RhoA Recruits Ect2 and Anillin to the Cortex where they interact to Maintain Furrow Ingression during Cytokinesis.

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Abstract

RhoA recruits Ect2 and anillin to the cortex where they interact to maintain furrow ingression during cytokinesis.

Paul Frenette

Cytokinesis is the division of one cell into two genetically identical daughter cells due to the ingression of an actin-myosin based contractile ring. Cytokinesis failure can lead to genomic instability and cancer. In human cells, contractile ring formation and ingression depends on active RhoA, which forms in a discrete region of the cortex during anaphase. Signals from the mitotic spindle shape the zone of active RhoA, but the mechanism is still poorly understood. We investigate how this discrete zone of active RhoA is formed to establish the division plane. Ect2, the guanine nucleotide exchange factor for RhoA, localizes to the anaphase spindle and activates RhoA after anaphase onset. We found that Ect2 is also recruited to the equatorial cortex in a RhoA-dependent manner, suggesting a positive feedback loop reinforces the activation of RhoA in the division plane. Ect2's cortical interaction is essential for its activity, and one hypothesis is that cortical Ect2 is more active vs. Ect2 in other locations of the cell. Furthermore, Ect2 can interact with anillin, a conserved scaffold protein that also localizes to the cell cortex during anaphase and is essential for cytokinesis. In anillin-depleted cells, the cortical pool of Ect2 is lost, which could lead to decreased localization of active RhoA and unstable furrowing. We hypothesize that in human cells, the Ect2-anillin interaction anchors anaphase spindle microtubules to the equatorial cortex to generate active RhoA in a discrete zone to form the contractile ring and maintain its ingression in the correct plane.

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Contributions of authors

Figure 1: The RhoA pathway model was designed by Drs. Alisa Piekny, Michael Werner and Michael Glotzer.

Figure 3: - Dr. Alisa Piekny contributed to figure 3A and B by taking the images.

Figure 4: - Dr. Alisa Piekny contributed to figure 4A by taking the images.

Figure 5: - Eric Haines/Paknoosh Pakarian contributed to figure 5B by performing the PIP strips.

- Dr. Alisa Piekny contributed to figure 5C by taking the images.

Figure 7: - Dr. Alisa Piekny contributed to figure 7A by taking the images.

Figure 8 - Dr. Alisa Piekny made all of the figures in figure 8 (A, B, C, D and E)

Figure 10: - Dr. Alisa Piekny contributed to figure 10A by taking the images and 10B by performing the quantitation.

Figure 11: Dr. Alisa Piekny designed the models.

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Table of Abbreviations

AA – Amino acids

AHD – Anillin homology domain

ANI – Anillin

Cdk1 – Cyclin dependent kinase 1

CPC – Chromosome passenger complex

DAPI - 4, 6-diamidino-2-phenylindole

DH – Dbl homology domain

DMEM – Dulbecco's Modified Eagle Medium

DTT – Dithiothreiol

Ect2 – Epithelial cell transformer 2

FBS – Fetal bovine serum

GAP – GTPase activating protein

GEF – Guanine nucleotide exchange factor

GDP – Guanosine diphosphate

GFP – Green fluorescent protein tagged vector

GST – Glutathione S-transferase

GTP – Gunaosine triphosphate

HEK 293 – Human embryonic kidney cells

Hela – Cervical cancer cell line

IF – Immunofluorescence

INCENP – Inner centromere protein

KIF4A – Kinesin family member 4A

MAPs – Microtubule associated proteins

MBP – Myelin basic protein vector

MKLP1 – Mitotic kinesin like protein

MYC – Myc tagged vector

NDS – Normal donkey serum

PCR – Polymerase Chain reaction

PH – Plekstrin homology domain

PIP – Phosphoinositol phosphate

Plk1 – Polo-like kinase 1

PBS (T) – Phosphate buffer saline (Triton X)

PRC1 – Protein regulator of cytokinesis 1

PS – Penicillin streptomycin

RBD – Rho GTP binding domain

RHO (A) – Ras homolog gene family, member (A)

RLC – Regulatory myosin light chains

RNAi – RNA interference

RNAi^{r} – resistant RNA interference

ROCK – Rho dependent kinase

Ser- Serine

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS (T) – Tris buffer saline (Triton X)

TCA – Trichloroacetic acid

Thr – Threonine

9E10 – a cell line expressing anit-Myc monoclonal anitbodies.

Introduction

1.1 Overview

Cytokinesis occurs at the end of mitosis to physically separate one cell into two genetically identical daughter cells. Cytokinesis failure can lead to changes in cell fate and developmental abnormalities, but also genomic instability, which can lead to cancer. Cytokinesis is highly coordinated with mitosis to ensure that division occurs only after segregation of sister chromatids. Mitosis proceeds by changes in cyclin dependent kinase (Cdk) activity, and errors in chromosome alignment will activate checkpoints to ensure the cell corrects the errors before completing mitosis, and initiating cytokinesis.

Mitosis is comprised of four main stages. During the first stage, prophase/prometaphase, genetic material condenses to form chromosomes and the nuclear envelope breaks down. Sister chromatids remain attached at their centromeres and replicated centrosomes initiate spindle assembly. During metaphase, microtubules from the mitotic spindle form attachments at the kinetochores of chromosomes and align them along the metaphase plate. When equal tension is achieved across the kinetochores, a checkpoint is released and sister chromatid pairs segregate toward their respective poles during anaphase. In telophase, after chromosome segregation, the nuclear envelope re-assembles around decondensing chromosomes. The signals that stimulate anaphase entry, *i.e.* release from mitotic checkpoints leading to the downregulation of Cdk1 activity, also initiate cytokinesis (Wheatley et al., 1997). During anaphase, an actin-myosin ring forms in a specified plane, and constricts during telophase to pinch the cell into separate daughter cells

1.2 Cytokinesis in animal Cells

An important aspect of cytokinesis is to determine the division plane to ensure that both daughter cells properly inherit DNA, cytosolic and cortical components. In animal cells, the division plane is determined by the position of the mitotic spindle, and is under spatial and temporal control. The astral and central spindle microtubules that form the mitotic spindle signal to the overlying cortex to form a discrete actin-myosin contractile ring in a plane that bisects the spindle and lies between the segregating chromosomes (Burgess and Chang, 2005). However, the "astral" and "central spindle" pathways have been shown to be redundant.

Astral microtubules emanate from centrosomes toward the polar cortex and deliver inhibitory cues to prevent the localization of contractile ring proteins in the poles of the cell, while permitting their localization in the centre of the cell (Bringmann et al., 2005; Dechant and Glotzer, 2003; Lewellyn et al., 2010). The central spindle is formed by bundled overlapping antiparallel microtubules between segregating chromosomes and stimulates contractile ring formation (centrally positioned astral microtubules may also stimulate furrowing; Glotzer, 2004; Glotzer, 2009). Both the astral and central spindle microtubules work together to define the cleavage plane, however, as long as one pathway is intact, a correctly positioned contractile ring often can form.

The GTPase, RhoA, is required to form the contractile ring and its activator, the Guanine nucleotide exchange factor (GEF) Ect2, is recruited to the central spindle where it generates active RhoA in the central plane of the cell. Exactly how a discrete zone of RhoA is generated and maintained in the overlying cortex, when its regulator is on the

central spindle, is not clear. Temporally, metazoan cytokinesis starts during anaphase when Cdk1 becomes inactive (Niiya et al., 2005; Shuster and Burgess, 1999). Once inactivated, targets of Cdk1, such as the central spindle proteins Mklp1 (kinesin-like protein) and Prc1 (protein regulator of cytokinesis 1), become dephosphorylated and form the central spindle (Glotzer, 2009). Other targets include Ect2, and its dephosphorylation is required to form the contractile ring (Glotzer, 2009). Controlling the function of these proteins by Cdk1-phosphorylation ensures that the timing of cytokinesis is coordinated with anaphase onset and chromosome segregation.

1.2.1 The Central Spindle

The central spindle is an important regulator of cytokinesis and is composed of antiparallel microtubules bundled by many proteins. These components work together to create an "on" switch for cytokinesis. Central spindle assembly and regulation relies on microtubule associated proteins (MAPs) including Prc1, the centralspindlin complex and the chromosome passenger complex (CPC; Jeyaprakash et al., 2007; Jiang et al., 1998; Mishima et al., 2004). Prc1 is a highly conserved microtubule bundling protein, which localizes to microtubules by binding to the kinesin motor protein KIF4A, and is essential for central spindle formation in *C. elegans*, *Drosophila* and human cells (Glotzer, 2009; Jiang et al, 1998). It also may act as a docking site for Plk1 (polo kinase 1, a mitotic kinase required for cytokinesis, see section 1.3.2; Glotzer, 2009). Cells lacking Prc1 cannot form central spindles and other central spindle proteins fail to localize (Mollinari et al., 2002). Despite its essential role for central spindle assembly, Prc1 is dispensable

for cytokinesis, likely since astral microtubules also restrict the localization of contractile ring proteins to form the division plane (Glotzer, 2009). The highly conserved centralspindlin complex is made up of Mklp1 and Cyk-4/MgcRacGAP (Glotzer, 2009; Mishima et al., 2002; Mishima et al., 2004). Both Mklp1 and Cyk-4 are required for stable central spindle formation in many animal cell types, however, some central spindle proteins can partially localize in their absence, including Prc1 (Glotzer, 2009). While contractile rings form in the absence of Mklp1, they do not form in the absence of Cyk-4, suggesting Cyk-4 has a more direct role in contractile ring formation (see section 1.3.2; Yuce et al., 2005). The chromosome passenger complex (CPC) is made up of INCENP (inner centromere protein), AuroraB kinase, survivin and borealin. Similar to the centralspindlin complex, the CPC is required for stable central spindle formation and spindle assembly, but some proteins partially localize in their absence. Furthermore, they are also dispensable for contractile ring formation (Glotzer, 2009).

As described above, decreased Cdk1 activity is the cue for anaphase onset and central spindle assembly. Cdk1 phosphorylates Prc1, which prevents its association with microtubules and KIF4A until its dephosphorylation (Jiang et al., 1998; Glotzer, 2009; Zhu et al., 2006). Furthermore, Prc1 only recruits Plk1 to the central spindle after dephosphorylation (Glotzer, 2009; Wolfe et al., 2009). Cdk1 phosphorylation of Mklp1 blocks its association with microtubules and Cyk-4, and prevents the centralspindlin complex from forming before it is needed (Glotzer, 2009; Mishima et al., 2004). Cdk1 also phosphorylates Ect2 and keeps it inactive until the phosphate is removed, after which it binds to Cyk-4 (Yuce et al., 2005; Kamijo, 2006). Therefore, Cdk1 is an essential

regulator of cytokinesis by coordinating the timing of central spindle assembly and RhoA activation (see 1.3.2) after mitotic exit.

1.2.2 The RhoA pathway

The key regulator of contractile ring formation and ingression is RhoA, a small GTPase related to the family of Ras-like GTPases. This family of GTPases includes Rho, Rac and Cdc42, and there are three isoforms of Rho; RhoA, RhoB and RhoC (Heasman and Ridley, 2008). However, only RhoA seems to be necessary for cytokinesis, since it is most conserved across metazoans and its depletion causes cytokinesis defects in organisms including *C. elegans*, *Drosophila*, *Xenopus* and humans (Piekny et al, 2005). Rho GTPases are active when bound to GTP and inactive when bound to GDP. Their GTP-bound state is regulated by guanine nucleotide exchange factors (GEFs) that exchange GDP for GTP and their GDP-bound state is regulated by GTPase activating proteins (GAPs), which stimulate hydrolysis of GTP to GDP. Ect2 is the main GEF for RhoA during cytokinesis, although in human cells other GEFs, including MyoGEF and GEF-H1, may contribute to RhoA activation (Asiedu et al., 2009; Birkenfield et al., 2007; Wu et al., 2006). The main GAP for RhoA during cytokinesis is not known, although candidates include RGA-3/4 in early *C. elegans* embryos, p190 RhoGAP in human cells or Cyk-4 (conserved among metazoans; Jantsch-Plunger et al., 2000; Manchinelly et al., 2010; Schonegg et al., 2007; Su et al., 2003). Studying the role of Cyk-4 in inactivating RhoA is complicated by the fact that it is also required to activate Ect2 (the GEF; see below).

Regulation of RhoA activity is crucial for contractile ring formation and ingression as shown in Figure 1. Upon anaphase onset, decreased Cdk1 activity permits central spindle assembly and the formation of complexes that regulate RhoA. Cyk-4 and Mklp1 form a complex and bundle microtubules to help form the central spindle (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007). Prc1, which also bundles central spindle microtubules, recruits Plk1, where it phosphorylates Cyk-4 (Zhu et al., 2006; Wolfe et al., 2009). Dephosphorylation of Ect2 allows it to bind Cyk-4, which recruits it to the central spindle and helps potentiate its activity to generate active RhoA in the central plane of the cell (Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b). Increased levels of GTP bound RhoA signal to downstream effectors, such as formins, Rho-dependent kinase (ROCK) and citron kinase. Formins induce actin polymerization and ROCK activates myosin to form a functional contractile ring (Piekny et al, 2005). Citron kinase is another Rho effector that phosphorylates myosin, but its function during cytokinesis is unclear. Citron kinase localizes to the cleavage furrow and its depletion in *Drosophila* cells causes late cytokinesis defects, suggesting it may help stabilize midbody formation (Echard et al., 2004; Eda et al., 2001; Gruneberg et al., 2006). After furrow ingression, it is not clear how long active RhoA is required. Ect2 relocalizes to the newly forming daughter nuclei, and its removal combined with increased levels of Cyk-4 suggests that RhoA would be turned off at this time (Piekny et al, 2005).

