and transfected with mCherry-tagged Neuromodulin (1-60aa) Ran (Q69L; red). The cell shown had less than 3000 levels of active Ran, but more than 1000, and has asymmetric furrow ingression. **D)** Time lapse of Hela cells stably expressing Anillin:GFP (green) and transfected with mCherry-tagged Neuromodulin (1-60aa) Ran (Q69L; red). Both cells shown had more than 3000 levels of active Ran. The top cell shows one of the observed phenotypes, when the breadth of Anillin increased and cells had asymmetric furrow ingression (62.5%, n=8). The bottom cell shows the second observed phenotype, when cells oscillate, similar to Anillin-depleted cells. The scale bars are 10 μm.

changes in the localization of myosin in cells lacking functional RCC1 (Ran GEF). To do this, I took advantage of a cell line called tsBN2, which contain a temperature-sensitive (ts) mutant of RCC1 in baby hamster kidney cells (BHK). RCC1 is inactive when the temperature is shifted to 39.7˚C, but has wild-type activity at 33˚C [Nishimoto and Basilico, 1978]. I transfected tsBN2 cells with GFP:MLC (active) and imaged them during cytokinesis. When endogenous Ran was active (at 33˚C), active MLC localized similar to Hela cells, and accumulated in the furrow during anaphase and was restricted in telophase (Figure 8A). In contrast, when the levels of active Ran decreased by upshifting RCC1 to 40˚C, active MLC localized broadly along the cortex in 65.2% (n=23) of the cells during cytokinesis, and displayed asymmetric furrow ingression (Figure 8A). Interestingly, 21.75 % (n=23) of the cells showed global MLC localization, which did not change 20 minutes after anaphase onset and cells failed to form furrows. This ‘early telophase arrest’ phenotype likely arose because cells failed to properly re-assemble the nuclear envelope

