Determining the Importin-Regulation of Ect2 Function During Cytokinesis

Joseph Del Corpo

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Signed by the Examining Committee:

_____ Chair

Dr. Michael Sacher

_____ External Examiner

Dr. Vladimir Titorenko

_____ Examiner

Dr. Michael Sacher

_____ Examiner

Dr. Brandon Helfield

_____ Supervisor

Dr. Alisa Piekny

Approved by _____

Dr. Robert Weladji, Graduate Program Director

_____ 2021 _____

Pascale Sicotte, Dean of Faculty

Abstract

Determining the Ran-regulation of Ect2 function during cytokinesis

Joseph Del Corpo

This work describes the potential requirement for a nuclear localization signal (NLS) in the regulation of Ect2, a RhoA GEF that activates RhoA for cytokinesis. The NLS has not previously been studied for its requirement in cells. Earlier studies led to a model where Ect2 is in an autoinhibited conformation mediated by Cdk1 phosphorylation, and that removal of this phosphate during anaphase is required for the open, active conformation. Further, Cyk4, a central spindle protein, binds to BRCT domains in the N-terminus of Ect2, which, together with lipid-binding is required for Ect2 activity. Interestingly the NLS is adjacent to the Cdk1 site, and *in vitro* studies showed that importin-binding is competed by phosphorylation. One model is that importin-binding facilitates the open conformation of Ect2 and work in concert with Cyk4-binding. Another, non-mutually exclusive model is that the NLS is required to mediate the localization of Ect2 to the re-forming nuclei in the daughter cells at the end of cytokinesis for abscission. To test these models, we generated point mutations in the NLS and performed rescue assays. We found that the NLS is required for the function of Ect2 in cytokinesis by regulating abscission. Further, point mutations in the Cdk1 phosphorylation site did cause obvious cytokinesis defects, suggesting this model needs to be revised.

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Dedications

I'd like to thank my family and friends and dedicate this thesis to them. Thank you to my family for your unwavering support throughout all my academic endeavours; you have encouraged me all along the way even though you had no idea what any of it meant. Thank you to my friends for all that you have done to help keep me sane during this process; your support has been immeasurable, and I truly cannot thank you all enough. Lastly, thank you to my partner. Julia, any praise that my work may receive is equally yours; I genuinely do not think that I could have done this without your constant love, support, motivation, and patience. I love you so much and thank you for everything that you do.

I'd like to dedicate this thesis to my grandfather. Spero che vi ho reso orgoglioso e che la mia ricerca possa contribuire a cambiare il mondo in meglio.

Contribution of Authors

Figure 8. This figure was made in collaboration with Mathieu Husser. Mathieu captured and provided the image used in Part B.

Figure 10. This figure was made in collaboration with Nhat Pham. Nhat captured and provided the mNeonGreen::Ect2 HeLa movie used in Part A

Figure 11. This figure was made in collaboration with Nhat Pham. Nhat captured and provided the mNeonGreen::Ect2 HeLa image used as the untreated control in Part A.

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List of Abbreviations

ADP	Adenosine diphosphate
ALIX	ALG-2 interacting protein-X
Arf	ADP-ribosylation factor
BRCT	BRCA 1 C-terminal
C2	Protein kinase C conserved lipid-binding region 2
Cdk1	Cyclin-dependent kinase 1
Cep55	Centrosomal protein of 55 kDa
CPC	Chromosomal passenger complex
Cyk4	Cytokinesis defect 4 (*C. elegans* homolog of MgcRacGAP)
DAD	Diaphanous autoregulatory domain
DAPI	4',6-diamindino-2'-phenylindole dihydrochloride
DH	Dbl (diffuse B-cell lymphoma) homology
DID	Diaphanous inhibitory domain
DNA	Deoxyribonucleic acid
Ect2	Epithelial cell transforming 2
ELC	Essential light chains
ESCRT	Endosomal sorting complex required for transport
FH1	Formin homology 1 (domain)
FH2	Formin homology 2 (domain)
GAP	GTPase activating protein
GBD	GTPase binding domain
GDP	Guanosine diphosphate

GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HeLa	Henrietta Lacks (cervical cancer cells derived from)
INCENP	Inner centromere protein
KIF4	Kinesin superfamily protein 4
MCAK	Mitotic centromere-associated kinase
mCherry	Monomeric cherry fluorescent protein
MDCK	Madin-Darby canine kidney (cells)
MgcRacGAP	Male germ cell RacGTPase activating protein
MKLP1	Mitotic kinesin like protein 1
MP-GAP	M-phase GAP
mNeonGreen	Monomeric Neon Green
NLS	Nuclear localization sequence
PBC	Poly-basic cluster
PH	Pleckstrin homology (domain)
Plk1	Polo-like kinase 1
PRC1	Protein regulator of cytokinesis 1
Rab11-FIP3	Ras-associated binding protein 11 family-interacting protein 3
Rac	Ras-related C3 botulinum toxin substrate protein
Ran	Ras-related nuclear protein
RanGAP	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor

RBD	RhoA GTP-binding domain
RCC1	Regulator of chromosome condensation 1
RhoA	Ras homolog family member A
RLC	Regulatory light chain
RNAi	Ribonucleic acid interference
S2	Schneider 2 (cell)
siRNA	Small interfering ribonucleic acid
TSG101	Tumor susceptibility gene 101 protein
VPS4	Vacuolar protein sorting-associated protein 4

Chapter 1. Introduction

Preamble

This work describes a novel mode of regulating Ect2 function in cytokinesis. Ect2 is a guanine nucleotide exchange factor (GEF) for RhoA that is conserved among metazoans. Ect2 is required to generate active RhoA to assemble a contractile ring for cytokinesis, the end-stage of mitosis where the cell pinches into two daughters. Ect2 was originally identified as an oncogene and new knowledge of its regulation could lead to novel insights to our understanding of cancers and how they develop.

1.1 Cytokinesis

Cytokinesis is the last step of the cell cycle and is characterized as the physical separation of a cell into two daughter cells (Figure 1). Cytokinesis must be tightly controlled to ensure the successful segregation of chromosomes and distribution of cell fate determinants. If cytokinesis fails, the daughter cells could have alternative fates and/or become aneuploidy, which are hallmarks of cancer. Cytokinesis occurs due to the assembly and ingression of an actomyosin ring (Figure 2). The mitotic spindle, along with other microtubule-independent cues spatiotemporally control the ring. After ingression, the ring transitions to a midbody, which is required for abscission of the daughter cells (Figure 1; Green et al., 2012; Glotzer, 2017).

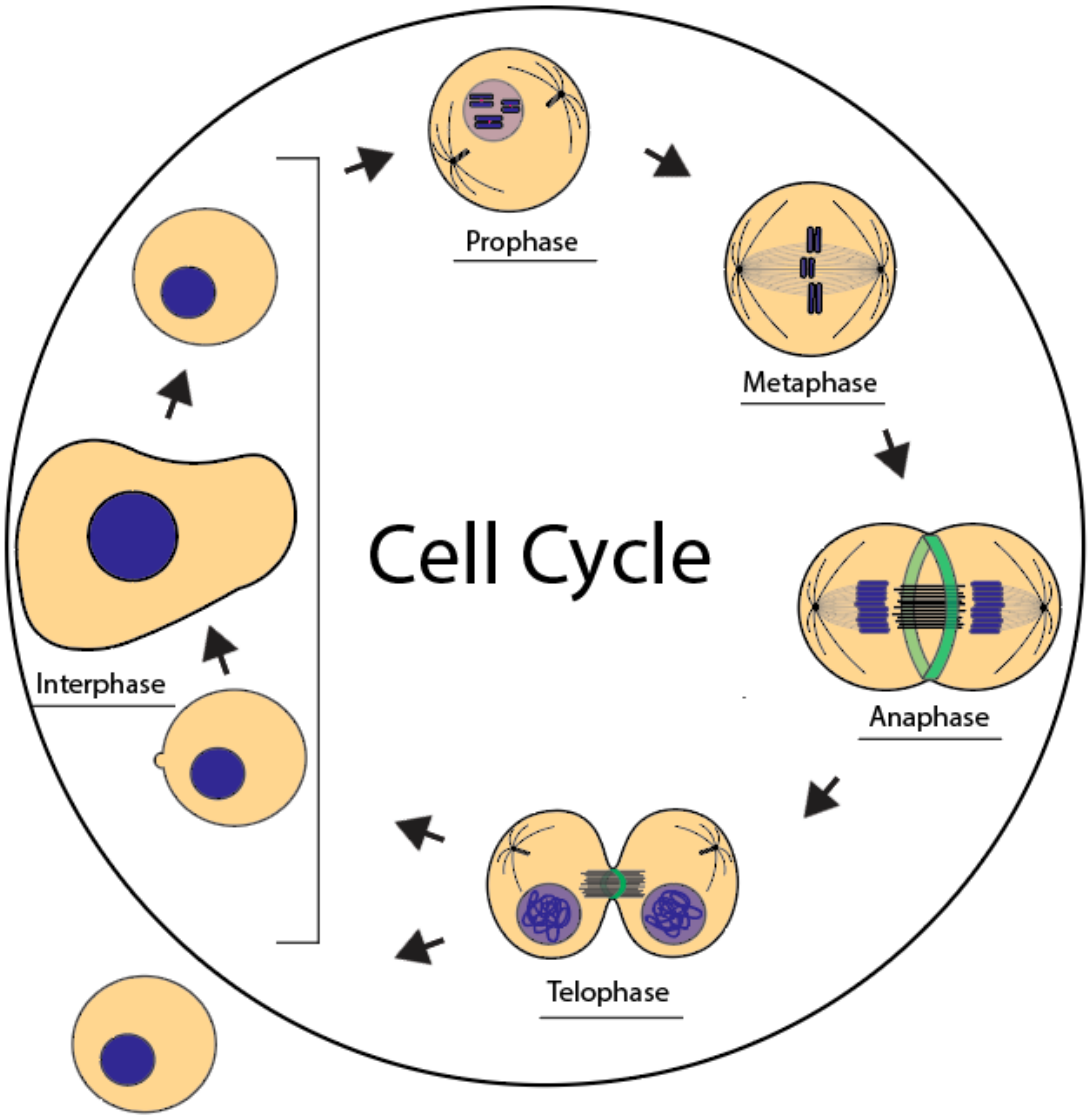


Figure 1 – Mitosis and the cell cycle. Mitosis begins with prophase where the cell rounds up, the nuclear envelope breaks down, chromosomes condense, duplicated centrosomes mature, and microtubules become more dynamic. During metaphase, a bipolar spindle forms with aligned sister chromatids along the midzone of the cell. As Cdk1 activity drops, cells exit mitosis into anaphase where a central spindle arises between segregating chromosomes, and a contractile ring assembles in the equatorial plane. The ring ingresses to pinch in the daughter cells during telophase, and the chromosomes decondense as the nuclear envelope re-assembles. Finally, the daughter cells undergo abscission to separate. Cytokinesis encompasses all stages of mitotic exit.