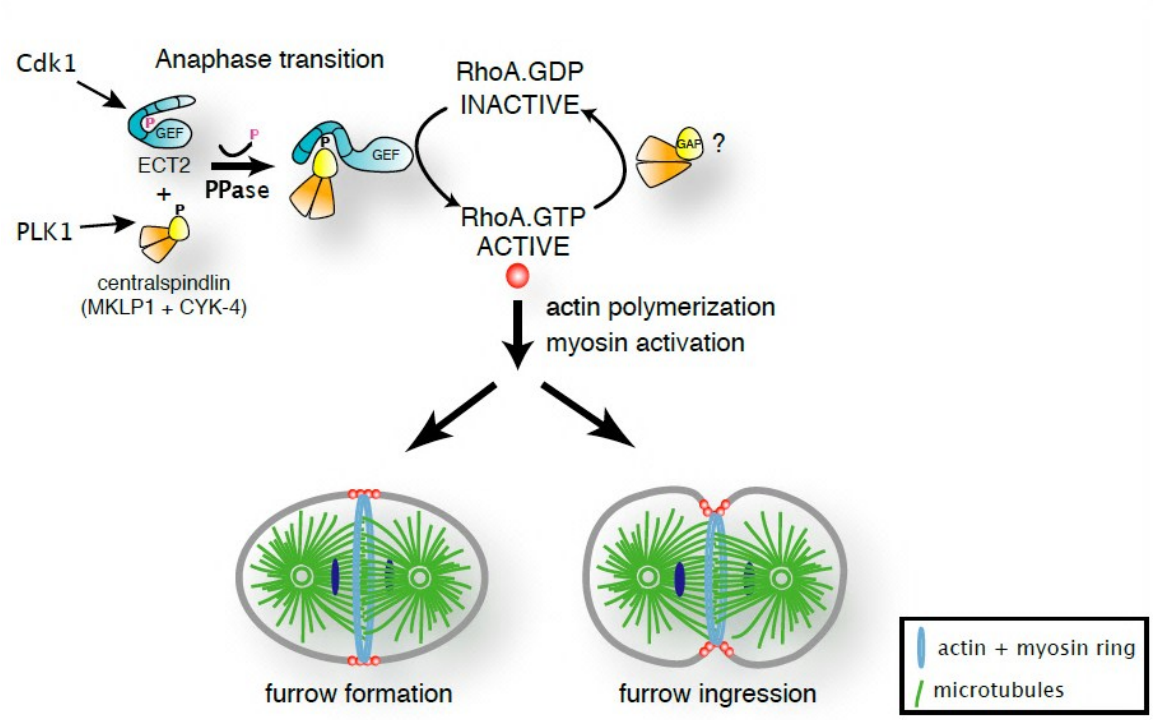


Figure 1 The RhoA pathway. Cdk1 phosphorylates and inactivates Ect2 before anaphase. After anaphase entry, Cdk1 activity decreases and Ect2 is dephosphorylated. Also, Plk1 phosphorylates the centralspindlin protein Cyk-4. Phosphorylated Cyk-4 recruits Ect2 to the central spindle, which is positioned between segregating chromosomes in the central plane of the cell. Ect2 then activates RhoA by exchanging GDP for GTP. Once RhoA is active, it regulates downstream effectors to polymerize actin and activate myosin to form a functional contractile ring.

1.2.3 The Contractile ring

During anaphase, increased pools of active RhoA will lead to the formation of an actin-myosin contractile ring. Actin filaments are polymerized by formins, proteins with multiple domains that permit the assembly of profilin-associated actin monomers into long filaments. Actin filaments align parallel to the cleavage furrow and come into close contact with the plasma membrane (Reichl et al., 2008). Nonmuscle myosin II is a motor protein that uses the power from ATP hydrolysis to slide actin filaments along each other to create the force necessary to pinch in the cell by a purse-string like mechanism (Matsumura, 2005; Pollard, 2010; Reichl et al, 2008). As described above, myosin activity is regulated by ROCK. Myosin filaments are formed from hexamers of two nonmuscle myosin II heavy chains, two essential myosin light chains and two regulatory myosin light chains (RLC). Phosphorylation of RLC at conserved sites (Ser-19/Thr-18) activates myosin's motor activity (Matsumura, 2005). Myosin phosphatase dephosphorylates RLC, causing downregulation of myosin activity (Matsumura, 2005). ROCK can phosphorylate and inactivate the regulatory subunit of myosin phosphatase, rendering it inactive, and can directly phosphorylate RLC (Matsumura, 2005). Therefore, ROCK activation leads to the upregulation of myosin activity for contractile ring assembly and ingression. In *C. elegans* embryos, ROCK mutants cause lower levels of phospho-specific myosin and embryos display cytokinesis defects, specifically, furrow ingression is slowed in comparison to wild-type embryos (Piekny and Mains, 2002). ROCK mutants are suppressed by mutations in the regulatory subunit of myosin phosphatase, supporting their antagonistic roles (Piekny and Mains, 2002).

1.3 Anillin

Anillin is a multidomain protein first isolated as an actin-binding protein from *Drosophila* embryonic cells and is conserved among metazoans (Field and Alberts, 1995; D'Avino, 2009; Piekny and Maddox, 2010). During cytokinesis, anillin colocalizes with actin, myosin and RhoA at the cleavage furrow and likely functions as a scaffold for the contractile ring (Figure 2; D'Avino, 2009; Piekny and Maddox, 2010). In its N-terminus, anillin has binding domains for F-actin and myosin (Field and Alberts, 1995; Straight et al., 2005). Although actin and myosin localize independently of anillin, anillin might restrict them to a defined plane during cytokinesis. In anillin-depleted cells the contractile ring forms and partially ingresses, but then laterally oscillates around the cell and regresses (Goldbach et al., 2010; Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008; Straight et al., 2005; Zhao and Fang, 2005a).

Anillin bundles F-actin and interacts with myosin (Field and Alberts, 1995; Straight et al., 2005). Anillin's ability to bundle F-actin is conserved in *Xenopus* and cultured human cells (Kinoshita et al., 2002). Septins assemble actin into ring-like structures *in vitro* and *in vivo*, and anillin's interaction with actin and septins helps form the linear filaments which may be important for contractile ring formation (Kinoshita et al., 2002; Piekny and Maddox, 2010). *Xenopus* anillin also directly interacts with non-muscle myosin II, and this interaction depends on RLC phosphorylation (Straight et al., 2005). The amino acid sequence of the myosin binding site is conserved in *C. elegans* ANI-1, *Drosophila* anillin and human anillin suggesting these isoforms also interact with myosin. The purpose of the interaction may be to crosslink myosin with other contractile ring components. In anillin-depleted cells, myosin initially localizes to the equatorial

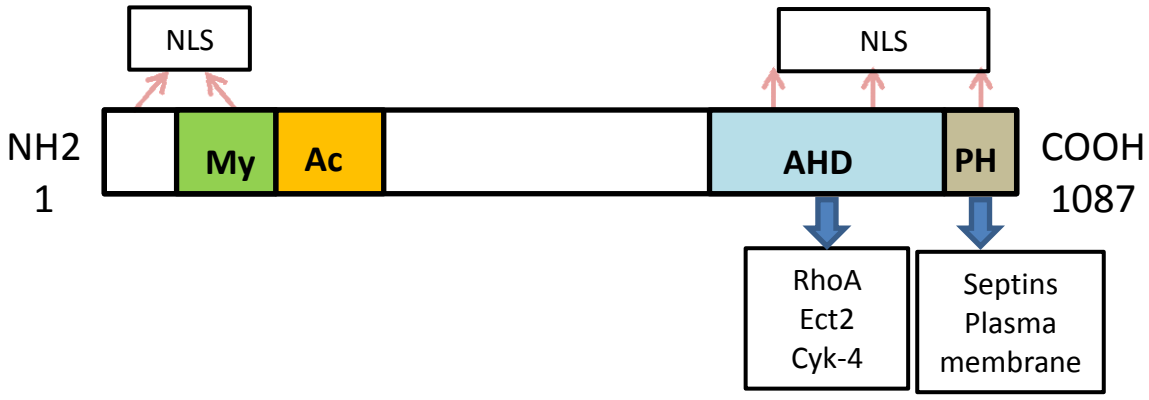


Figure 2 A hypothetical structure of the protein anillin. In the N-terminal region are the proposed binding regions of myosin (My), actin (Ac) along with two nuclear localization sequences (NLS). In the C-terminal region are the anillin homology domain (AHD) and the plekstrin homology domain (PH) along with three nuclear localization sequences. The AHD domain interacts with RhoA, Ect2 and Cyk-4 while the PH domain will interact with septins and the plasma membrane.

plane, but then mislocalizes around the polar cortex, likely driving their oscillation behavior (Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008; Straight et al., 2005; Zhao and Fang, 2005a). Interestingly, neither the actin nor myosin binding domains of anillin are required for anillin's cortical localization. Mutants lacking the actin and myosin domains still localize cortically, but they do not rescue loss of endogenous anillin (Oegema et al., 2000; Piekny and Glotzer, 2008).

In its C-terminus, anillin has a conserved anillin homology domain (AHD) that directly interacts with human RhoA (Figure 2; Piekny and Glotzer, 2008; Piekny and Maddox, 2010). This region shares homology with Rhotekin, a protein that specifically binds to active (GTP-bound) RhoA (Piekny and Glotzer, 2008). Anillin and RhoA colocalize during cytokinesis, and although RhoA is upstream of anillin (required for contractile ring formation and ingression; Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008), anillin also is required to stabilize RhoA as visualized by TCA fixation (Piekny and Glotzer, 2008; Zhao and Fang, 2005a). Over-expression of anillin in human lung cancer cells causes the generation of active RhoA (Suzuki et al., 2005). These results illustrate that anillin may contribute to the localization or generation of active RhoA to help define the division plane.

The AHD also interacts with RacGAP50C, the *Drosophila* Cyk-4 homologue (Figure 2; D'Avino et al., 2008; Gregory et al., 2008; Piekny and Maddox, 2010). C-terminal fragments of anillin directly bind to N-terminal fragments of RacGAP50C *in vitro* (D'Avino et al., 2008) and fixed FRET studies support the anillin-RacGAP50C interaction *in vivo* (Gregory et al., 2008). In anillin-depleted *Drosophila* larval brain cells, the central spindle failed to remain associated with the cortex (Gregory et al., 2008).

Therefore, anillin may help anchor the central spindle to the equatorial cortex to orient the division plane during cytokinesis (D'Avino, 2009).

Anillin may also interact with microtubules. *Drosophila* anillin was purified based on its ability to bind to both F-actin and microtubules (Sisson et al., 2000). Also, in cells treated with Latrunculin A to disrupt actin filaments, anillin structures form at the plus ends of microtubules (Hickson and O'Farrell, 2008). It is not clear what role anillin-microtubule binding might have, and whether this binding helps anillin organize the cortex to define the division plane (Piekny and Maddox, 2010; Tse et al., 2011).

The C-terminus of anillin, particularly the pleckstrin homology (PH) domain, interacts with septins, a conserved family of GTP-binding proteins (Figure 2). Anillin and septins colocalize at the contractile ring in *C. elegans* and human cells (Maddox et al., 2007; Oegema et al., 2000). As mentioned above, anillin and septins directly interact, and are able to direct the formation of F-actin into linear filaments *in vitro* (Kinoshita et al., 2002). Septin localization depends on anillin and in *C. elegans*, *Drosophila* and human cells, anillin-depletion or mutations prevents the recruitment of septins to the contractile ring (Field et al., 2005; Hickson and O'Farrell, 2008; Maddox et al., 2007; Oegema et al., 2000). Septins may also interact with phospholipids (Casamayor and Snyder, 2003; Zhang et al., 1999) and areas of concentrated septins (septin sheets) have been seen close to the plasma membrane (Rodal et al., 2005). Perhaps septins join the cytoskeleton to the plasma membrane through their interaction with anillin.

In summary, through its many interactions, anillin is a key regulator of cytokinesis. Anillin can crosslink all the major components of the cell including the

plasma membrane (through septins), the mitotic spindle (through Cyk-4), actin-myosin filaments and their upstream regulator, RhoA, to reinforce and maintain the division plane.

1.4 Ect2

Ect2 (Epithelial cell transformer protein 2) is an essential protein for cytokinesis in metazoans and the *Drosophila* homologue, Pebble, was first identified in a screen for recessive embryonic lethal mutations (Jugens et al., 1984; Prokopenko et al., 1999). Ect2 was subsequently identified in human cells as a proto-oncogene from a murine keratinocyte cDNA expression library (Miki et al., 1993). Human Ect2 is 883 amino acids with a predicted molecular weight of 104 kDa. Ect2 belongs to the Dbl family of GEF proteins that share homology in two regions; the DH (Dbl homology) and PH (Pleckstrin homology) domains (Rossman et al., 2005). Ect2's DH region coordinates the exchange of GDP for GTP on RhoA; however, the exact function of the PH domain is unclear. PH domains in other proteins interact with phospholipids or other proteins, and these interactions may mediate the cortical localization of Ect2 to bring it into close proximity to its substrate (Chalamalasetty et al., 2006). In addition, the PH domain may contribute to Ect2's GEF activity based on *in vitro* experiments (Solski et al., 2004). The N-terminus of Ect2 serves a regulatory function and contains domains with homologies to other cell cycle control and repair proteins (Saito et al., 2003). In particular, Ect2 contains two BRCT (breast cancer gene 1 carboxy terminal) motifs that form a phosphopeptide-binding module (Saito et al., 2003; Saito et al., 2004). This module interacts with itself in

an autoregulatory manner (see below; Saito et al., 2004), and also with the central spindle protein Cyk-4, which potentiates Ect2's activation and localizes it to the central spindle (Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b). Between the N and C-terminal halves, Ect2 has two nuclear localization signals (NLS) that keep it in the nucleus until nuclear envelope breakdown down during mitosis, and direct its relocalization into daughter nuclei after furrow ingression (Saito et al., 2004).

Recent studies revealed the mechanism that activates Ect2 during cytokinesis. In metaphase, Ect2 is held in an inactive conformation by Cdk1 phosphorylation at a site that is adjacent to the DH PH regions, which forms a phosphopeptide motif that interacts with Ect2's BRCT domains (Hara et al., 2006; Saito et al., 2004; Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b). Constructs lacking the N-terminus of Ect2 are constitutively active and cannot self-regulate (Kim et al., 2005; Saito et al., 2003). During anaphase, Cdk1 activity decreases due to Cyclin B degradation, and the levels of phosphorylated Ect2 decrease (Yuce et al., 2005). In addition, Cyk-4 is phosphorylated by Plk1, after which it binds the N-terminus of Ect2 (Brennan et al., 2007; Petronczki et al., 2007; Wolfe et al., 2009). Formation of the Ect2-Cyk-4 complex helps recruit Ect2 to the central spindle where it may activate RhoA in the central plane of the cell. Cyk-4 likely is required for Ect2 activation since Cyk-4 depletion causes cytokinesis defects and loss of RhoA localization similar to Ect2 depletion (Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b).

Ect2's localization throughout the cell cycle is highly dynamic. During interphase, it is nuclear and after nuclear envelope breakdown it becomes cytosolic. During anaphase and telophase Ect2 is localized predominantly on the central spindle, although cortical-

enriched pools are also observed. After furrow ingression Ect2 localizes to an electron dense structure that forms at the end of cytokinesis known as the midbody and will then re-localizes to the newly formed daughter nuclei (Chalamalasetty et al, 2006; Simon et al., 2008; Yuce et al., 2005). Despite its nuclear localization in cultured human cells (*i.e.* Hela cells), Ect2 may have more diverse localization patterns in other cell types (*i.e.* MDCK cells) where it has been shown to have non-mitotic functions in cell polarity (Liu et al., 2006). Although Ect2 primarily acts on RhoA for cytokinesis (Miki et al., 1993; Saito et al., 2004; Tatsumoto et al., 1999), it may regulate the activity of other GTPases for non-mitotic functions (Justilien and Fields, 2009; Oceguera-Yanez et al., 2005).