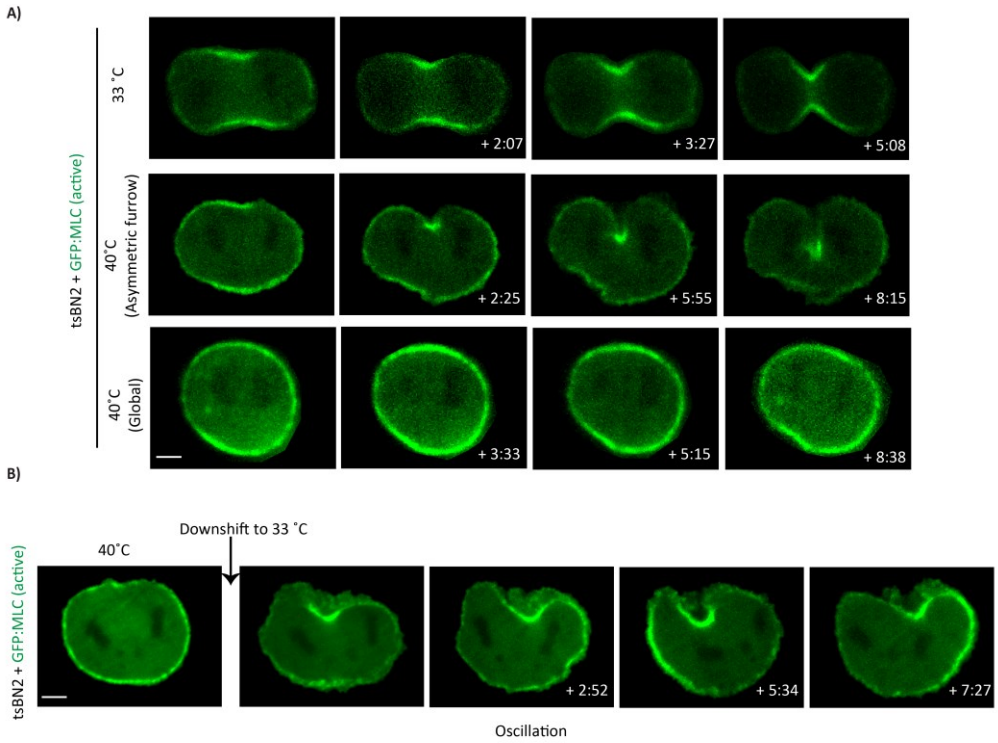


Figure 8. Decreasing the levels of active Ran leads to the global accumulation of GFP:MLC (active) during cytokinesis. A) Time lapse of temperature-sensitive RCC1 mutant baby hamster kidney (tsBN2) cells transfected with GFP:MLC (active). The top cell shows the localization of GFP:MLC (active) when tsBN2 cells were kept at 33˚C. MLC localized similar to Hela cells, and accumulated at the equatorial cortex during anaphase and remained restricted through telophase. The next cell shows the localization of GFP:MLC (active), when the levels of active Ran were decreased by upshifting RCC1 to 40˚C. MLC localized broadly in mid-anaphase, but was restricted in telophase and displayed asymmetric furrow ingression (65.2%, n=23). The next cell also was upshifted to 40˚C, but was exposed to higher temperatures for longer time. MLC localized globally around the entire cortex, and remained there for 20 minutes after anaphase onset preventing furrow ingression; 21.75%, n=23). **B)** tsBN2 cells kept at 40˚C and that had globally localized GFP:MLC (active) were downshifted to 33˚C. MLC localization immediately started to clear and the cell oscillated (100%, n=2). The scale bars are 10 µm.

during telophase in the absence of active Ran [Clarke and Zhang, 2008]. I also took advantage of the temperature sensitivity of the allele to perform a downshift experiment to determine what would happen to globally localized active MLC once the levels of Ran-GTP were restored. When anaphase cells with globally localized active MLC at 40˚C were downshifted to 33˚C, active MLC localization shifted and the cells oscillated (n=2; Figure 8B). As chromatin moved closer to the cortex, MLC delocalized from the nearest cortex, and accumulated at sites away from chromatin. These results strongly support the idea that Ran-GTP functions to localize the contractile proteins during cytokinesis.