Ring assembly is initiated by RhoA (Ras homolog family member A), a small GTPase that is considered to be the master regulator of cytokinesis (Glotzer, 2017). RhoA exists in both an inactive, GDP (Guanosine diphosphate)-bound state and an active, GTP (Guanosine triphosphate)-bound state. While MP-GAP (M-phase-GTPase activating protein) globally stimulates GTP hydrolysis to generate inactive RhoA around the cortex, the GEF Ect2 (epithelial cell transforming 2) generates active RhoA in the equatorial plane (Yüce et al., 2005; Zanin et al., 2013). Ect2 requires Cyk4 (cytokinesis defect 4)- and phospholipid-binding for its activity, which is described in extensive detail in a subsequent section (Section 1.2). This regulation ensures the correct spatiotemporal control over the generation of active RhoA for ring assembly. When MP-GAP is depleted from cultured HeLa cells, ectopic active RhoA is generated in regions of the cortex outside the furrow causing excessive, uncontrolled blebbing and contractility (Zanin et al., 2013). Conversely, in HeLa (Henrietta Lacks; cervical cancer derived from) or S2 (Schneider 2) cells depleted of Ect2 or RhoA, the contractile ring fails to assemble and cytokinesis fails (Yüce et al., 2005).

Active RhoA initiates ring formation by mediating F-actin assembly and nonmuscle myosin II activation (Figure 2). Formin nucleates the assembly of unbranched actin filaments through its FH1 (formin homology 1) and FH2 (formin homology 2) domains (Romero et al., 2004). The domains function as a dimer, which is autoinhibited by the DAD (diaphanous autoregulatory domain) at the C-terminus, which binds to a DID (diaphanous inhibitory domain) in the N-terminus. The binding of RhoA-GTP to the GBD (GTPase binding domain), which overlaps the DID, relieves this autoinhibition and enables the FH1 domains to bind to profilin-actin which brings them to the FH2 domains for polymerization. Actin assembly occurs at the barbed end of the growing filament, and requires ATP (adenosine triphosphate; Romero et al.,

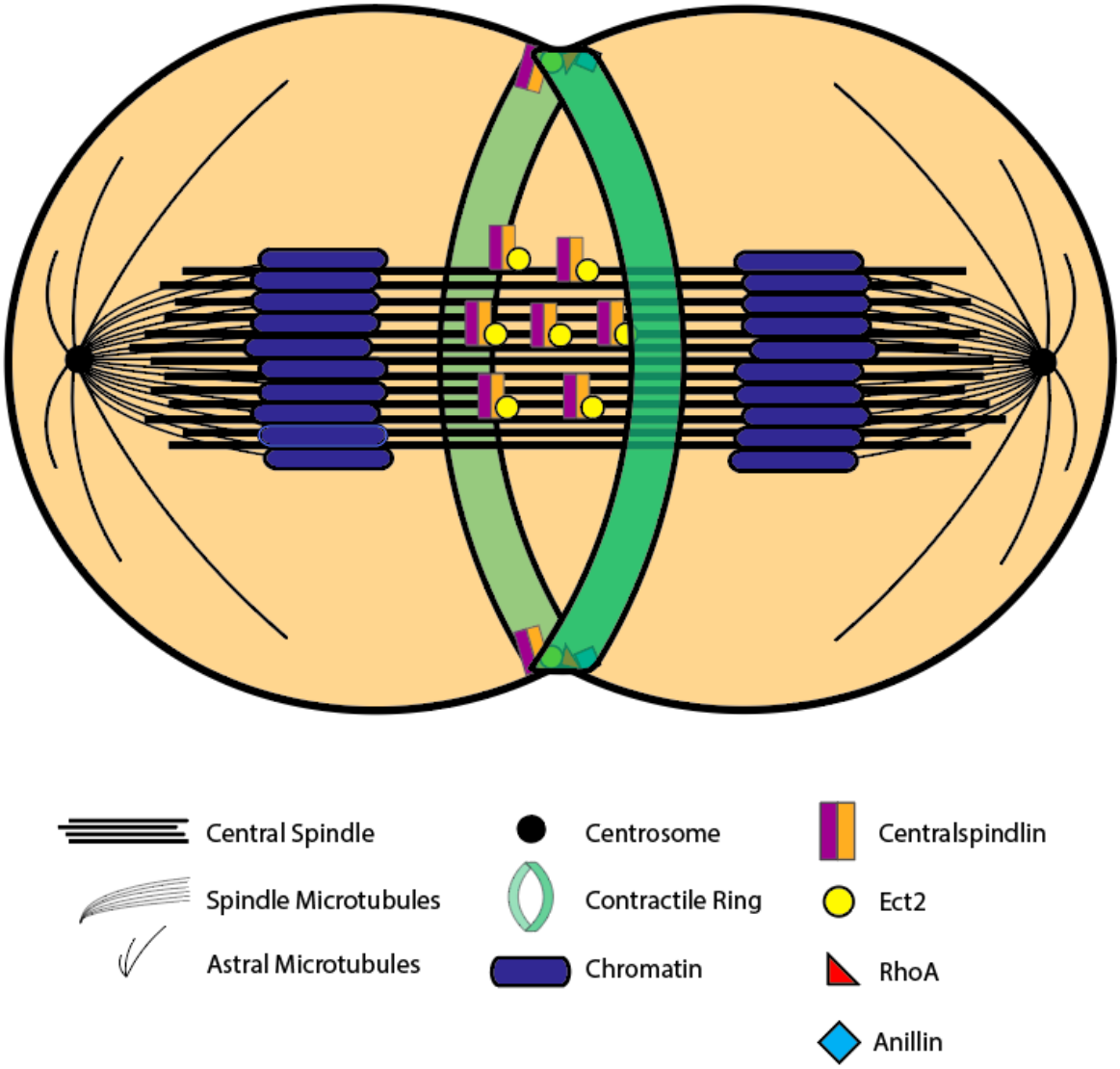


Figure 2 – The contractile ring and some of its regulators in early telophase. The actomyosin contractile ring (in green) is generated in the equatorial plane upon RhoA (red) activation by the GEF, Ect2 (yellow). Ect2 binds to Cyk4, part of the centralspindlin complex (Cyk4 in orange, MKLP1 in purple), which is required for its activation and localization to the central spindle (thick black lines) formed between segregating chromosomes (dark blue). The generation of active RhoA in the overlying cortex requires membrane-localized Ect2-centralspindlin complexes. Active RhoA then stimulates F-actin assembly and myosin activation, in addition to recruiting effectors that control ring position and ingression including anillin (blue).

2004; Bement et al., 2005). RhoA-GTP also binds to the effector Rho kinase to mediate the assembly and activation of nonmuscle myosin II filaments (Piekny and Mains, 2002; Piekny et al., 2003; Matsumura, 2005; Otomo et al., 2005). Myosin II is a hexamer formed from two myosin heavy chains, two regulatory light chains (RLC) and two essential light chains (ELC). Myosin assembles into bipolar oligomers through the coiled-coil regions of the heavy chains, which requires RLC phosphorylation (Piekny and Mains, 2002; Piekny et al., 2003; Matsumura, 2005). Myosin filaments can bind to and/or translocate actin filaments via their motor domains to drive assembly and constriction of the contractile ring (Glotzer, 2005).

Active RhoA also recruits other effectors required for ring assembly and/or ingression including anillin (Figures 2, 3). Anillin is a scaffold with binding sites for several cytokinesis proteins and regulators including actin, myosin, phospholipids, microtubules, Ect2, active RhoA and septins. Through these interactions, anillin is thought to coordinate different signals required for cytokinesis for positioning the ring and anchoring it to the membrane (Yonemura et al., 2004; Bement et al., 2006; Piekny and Glotzer, 2008). In support of this proposed function, depleting anillin in HeLa or S2 cells causes the ring to oscillate and cytokinesis failure (Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008).

As the ring constricts and the perimeter of the cell decreases, contractile ring components such as anillin, septins, and myosin, are lost from the ring; this loss of components occurs at a rate that is proportional to the decrease in ring circumference, although how this is regulated is still not well-understood (Carvalho et al., 2009). This transition ultimately results in the formation of the midbody and leads to abscission, which is the last step in cytokinesis (Kechad et al., 2012). Abscission is the final step required to separate the dividing cell into two daughters and is driven

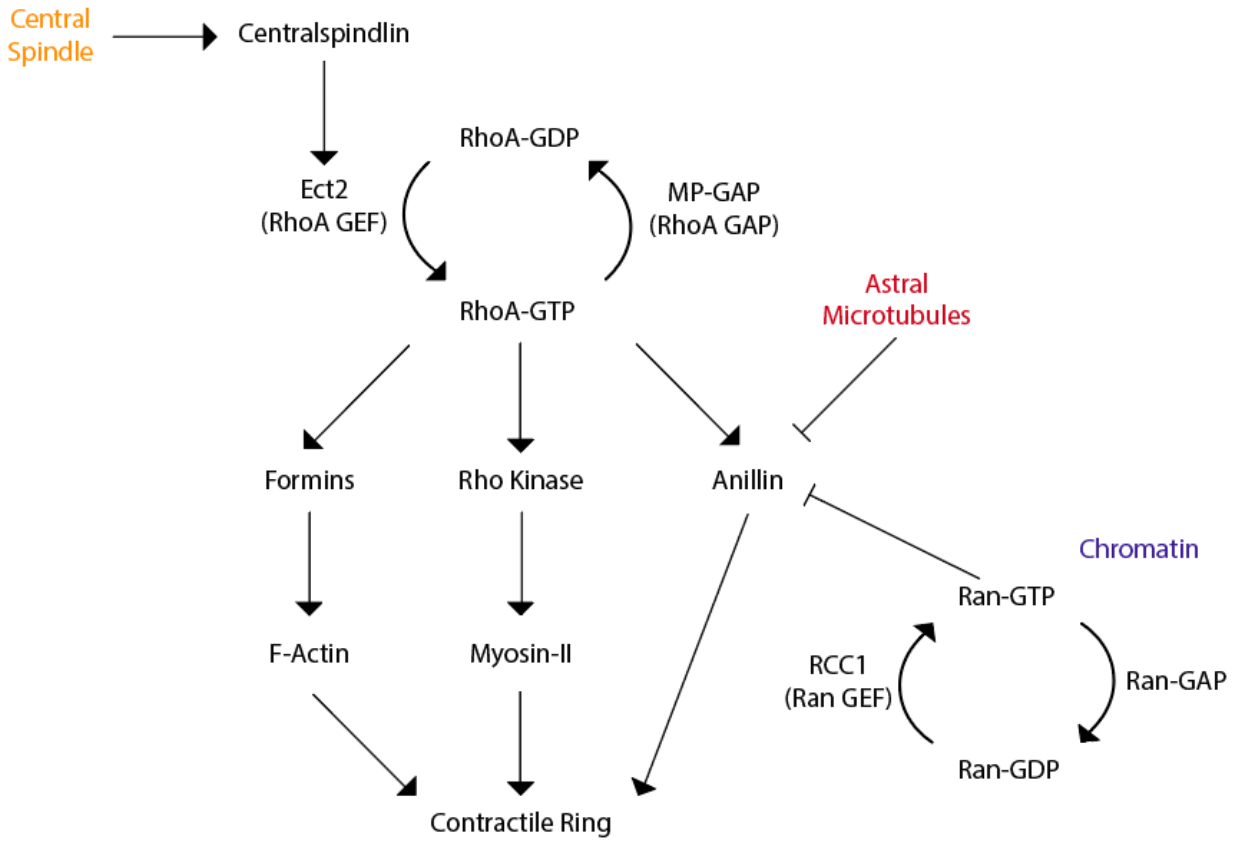


Figure 3 – Pathways regulating contractile ring assembly and/or position. The contractile ring is regulated and positioned by a number of different proteins. RhoA is the master regulator of cytokinesis and initiates contractile ring assembly via effectors such as formins and Rho kinase which assemble F-actin and activates nonmuscle myosin II, respectively. Ect2 function requires binding to Cyk4, which is part of the centralspindlin complex with MKLP1 (central spindle pathway, orange). Active RhoA recruits anillin, which is required to stabilize and anchor the contractile ring to the cell membrane. Anillin is regulated by astral microtubules (red) and chromatin (blue), via Ran-GTP.

by a combination of vesicle trafficking, such as Rab11-FIP3 (Ras-associated binding protein 11 family-interacting protein 3) endosomes, membrane shedding and fission via ESCRTs (endosomal sorting complex required for transport; El Amine et al., 2013; König et al., 2017).