1.5 Cancer

As previously mentioned, Ect2 was first described in human cells as a proto-oncogene and truncations of Ect2's N-terminus disrupt its auto-regulation and are sufficient to cause cellular transformation (Miki et al., 1993; Saito et al., 2003; Saito et al., 2004). Any mutation that disrupts the regulation of Ect2, such as mutations in the NLS that cause Ect2 to become cytosolic, or N-terminal truncations leading to constitutively active forms of Ect2, could activate various GTPases when it is not supposed to and lead to altered cell morphology and metastasis. Recent data has shown correlations between Ect2's mRNA and protein levels in certain cancer types, perhaps due to its ability to induce changes in the cytoskeleton. Ect2 is over-expressed in tumours isolated from different tissues including lung, brain, pancreatic, bladder, esophageal and ovarian (Fields and Justilien, 2010; Hirata et al., 2009; Salihia et al., 2008; Sano et al.,

2006; Zhang et al., 2008). In primary non-small cell lung cancer (NSCLC) cells and glioblastoma tumours, Ect2 is strongly mislocalized and is no longer nuclear (Fields and Justilien, 2010; Hirata et al., 2009; Saliha et al., 2008). Although Ect2 is elevated in pancreatic primary tumour cells, it is uncertain if it plays a direct role in transformation (Zhang et al., 2008). The mechanism that leads to the specific upregulation of Ect2 in tumours is not clear. The Ect2 gene maps to chromosome 3q26 and studies using lung, esophageal and ovarian tumours showed that the Ect2 gene is amplified in these tumours. However post-translational modifications that alter Ect2's activity likely are also important for its ability to cause transformation (Fields and Justilien, 2010).

Aside from Ect2's well-known role in cytokinesis (see above), other studies showed that Ect2-depletion can alter cell polarity and adhesion (Justilien and Fields, 2009; Salihia et al., 2008; Sano et al., 2006). Interestingly, depletion of Ect2 in NSCLC cells failed to cause an increase in the percentage of multinucleated cells, a hallmark of cytokinesis failure, in comparison to control cells. This indicates that Ect2 may have a different function in tumour cells vs. its role in cytokinesis (Justilien and Fields, 2009). These cells may undergo cytokinesis using a Rho-independent mechanism that involves 'traction' to pull cells apart, a mechanism that is often used in adherent cells. Alternatively, the upregulation of Ect2 in these cells may make them more resistant to RNAi, and a threshold minimum of protein may remain to drive RhoA activation. An emerging theme from these studies is that each cell type differs from the next and Ect2 has the potential to play a myriad of roles in the cell.

To summarize, Ect2 is an important GEF for the GTPase RhoA during cytokinesis and identifying its mechanism of localization and subsequent activation

during contractile ring formation and ingression is paramount to our understanding of cytokinesis. The C-terminus of Ect2 contains conserved DH and PH domains, which are essential for its GEF activity *in vitro* (Solski et al., 2004). While the DH region catalyzes nucleotide exchange on RhoA, the role of the PH region is unclear. Here, we describe the role of the PH region in regulating Ect2's cortical localization and activity *in vivo*. Ect2 is recruited to the cortex by active RhoA, perhaps in a feed-forward pathway, to further generate a discrete pool of active RhoA for successful cytokinesis. Moreover, cortical Ect2 interacts with anillin, which could establish a physical link between the central spindle and the contractile ring and may be functionally analogous to the *Drosophila* RacGAP50C/Cyk-4-Anillin interaction. Anchoring the central spindle to the cortex likely helps stabilize the activation of RhoA in a discrete zone to maintain the division plane.

Materials and Methods

2.1 Tissue Culture

HEK 293 and Hela cells were maintained in Dulbecco's modified eagle high glucose media (DMEM; Wisent) supplemented with 2 mM L-glutamine, 100 u penicillin and 0.1 mg/mL streptomycin (PS; Wisent) and 10% fetal bovine serum (FBS; Wisent). Cells were grown in a humidified 5% CO_2 incubator at 37 °C for 2-3 days until they were 100% confluent. Stock plates were maintained by seeding new plates with 10% cells from the confluent plates. Specifically, confluent plates were washed with 1 X phosphate buffered saline (PBS; 150 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and KH_2PO_4; Wisent), then 400 µL trypsin (Wisent) was added. Cells were incubated at 37 °C for five minutes. Then 10 mL pre-warmed, fresh DMEM media was added to the cells, which were thoroughly resuspended and 1/10 of the cells were added to plates with pre-warmed, fresh DMEM media.

2.2 Transfections

Hela cells transfected with siRNAs and/or co-transfected with DNA were plated in DMEM media without antibiotics (PS-) using Lipofectamine 2000 (Invitrogen) as per manufacturer instructions. For optimal transfection efficiency and limited lethality, ~4-5-fold less Lipofectamine reagent was used and cells were transfected at ~50-60% confluency. Cells were treated with siRNAs (2 µL of 75 nM stock per 2 mL of a 6-well dish or 10 µL per 10 cm plate; see below; or co-transfected with DNA) for ~30 hours

(Yuce et al., 2005), and with DNA for a minimum of 24 hours. For 10 cm plates, 4 – 6 µg of DNA was transfected, while 0.5 - 2 µg of DNA was used per well of a 6-well dish.

In order to deplete endogenous protein levels, the following dsRNA sequences were used to target the mRNA product:

Anillin RNAi: *CGAUGCCUCUUUGAAUAAA* (Dharmacon)

Ect2 RNAi: *GGCGGAAUGAACAGGAUUU* (Dharmacon)

RhoA RNAi: *GCCGGUGAAACCUGAAGAA* (Dharmacon)

HEK 293 cells were transfected with DNA using calcium phosphate. For 10 cm plates, the desired DNA was aliquoted into a microfuge tube with 61 µL of 2.5 M calcium chloride and the volume was brought to 500 µL with water. In a separate microfuge tube, 500 µL of 2 X stock HBS (280 mM NaCl, 1.5 mM Na_2PO_4, 10 mM KCl, 50 mM HEPES, pH to 7.0) was added. The DNA solution was incubated for 5 minutes and then was added to the HBS tube slowly, while swirling. The mixed solution was then immediately added to the cells. Cells were lysed 24-30 hours after transfection.

2.3 Drug Treatments

The following drugs were used in this study; 2.5 mM thymidine (Bioshop) to block cells in S phase, 40 ng/mL nocodazole (Sigma), which prevents microtubule polymerization and blocks cells in metaphase, and 30 nM purvalanol (Sigma), which inhibits Cdk1 activity to promote mitotic exit.

Cells were synchronized by first treating them with 2.5 mM thymidine for 12 - 16 hours to block them in S phase. Cells were then released by washing three times with PBS and recovered for eight hours in fresh DMEM. Cells were treated a second time with thymidine, and released again the following day. Six - seven hours after the second release, cells were treated with 40 ng/mL of nocodazole for three hours to block them in metaphase. Cells were washed five times with PBS, then released into fresh DMEM media for 45 minutes, after which time the cells were in anaphase. Alternatively, cells were not released from nocodazole and 30 nM purvalanol was added to promote mitotic exit. Cells transfected with DNA were treated with 40 ng/mL nocodazole for six hours (and not previously with thymidine), then washed five times with PBS and released for 45 minutes with fresh DMEM to permit anaphase entry. Alternatively, 30 nM purvalanol was added to the nocodazole-treated cells without release.

2.4 Plasmid Constructs

The following anillin constructs were used in this study and were previously generated (the GFP vector is kanamycin resistant; MBP and GST vectors are ampicillin-resistant):

- GFP: Anillin (608-1087; C-term)
- MBP: Anillin (608-1087; C-term)
- MBP: Anillin (100-460; A1)
- MBP: Anillin (608-940; A2)
- GST: Anillin (608-940; A2)
- MBP: Anillin (671-772; A3)

- MBP: Anillin (772-940; A4)
- GST: Anillin (820-940; A5)
- GST: Anillin (890-940; A6)

The following Ect2 constructs were used in this study and were previously generated (the Myc vector is ampicillin-resistant):

- Myc: Ect2 (1-883; FL)
- Myc: Ect2 (1-420; E1)
- Myc: Ect2 (1-227; E2)
- Myc: Ect2 (421-883; E3)
- Myc: Ect2 (421-774; E4)
- Myc: Ect2 (662-883; E5)
- Myc: Ect2 (421-621; E6)
- Myc: Ect2 (622-774; E7)

Quickchange Mutagenesis was used to create silent mutations in full-length Ect2, in the site recognized by Ect2 RNAi, to make the full-length Ect2 construct RNAi-resistant. In addition, quickchange mutagenesis was used to generate the following Ect2 mutants in FL and E3 constructs:

-Ect2 N608A-N609A mutant

Forward primer:

5’GTAATGACGCATATTGCTGCGGATAAGAGAAAAACAG3’

Reverse Primer: 5’

CTGTTTTTCTCTTATCCGCAGCAATATGCGTCATTAC3’

-Ect2 D668G mutant (also generated in E5 constructs)

Forward Primer:

5’TAACTCTCTTCCTCTTCAATGGATGCCTAGAGATAGCAAGAAA3’

Reverse Primer:

5’TTTCTTGCTATCTCTAGGCATCCATTGAAGAGGAAGAGAGTTA3’

Other Ect2 mutants were generated by quickchange mutagenesis in E3 constructs and showed a reduction in anillin binding similar to the D668G mutant:

- 667 NDC 669 to AGG
- D668R
- D668K

Additional Ect2 mutants were created but showed no change in anillin binding:

- 627 EVD 629 to AGA
- 638 SHRS 641 to AAGG
- 674 RKRHK 678 to GAGHA
- 708 KK 709 to AA
- 717 EDCHN 721 to AAAAA
- 744 SDE 746 to AGA.

The Ras tail (EKMSKDGKKKKKKSKTKCVIM) was added onto FL (wild-type and D668G) and E3 (wild-type and D668G) Ect2 constructs.

Other constructs used in this study were previously generated, GFP:hsRhoA (kanamycin-resistant), GST:RBD (7-89 AA from Rhotekin; ampicillin-resistant).

All newly generated constructs were verified by DNA sequencing at McGill University and Genome Quebec Innovation Centre.

2.5 Protein Purification

2.5.1 GST:anillin

To purify the GST-tagged anillin proteins, a modified protocol from Amersham Bioscience was used. BL21 cells transformed with the desired GST:anillin construct were used to inoculate overnight 5 mL cultures of LB (Luria Broth; 10 grams Tryptone, 10 grams NaCl, 5 grams yeast extract) ampicillin (Amp; 50 µg/mL) and were grown at 37 °C overnight (12 - 18 hours) with shaking (~250 - 300 rpm). A 1:100 dilution of each overnight culture was used to inoculate fresh 200 mL LB Amp cultures and grown at 37 °C with proper aeration while shaking until an OD_{600} of 0.3 - 0.6 was reached. The temperature was then decreased to 28 °C and protein expression was induced by adding 1 mM IPTG (Bio Basic) for three-four hours. Cultures were then transferred to centrifuge bottles and cells were pelleted by centrifugation at 4,000 rpm for 25 minutes at 4 °C. Cells were washed by resuspending them in 25 mL 1 X PBS and then were pelleted again by centrifugation (same as previously mentioned). The supernatant was removed and the pellets were flash frozen in liquid nitrogen and stored at -80 °C until purification.

To purify protein from a frozen pellet, it was resuspended in 30 mL lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$ and 1% Triton-X100) supplemented with 1 mM DTT and protease inhibitors 10 µg/mL leupeptin (Bioshop), 10 µg/mL

pepstatin (Bioshop) and 1 mM PMSF. After resuspension, 5 mM lysozyme was added and incubated on ice for 30 minutes. The solution was then sonicated three times, each for 30 seconds with alternating pulses of 1 second on and 1 second off at 40% amplitude on ice (and 1 - 2 minute pauses between each sonication). The solution was then centrifuged at 13,000 rpm for 15 - 25 minutes at 4 °C. The supernatant was transferred to a pre-chilled 50 mL conical tube and 600 µL of 50 % Glutathione Sepharose beads (GE), pre-equilibrated and in lysis buffer, was added and incubated at 4 °C for 3 hours with gentle rotation. The beads were then pelleted by centrifugation at 4,000 rpm, 4 °C for 2 – 5 minutes and supernatant was removed, until a volume ~1 – 2 mL remained. The beads were then transferred to a 2 mL microcentrifuge tube and washed 3 – 4 times using lysis buffer. After the last wash, supernatant was removed until beads were in a 50% slurry (equal volume of liquid: beads). The purity and concentration of bound-protein was checked by running 5 and 10 µL aliquots of the 50% slurry by SDS-PAGE, together with a known amount of BSA (2 µg), and staining the gel with coommassie. Gels were scanned and the pixel intensities of each lane were measured using ImageJ software to quantitate protein concentration. Aliquots of the purified beads were made to5 µg were subsequently frozen in liquid nitrogen and stored at -80 °C for future use.

2.5.2 GST:RBD

The procedure to purify GST:RBD protein is similar to GST:anillin, except that RBD is extremely labile and must be made fresh the same day of use (*i.e.* although cell pellets can be stored at -80 °C, purified protein cannot be stored) and protein is induced at 31-32 °C instead of 28 °C.

2.5.3 MBP:anillin

The protocol to purify MBP-tagged anillin is similar to the protocol described for GST:anillin, except 600 µL of 50% amylose resin beads (NEB; pre-equilibrated in lysis buffer) is used instead of Glutathione Sepharose (GE).

2.6 Pull Down Assay

2.6.1 In vitro binding assay with MBP and GST-tagged anillin

Transfected HEK 293 cells were lysed with 400 µL lysis buffer (10 cm plate, or 150 µL per 6 cm plate) using a scraper and transferred to cold 1.5 mL microcentrifuge tubes. Lysates were centrifuged at 13,000 rpm for 5 - 10 minutes. Supernatants were transferred to fresh microcentrifuge tubes. 20 µL of the supernatant was transferred to a new microcentrifuge tube and labeled 'input'. Purified anillin protein (5 µg of 50% slurry prepared as described above) was added to equal volumes of supernatants, and a separate tube was prepared with an equal volume of supernatant and 2 -3 µg of GST or 4 - 5 µg MBP protein (depending on the experiment, a mix of supernatants was used). The tubes were incubated with gentle rotation overnight at 4 °C, and then washed three – four times with 1 mL lysis/wash buffer. After the last wash, the supernatant was removed and the beads were resuspended in 10 µL sample buffer (1/10 v/v β-mercaptoethanol + 4 X SDS-sample buffer {0.8 grams SDS, 2.0 mL 1M Tris pH 6.8, 4.0 mL 100% glycerol, 1.0 mL EDTA and 8 mg bromophenol blue diluted in distilled water}), along with the original input tubes, then heat denatured at 96 °C for 2 - 3 minutes. The denatured samples were centrifuged and stored at -20 °C until they were run by SDS-PAGE and bound proteins were detected by immunoblotting (see below).