Chapter 4. Discussion

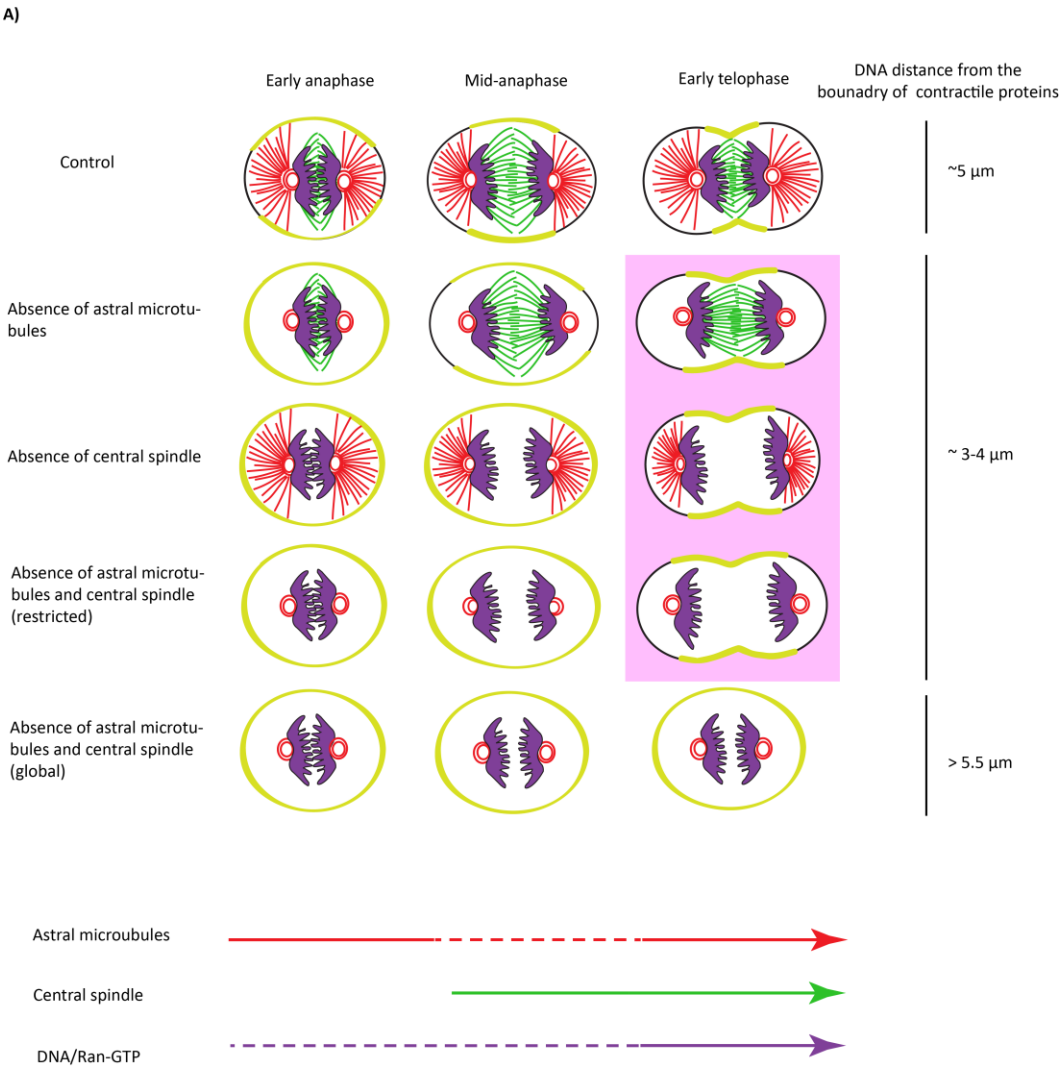
4.1 Summary

In this study I provide evidence to support the role of Ran-GTP in regulating the localization of contractile proteins during cytokinesis. In early telophase cells, the zone of active myosin and anillin at the equatorial cortex correlates with chromatin position. When the central spindle and/or astral microtubules are disrupted, contractile proteins are more broadly localized. In these cells, chromatin moves closer to the polar cortex and the distance between the boundary of accumulated contractile proteins and chromatin decreases in comparison to control cells. Removal of both the central spindle and astral microtubules in bipolar cells shows that chromatin is sufficient to alter the localization of anillin, as long as it is within 3-4 um of the cortex. This distance varies depending on cell shape and polarity, as it is higher and more variable in "non-bipolar" cells. In cells where chromatin is more centrally positioned and is at a greater distance from the cortex, anillin localizes globally in both bipolar and "non-bipolar" systems. One of the signals associated with chromatin is Ran, and over-expressing constitutively active Ran (Q69L) targeted to the equatorial membrane changes the boundary of anillin localization and causes asymmetric furrow ingression or oscillation. Interestingly, inactivating endogenous Ran using a tsRCC1 mutant leads to the global localization of myosin in anaphase and alters furrowing. Since Ran-GTP exists as gradient in cytoplasm with higher levels in the vicinity of chromatin during mitosis, these data suggest that in cells lacking functional central spindle and/or astral microtubules, chromatin becomes essential to properly position contractile proteins. In these cells it moves closer to the cell cortex, providing higher levels of active Ran near the cell cortex which in turn contribute in restricting the contractile proteins along the cell cortex. This

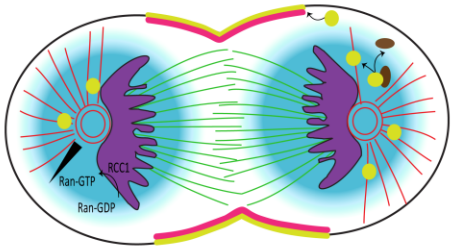
evidence not only supports a role for Ran-GTP, but also highlights the fact that different pathways work together to precisely position the contractile ring.