1.2 Microtubule-dependent regulation of ring assembly and position

Microtubule-dependent and -independent pathways control assembly and/or position of the contractile ring (Figure 3). The anaphase spindle is composed of astral microtubules that emanate toward the polar cortex, and a central spindle which is composed of antiparallel bundled microtubules that arises between the segregating chromosomes (Figures 2, 3; Glotzer, 2005). The astral microtubules are thought to have inhibitory roles in regulating the ring, while the central spindle provides stimulatory cues. For example, loss of the astral microtubules results in a broader zone of active RhoA and anillin (van Oostende Triplet et al., 2014; Beaudet et al., 2017). There are several non-mutually exclusive models to explain this effect of microtubules on ring proteins. One is that microtubule depolymerization releases active GEF-H1, which can generate active RhoA outside the equatorial plane (Birkenfeld et al., 2007). Another is that anillin has an increased affinity for astral microtubules in the poles, which promotes its removal along with associated proteins from cortical regions outside the equatorial plane (Figure 3; Tse et al., 2011; van Oostende Triplet et al., 2014). In support of this model, depletion of MCAK (mitotic centromere-associated kinase) which depolymerizes microtubules, causes an increase in the length of astral microtubules and a narrower zone of anillin and associated contractile ring proteins in the equatorial plane (Zanin et al., 2013; van Oostende Triplet et al., 2014).

The central spindle pathway plays an important role in controlling Ect2 function and generating active RhoA in the equatorial plane during anaphase (Figures 2, 3). Central spindle assembly initiates during anaphase and requires several complexes including PRC1 (protein

regulator of cytokinesis 1) and KIF4 (kinesin superfamily protein 4), centralspindlin which is a heterotetramer formed from MKLP1 (mitotic kinesin-like protein) and Cyk4, and the chromosomal passenger complex (CPC) which consists of INCENP (inner centromere protein), Borealin, Survivin and Aurora B kinase (Mishima et al., 2002; Schroeder, 1990; Tatsumoto et al., 1999; Yoshizaki et al., 2004). Formation of the PRC1 and KIF4, and centralspindlin complexes are negatively regulated by Cdk1 (cyclin-dependent kinase 1) phosphorylation, and their assembly only occurs after Cdk1 activity decreases during anaphase (Mishima et al., 2002, 2004; Kurasawa et al., 2004; Hu et al., 2011). Inhibiting Cdk1 activity in metaphase is sufficient to generate a central spindle (Mishima et al., 2004; Niiya et al., 2005). Centralspindlin is also controlled by the CPC, where Aurora B kinase phosphorylates MKLP1 to prevent 14-3-3-binding and permits higher order assembly of complexes and midbody formation (Glotzer, 2009; Douglas et al., 2010). Cells lacking any of these proteins will experience some sort of defect in central spindle assembly (Mishima et al., 2002; Kurasawa et al., 2004; Mollinari et al., 2005). The loss of PRC1 in cells causes the mislocalization of centralspindlin and the CPC in a broad, diffuse manner (Mollinari et al., 2005).

The central spindle provides stimulatory cues for ring assembly (Figures 2, 3). Centralspindlin binds to and activates Ect2 in the equatorial plane (Yüce et al., 2005; Zhao and Fang, 2005; Chalamalasetty et al., 2006). Specifically, Cyk4 is phosphorylated by Plk1 (Polo-like kinase 1) and subsequently binds to the N-terminal BRCT (BRCA 1 C-terminal) domains of Ect2 (Figure 4; Yuce et al., 2005). This is thought to relieve autoinhibition of the C-terminal GEF (DH; Dbl homology) domain of Ect2 (Hara et al., 2006; Niiya et al., 2006). Prior to anaphase, Cdk1 phosphorylation of Ect2 was proposed to mediate this autoinhibition, and the loss of this phosphorylation in anaphase combined with Cyk4-binding helps Ect2 to become fully active

(Yuce et al., 2005; Zhang and Glotzer, 2015). In its C-terminus, Ect2 also has a Pleckstrin homology (PH) domain and neighbouring polybasic cluster (PBC), which mediate phospholipid binding and are also required for the activity of Ect2 (Figure 4; Burkard et al., 2007; Frenette et al., 2012; Lekomtsev et al., 2012).

The role of the central spindle microtubules in regulating Ect2 is not clear. Despite the obvious role that centralspindlin has in recruiting Ect2 to the equatorial plane, the central spindle itself is dispensable for Ect2 function. In HeLa cells or early *C. elegans* embryos, the loss of PRC1, Aurora B Kinase or MKLP1 does not cause failed ring assembly, but only causes later defects in abscission (Basant et al., 2015; Glotzer, 2009; Kurasawa et al., 2004; Mollinari et al., 2005; Petronczki et al., 2007). This phenotype is quite different from the loss of Cyk4 or Plk1 inhibition which causes an early failure in ring assembly (Adriaans et al., 2019; Yüce et al., 2005; Zhang and Glotzer, 2015). Other studies showed that Plk1 requires PRC-binding at the central spindle (Mollinari et al., 2005; Zhu et al., 2006), and one model is that the central spindle microtubules act as a sink for centralspindlin, and when Cyk4 is phosphorylated by Plk1, the complex is released to the overlying membrane.

If the central spindle is not required for ring assembly, then it is not clear how active RhoA is generated in the equatorial plane. The PH and PBC domains are required to mediate the recruitment of Ect2 to the membrane and for its activity (Frenette et al., 2012; Lekomtsev et al., 2012; Petronczki et al., 2007). This could be because Ect2 is recruited to regions of the membrane where RhoA preferentially binds, which facilitates the rapid turnover and generation of active RhoA (Frenette et al., 2012; Lekomtsev et al., 2012; Petronczki et al., 2007). A recent study proposed that the PH domain inhibits the neighbouring DH domain (GEF region), which is relieved by binding to active RhoA to release a fully active DH domain (Chen et al., 2020). However, it is

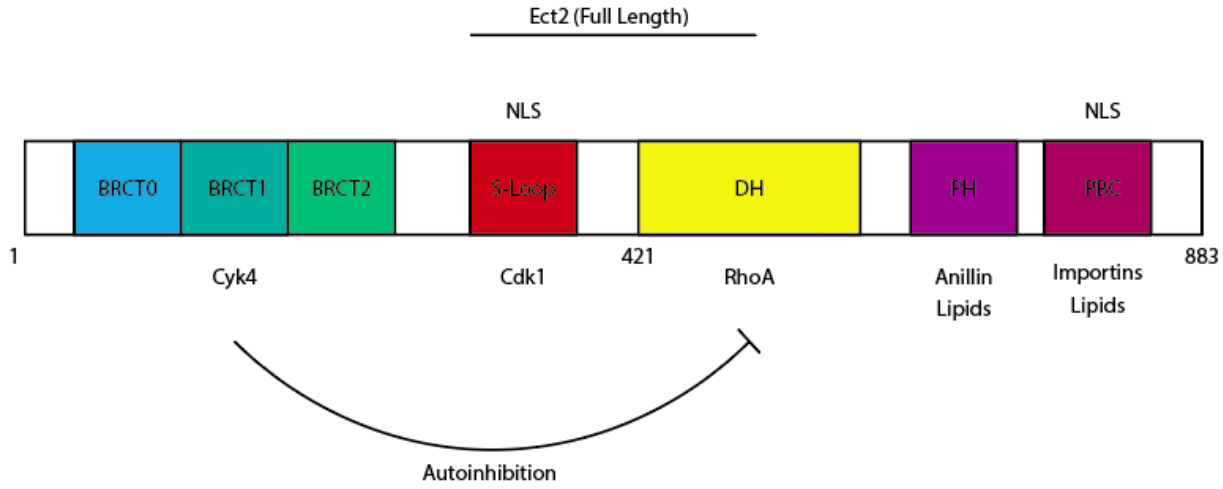


Figure 4 – The structure of Ect2. A schematic of Ect2 shows its various domains along with the interactions that occur with other proteins and cell components. The autoinhibition of Ect2 is putatively regulated by the interaction between the N-terminal BRCT domains (BRCT 0 in blue, BRCT1 in teal and BRCT2 in green) and Cdk1 phosphorylation in the S-loop (red), which could prevent GDP-GTP exchange on RhoA via the DH domain (yellow). Cyk4 is phosphorylated by Plk1, which facilitates binding to the BRCT1 domains and together with the loss of Cdk1 phosphorylation could relieve autoinhibition. The C-terminal PH (purple) and PBC (maroon) domains bind to lipids and are also required for Ect2 function, but their role is less clear. There are two putative NLS sites in Ect2, one is in the S-loop and the other is in the PBC.

extremely challenging to test this model given that the loss of RhoA or Ect2 causes a complete block in ring assembly. Further, point mutations in the PH domain or PBC are sufficient to completely disrupt the GEF activity of Ect2, suggesting that one/both are stimulatory vs. inhibitory (Frenette et al., 2012; Lekomtsev et al., 2012; Petronczki et al., 2007). There likely are additional microtubule-dependent and -independent pathways that also regulate the generation of active RhoA in the equatorial plane for ring assembly and/or positioning. For example, Cyk4 has lipid-binding domains and centralspindlin complexes can assemble at the plasma membrane independent of Plk1 activity (Adriaans et al., 2019). Aurora B kinase phosphorylates MKLP1 at the equatorial membrane to promote centralspindlin complex assembly via inhibiting 14-3-3 binding, which otherwise binds to MKLP1 and prevents the assembly of higher order complexes (Douglas et al., 2010; Capalbo et al., 2012).