2.6.2 In vitro binding assay with GST:RBD

To pull down endogenous active RhoA with GST:RBD beads, a similar procedure was used as described above, except TBS (Tris-buffered saline 150 mM NaCl 50 mM Tris pH 7.6) was used to wash the cells before lysing instead of PBS, cell lysates were centrifuged for 2 – 5 minutes vs. 5 – 10 minutes, and the beads were incubated for exactly 90 minutes instead of overnight before washing and adding sample buffer.

2.7 Immunoblotting

Denatured protein samples were fractionated by SDS-PAGE and proteins were detected by immunoblotting. The fractionated proteins were transferred to nitrocellulose membranes (GE), pre-incubated with transfer buffer (100 mL 10 X Running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) + 200 mL methanol + 700 mL distilled water) for one hour using wet (immersed in transfer buffer; BioRad) or semi-dry transfer (wet, but not immersed; Bio-Rad). The membranes were blocked for ~ 20 minutes using 5% skim milk in PBST (PBS + 0.2 % Tween-20), then incubated with primary antibodies in 5% skim milk in PBST, or just PBST (if being re-used), for a minimum of one hour at room temperature (or overnight at 4 °C) with gentle rocking. Membranes were washed three times with PBST with ten minute intervals, then incubated for a minimum of one hour with 1:2500 dilution secondary antibodies {(anti-rabbit or anti-mouse 680 or 800 (Rockland); or anti-rabbit or anti-mouse 488 (Invitrogen)} in PBST. Membranes were washed three times with PBST and then scanned using the Odyssey (Li-Cor) scanner at 700 or 800 wavelengths, or with the Typhoon Trio phosphoimager (GE) at 488 wavelengths at 600 PVT at either medium or high quality. The original 16-bit images

were opened in ImageJ for quantitation using selected regions of interest and measuring pixel intensity in comparison to the inputs. To make figures, the images were adjusted and cropped in ImageJ, then converted into 8-bit images and imported into Adobe Illustrator as TIFFs.

2.7.1 Antibodies

The following primary antibodies were used for immunoblotting:

- 1:2 supernatant of mouse anti-Myc 9E10 monoclonal antibodies (gift from Dr. Sacher, Concordia University, Montreal, QC)
- 1:100 mouse anti-RhoA monoclonal antibodies (Santa-Cruz)
- 1:1000 mouse anti-GFP monoclonal antibodies (Roche)
- 1:1000 rabbit anti-Ect2 polyclonal antibodies (gift from Dr. Glotzer, University of Chicago, Chicago, IL)

2.7.2 PIP Strips

Proteins were assayed for their ability to bind to phospholipids using PIP strips (Echelon BioSciences). Transfected HEK 293 cells were lysed and prepared as described above, and lysates from 3 X 10 cm plates were combined and incubated with each PIP strip overnight at 4 °C. A control was performed using lysates from cells transfected with GFP. The strips were probed with secondary antibodies and scanned as described for immunoblotting. In addition to using lysates, strips were also incubated with purified proteins (concentration range was 100 – 500 nM purified protein), and probed with mouse anti-GST or mouse anti-MBP antibodies (NEB).

2.8 Fixation and Immunostaining

Hela cells grown and transfected on cover slips were fixed and stained for microscopy. To fix cells, media was removed, then cells were washed with pre-warmed 1 X cytoskeletal buffer (80 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA), then fixed using ice-cold 100% methanol or freshly prepared ice-cold 10% TCA (trichloroacetic acid). Cells were incubated with either fixative for 20 minutes at 4 °C, then washed four times with 1 X TBST (TBS with 0.5% Tween-20). To stain, coverslips were placed on a piece of parafilm in a 'wet chamber' composed of a plastic container with moistened towels to prevent evaporation. First, cells were incubated with 5% v/v normal donkey serum (NDS) in 1 X TBST for 20 minutes to block non-specific binding. Then cells were incubated with primary antibodies diluted in 1 X TBST 5% NDS for two hours at room temperature. Cells were washed three times with 1 X TBST, then incubated with 1:250 secondary antibodies, anti-mouse or rabbit 488 or anti-mouse or rabbit 568 (Invitrogen), in 1 X TBST and 5% NDS for two hours at room temperature. After removing the third wash, cells were incubated with 1:1000 dilution DAPI (1 mg/mL stock) in TBST for five minutes, washed once with 1 X TBST, then washed again with 0.1 M Tris pH 8.8. After removing Tris, a single drop of pre-warmed mounting media (5% n-propyl gallate (Alfa Aesar) 50% glycerol 50 mM Tris pH 9.0) was added, and coverslips were carefully placed on pre-washed glass slides, excess fluid was removed and they were sealed with nail polish. The sealed slides were stored at -20 °C.

2.8.1 Antibodies

The following primary antibodies and dilutions were used for immunofluorescence (IF):

- 1:200 rabbit anti-GFP polyclonal antibodies (gift from Dr. Glotzer, University of Chicago, Chicago, IL).
- 1:50 mouse anti-Plk1 monoclonal antibodies (Santa Cruz)
- 1:200 rabbit anti-anillin polyclonal antibodies (Piekny and Glotzer, 2008)
- 1:200 mouse anti-tubulin polyclonal antibodies (DM1A, Sigma-Aldrich)
- 1:2 mouse anti-Myc 9E10 monoclonal antibodies (gift from Dr. Sacher, Concordia University, Montreal, QC)
- 1:200 rabbit anti-Ect2 polyclonal antibodies (gift from Dr. Glotzer, University of Chicago, Chicago, IL)
- 1:100 mouse anti-RhoA monoclonal antibodies (Santa Cruz)

2.9 Microscopy

Images of fixed cells were collected using the Leica DMI 6000B inverted microscope with 40x /0.75 or 63x /1.4 NA objectives using an OrcaR2 (Hamamatsu) camera with Volocity acquisition software (PerkinElmer). Gain settings and exposure times were kept constant for all images based on control slides (optimized for maximal dynamic range from 0 – 4095 pixels). 0.2 - 0.5 μm Z-sections were collected using the ASI Piezo Z stage (MadCityLabs). Images were exported as 16-bit TIFFs and opened in ImageJ for quantitation, adjustments, color overlays, generating Z-stack projections and cropping. For example, pixel intensities were measured based on control cells and

background. Files were then converted into 8-bit TIFFs for import into Adobe Photoshop and/or Adobe Illustrator to prepare figures.

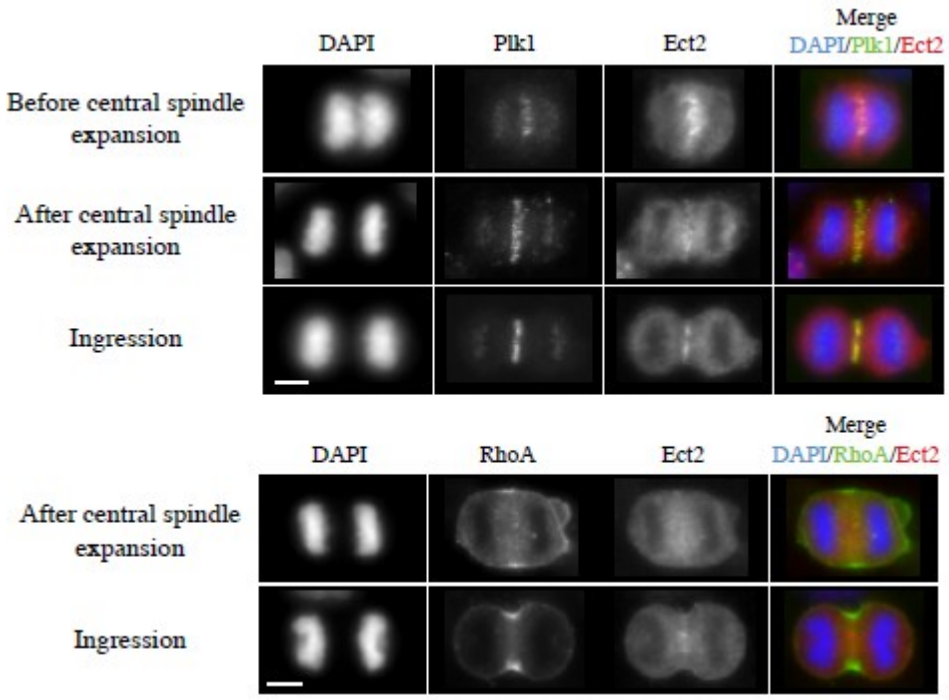
Results

3.1 Ect2 localizes to the cortex during furrow ingression.

Chalamasetty et al. (2006) showed that full length Ect2 localizes to both the central spindle and cortex. Upon anaphase transition, the N-terminal region of Ect2 interacts with Cyk-4, which recruits it to the central spindle. Ect2's C-terminal region localizes cortically and contains the catalytic domain necessary for RhoA activation. We further characterized Ect2's cortical localization during cytokinesis. In early anaphase cells, when sister chromatid separation had just begun and the central spindle was newly formed, Ect2 concentrated on the central spindle and co-localized with Plk1 (Figure 3A). Little cortical Ect2 localization was visible in these cells. After cells progressed through anaphase and the central spindle expanded toward the cortex, Ect2 dispersed along the central spindle and small pools were visible at the cortex (Figure 3A). As cytokinesis progressed and cells furrowed, Ect2's localization at the cortex was more obvious, although it retained localization at the central spindle (Figure 3A). These results indicate that Ect2 first becomes cortical after central spindle expansion, but its cortical localization increases during cleavage furrow ingression. This increased cortical localization coincides with RhoA activation for contractile ring formation and ingression. To further examine the overlap between Ect2 and the contractile ring, we examined its co-localization with anillin (Figure 3B). In an end-on view, a ring of co-localization (in yellow) between Ect2 and anillin was detected on the inner part of the contractile ring (facing the central spindle). Therefore, as cytokinesis progresses, Ect2, which originally is in a physically distinct location from the highly cortical contractile ring proteins (data

not shown), overlaps with contractile ring components, and is in a position to regulate the levels of active RhoA.

A)



B)

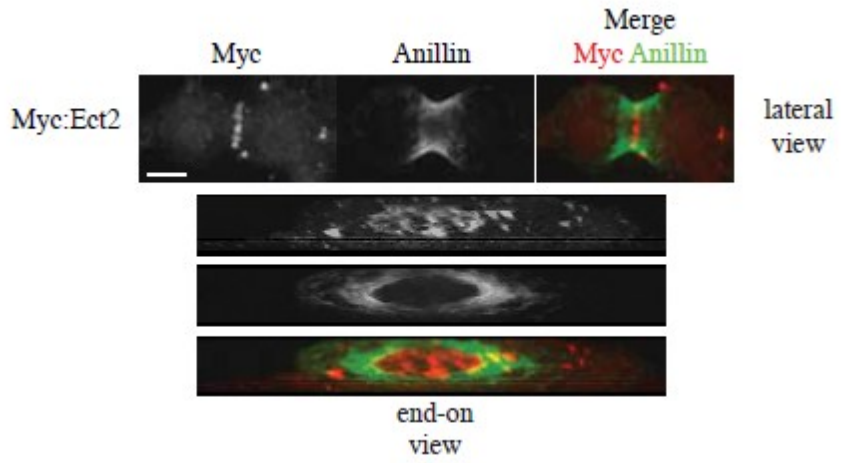
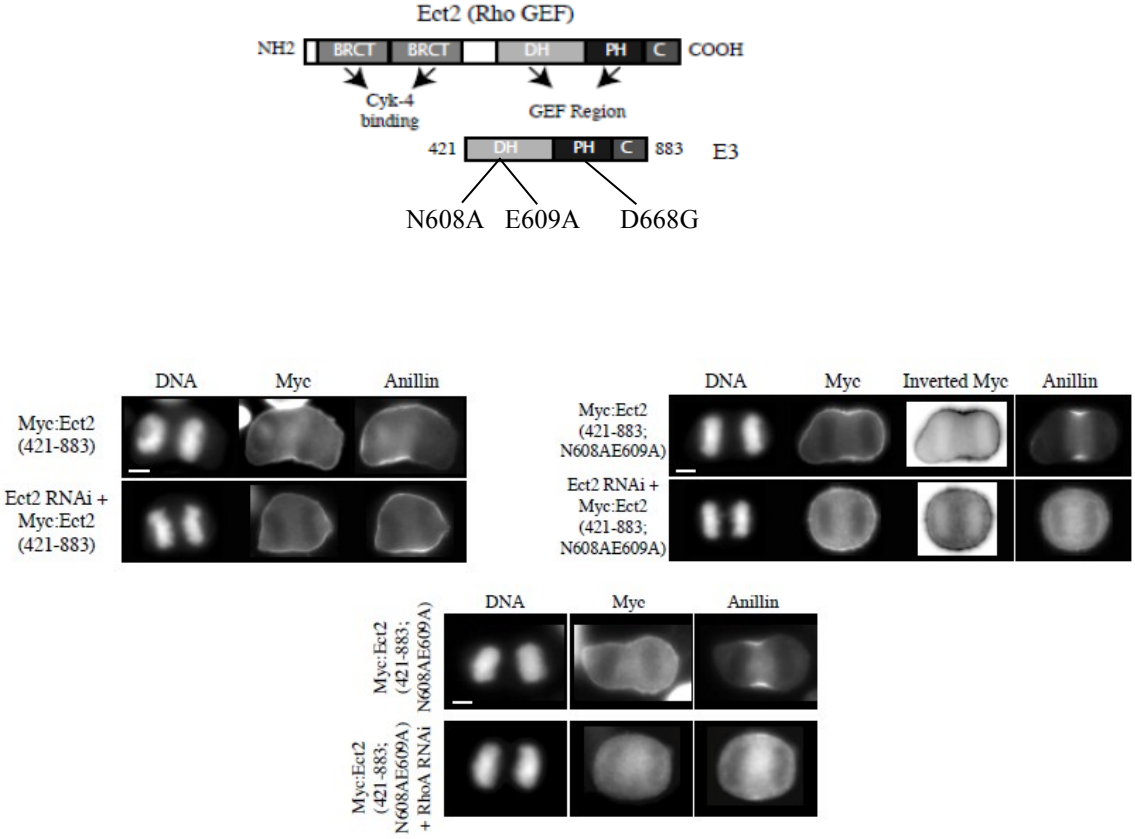


Figure 3 A) Hela cells were fixed and co-stained with Plk1 (green), RhoA (green) or Ect2 (red) antibodies. Z-stack projections of cells from before, during, and after central spindle expansion are shown. B) Hela cells were transfected with full length Myc:Ect2, fixed and co-stained with Myc (red) and anillin (green) antibodies. Z-stack projections of a cell during furrow ingression are shown from a side (top) and an end-on view (bottom; zoomed in). Co-localization is seen in yellow. Scale bars are 10 μm. Images were taken by Dr. Piekny.