Ran-GTP likely coordinates with the central spindle and astral microtubules to restrict the localization of contractile ring components during anaphase and early telophase (Figure 9). Based on previous studies, astral microtubules contribute signals to restrict the localization of contractile proteins throughout anaphase and telophase. Disrupting the astral microtubules at any stage during anaphase and ingression results in an increase in the breadth of contractile ring proteins [Murthy and Wadsworth, 2008; van Oostende, Haji Bik and Piekny, unpublished observations]. Since the central spindle does not fully form and extend to the cortex until mid-anaphase, it is likely that prior to this, signals that mediate the localization of contractile proteins come from other pathways [Glotzer, 2009]. However, during mid-anaphase, the central spindle directs the activation of RhoA in a defined zone, and disruption of the central spindle causes global localization of active RhoA [Bement *et al*., 2005; Yuce *et al*., 2005]. Yet, contractile proteins are cleared from the polar cortex as these cells progress through telophase, although their localization remains broad along the equatorial cortex [Yuce *et al*., 2005; Piekny and Glotzer, 2008; van Oostende, Haji Bik and Piekny, unpublished observations]. This suggests that other pathways regulate the localization of contractile proteins after central spindle disruption, particularly during early telophase. Disruption of the central spindle in combination with removal of astral microtubules in bipolar cells causes contractile proteins to spread along the entire cortex in ~57% of the cells. However, in the other 43% of cells, contractile proteins are still cleared from the polar cortex. Based on my results and studies by Kiyomitsu and Cheeseman (2013), chromatin may move closer to the polar cortex in these cells to inhibit the localization of contractile proteins via Ran-GTP.

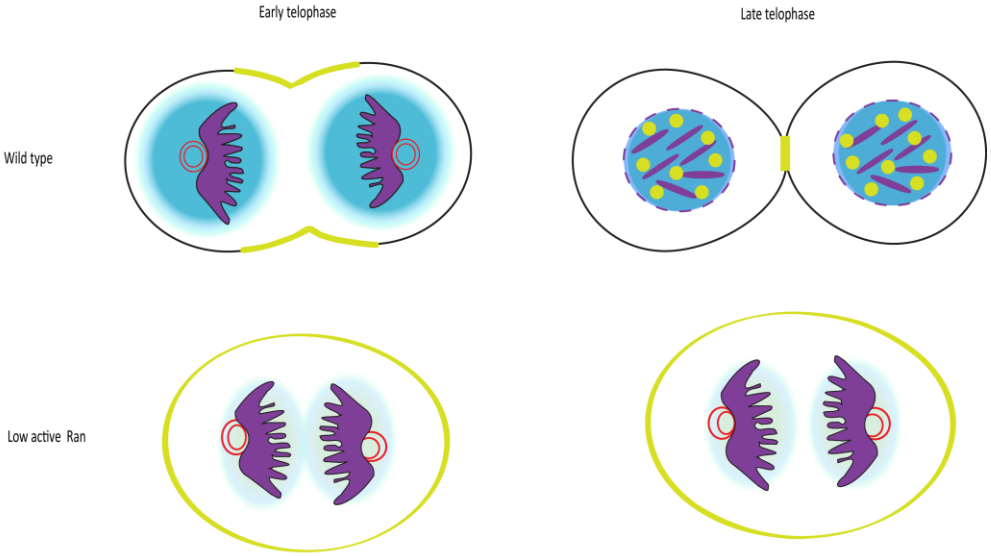
To summarize, my model suggests that in early anaphase astral microtubules act as the major mechanism to exclude contractile proteins from the


A)
Early anaphase
Mid-anaphase
Early telophase
DNA distance from the bounadry of contractile proteins
Control
~5 µm
Absence of astral microtu-bules
Absence of central spindle
~ 3-4 µm
Absence of astral microtu-bules and central spindle (restricted)
Absence of astral microtu-bules and central spindle (global)
> 5.5 µm
Astral microubules
Central spindle
DNA/Ran-GTP