1.3 Microtubule-independent regulation of ring assembly and position

Microtubule-independent pathways also regulate ring assembly and/or position, and one that our lab helped to discover is the chromatin pathway. The chromatin pathway relies on a signal that is generated by chromatin in the form of active Ran (Ras-related nuclear protein; Kalab et al., 2006; Kalab and Heald, 2008). Essentially, a gradient of active Ran forms around the segregating chromosomes during anaphase, with a reciprocal gradient of importins enriched in the equatorial plane that controls the localization of contractile proteins for cytokinesis (Figures 3, 5; Beaudet et al., 2017, 2020; Ozugergin and Piekny, 2021; Ozugergin et al., 2021).

Ran is best known for its role in nucleocytoplasmic transport, but also has a well described role in regulating mitotic spindle assembly (Cavazza and Vernos, 2016; Clarke and Zhang, 2008; Kalab, 2002; Kalab et al., 2006; Kalab and Heald, 2008; Ozugergin and Piekny, 2020). Ran

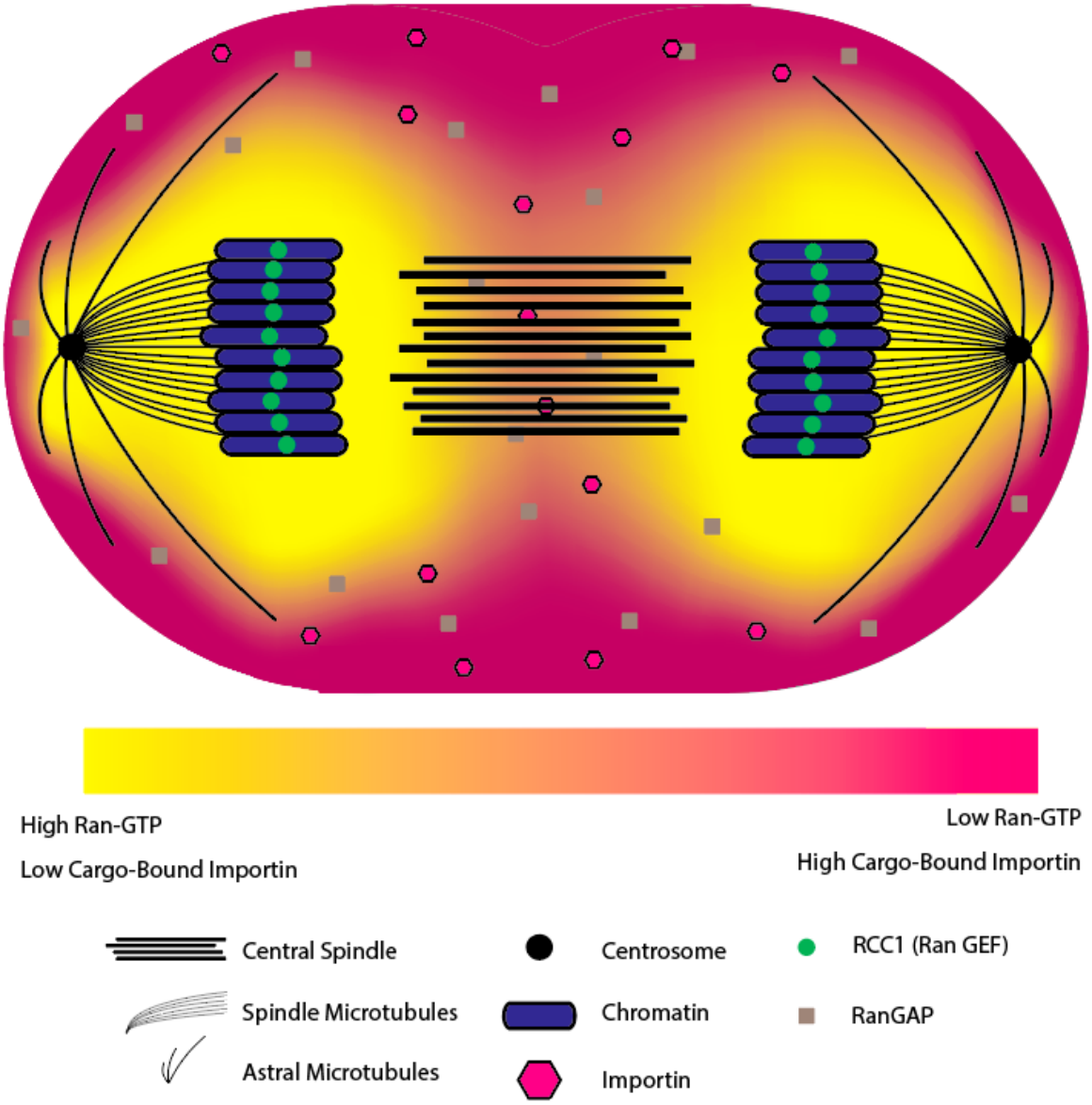


Figure 5 – The Ran-GTP and importin gradient during mitotic exit. An inverse gradient of Ran-GTP and importin forms near chromatin (high Ran-GTP in yellow) and the equatorial cortex (high cargo-bound importins in pink), respectively. The Ran-GTP gradient forms because the Ran GEF, RCC1 (green), is tethered to histones on chromatin, while RanGAP (grey) is cytosolic. Importins can bind to cargo where active Ran levels are low, but not where active Ran levels are high. Adapted from Ozugergin and Piekny, 2021.

regulates the localization or function of proteins with nuclear localization sequences (NLSs) through proteins called importins (Clarke and Zhang, 2008). Importins can bind to the NLS in proteins when active Ran levels are low, but not when active Ran is high (Clarke and Zhang, 2008).

In the cell, inverse gradients of importins and active Ran form where active Ran is high in the nucleus and low in the cytosol (Clarke and Zhang, 2008). This is because RCC1 (regulator of chromosome condensation 1), the GEF for Ran is tethered to histones, while RanGAP is found in the cytosol (Clarke and Zhang, 2008). In this way, importins control the import of NLS-containing proteins into the nucleus as they can interact with proteins in nuclear pore complexes for nuclear entry. When in the nucleus, high levels of active Ran release these proteins from importin-binding (Clarke and Zhang, 2008). During nuclear envelope breakdown, active Ran is continuously generated near chromatin to maintain a gradient of active Ran even in the absence of a physical partition between DNA and the cytosol (Kalab et al., 2006; Kalab and Heald, 2008). During mitosis, importin-binding prevents complexes from forming that are required for mitotic spindle assembly, and this inhibition is relieved in the vicinity of chromatin where active Ran is high (e.g. Figure 5; Kalab et al., 2006; Kalab and Heald, 2008).

Our lab found that importin-binding facilitates the cortical localization of anillin for its function in cytokinesis. This important finding suggested that the inverse gradient of importins and active Ran could be multifunctional – while promoting spindle assembly near chromatin, it also can promote ring assembly in the equatorial plane (Figure 5; Ozugergin and Piekny, 2021). As previously mentioned, anillin is a scaffold that crosslinks and coordinates components and regulators of the ring including actin, myosin, RhoA and phospholipids (Piekny and Glotzer, 2008; Piekny and Maddox, 2010; Frenette et al., 2012; Beaudet et al., 2017, 2020). The F-actin and nonmuscle myosin-II binding domains are in the N-terminus of anillin, while the RhoA-GTP-

binding domain (RBD), C2 domain with binding sites for microtubules, phospholipids and Ect2, and PH domain with binding sites for phospholipids and septins are in the C-terminus (Piekny and Glotzer, 2008). We found that anillin contains a highly conserved NLS in the C2 domain that is required for importin-binding and for its localization and function in cytokinesis in HeLa cells (Beaudet et al., 2017, 2020). We also found that the RBD and C2 domain interact to impede anillin function. Binding to active RhoA relieves this autoinhibition, and binding to importin stabilizes the open conformation for phospholipid-binding (Beaudet et al., 2020). We propose that other cytokinesis proteins could be regulated by importin-binding. In early *C. elegans* embryos, we found that ANI-1, the anillin homologue, is regulated by the chromatin pathway in AB cells fated to become multiple tissues, but not in P1 cells fated to become germline (Ozugergin et al., 2021). Since Ect2 has several conserved NLS sequences in the protein (Figure 4), we propose that importin-binding could similarly regulate its cortical recruitment in the equatorial plane. This model is described in more detail in section 1.5.

1.4 Midbody formation and abscission

After ingression, the ring and central spindle compress and transition into the midbody (Green et al., 2012; Frémont and Echard, 2018). Proteins are either repartitioned between the ring and spindle or are removed or released. Proteins like the PRC1 and KIF4 complex, and centraspindlin remain at the midbody core, while others such as CPC will partition to either side of the midbody (Capalbo et al., 2012; Frémont and Echard, 2018; Henne et al., 2011; Mierzwa and Gerlich, 2014). Myosin, anillin and septins are shed from the ring, while Ect2 relocalizes to the nuclei of the daughter cells. Failure to shed ring components causes cytokinesis failure and abscission defects (El Amine et al., 2013). It is important to note that although proteins like anillin are shed from the

ring, the presence of these proteins are crucial for the ring to midbody transition (El Amine et al., 2013). Other proteins are recruited to the midbody and include Rab (Ras-associated binding) GTPases, FIP3 complexes, Cep55 (centrosomal protein of 55kDa), ESCRT-I/ALIX (ALG-2 interacting protein-X), ESCRT-III, and vacuolar protein sorting-associated protein 4 (VPS4) among others (Carlton et al., 2008; Fabbro et al., 2005; Morita et al., 2007; Schiel et al., 2012; Simon et al., 2008). The recruitment of endosomes, combined with the shedding of membrane and proteins and fission via ESCRTs are all required for successful abscission (König et al., 2017; El Amine et al., 2013; Kechad et al., 2012).

The midbody acts as a template for the abscission machinery to pinch off the constricted membrane on one side of the intercellular bridge (Henne et al., 2011; Hu et al., 2012). Cep55 interacts with the ESCRT-I component TSG101 (tumor susceptibility gene 101 protein) and ALIX at the midbody, and the interaction with ALIX and ESCRT-III is required for abscission (Carlton et al., 2008; Morita et al., 2007). TSG101 forms two rings adjacent to the core midbody via recruitment by Cep55, and ESCRT-III subsequently forms additional helical ring-like structures on one side (Carlton and Martin-Serrano, 2007; Morita et al., 2007). Remodeling at these sites is associated with secondary constriction and likely involves VPS4 (Mierzwa and Gerlich, 2014; Guizetti et al., 2011; Schiel et al., 2012). Secondary constriction also involves recycling endosomes, which could cause changes in curvature that help drive ESCRT-III polymerization into helical filaments. (Carlton et al., 2008; Elia et al., 2011; Fabbro et al., 2005; Henne et al., 2011; Morita et al., 2007). As ESCRT-III constricts the membrane on one side of the midbody, it is depolymerized by VPS4 (Elia et al., 2011). The membrane at that end undergoes fission to finalize the separation of the two daughter cells (Elia et al., 2011). This has been observed in

MDCK (Madin-Darby canine kidney) cells, where tubulin was observed being removed from one side of the midbody as the ring continues to constrict (Elia et al., 2011).