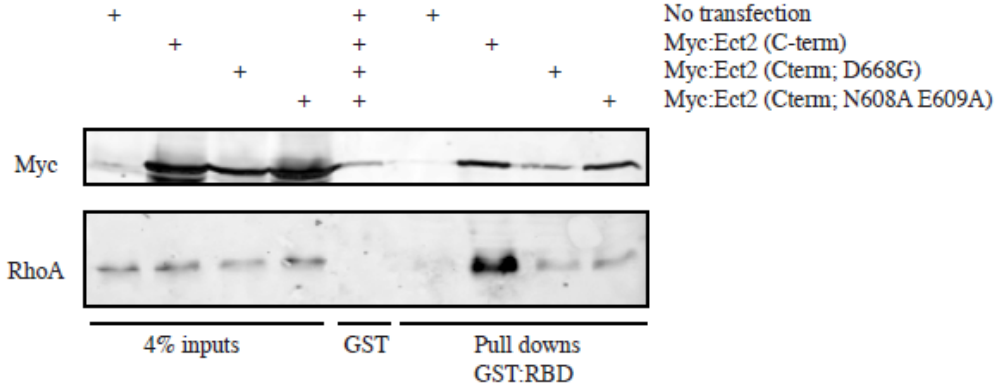
3.2 Cortical Ect2 localization depends on active RhoA.

The C-terminus of Ect2 (E3; 421-883) localizes to the cortex (Chalamasetty et al., 2006) and not to the central spindle, because it does not contain the N-terminal Cyk-4 binding region. Cortical proteins, such as anillin, depend on RhoA for their recruitment in human cells (Piekny and Glotzer, 2008; Watanabe et al., 2010; Yuce et al., 2005; Zhao and Fang, 2005a) and we wanted to determine if Ect2's cortical localization is also Rho-dependent. In order to do this, we used C-terminal Ect2 constructs and determined their localization in the absence of active RhoA. C-terminal Ect2 activates RhoA and localizes around the entire cortex of Hela cells. This construct caused anillin to become ectopically localized around the cortex regardless of the presence of endogenous Ect2 (Figure 4A). A set of mutations in the DH region of Ect2 (N608A E609A) decreased Ect2's ability to activate RhoA (Figure 4B) and although localized cortically in the presence of endogenous Ect2 and RhoA, failed to do so in their absence (Figure 4A). These findings suggest that in order for Ect2 to localize to the cortex, RhoA must be present and active.

A)


Ect2 (Rho GEF)
NH2
BRCT
BRCT
DH
PH
C
COOH
Cyk-4 binding
GEF Region
421
DH
PH
C
883
E3
N608A
E609A
D668G
DNA
Myc
Anillin
Myc:Ect2 (421-883)
Ect2 RNAi + Myc:Ect2 (421-883)
DNA
Myc
Inverted Myc
Anillin
Myc:Ect2 (421-883; N608AE609A)
Ect2 RNAi + Myc:Ect2 (421-883; N608AE609A)
DNA
Myc
Anillin
Myc:Ect2 (421-883; N608AE609A)
Myc:Ect2 (421-883; N608AE609A) + RhoA RNAi

B)


No transfection
Myc:Ect2 (C-term)
Myc:Ect2 (Cterm; D668G)
Myc:Ect2 (Cterm; N608A E609A)
Myc
RhoA
4% inputs
GST
Pull downs
GST:RBD

C)

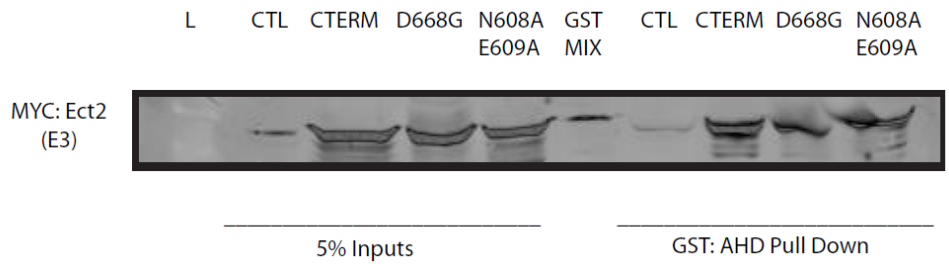


Figure 4 A) Schematic diagram of the proposed structure of Ect2 with the locations of the mutations. Images of fixed Hela cells transfected with Myc-tagged Ect2 constructs, with or without Ect2 RNAi or RhoA RNAi, co-stained with DAPI, Myc and anillin antibodies. Scale bar is 10 μm. Images were taken by Dr. Piekny. B) A western blot of lysates from HEK 293 cells transfected with Myc-tagged Ect2 constructs pulled down using GST-tagged RBD and probed with RhoA and Myc antibodies. C) A western blot of lysates from HEK 293 cells transfected with Myc-tagged Ect2 constructs and pulled down using GST-tagged AHD from anillin and probed with Myc antibodies. E3= C-terminus of Ect2.

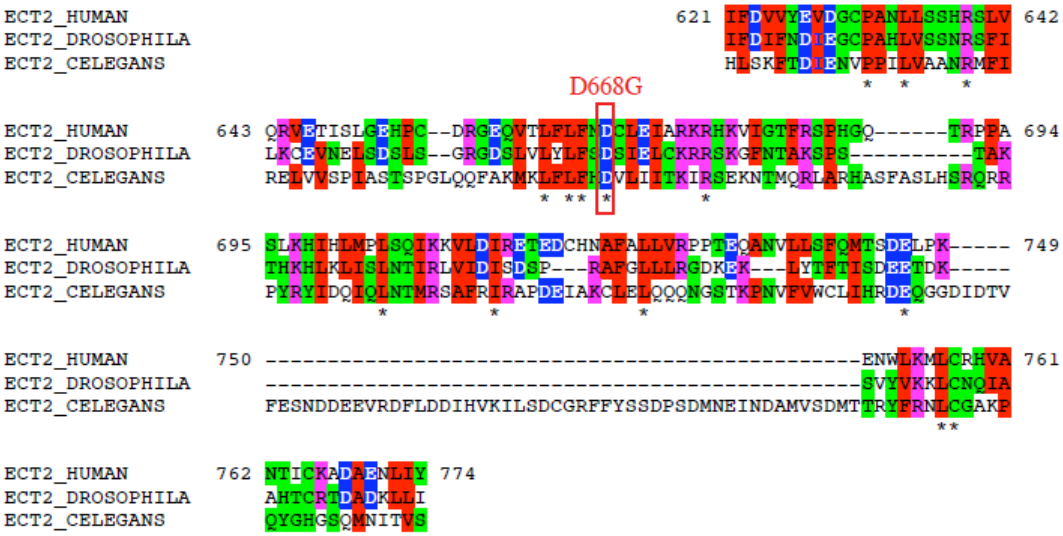
3.3 The PH region of Ect2 is required for its cortical localization.

Ect2 is conserved among metazoans, and has high sequence homology in the DH region in the C-terminus (not shown). While the DH region exchanges nucleotide on RhoA, the function of the PH region is less clear. Studies showed that the PH region is required for Ect2's GEF activity *in vitro* (Solski et al., 2004), and PH regions are known to mediate phospholipid interactions (Marcia et al., 2008). We hypothesize that the PH region of Ect2 may be important for regulating its cortical localization, by mediating its interaction with lipids (or other proteins). To assess the homology of the PH region, a sequence alignment was done using Clustal W (Figure 5A). Human Ect2 was compared to its homologues in *Drosophila* (Pebble) and *C. elegans* (ECT-2). Seven mutations (data not shown) were generated in Myc-tagged C-terminal Ect2 constructs. One of these mutations, a conserved aspartic acid (D) at position 668 mutated to a glycine (G; Figure 5A), caused a reduction in Ect2's ability to generate active RhoA (determined by pull down experiments using 1 μM of GST tagged Rho-GTP binding domain from Rhotekin; Ren et al., 1999; Figure 4B) and disrupted its interaction with phospholipids (Figure 5B). This suggests that the PH region of Ect2 is important for mediating interaction with lipids and for RhoA activation.

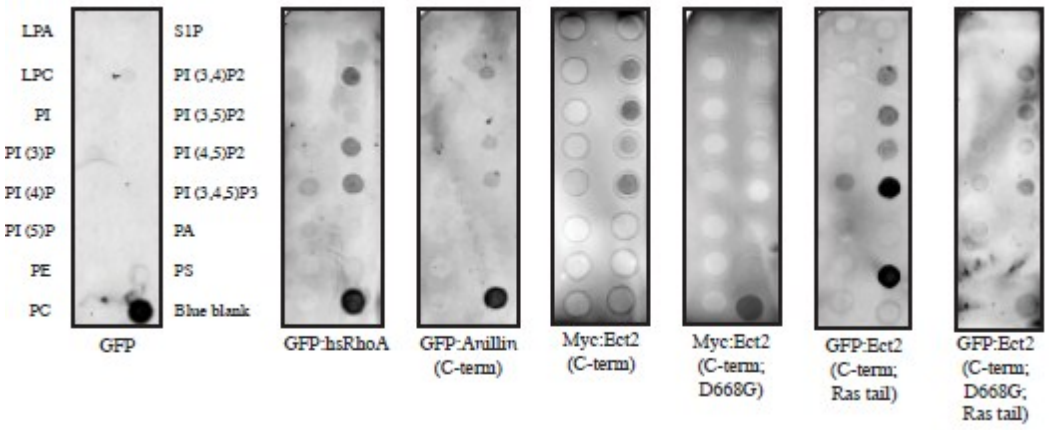
To determine if the PH region of Ect2 is required for its cortical localization, we examined localization of the D668G mutant and its ability to ectopically localize anillin. As shown before, the C-terminus of Ect2 was cortical and drove the ectopic localization of anillin (Figure 4A, 5C). The D668G mutant failed to localize cortically (even in the presence of endogenous Ect2) and was less effective at ectopically localizing anillin (>30% of the breadth of the cell; Figure 5C, D). In the absence of endogenous Ect2, the

ability of D668G to drive ectopic anillin correlated with high expression levels (Figure 5D). These results suggest that the PH region of Ect2 is essential for its cortical localization and for its ability to activate RhoA *in vivo*, as determined by ectopic anillin localization.

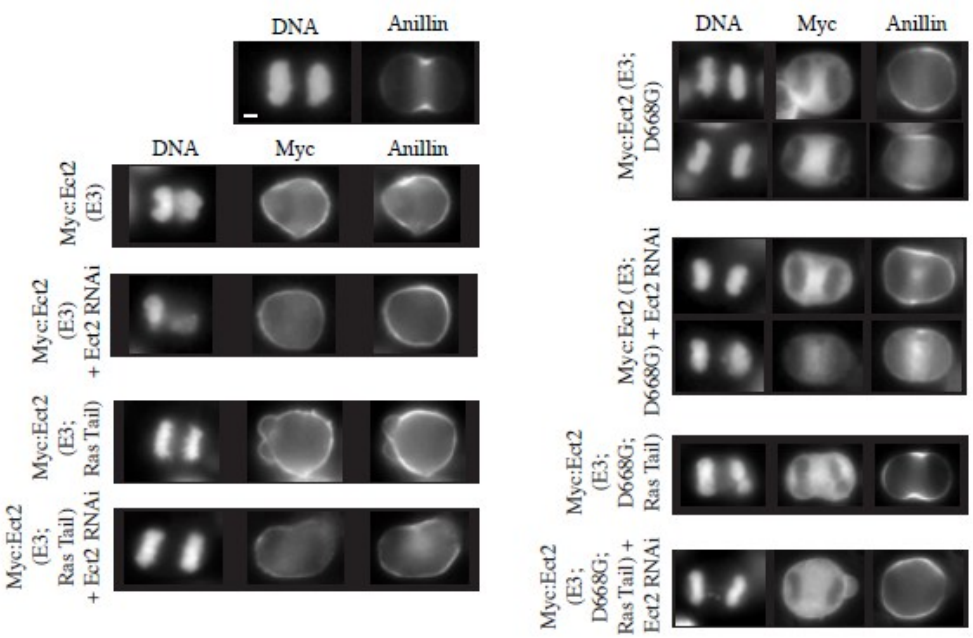
A)


ECT2_HUMAN
ECT2_DROSOPHILA
ECT2_CELEGANS
D668G

B)


LPA
LPC
PI
PI (3)P
PI (4)P
PI (5)P
PE
PC
S1P
PI (3,4)P2
PI (3,5)P2
PI (4,5)P2
PI (3,4,5)P3
PA
PS
Blue blank
GFP
GFP:hsRhoA
GFP:Anillin (C-term)
Myc:Ect2 (C-term)
Myc:Ect2 (C-term; D668G)
GFP:Ect2 (C-term; Ras tail)
GFP:Ect2 (C-term; D668G; Ras tail)

C)



D)

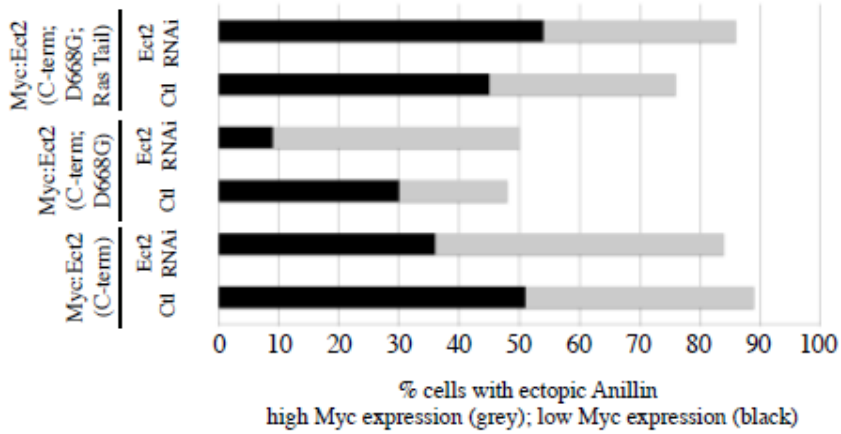


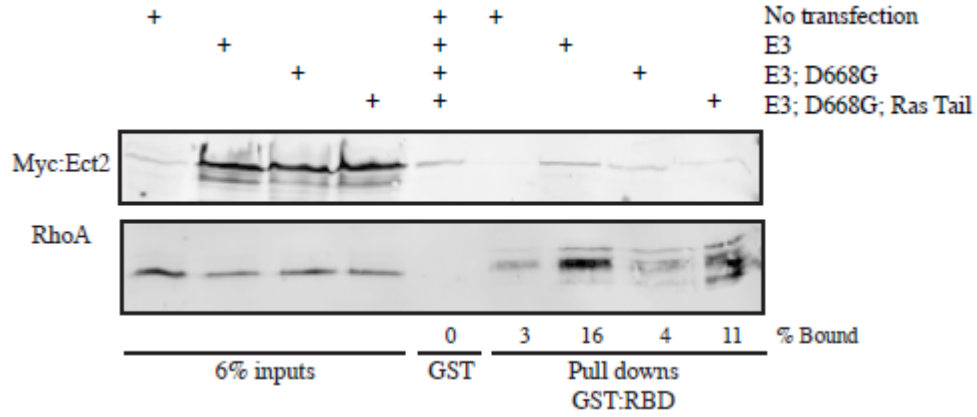
Figure 5 A) Residues in red show hydrophobic amino acids, blue are acidic amino acids, purple are basic amino acids and green are neutral amino acids. * Indicates conserved amino acids. A mutation at position 668 was made using site directed mutagenesis; aspartic acid was changed to glycine. B) Western blots of PIP strips (the different phosphoinositols are shown beside the strip on the left) incubated with lysates from HEK 293 transfected with GFP, GFP:RhoA, GFP:Anillin, Myc:Ect2, Myc:Ect2; D668G, GFP:Ect2; Ras Tail and GFP:Ect2; D668G; Ras Tail and probed with GFP or Myc antibodies as indicated. Performed by Eric Haines and Paknoosh Pakarian. C) Images of Hela cells transfected with Myc-tagged Ect2 constructs +/- RNAi, fixed and stained with DAPI, Myc and anillin antibodies. Scale bar is 10 µm. E3= C-terminus of Ect2. Images were taken by Dr. Piekny. D) The percentage of Hela cells transfected with the indicated

Myc-tagged Ect2 constructs +/- Ect2 RNAi, with ectopic anillin localization (>30% of the breadth of the cell) is shown in the graph.