B)


Ran-GTP
RCC1
Ran-GDP

C)


Early telophase
Late telophase
Wild type
Low active Ran

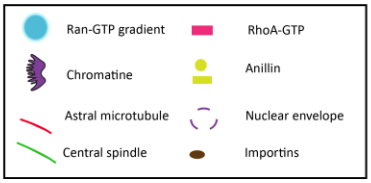

Ran-GTP gradient
RhoA-GTP
Chromatine
Anillin
Astral microtubule
Nuclear envelope
Central spindle
Importins

Figure 9. Model showing how multiple pathways regulate the localization of contractile proteins. A) Cartoon schematics show how the astral microtubules, central spindle, and chromatin could work together to precisely position the contractile ring. In early anaphase, astral microtubules may function as the major mechanism to exclude contractile proteins from the cell cortex. Disruption of astral microtubules in any part of anaphase or telophase cause contractile proteins to localize more broadly. As cells progress through anaphase, the central spindle forms and is the major pathway that directs the localization of active RhoA at the equatorial cortex. Central spindle disruption causes contractile ring proteins to localize globally in mid-anaphase, and broadly in early telophase. chromatin may provide cues early, since decreasing Ran-GTP in early anaphase cells caused myosin to localize globally. chromatin also provides cues as cells progress through anaphase and telophase. In the absence of both astral microtubules and the central spindle, it moves closer to the cell cortex (within 3-4 μm), providing higher levels of active Ran near the cell cortex which in turn contribute in restricting the contractile proteins along the cell cortex. **B)** A model suggesting how Ran can regulate contractile ring position via regulating anillin. Ran-GTP releases anillin from importins, this frees it to bind to microtubules or RhoA. At the equatorial cortex, high levels of active RhoA outcompete microtubules for anillin binding, while in other regions of the cell active RhoA levels are too low to compete for anillin. **C)** A model shows that as cells progress through telophase, the high levels of Ran-GTP associated with chromatin helps reform the nuclear envelope. Proteins with NLS sites including anillin and Ect2 are sequestered in the nucleus as cells exit mitosis. In cells with low levels of active Ran, contractile ring proteins localize globally and the nuclear envelope likely fails to re-assemble. Thus, contractile proteins such as anillin have continued access to the cortex and cause an early telophase arrest.

cell cortex (Figure 9). As cells progress through anaphase, the central spindle forms and is the major pathway that directs the localization of active RhoA at the equatorial cortex. As cells progress through telophase, signals from the astral microtubules and central spindle regulate the localization of contractile proteins, but importantly, chromatin also functions to restrict their localization from the

polar cortex. However, further experiments are required to determine the relative requirements for the different pathways

4.2 Chromatin position correlates with the localization of contractile proteins

Chromatin may regulate the localization of contractile proteins in a distance-dependent manner. I measured the distance of chromatin from the boundary of contractile proteins in bipolar and "non-bipolar" systems under different conditions. My results suggest that chromatin may restrict the localization of contractile proteins when it is within 3-4 µm from the cortex. Although this distance is smaller in comparison to control cells, the 'minimum' distance of 3-4 µm was observed after central spindle disruption, astral microtubules, and after co-disruption of both the central spindle and astral microtubules. For example, in cells with disrupted central spindles, the distance from chromatin to the boundary of accumulated anillin was 3.46 (+/- 0.41) µm vs. 5.07 (+/- 0.45) µm for control cells (Figure 4C). When astral microtubules were depolymerized in cells with disrupted central spindles, the distance from chromatin to the boundary of accumulated anillin (restricted localization) was 3.93 (+/- 0.55) µm (Figure 4C). In cells where chromatin remained more centrally located and was further from the cortex (5.5 +/-0.6 µm), anillin was not removed from the polar cortex (Figure 4A). These data suggest that chromatin may not provide the primary signal for restricting the localization of contractile proteins during early stages of cytokinesis, when the chromatin is more centrally located in the cell (e.g. during early anaphase). However, as chromatin moves closer to the cortex, it could come sufficiently close to restrict the localization of contractile proteins. The chromatin-associated mechanism for regulating contractile proteins becomes particularly important when other pathways are disrupted, and contractile proteins spread along the cortex. My data suggests that chromatin could contribute to the exclusion of contractile proteins