The recycling endosomes involved in abscission have Rab11-FIP3 complexes and dock at the intercellular bridge via interaction with the exocyst and Arf (ADP-ribosylation factor) GTPases (Fielding et al., 2005; Schiel et al., 2012; Simon et al., 2008). These Rab11-FIP3 endosomes are required for the transport of a number of key cargo proteins to the intercellular bridge including recruitment of the ESCRT machinery (Schiel et al., 2012). FIP3 also binds to Cyk4, which presumably occurs after Ect2 localizes to the forming nuclei of the daughter cells (Figure 6). There is a gain in the concentration of Cyk4-Rab11-FIP3 complexes as Cyk4-Ect2 complexes are lost during maturation of the midbody (Simon et al., 2008). Cyk4 contains competing sites for Ect2 and FIP3, and as Ect2 localizes to the nuclei, this putatively frees up binding sites for FIP3 (Simon et al., 2008). However, it is not clear if FIP3 outcompetes Ect2 from Cyk4-binding, which subsequently permits Ect2 to localize to the nuclei or if the nuclear localization of Ect2 is required to free up binding sites for FIP3 (e.g. Figure 6). In the case of the latter, preventing the nuclear localization of Ect2 could cause abscission delays or failure by preventing formation of the Cyk4-FIP3 complexes. In a previous study, cells overexpressing an N-terminal construct of Ect2 that lacked the NLS did not interfere with ingression, but caused abscission failure and the formation of binucleated cells (Chalamalasetty et al., 2006).

1.5 **Models for Ect2 regulation**

As described earlier, Ect2 is a critical regulator of cytokinesis and requires tight regulation. Ect2 is nuclear in interphase cells and becomes enriched in the cytosol after nuclear envelope breakdown in prometaphase. It has an NLS (includes residues 347-351) in the central S-loop,

which is adjacent to several putative Cdk1 phosphorylation sites (Niiya et al., 2006; Saito et al., 2004; Suzuki et al., 2015). While the NLS was assumed to mediate nuclear import, an early hypothesis derived from prior studies was that Cdk1 phosphorylation of the T342 site regulates the activity of Ect2 (Burkard et al., 2009; Chalamalasetty et al., 2006; Liot et al., 2011; Suzuki et al., 2015; Yüce et al., 2005). The model is that Ect2 is autoinhibited during metaphase because the phosphorylated residues bind to basic residues within the N-terminal BRCT domains, rendering the DH domain inaccessible to RhoA (Burkard et al., 2009; Kim et al., 2005). As Cdk1 activity decreases in anaphase, the decrease in phosphorylation at this site frees the BRCT domains for phosphor-Cyk4 binding, relieving autoinhibition (Burkard et al., 2009; Kim et al., 2005). In support of this model, Cyk4 only forms a complex with dephosphorylated Ect2 after anaphase onset (Yüce et al., 2005). Further, Cdk1 inhibition is sufficient to promote Cyk4-binding (Yüce et al., 2005). In addition, over-expression of the C-terminus of Ect2 causes ectopic, uncontrolled GEF activity with transforming capacity, while over-expression of the N-terminus of Ect2 causes a dominant negative phenotype with loss of ring assembly that is similar to loss-of-function phenotypes caused by RNAi (Chalamalasetty et al., 2006; Kim et al., 2005; Liot et al., 2011; Niiya et al., 2006). However, phosphorylation mutations did not appear to cause any obvious phenotypes, although no rescue assays were performed making this data hard to interpret (Hara et al., 2006; Niiya et al., 2006; Suzuki et al., 2015). Also, it was not known if the nuclear localization of Ect2 is required for cytokinesis, or if the NLS has any other role in regulating its function. Several recent studies suggested that importin-binding to the NLS is negatively regulated by phosphorylation (Suzuki et al., 2015). For example, a peptide containing the NLS with a phosphomimetic mutation was unable to bind to importins (Suzuki et al., 2015). Further, there are two Cdk1 sites close together which could be partially redundant and/or work in concert to control importin-binding

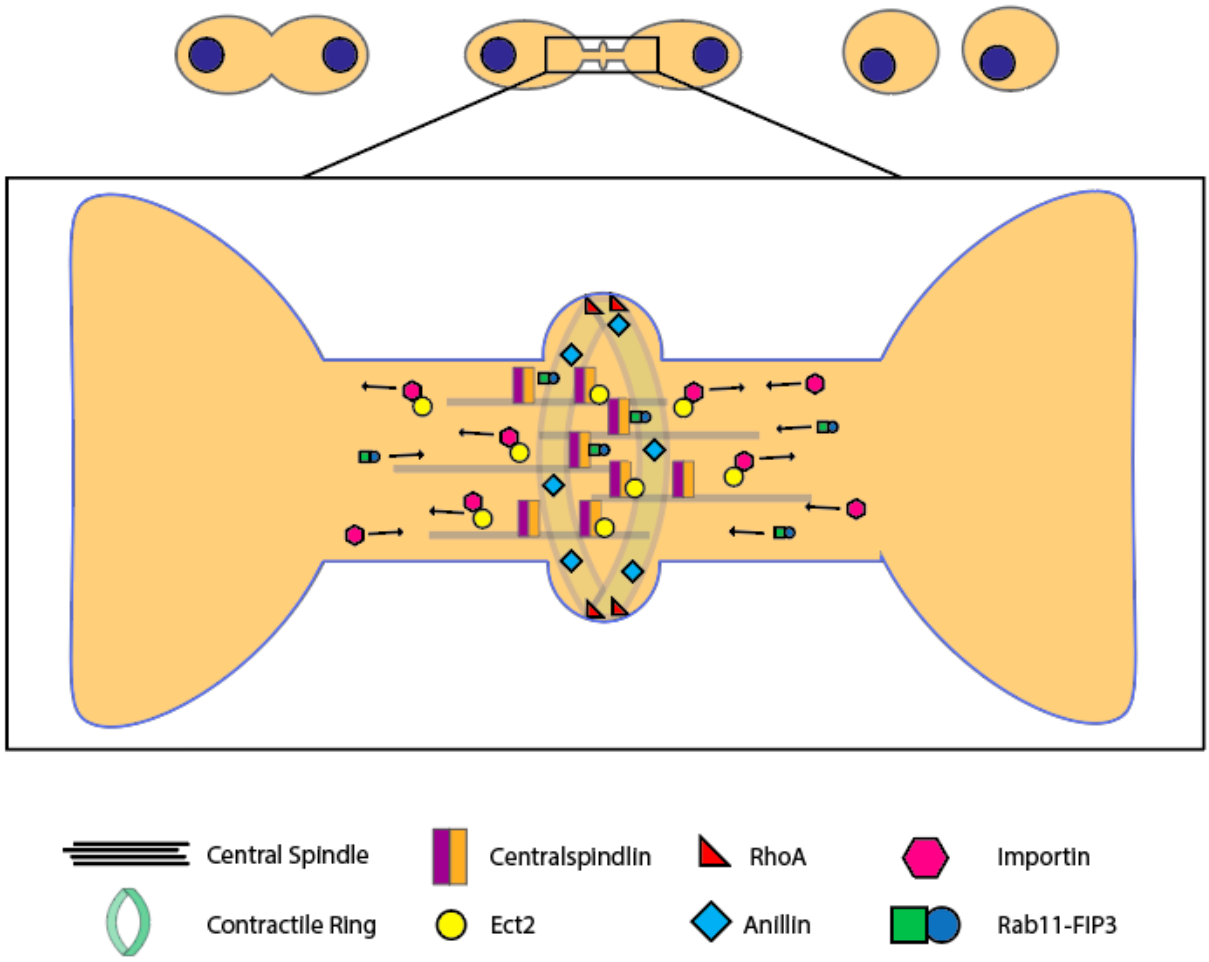


Figure 6 – Exchange of machinery at the intercellular bridge mediates abscission. The intercellular bridge is the site where the midbody matures and where abscission will occur. After ingression, the ring (RhoA in red, anillin in blue) and central spindle components transition to a midbody. Some ring components (e.g. anillin, septins) are shed, while additional machinery is recruited for secondary ingression and abscission. The centralspindlin complex (Cyk4 in orange, MKLP1 in purple) must transition from binding Ect2 (yellow) to binding Rab11-RIP3 (green and blue, respectively). Importin-binding (pink) transports Ect2 to the reassembling nuclei in the newly forming daughter cells.

(Suzuki et al., 2015). However, the physiological relevance of these Cdk1 sites and their effect on importin-binding were not tested in cells. Another study proposed that Cdk1 phosphorylation could block importin-binding to keep Ect2 in the cytosol prior to nuclear envelope breakdown, so that it could increase active RhoA to mediate cell rounding for mitosis (Matthews et al., 2012). However, they provided no functional data to support this model. Further, while they claimed that phosphorylated Ect2 could form a complex with Cyk4 prior to anaphase, this was not seen in other studies (Gómez-Cavazos et al., 2020; Kim et al., 2014; Schneid et al., 2021; Yüce et al., 2005).

We propose that importin-binding to the NLS in the S-loop could coordinate regulation of the N- and C-terminus of Ect2. One model is that importin-binding could help alleviate autoinhibition between the N- and C-terminus to facilitate Cyk4-binding, or to stabilize the open conformation after Cyk4-binding for its activity and/or localization. A gradient of importins free to bind to NLS proteins near the equatorial cortex could create an equatorial gradient of active Ect2. Cdk1 phosphorylation could control the importin-interaction and temporally couple Ect2 activation with anaphase, when active RhoA is needed in the equatorial plane to build a contractile ring. In metaphase, Cdk1 phosphorylation could block importin-binding to help Ect2 enrich in the cytosol, where it would remain inactive until anaphase when this phosphorylation is lost and importin- and Cyk4-binding is required to activate Ect2 in the equatorial plane (Su et al., 2011; Kotýnková et al., 2016). We predict that if importin-binding is required to stabilize an active Ect2 conformation, then mutations in the NLS that block importin-binding should cause a reduction in the cortical enrichment of Ect2 and a decrease in active RhoA. If Cyk4-binding is sufficient for Ect2 activation, then it may not prevent ring assembly per se, but could make cells sensitive to failure upon other perturbations. If Cdk1 phosphorylation competes with importin-binding, then

we predict a phosphor-mimetic mutant to phenocopy the NLS mutant, while the phosphor-deficient mutant might cause ectopic cortical recruitment.