3.4 Ect2's membrane association is required for its ability to activate RhoA.

Ect2's PH domain is necessary for its cortical localization and for its ability to activate RhoA. The PH region may mediate interactions with phospholipids, because D668G no longer interacts with lipids on a PIP strip. To determine if lipid binding is essential for Ect2 function, we added the hydrophobic Ras tail onto the 3' end of D668G and assayed its ability to activate RhoA. Adding the Ras tail to D668G restored lipid interactions and partially restored Ect2's cortical localization (Figure 5B, C). Over-expression of the C-terminus of Ect2 generated active RhoA above endogenous Ect2 (Figure 4B, 6A) and, as described earlier, the D668G mutant generated lower levels of active RhoA (Figure 4B, 6A). Adding the Ras tail onto D668G partially restored Ect2's ability to generate active RhoA (Figure 6A). Adding the tail onto non-mutant Ect2 also enhanced its ability to generate active RhoA (assessed using low levels of expression; Figure 6B). The Ras tail also restored the ability of D668G to ectopically localize anillin (Figure 5C, D), supporting its ability to generate active RhoA. These results suggest that membrane association is essential for Ect2's function. Since RhoA localizes to the membrane during cytokinesis and is the substrate for Ect2, it is not surprising that Ect2 also needs to be at the membrane to activate RhoA. Furthermore, forcing more Ect2 to the membrane, in close proximity to RhoA, should enhance the generation of active RhoA.

A)



B)

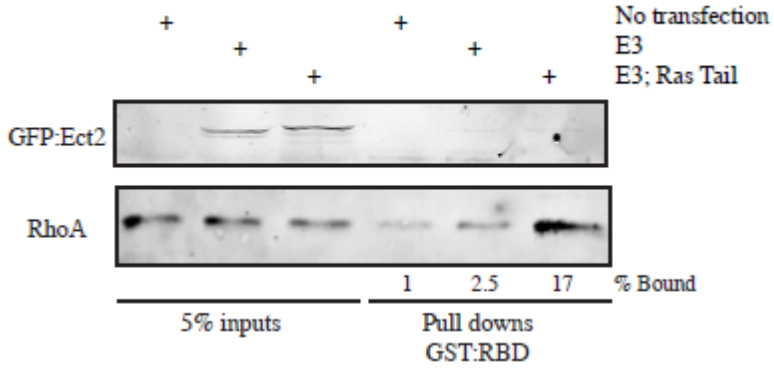


Figure 6 A) A western blot of lysates from HEK 293 cells transfected with Myc-tagged Ect2 constructs pulled down using GST-tagged RhoA-GTP binding domain (RBD) from Rhotekin, which specifically binds to GTP-bound RhoA. The membrane was probed with RhoA and Myc antibodies. B) A western blot of lysates from HEK 293 cells transfected with GFP-tagged Ect2 constructs and pulled down using GST-tagged RBD and probed with RhoA and Myc antibodies. E3= C-terminus of Ect2.

3.5 The PH region of Ect2 is required for cytokinesis.

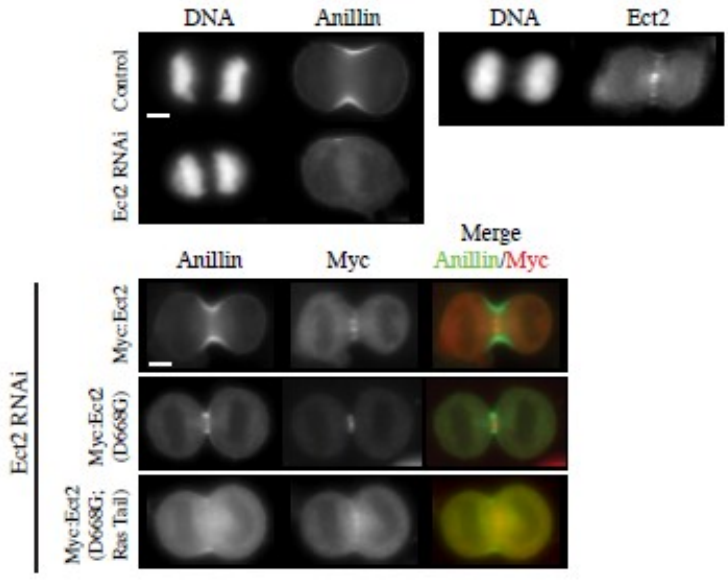
The PH domain of Ect2 is essential for its cortical localization and its ability to generate active RhoA. In order to assess the importance of the interaction *in vivo* we determined if full length Ect2 carrying the D668G mutant was able to rescue the loss of endogenous Ect2. We co-transfected Ect2 RNAi with Myc-tagged full length RNAi resistant ($RNAi^r$) Ect2 constructs in Hela cells and assayed 'rescue' by counting the percentage of binucleate cells (Ect2 is required for cytokinesis and RNAi causes the formation of binucleate cells). While cells co-expressing full length Ect2 had a reduced number of binucleate cells (25%) vs. Ect2 RNAi alone (45%), cells co-expressing D668G had 44% binucleate cells and those expressing D668G Ras tail had 45% ($n > 400$ cells per construct; Figure 7A, B). Non-mutant full length Ect2 did not completely rescue binucleate cells because the mere over-expression of Ect2 caused some cells to become binucleate (14% for non-mutant, 13% for D668G and 16% for D668G; Ras tail; Figure 7B). The decreased ability of D668G (or D668G; Ras tail) to rescue the number of binucleate cells compared to non-mutant Ect2, suggests that D668 is important for Ect2's function *in vivo*. Furthermore, since adding the Ras tail onto D668G failed to rescue Ect2, there may be properties of the PH region that cannot easily be compensated for, for example the proportions and/or types of phospholipids it interacts with could vary vs. the Ras tail.

To determine if the PH region causes cytokinesis failure because of decreased RhoA activation, we analyzed the localization of anillin in cells co-transfected with the $RNAi^r$ full-length Ect2 constructs and Ect2 RNAi. Full length $RNAi^r$ Ect2 rescued anillin localization as determined by anillin's fluorescence intensity levels ($n = 19$; Figure 7A).

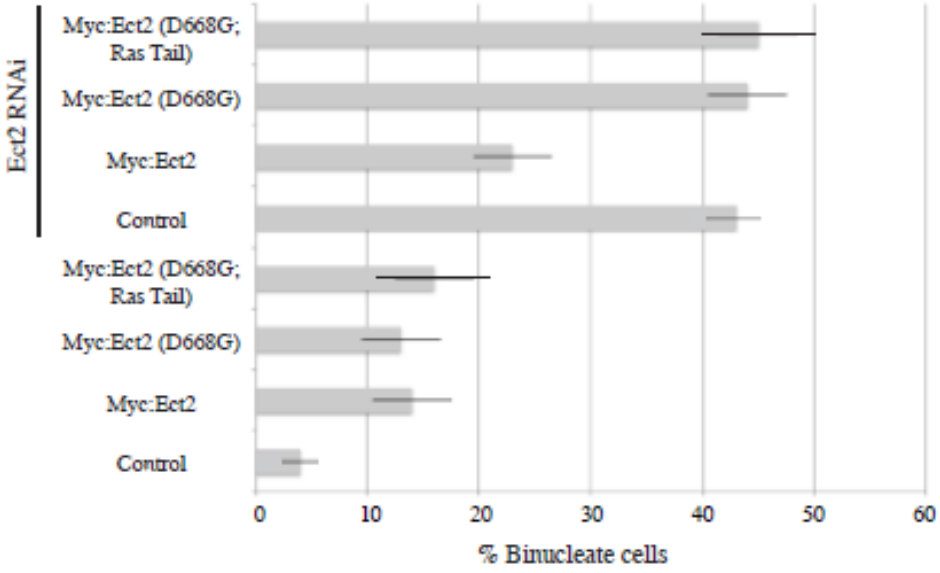
The RNAi^r D668G and the RNAi^r D668G: Ras tail mutants only partially restored anillin localization as compared to non-mutant Ect2 (n = 8 or 9 respectively; Figure 7A). Despite having lower levels of anillin, cells expressing the D668G mutant showed extensive furrowing, which supports our previous findings that the D668G mutant may not be null in its ability to activate RhoA. The failure of the Ras tail to fully rescue anillin localization supports that the tail is not sufficient to restore Ect2 function. The localization of D668G was less cortical vs. non-mutant Ect2, but both had similar central spindle localization, and D668G Ras tail had stronger cortical localization, but reduced central spindle localization (data not shown). Therefore, the Ras tail could interfere with Ect2's ability to localize to the central spindle, which would decrease its ability to activate RhoA.

We also determined the effect of D668G on the levels of endogenous active RhoA using an RBD pull down assay. Over-expressing full length Ect2 caused a small increase in the generation of active RhoA, but not to the same extent as C-terminal Ect2 (Figure 7C). Since this was done in the presence of endogenous Ect2, there likely are a limited number of associations with Cyk-4 and not all exogenous Ect2 is properly localized and activated. However, the D668G mutant generated less active RhoA compared to endogenous Ect2 or the non-mutant (Figure 7C). The D668G mutant likely maintains its Cyk-4 interaction (shown by its ability to still localize to the central spindle) and could compete with endogenous Ect2's localization to Cyk-4 and its ability to generate active RhoA. This result suggests that cortical association is crucial for Ect2's GEF activity. Also, Ect2 must interact both with the cortex and the central spindle for it to be fully functional and the PH domain plays a pivotal role in mediating its cortical interactions.

A)


DNA
Anillin
DNA
Ect2
Control
Ect2 RNAi
Merge
Anillin/Myc
Anillin
Myc
Ect2 RNAi
Myc:Ect2
Myc:Ect2 (D668G)
Myc:Ect2 (D668G; Ras Tail)

B)


Ect2 RNAi
Myc:Ect2 (D668G; Ras Tail)
Myc:Ect2 (D668G)
Myc:Ect2
Control
Myc:Ect2 (D668G; Ras Tail)
Myc:Ect2 (D668G)
Myc:Ect2
Control
0
10
20
30
40
50
60
% Binucleate cells

C)

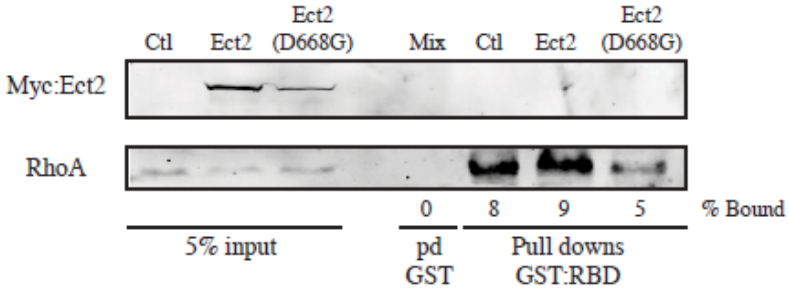


Figure 7 A) Images of Hela cells transfected with Myc-tagged Ect2 constructs and Ect2 RNAi co-stained with DAPI, Ect2, Myc (red) and anillin (green) antibodies. Scale bars are 10 μm. Images were taken by Dr. Piekny. B) A bar graph shows the percentage of binucleate cells for the indicated Myc-tagged constructs with or without Ect2 RNAi (lines indicate standard deviation). C) A western blot of lysates from HEK 293 transfected with Myc-tagged Ect2 constructs and pulled down using GST-tagged RBD and probed with RhoA and Myc antibodies.

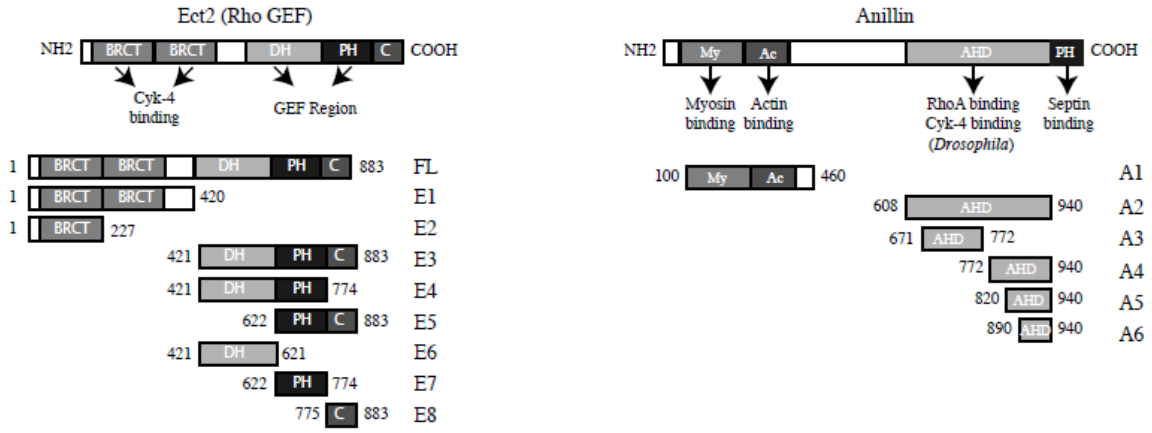
3.6 The PH domain of Ect2 interacts with the anillin homology domain (AHD) of anillin.