in combination, or redundantly with other pathways, but requires proximity to the cortex. Ran-GTP could be the diffusible signal associated with chromatin that regulates the position of the contractile ring (see section 4.3). RCC1 (RanGEF) binds to chromatin, and generates high levels of Ran-GTP in the vicinity. This could create a gradient of Ran-GTP that only reaches threshold levels at the cortex when the chromosomes are positioned nearby. When other pathways are perturbed, higher levels of active Ran may be required to restrict the localization of contractile proteins (e.g. to oppose RhoA pathway).

Multiple pathways also likely function to restrict the localization of contractile proteins in monopolar cells. These cells had a minimum distance of 6.6 (+/- 0.65) µm from chromatin to the boundary of accumulated anillin (Figure 5B). Although this distance was greater than seen in the control bipolar cells, these cells are structurally different, which could cause a change in the relative contributions of some pathways vs. others. For example, they do not have a 'central spindle' comprised of bundled anti-parallel microtubules, and instead have a single axis of parallel bundled microtubules that lie beneath the polarized cortex, with chromatin at the opposite side of the cell. Central spindle proteins accumulate at the ends of these microtubules where they can directly signal to the overlying cortex. In these cells, chromatin may not play as much of a role in regulating the localization of contractile proteins, given its distance from the polarized cortex.

Chromatin is sufficient to restrict the localization of contractile proteins in cells lacking microtubules and that have random geometry. Cells treated for several hours with 100 nM Nocodazole to fully disrupt microtubules and forced to exit mitosis showed two phenotypes depending on the position of chromatin (Figure 6B). Similar to bipolar cells, when chromatin was located more centrally, anillin localized globally around the cortex, and when chromatin was located closer to the cortex, anillin cleared from the overlying areas. In these cells, the distance of chromatin to the boundary of accumulated anillin was 5.04 +/- 2 µm

(Figure 6C). It is difficult to compare the chromatin distance in these cells with the bipolar cells described above, due to high variability in the data, likely because the boundaries of anillin formed more randomly in this system and were more difficult to resolve spatially. However, cell shape could be important in helping to stabilize contractile proteins. A more random localization of anillin or myosin could impart uneven contractions at different regions of the cortex, which could cause changes in cortical flow. If there is a diffusible signal associated chromatin that can regulate contractile proteins, then the change in flows could alter how quickly the signal is able to reach the cortex.

There were some caveats to these experiments that must be taken into consideration and could have altered the outcome of the data. For example, although I took care to match the timing of cells after the various treatments, there could be changes in chromatin-boundary distance measurements due to changes in the timing of cytokinesis in comparison to control cells. Furthermore, the GFP levels from H2B:GFP were often very high, which may have obscured weaker signals from Anillin:GFP, causing a shift in the 'boundary' of accumulated anillin. I corrected this by using H2B:mRuby in place of H2B:GFP, but did not have time to repeat all of the experiments using this probe.

4.3. Ran-GTP regulates the localization of contractile proteins

Ran-GTP regulates the localization of contractile proteins during cytokinesis. When active Ran was targeted to the equatorial membrane, it caused a change in the breadth of Anillin:GFP localization and caused asymmetric furrow ingression or oscillations in a level-dependent manner (Figure 7B, C, and D). For example, when the levels of Ran (Q69L) increased, it caused stronger changes in anillin localization and cytokinesis phenotypes. Also, decreasing endogenous Ran-GTP by inactivating its GEF, RCC1, caused broad or global localization of myosin, and asymmetric or failed furrow ingression, likely depending on the levels of

inactive Ran (Figure 8A). However, cells with global MLC localization may not have had properly assembled nuclear envelopes in telophase. Thus, contractile proteins such as anillin and Ect2, which are normally sequestered in the nucleus during interphase, have continued access to the cortex. All of these data suggest that Ran-GTP negatively regulates the localization of anillin and myosin.