Another potential role for importins may be to localize Ect2 to the newly formed daughter nuclei to either 1) free Cyk4 to form a complex with Rab11-FIP3, an endosome cycling complex required for abscission and/or 2) decrease active RhoA to decrease ring components. As described earlier, there is a correlation with the assembly of FIP3-Cyk4 complexes with the disassembly of Ect2-Cyk4 complexes, which may be required to drive the later stages of abscission (Simon et al., 2008). Thus, the failure to localize Ect2 to the nuclei could prevent the docking of recycling endosomes and the delivery of cargo required for abscission. Also, ring proteins need to be repartitioned or removed to allow for transition to later stages of cytokinesis and abscission, and failure to remove these components cause abscission failure (Fabbro et al., 2005; Chalamalasetty et al., 2006). Thus, if Ect2 persists at the midbody, it may continue to generate active RhoA which could lead to the continued generation of ring components. In HeLa cells where N-terminal Ect2 constructs lacking an NLS, or containing a mutated NLS were overexpressed, abscission phenotypes were observed (Chalamalasetty et al., 2006). However, over-expression studies are difficult to interpret and they did not do any loss-of-function or rescue studies to study the requirement of the NLS for the function of Ect2 in cytokinesis (Chalamalasetty et al., 2006). If Ect2 needs to be removed from the midbody for abscission, then we predict that NLS mutations that prevent its localization to the nuclei should cause abscission failure, either because ectopic RhoA causes an excess of ring components at the midbody, and/or because of a failure to form Cyk4- FIP3 complexes. To distinguish these, we could examine changes in the localization of ring and abscission components at the midbody.

1.6 Thesis summary

Ect2 is a highly conserved crucial regulator of contractile ring assembly in metazoans, yet it is not clear how active Ect2 is generated at the equatorial cortex, nor is it known if the S-loop NLS is required for the function of Ect2 in cytokinesis. It is also not clear how Cdk1 phosphorylation could affect the NLS during cytokinesis. To address these questions, I generated several mutant Ect2 constructs and performed rescue studies to determine if the NLS and Cdk1 phosphorylation are required for the function of Ect2. Since Ect2 fusion constructs have been notoriously difficult to express in cells, we designed a linker that would permit improved visualization of Ect2 localization. Next, we introduced mutations in the NLS in constructs that were RNAi-resistant to perform rescue assays. We found that mutating 3 or 5 residues in the NLS from basic to uncharged residues failed to rescue loss of endogenous Ect2 and caused cytokinesis failure, supporting an important role for the NLS in regulating the function of Ect2. Although preliminary, the majority of cells that failed cytokinesis displayed abscission phenotypes supporting a role for the NLS in mediating the localization of Ect2 to the reforming nuclei. Interestingly, mutating both Cdk1 phosphorylation sites in the S-loop caused no obvious cytokinesis phenotypes suggesting that the model for the autoinhibition of Ect2 needs to be revised.

Chapter 2. Materials & Methods

2.1 Constructs

The Myc::Ect2 constructs (full-length and N-terminal; 1-421) were previously generated (Yüce et al., 2005). The mCherry:Ect2 construct was generated by cloning Ect2 cDNA from pCMV-(myc)3:Ect2 and mCherry cDNA into the eGFP-C1 vector (where the GFP cDNA had been removed) with a EAAAKx3 linker through HiFi assembly (courtesy of Nhat Pham). The following mutant Ect2 constructs were generated in Myc or mCherry::Ect2 full-length or N-terminus by PCR site-directed mutagenesis: NLS 5A (347-RKRRR-351 → 347-AAAAA-351), NLS 3A (347-RKRRR-351 → 347-RAAAR-351), phosphodeficient TASA (T342A/S345A), and phosphomimetic TDSD (T342D/S345D) (Suzuki et al., 2015). All constructs were verified by sequencing before experimental use.

2.2 Cell culture, transfection and immunostaining

A HeLa cell line where endogenous Ect2 is tagged with mNeonGreen (mNeonGreen::Ect2; mNG::Ect2) was generated using CRISPR-Cas9 (courtesy of Mathieu Husser). Unmodified HeLa and mNG::Ect2 HeLa cells were cultured in Dubelco's Modified Eagles's Medium (DMEM; Wisent) containing phenol red, sodium pyruvate, and L-glutamine supplemented with 10% cosmic calf serum (CCS; Hyclone) and 2 mM L-glutamine (Wisent). For fixed-cell imaging, HeLa cells were seeded to ~50% confluency on square glass coverslips etched using 0.1 M HCl. The cells were transfected using Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions with 2.5 nM Ect2 siRNAs and/or 2 µg of an RNAi$^{resistant(r)}$Myc::Ect2 construct (Yüce et al., 2005). After 30 hours, the cells were fixed using fresh, ice-cold 10% w/v trichloroacetic acid for 14 minutes before being washed three times with PBST (1 X PBS with 0.5% Triton X-100) as

previously described (Yüce et al., 2005). Cells transfected with Myc::Ect2 constructs were immunostained for Myc using primary anti-Myc monoclonal antibodies (1:250; DSHB) and anti-mouse Alexa-568 (Invitrogen; 1:500), anillin using primary anti-anillin rabbit polyclonal antibodies (1:250; Piekny and Glotzer, 2008) and secondary anti-rabbit Alexa-488 antibodies Invitrogen; 1:400), and DAPI for DNA visualization (1/1000 dilution from 1mg/mL stock; Sigma). Cells were blocked the following day with normal donkey serum (NDS; 5% in PBST) for 20 minutes before being incubated with the primary and secondary antibodies for 2 hours each, washing with PBST between antibodies, and incubating with DAPI for 5 minutes. Coverslips were mounted onto glass slides with mounting media (4% n-propyl gallate in 50% glycerol diluted in 50 mM Tris pH 9 and water) and sealed with nail polish to prevent drying.

For live-cell imaging, mNG:Ect2 HeLa cells were seeded onto 25 mm circular glass (No. 1.5) coverslips. Transfection was performed as described above using siRNAs and the mCherry::Ect2 constructs. After 24 hours, cells were treated with 0.81 nM of Hoechst 33342 (Thermo Fisher Scientific) to visualize DNA. We also imaged cells using ibidi dishes. Cells were seeded to a confluency between 50-60% in 4-well chamber slides (ibidi) with 500 µL of media per well. Cells were similarly treated with Hoechst 33342 as above after 24 hours before being imaged for 12 hours to ensure that phenotypes were captured as cells started failing cytokinesis ~ the 30-hour mark.

2.3 Microscopy

Fixed cells transfected with Myc::Ect2 and co-stained for anillin and DNA (DAPI) were imaged using the Leica DMI6000B inverted epifluorescent microscope equipped with Hamamatsu OrcaR2 camera and piezo Z stage (MadCityLabs) using the 63X immersion oil objective. Images

were collected using differential interference contrast, and the appropriate filters. Z-planes were captured every 0.3 μm to generate a Z-stack using Volocity software (PerkinElmer). The files were saved and exported as TIFF files and were opened and analyzed using ImageJ (NIH).

For live imaging, mNeonGreen-tagged Ect2 HeLa cells co-transfected with siRNAs and rescuing mCherry::Ect2 plated on round glass coverslips were placed in a magnetic chamber (Quorum) with 1 mL fresh media. Cells were imaged using the Nikon Eclipse Ti microscope with a Livescan Sweptfield confocal unit. Cell were treated with 0.81 nM of Hoechst 33342 to visualize DNA and was captured using a 405 nm laser. Endogenous Ect2 was detected via its mNeonGreen tag using a 488 nm laser and mCherry::Ect2 was detected using a 561 nm laser. Cells were kept at 37˚C with 5% CO_2 using an INU-TiZ-Fi chamber (MadCityLabs). Images were captured using NIS-Elements (Nikon) software with 1 μm sections using a piezo Z stage, a 60X objective, NA of 1.4 and an EMCCD iZON897 camera (Andor). Images were taken every 6 minutes for 12 hours beginning ~24 hours post-transfection.

2.4 Analysis

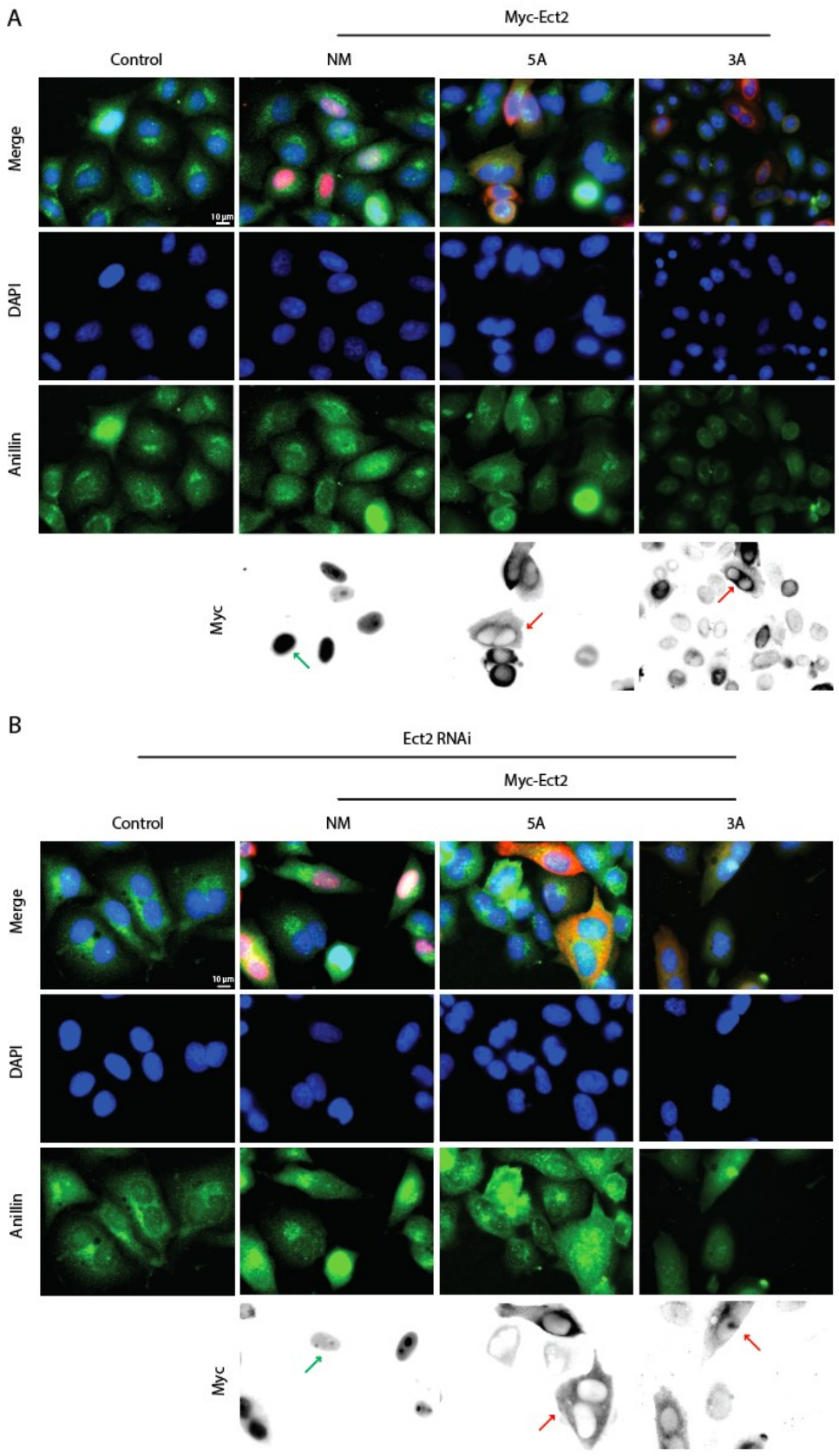
Images were analyzed using ImageJ. The observed signal intensities were used to determine threshold expression levels of endogenous Ect2 (<500) and transgenic Ect2 (between 500-2000) were used to determine that a cell had been adequately knocked down for endogenous Ect2, and had sufficient expression of exogenous Ect2, respectively. These cells were classified as mononucleate (single nucleus) vs. binucleate (two nuclei). The data was statistically analyzed using a One-Way ANOVA with Tukey Post Hoc using Prism 9 (GraphPad) for population counts.