Ect2 depends on active RhoA for its cortical localization during cytokinesis and the temporal recruitment of Ect2 to the cortex coincides with anillin's recruitment to the contractile ring. Anillin interacts with RhoA-GTP, and RhoA localization visualized by TCA fixation is lost in anillin-depleted cells. Therefore, we determined if anillin interacts with Ect2. The structures of anillin and Ect2 and the corresponding regions used in our study are shown in Figure 8A. We observed a weak interaction between the AHD (chosen because this is the region that interacts with RhoA-GTP in human cells and RacGAP50C/Cyk-4 in Drosophila; D'Avino et al., 2008; Gregory et al., 2008; Piekny and Glotzer, 2008) and endogenous Ect2 (Figure 8B). The interaction occurred after Cdk1 inhibition, suggesting that anillin preferentially binds to the 'open' conformation of Ect2. As mentioned earlier, Ect2 is auto-inhibited by Cdk1 phosphorylation until anaphase. Cdk1 inhibition causes Ect2 to unfold, permitting its interaction with Cyk-4 in the N-terminus and RhoA in the C-terminus (Hara et al., 2006; Saito et al., 2004; Yuce et al., 2005). To narrow down the regions of Ect2 that anillin interacts with, we used fragments corresponding to the N-terminus or C-terminus of Ect2. These Myc-tagged fragments were expressed in HEK 293 cells and pulled down using bacterially purified MBP-tagged AHD of anillin (A2). The AHD of anillin interacted most strongly with the C-terminus of Ect2 (Figure 8C) and did not interact with the N-terminus. To further identify the minimal binding region on Ect2, different C-terminal constructs were used. Any Ect2 fragments that contained the PH region (E3, E4, E5 and E7) interacted with

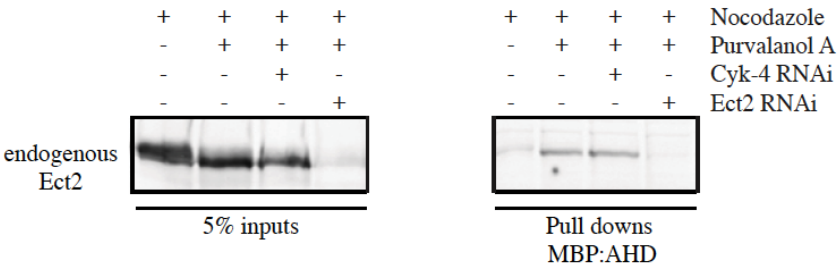
anillin whereas those lacking the PH region (E6 and E8) did not interact (Figure 8D). This data suggests that the AHD of anillin interacts with the PH region of Ect2.

To determine the minimal binding domain on anillin that Ect2 interacts with, multiple bacterially expressed anillin proteins were made and used to pull down Myc-tagged C-terminal Ect2 constructs (E3 and E5) from HEK 293 cell lysates. The C-terminal part of the AHD region (A6) pulled down Ect2 with equal strength to the largest fragment of the AHD (A2), but an N-terminal part of the AHD as well as the N-terminus of anillin did not (A3 and A1; Figure 8E).

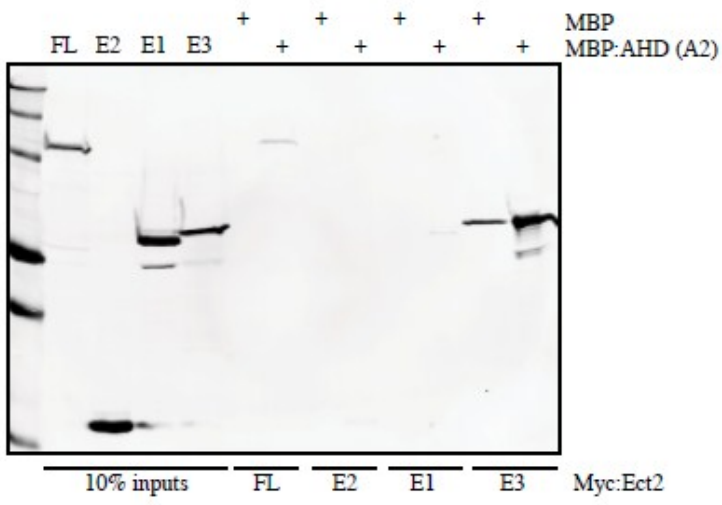
A)


Ect2 (Rho GEF)
NH2
BRCT
BRCT
DH
PH
C
COOH
Cyk-4 binding
GEF Region
1 BRCT BRCT DH PH C 883 FL
1 BRCT BRCT 420 E1
1 BRCT 227 E2
421 DH PH C 883 E3
421 DH PH 774 E4
622 PH C 883 E5
421 DH 621 E6
622 PH 774 E7
775 C 883 E8
Anillin
NH2
My
Ac
AHD
PH
COOH
Myosin binding
Actin binding
RhoA binding
Cyk-4 binding
(Drosophila)
Septin binding
100 My Ac 460 A1
608 AHD 940 A2
671 AHD 772 A3
772 AHD 940 A4
820 AHD 940 A5
890 AHD 940 A6

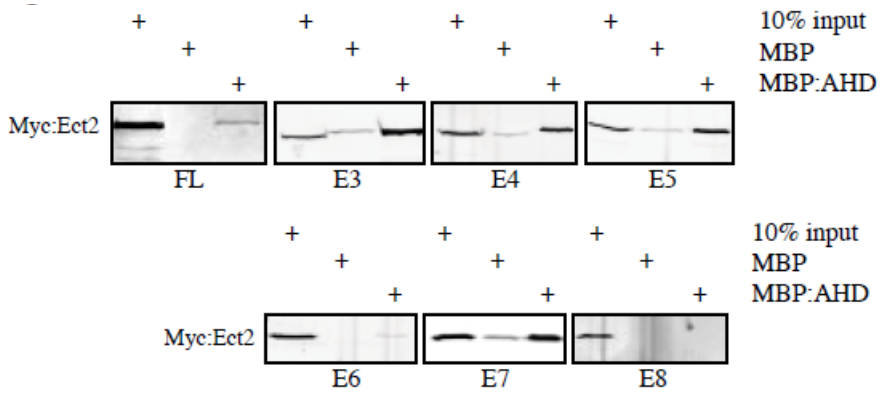
B)


Nocodazole
Purvalanol A
Cyk-4 RNAi
Ect2 RNAi
endogenous Ect2
5% inputs
Pull downs MBP:AHD

C)


MBP
MBP:AHD (A2)
FL E2 E1 E3
10% inputs
FL
E2
E1
E3
Myc:Ect2

D)



E)

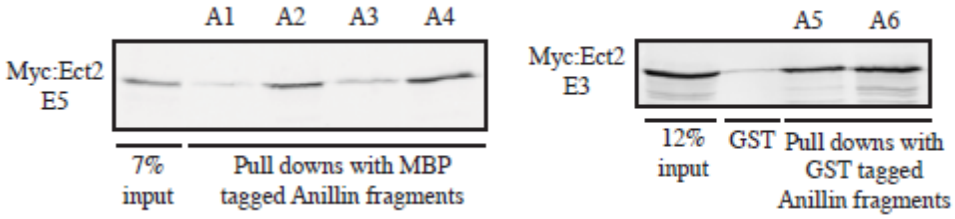
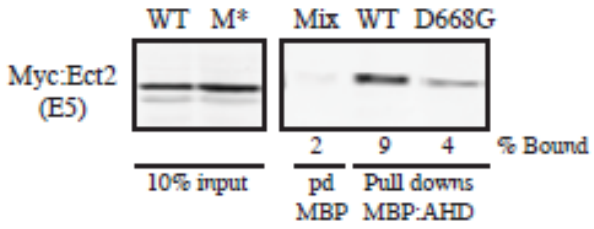


Figure 8 A) Schematic diagrams of the proposed structures of Ect2 and anillin are shown along with the different constructs used in this study. B) A western blot of lysates from Hela cells synchronized with nocodazole (arrests cells in metaphase) +/- purvalanol (to inhibit Cdk1 activity), and treated with Cyk-4 or Ect2 RNAi, pulled down with the AHD of anillin and probed with Ect2 antibodies. C) A western blot of lysates from HEK 293 transfected with Myc-tagged Ect2 constructs pulled down using MBP tagged AHD of anillin and probed with Myc antibodies. D) Western blots of lysates from HEK 293 transfected with Myc tagged Ect2 constructs pulled down with MBP tagged AHD of anillin and probed with Myc antibodies. E) Western blots of lysates from HEK 293 transfected with C-terminal Myc tagged Ect2 and pulled down using the indicated MBP tagged anillin constructs or GST tagged anillin constructs and probed with Myc antibodies. Figures A, B, C and D were done by Dr. Piekny.

3.7 Ect2 preferentially interacts with anillin at the membrane.

Since anillin interacts with the PH region of Ect2, which is required for its cortical localization and contributes to RhoA activation, we tested the ability of D668G to interact with anillin. C-terminal Ect2 constructs expressed in HEK 293 cells were pulled down with bacterially expressed AHD anillin. The D668G mutant had reduced anillin binding (~50% lower vs. non-mutant; Figure 9A). To determine if reduced binding with the mutant was due to its inability to localize to the membrane, we tested if adding the Ras tail (to force it back to the membrane) restores anillin binding. The Ras tail was able to partially restore D668G mutant interaction with anillin, however, it was still lower vs. non-mutant Ect2 (Figure 9B). Interestingly, adding the Ras tail onto non-mutant Ect2 enhanced anillin binding (Figure 9B). This result shows that both Ect2's localization at the membrane and the aspartic acid at position 668 are important for Ect2-anillin binding. We were unable to detect direct interactions between anillin and Ect2 *in vitro* (data not shown), and two possibilities are that another protein bridges the interaction, or phospholipids bridge the interaction between Ect2 and anillin *in vivo*. However, the interaction between Ect2 and anillin likely may not be through RhoA, because mutations in anillin that decrease RhoA binding have no effect on its interaction with Ect2 (data not shown). In addition, the Ect2 mutant N608A E609A that cannot effectively exchange nucleotide on RhoA binds to anillin similar to non-mutant Ect2 (Figure 4C).

A)



B)

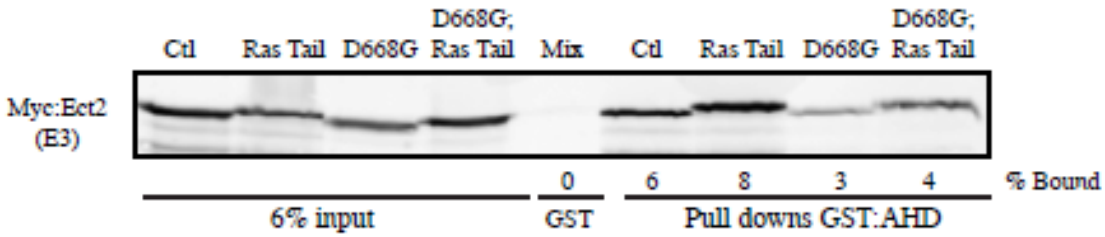


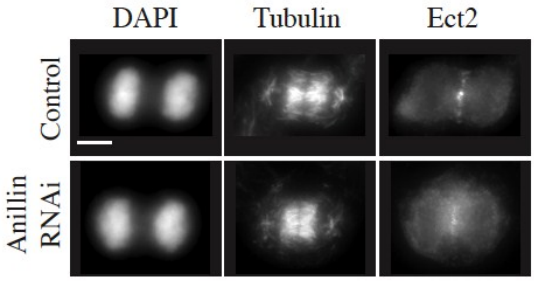
Figure 9 A) A western blot with lysates from HEK 293 transfected with Myc-tagged C-terminal Ect2 constructs pulled down with MBP-tagged AHD domain of anillin, and probed with Myc antibodies. B) A western blot with lysates from HEK 293 transfected with Myc-tagged Ect2 constructs and pulled down using GST-tagged AHD domain of anillin and probed with Myc antibodies. E5= 622-883 of Ect2 & E3= C-terminus of Ect2.

3.8 Anillin is required for the cortical localization of Ect2.

Our results thus far suggest that there is an interaction between Ect2 and anillin at the cortex but the *in vivo* relevance of their interaction is unclear. Anillin is required to scaffold components of the contractile ring, to maintain its position and stability during ingression. Anillin depletion in human or *Drosophila* S2 cells caused lateral instability of the contractile ring followed by cleavage furrow regression and cytokinesis failure (Hickson and O'Farrell, 2008; Piekny et al., 2008; Straight et al., 2005; Zhao and Fang, 2005a). Also, anillin is required for the stable localization of RhoA as visualized by TCA fixation, suggesting there may be a feedback loop between anillin and RhoA (Piekny et al., 2008; Zhao and Fang, 2005a). In *Drosophila*, anillin interacts with RacGAP50 (Cyk-4; D'Avino et al., 2008; Gregory, 2008) and this interaction may crosslink the central spindle to the cortex. We hypothesize that anillin's interaction with Ect2 may work similar to the anillin/RacGAP50 interaction in *Drosophila,* to help anchor the central spindle to the equatorial cortex in human cells. This would keep Ect2 in a stable position for the efficient generation of active RhoA, to maintain the division plane and furrow ingression. We examined the localization of Ect2 in anillin-depleted cells and found that little Ect2 was visible at the cortex after anillin RNAi in comparison to control cells (Figure 10A). Using line plots, we measured the fluorescence intensity of Ect2 across the equatorial plane of control and anillin depleted cells. While spikes of Ect2 signal were apparent at the cortex and on the central spindle in control cells, there were no cortical spikes and dampened central spindle spikes in anillin-depleted cells (n = 11 for anillin-depleted cells; n = 9 for control cells; Figure 10B). This result suggests that anillin is

required to sustain the cortical-central spindle interaction in human cells, and may do this through its interaction with Ect2.