The mechanism by which Ran alters the localization of contractile proteins is not known. However, I will speculate on several possibilities based on previously published data. First, Ran could specifically affect the localization of anillin. Anillin contains NLS elements in its N-terminus (predominant one) and C-terminus, which bind to importin alpha and beta [Oegema *et al*., 2000; Silverman-Gavrila *et al*., 2008; Piekny and Maddox, 2010]. The site in the C-terminus overlaps with the septin binding domain on anillin, which is important for recruiting septins to the cleavage furrow. Indeed, importin alpha and beta can compete with septin binding to anillin in vitro [Silverman-Gavrila *et al*., 2008]. Based on these data, one model is that when Ran-GTP levels are high in the cytosol during mitosis, it releases importins from anillin, freeing its binding site for septins, which recruits septins to the furrow. However, this model suggests a positive role for Ran-GTP in regulating the localization of contractile proteins, and does not fit with my data or with Kiyomitsu and Cheeseman's (2013) results supporting an inhibitory role for Ran-GTP. A modification of this model is that when Ran-GTP releases anillin from importins, this frees it to bind to microtubules or RhoA. Previous studies in our lab have shown that anillin may bind to active RhoA and microtubules. Their binding sites could be close together, and there could be competition between active RhoA and microtubules for binding to anillin [Jaramillo Garcia, Haji Bik and Piekny, unpublished observations]. Therefore, at the equatorial cortex, high levels of active RhoA outcompete microtubules for anillin binding, while in other regions of the cell active RhoA levels are too low to compete for anillin.

Another possibility is that Ran-GTP could regulate proteins that oppose RhoA activity, for example by regulating MP-GAP, which down-regulates RhoA

activity at the polar cortex [Zanin *et al*., 2013]. Interestingly, depleting MP-GAP causes the formation of ectopic cortical protrusions, but does not stably increase the zone of localized active RhoA [Zanin *et al*., 2013]. However, co-disruption of astral microtubules causes the global localization of contractile proteins [Zanin *et al.*, 2013]. This redundancy fits with my data suggesting that chromatin or Ran-GTP functions redundantly to regulate the localization of contractile proteins. However, it is not clear how Ran could mechanistically affect MP-GAP. Perhaps through binding to importins, which may normally sequester MP-GAP, but frees it to interact with the cortex in the presence of Ran-GTP. MP-GAPs were just recently described and have low amino acid sequence conservation with other GAPs, and across metazoans. Further studies on MP-GAP structure could shed light on possible mechanisms for its regulation. Yet another way in which Ran could oppose RhoA is to activate the Rac pathway. In support of this idea, Ran-GTP has been shown to activate Rac and Cdc42 during meiosis in mouse oocytes [Deng *et al*., 2007; Dehapiot *et al*., 2013]. Cells form blebs at the polar cortex, which function as valves to release cortical tension and stabilize the division plane [Sedzinski *et al*., 2011]. It is possible that Ran promotes changes in cortical stiffness by activating Rac to form Arp2/3-dependent branched F-actin. The mechanism by which Ran could regulate Rac activity is not clear, although it could compete with importins to release a GEF for Rac at the polar cortex.

To summarize, my data sheds light on a novel pathway that regulates the localization of contractile proteins to properly position the contractile ring at the right time and place. This pathway works in combination with other pathways to regulate the localization of contractile proteins, and becomes essential when the other pathways are perturbed. Importantly, linking the localization of contractile proteins to chromatin position ensures that the contractile ring always forms in a location that would permit the proper inheritance of each set of chromosomes into the daughter cells.

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