Chapter 3. Results

3.1 The NLS is required for Ect2 function during cytokinesis

We found that the Ran pathway controls the cortical localization and function of anillin in cytokinesis via its NLS (Beaudet et al., 2017, 2020), and determined if the Ran pathway similarly regulates Ect2. To test this, we generated two different NLS mutant constructs where five vs. three basic residues (Arginine and Lysine) were changed to Alanine (5A and 3A, respectively). We chose to make both because this region is close to a Cdk1 phosphorylation site, and we wanted to avoid potentially disrupting this function. Further, we chose this location because it had been previously shown to bind to importins, although has never been studied in the context of full-length Ect2 in cells (Suzuki et al., 2015). The 5A and 3A NLS mutant Myc-tagged Ect2 constructs localized to the cytosol in interphase cells, unlike the non-mutant, which is nuclear (Figure 7A). Thus, the site is indeed required for nuclear localization in cells.

Next, we determined if the NLS mutants could 'rescue' cytokinesis defects caused by loss of Ect2. To do this, I performed rescue assays whereby endogenous Ect2 was knocked down using siRNAs and Myc-tagged RNAi-resistant Ect2 constructs were co-expressed, and the number of binucleate (two nuclei) vs. mononucleate (one nucleus) cells were counted as a measure of cytokinesis defects. Knocking down endogenous Ect2 causes an increase in the proportion of binucleate cells (Yuce et al., 2005). Indeed, in my experiment, 60.8% of Ect2-depleted cells were binucleate compared to the untreated control, where the population exhibited less than 1% binucleates (Figure 7C). Of the cells expressing the 3A or 5A NLS mutant constructs, 28.2 and 19.4% were binucleate, respectively, suggesting that cells are sensitive to overexpression and have the potential to cause dominant negative effects (Figure 7C). Of the cells co-expressing Ect2 siRNAs and RNAi-resistant full-length Myc-tagged Ect2, 15.7% were binucleate, while those


A
Myc-Ect2
Control
NM
5A
3A
Merge
DAPI
Anillin
Myc
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B
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Myc-Ect2
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Anillin
Myc
10 μm

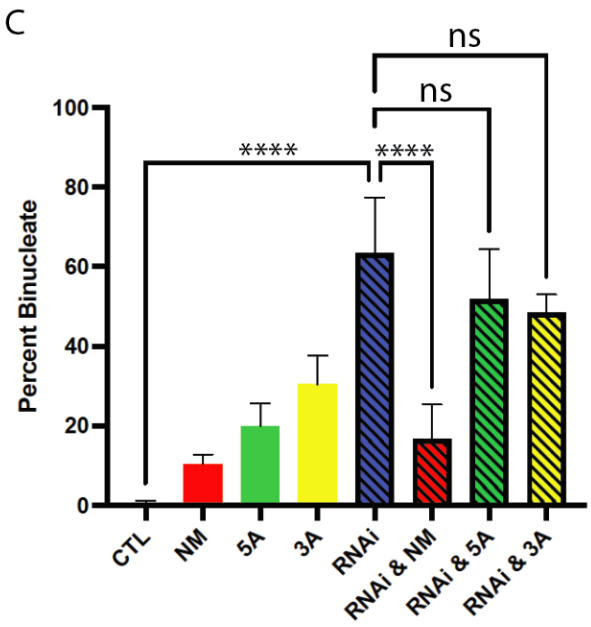


Figure 7 – The NLS in Ect2 is required for cytokinesis. A) Images show fixed HeLa cells expressing Myc-tagged non-mutant Ect2 (NM), NLS mutant 5A, and 3A (red) during interphase compared to control cells. The green arrow indicates nuclear localization of Myc-Ect2 while the red arrows indicate cytosolic localization. (**B**) Images show fixed HeLa cells expressing Myc-tagged non-mutant Ect2 (NM), NLS mutant 5A, and 3A (red) during interphase compared to control cells after Ect2 RNAi. The green arrow indicates nuclear localization of Myc-Ect2 while the red arrows indicate cytosolic localization. **C)** A bar graph shows the percent of binucleate cells, indicating failed cytokinesis for the different conditions as shown (control, blue; non-mutant, red; 5A NLS mutant, green; and 3A NLS mutant, yellow, where the lines indicate Ect2 RNAi). Statistical significance is indicated using asterisks as follows: $p > 0.05$ = ns (non-significant), $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

co-expressing the 3A or 5A mutant were 48.4 and 53.2% binucleate, respectively (Figure 7C). There was a significant difference between Ect2 RNAi rescued by the non-mutant Myc-Ect2 compared to 5A or 3A NLS mutants. This data suggests that the NLS site is at least partially required for cytokinesis. Since the results obtained from the rescue assay were not significantly different between the 5A and 3A NLS mutants, future experiments were conducted using the 3A mutant.

3.2 Generating new tools for Ect2 visualization and quantification

While our preliminary data was encouraging and supported a role for the NLS in regulating the function of Ect2 for cytokinesis, we needed to improve our methods. First, we wanted to quantify the knock down of endogenous Ect2, and second, we wanted to visualize 'rescuing' Ect2 in live cells, which would permit us to monitor the requirement of Ect2 during cytokinesis. Cells are sensitive to Ect2 over-expression, and prior constructs using fluorophores failed to express sufficiently in cells. We generated the first HeLa cell line were endogenous Ect2 was tagged with a fluorophore using CRISPR-Cas9 (experiments done by Mathieu Husser and Nhat Pham; Figure 8A). This gave us a tool to monitor endogenous levels after RNAi, and to only consider cells where levels have dropped below a defined threshold. Secondly, we generated mCherry-tagged Ect2 constructs containing an extended linker, which showed improved expression in cells (with Nhat Pham; Figure 8B). Using the newly generated mNeonGreen::Ect2 cell line, I developed a protocol to successfully visualize endogenous Ect2 using the sweptfield confocal microscope outfitted with an EMCCD camera in the CMCI. I ensured that I could consistently measure ~2,000 levels and compared this with when endogenous Ect2 was knocked down with siRNAs. I found that in most