A)



B)

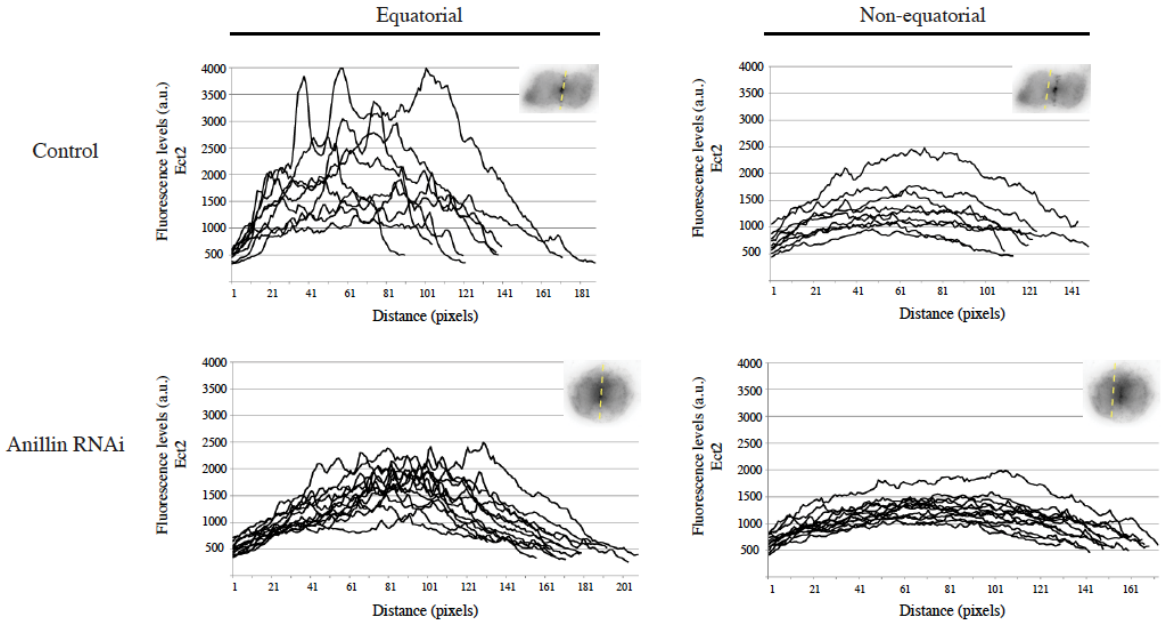


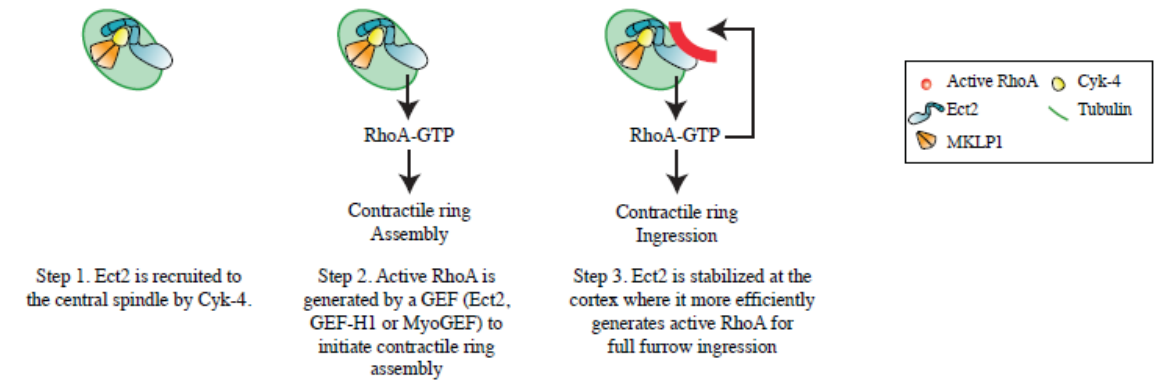
Figure 10 A) Z-stack projections of fixed Hela cells transfected with anillin RNAi and then co-stained with DAPI, tubulin, Ect2 antibodies. B) Line plots of multiple cells shown for control or anillin RNAi cells. The X-axis shows the distance across the cell in the indicated plane (equatorial or non-equatorial; yellow dotted lines) and the Y axis shows the levels of Ect2 fluorescence. Scale bar is 10 μm. Figure created by Dr. Piekny.

Discussion

We show that Ect2's cortical localization is required to generate sufficient levels of active RhoA for successful cytokinesis. Ect2 is recruited to the cortex by active RhoA, where it interacts with anillin, possibly to stabilize central spindle-cortical associations. This feedback loop suggests that even small amounts of active RhoA could switch Ect2 'on' at discrete cortical locations. As shown in Figure 11A, upon anaphase transition, Ect2 interacts with Cyk-4, and this interaction recruits Ect2 to the central spindle. After central spindle expansion and during furrow ingression, Ect2 is also cortically localized, which may increase Ect2's activation of RhoA during cytokinesis, particularly during furrow ingression.

Ect2 is a vital regulator of cytokinesis and generates active RhoA for contractile ring formation and ingression (Piekny et al., 2005). Despite this essential role, it is not clear how Ect2 regulates RhoA activity when its best-described localization is on the central spindle. We know that Ect2's interaction with Cyk-4 is essential for its function. Ect2 activation requires dephosphorylation, which permits its interaction with Cyk-4 and localization to the central spindle (Somers and Saints, 2003; Yuce et al., 2005; Zhao and Fang, 2005b). Depleting endogenous Cyk-4 has similar effects to Ect2 depletion; the contractile ring will not form and its ingression is blocked (Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b). In addition, Plk1 has to phosphorylate Cyk-4 for its interaction with Ect2; if Plk1 is inhibited then Cyk-4 cannot interact with Ect2 and cytokinesis fails (Petronczki et al., 2007; Wolf et al., 2009). However, the Cyk-4-Ect2

A)



B)

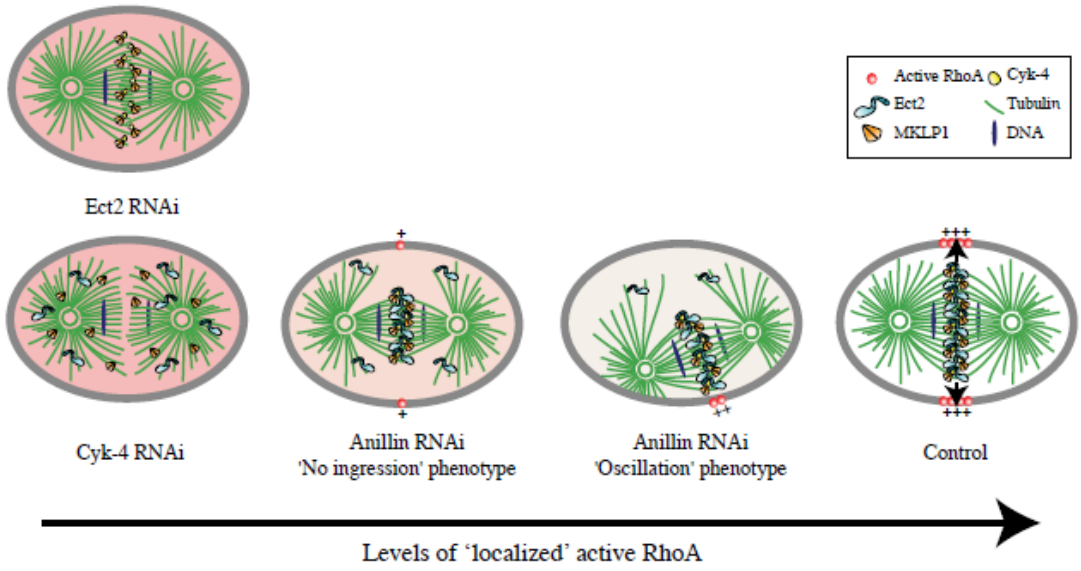


Figure 11 A) A proposed model shows the feed-forward pathway of Ect2 activation by active RhoA during cytokinesis. B) Schematic model showing that although Ect2 and Cyk-4 depletion lead to a strong decrease in active RhoA, anillin RNAi may cause reduced levels of localized active RhoA. In anillin depleted cells, the central spindle is not anchored properly, so Ect2 cannot be effectively maintained at the cortex to effectively generate active RhoA. Images and models are from Dr. Piekny.

interaction does not explain how Ect2 can activate RhoA at specific areas of the cortex during cytokinesis. During later stages of cytokinesis we noticed that Ect2 is more prominent at the equatorial cortex (Figure 3). Cortical pools of Ect2 are in a location more consistent with its substrate, and may more efficiently activate RhoA.

Many cortical proteins are recruited by active RhoA, and we found that Ect2's cortical localization also requires active RhoA (Figure 4). This dependence on RhoA suggests that once RhoA is activated, there is a feedback loop that will recruit even more Ect2 to the cortex, however it is unknown how this may work in other organisms. This loop may be essential to ensure that Ect2 activity is reinforced in one plane to ensure the generation of sufficient levels of active RhoA throughout cytokinesis. Ect2 depletion causes contractile ring assembly phenotypes, but also gives rise to additional phenotypes consistent with multiple requirements for Ect2 during ingression and abscission (data not shown; Chalamalasetty et al, 2006; Yuce et al., 2005). Ect2 RNAi may not eliminate 100% of endogenous Ect2, and it is not clear if other GEFs function partially redundantly with Ect2 during cytokinesis. Previous studies have shown that MyoGEF or GEF-H1 could be the culprits in human cells, although it is not clear if other GEFs contribute to cytokinesis in other eukaryotes (Asiedu et al., 2009; Birkenfeld et al., 2007; Wu et al., 2006). Interestingly, in *C. elegans* embryos, Ect2 is cortical (Motegi and Sugimoto, 2006) and in *Drosophila* S2 cells, the Ect2 homologue Pebble appears to be more cortical vs. human cells. Therefore, Ect2 could be more active in these organisms, and at an earlier time vs. human cells. The mechanism for restricting active RhoA to define the cleavage plane likely also would differ in these organisms *i.e.* in *C. elegans* the cortical GAPs, RGA-3/4, regulate the levels of active RhoA in the early embryo (Schmutz et al., 2007;

Schonegg et al., 2007). Cytokinesis is essential for successful cell division and it is not difficult to believe that there may be multiple GEFs and/or GAPs that work together to ensure threshold levels of active RhoA are generated in a discrete zone for cytokinesis.

As shown by Chalamasetty et al 2006, Ect2's cortical localization depends on its PH region and this is required for Pebble's cortical localization in *Drosophila* (Van Impel et al, 2009). A mutation in the PH region greatly diminishes Ect2's ability to interact with phospholipids and decreases its cortical localization (Figure 5). This same mutation also decreases Ect2's GEF activity towards RhoA (Figure 6). Adding a lipid tail to restore lipid association of the PH mutant partially restored Ect2's ability to activate RhoA (Figure 6). This bolsters our theory that Ect2 is most active at the cortex, possibly because Ect2 is in closer proximity to its substrate, RhoA, or because there is an Ect2-lipid interaction or another Ect2-protein interaction that potentiates Ect2's GEF activity. The ability of active RhoA to recruit Ect2 to the cortex and activate it supports the presence of a feedback loop. Even low levels of active RhoA at the equatorial plane could recruit Ect2 to generate more active RhoA, similar to turning on a switch. This mechanism could reinforce the equatorial plane through furrow ingression.

Ect2's cortical localization is also essential for its function *in vivo*. The PH domain mutant was unable to rescue cytokinesis defects caused by loss of endogenous Ect2 (Figure 7). We found that although anillin localization was partially restored in the majority of cells, which displayed extensive ingression, there may have been insufficient levels of active RhoA to successfully complete cytokinesis. Also, full-length Ect2 containing the PH mutation caused dominant-negative effects by competing for Cyk-4 binding and blocking the ability of endogenous Ect2 to generate active RhoA. This is

consistent with our data using the C-terminus of Ect2 and demonstrates the importance of Ect2's cortical association, either via lipid or protein interactions, for cytokinesis.

The PH region of Ect2 also interacts with the AHD of anillin, previously shown to interact with active RhoA (Figure 8). This interaction occurs at the cortex, likely after Ect2 and anillin are independently recruited by active RhoA. The PH domain mutant decreases binding with anillin, and adding a lipid tail onto this region partially restores the interaction (Figure 9). *Drosophila* anillin directly interacts with the central spindle protein RacGAP50C (Cyk-4 homologue), which may scaffold the central spindle to the cortex (D'Avino et al., 2008; Gregory et al., 2008). In human cells we could only detect weak interactions between Cyk-4 and anillin (data not shown). We propose that the Ect2-anillin interaction in humans is functionally analogous to the Cyk-4-anillin interaction in *Drosophila*. In anillin-depleted cells, Ect2 localization is lost near the cortex (Figure 10). The same phenotype was observed for tubulin and Plk1 suggesting that all of the central spindle-labeled microtubules are no longer positioned near the cortex (data not shown). This phenotype suggests that these cells should have lower levels of active RhoA (see model, Figure 11B). Past work showed that endogenous RhoA visualized by TCA fixation was not stable in anillin-depleted cells (Piekny and Glotzer, 2008; Zhao and Fang, 2005a). Furthermore, although the majority of anillin-depleted cells show oscillation phenotypes, a small proportion (~15%) of cells show failed ingression phenotypes consistent with RhoA or Ect2-depleted cells (data not shown). Therefore, we hypothesize that the Ect2-anillin interaction helps anchor central spindle-labeled microtubules at the equatorial cortex to position Ect2 for its activation and generation of active RhoA.

Perhaps there is a trimeric protein complex forming between anillin, Ect2 and RhoA. Anillin also interacts with active RhoA, and could scaffold Ect2 and RhoA to increase Ect2's proximity to its substrate. Alternatively, it is possible that anillin directly regulates Ect2's GEF activity. However, in anillin-depleted cells, enough active RhoA is still generated to drive the oscillation phenotype, which requires active myosin, and the C-terminus of Ect2 still localizes cortically suggesting sufficient levels of active RhoA are still generated (data not shown; Piekny and Glotzer, 2008).

To summarize, we show that the cortical recruitment of Ect2, possibly by active RhoA, is important for its function during cytokinesis. In order for anaphase to begin, Cdk1 must be inhibited, which permits Ect2 to interact with Cyk-4 for its recruitment to the central spindle (Figure 11A). The Ect2-Cyk-4 interaction is essential for Ect2's activation, likely to relieve Ect2's autoinhibition. This association may be sufficient to generate threshold levels of active RhoA (or other GEFs could contribute to the generation of active RhoA) to initiate contractile ring formation. As cells progress through mitosis, Ect2 is recruited to the cortex, where it generates more active RhoA for furrow ingression. At the cortex, Ect2 also interacts with anillin, and this interaction may stabilize central spindle-labeled microtubules at the equatorial cortex to reinforce the division plane. By anchoring microtubules in this plane, Ect2 is kept in close proximity to the cortex, to reinforce the generation of active RhoA. In anillin-depleted cells, the loss of central spindle-labeled microtubules at the equatorial cortex causes cortical instability, likely caused by unbalanced RhoA activation (Figure 11B).

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