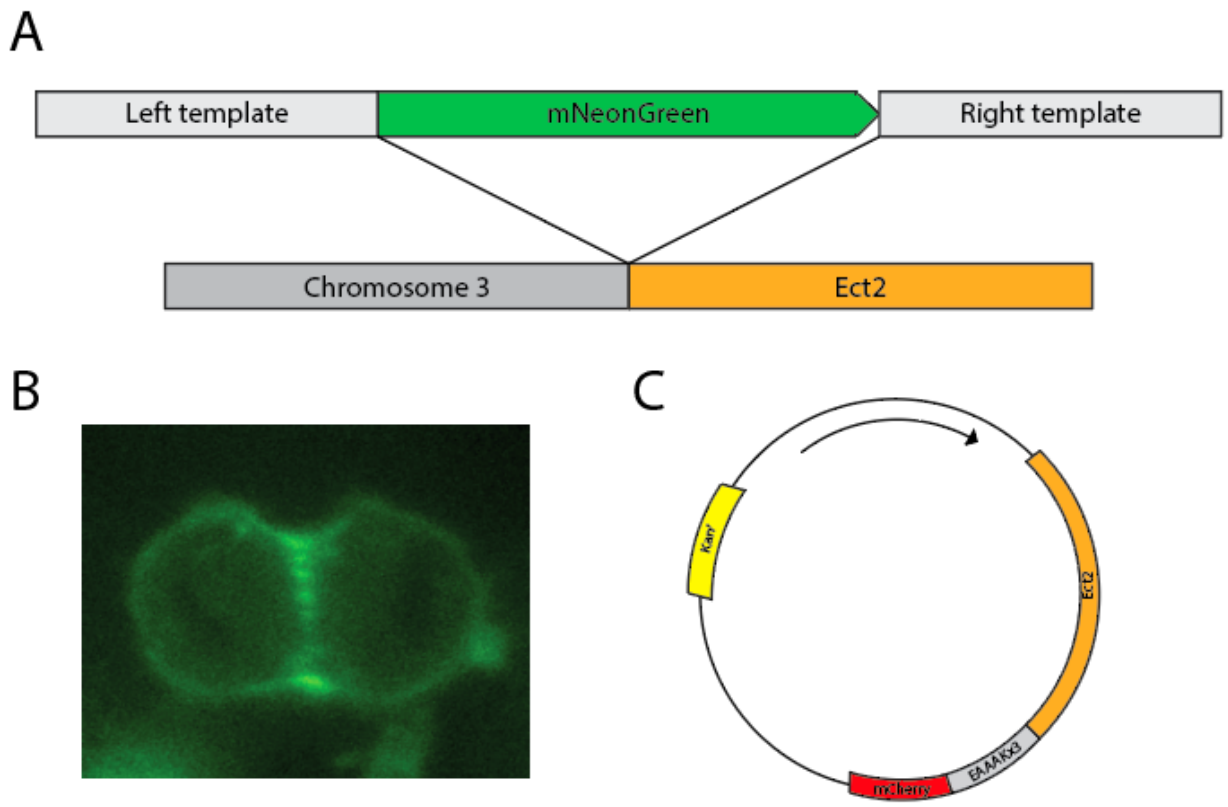


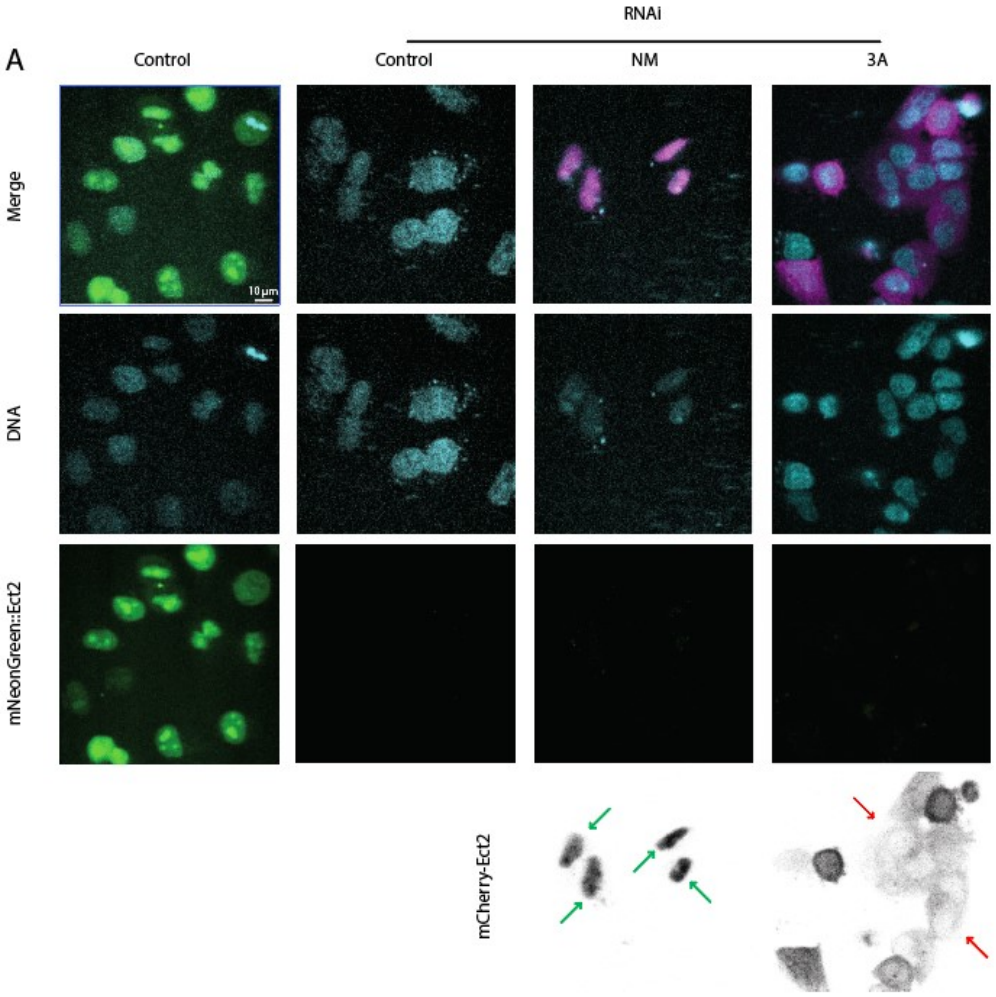
Figure 8 – Visualization of endogenous and exogenous Ect2 in cells. A) A schematic shows the insertion of the mNeonGreen fluorophore into the Ect2 locus at chromosome 3 in HeLa cells; the generated cell line has been tagged homozygously (courtesy of Mathieu Husser & Nhat Pham). **B)** Image of a mNeonGreen::Ect2 HeLa cell undergoing cytokinesis (courtesy of Mathieu Husser & Nhat Pham). **C)** A schematic shows the vector containing the mCherry-tagged Ect2 with its linker (courtesy of Nhat Pham).

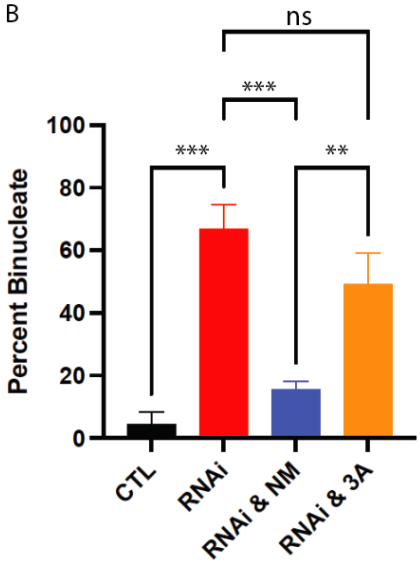
fields of view, binucleate cells had <500 levels remaining of endogenous Ect2 after knockdown demonstrating that the siRNAs and timescale for our rescue experiments was appropriate.

Next, I developed a protocol to visualize transgenic mCherry-tagged Ect2. As previously mentioned, no group had successfully reported visualization of exogenous Ect2 fused to a fluorophore without using maximum laser power, and even then, the levels were too low to quantify even with an EMCCD. We were able to establish an imaging protocol using more traditional laser power (e.g. 20-30%) and with exposure times that were more in line with what we do for other fluorophore tagged proteins (e.g. 50-100 ms). With these settings, we can be sure that phenotypes will not arise due to artefacts from harsh imaging conditions.

3.3 Using new tools, the NLS of Ect2 is required for cytokinesis

I repeated the experiments described in 3.1 using our new cell line with endogenously tagged Ect2, using co-transfection of siRNAs with rescuing (RNAi resistant) mCherry-tagged Ect2 to verify that the NLS is required for cytokinesis. In our previous study, the knockdown could have been less efficient in some of the cells, or the rescuing constructs could have been expressed at levels that were too low or too high, which could have impacted our findings. Imaging live cells revealed that the mCherry-tagged Ect2 3A NLS mutant was cytosolic, similar to the Myc-tagged construct in 3.1 (Figure 9A). While 4.4% of control cells were binucleate, I found that 67.0% were binucleate after Ect2 knockdown to <500 levels (vs. ~2000 for control), while 15.2% were binucleate after co-expression of RNAi-resistant mCherry-tagged non-mutant Ect2, where levels were between 300 and 2000 (Figure 9B). Using similar settings, I found that 49.5% were binucleate after co-expression of RNAi-resistant mCherry-tagged 3A Ect2 (Figure 9B). Therefore, using our new methods revealed that the NLS is indeed required (at least partially) for successful cytokinesis.


A
RNAi
Control
Control
NM
3A
Merge
DNA
mNeonGreen::Ect2
mCherry-Ect2
10 µm


B
ns
***
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Percent Binucleate
100
80
60
40
20
0
CTL
RNAi
RNAi & NM
RNAi & 3A

Figure 9 – The NLS is required for the function of Ect2 in cytokinesis. A) Images show live HeLa cells with endogenous mNeonGreen::Ect2 (green) before and after Ect2 RNAi, and co-expressing mCherry-tagged non-mutant (NM) or the 3A NLS mutant Ect2 (magenta), with DNA in cyan. **B)** A bar graph shows the percentage of binucleate cells indicating cytokinesis failure for the conditions as indicated with control cells in black, RNAi in red, mCherry-tagged non-mutant Ect2 in blue and mCherry-tagged 3A NLS mutant Ect2 in orange. The *p* values were obtained using a one-way ANOVA with Tukey Post Hoc test. Statistical significance in the graph is indicated via asterisks as follows: $p > 0.05$ = ns (non-significant), $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

These data are in line with our previous fixed-cell data, but are more reliable in that each cell has more carefully defined thresholds for knockdown and rescue/overexpression.

3.4 The NLS is required for abscission

Next, I wanted to determine how mutating the NLS of Ect2 causes cells to fail cytokinesis. As discussed in the introduction, the NLS could be required earlier during anaphase to modulate the cortical localization of Ect2, by either facilitating Cyk4-binding or stabilizing the open conformation for lipid binding. Alternatively, the NLS could be required during later stages of cytokinesis for Ect2 to localize to the reforming daughter nuclei and to permit the transition of midbody complexes to those required for abscission. The former would cause failed ring assembly or ingression phenotypes, while the latter would cause abscission phenotypes.

Cells were transfected as in 3.3, and imaged with 6-minute intervals over a 12-hour period starting 24 hours after transfection to capture phenotypes. We previously observed the majority of Ect2 RNAi phenotypes occurring ~30 hours after transfection (data not shown). Control cells, where cells were not treated or were depleted of endogenous Ect2 and rescued with mCherry-tagged non mutant Ect2 successfully completed cytokinesis with no obvious phenotypes (n=22 and 2, respectively; Figure 10A and B). However, cells co-expressing the NLS 3A mutant showed two different cytokinesis phenotypes (Figure 10B). One set of cells had persistent Ect2 localization at the midbody and abscission failure (7/10 cells), while in the other set of cells, the furrow partially ingressed, then oscillated followed by regression (3/10 cells; Figure 10B). In all cells, the NLS 3A mutant did not localize to the nuclei as expected. Although more control cells need to be filmed, the phenotypes observed with the NLS 3A mutant are consistent with both early and late requirements for importin-binding in regulating Ect2 function for cytokinesis.

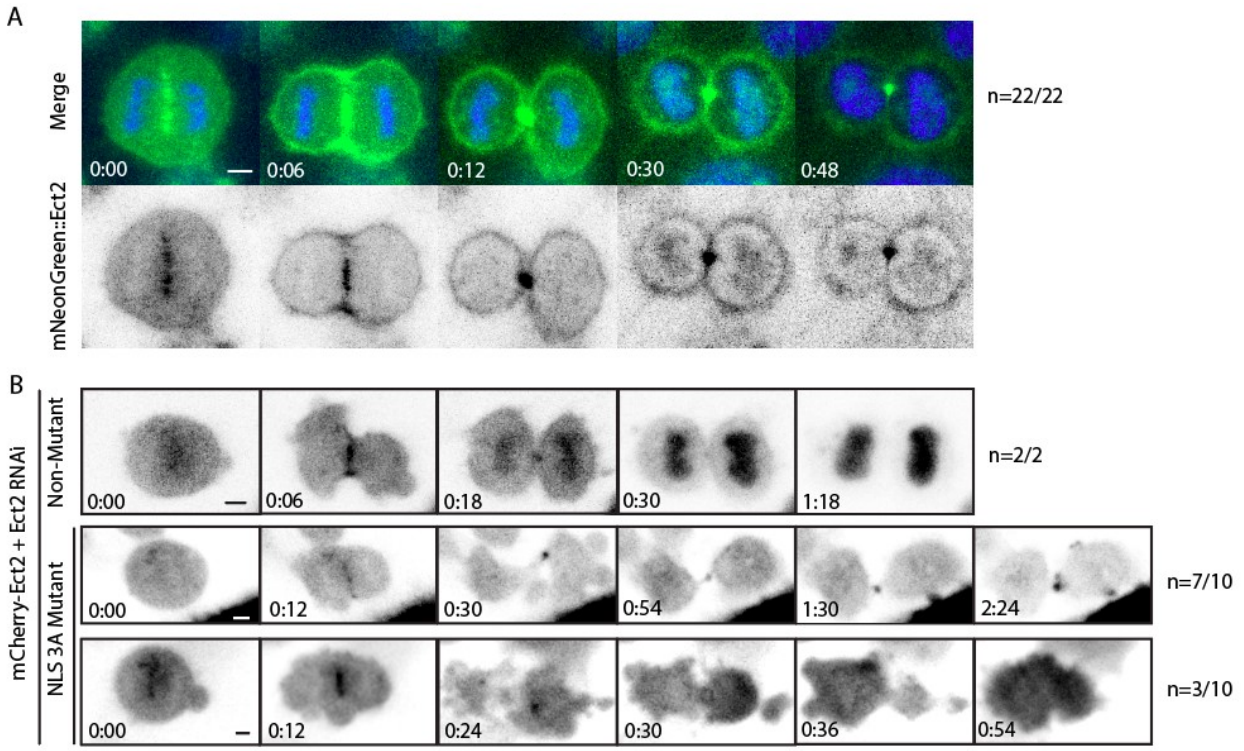
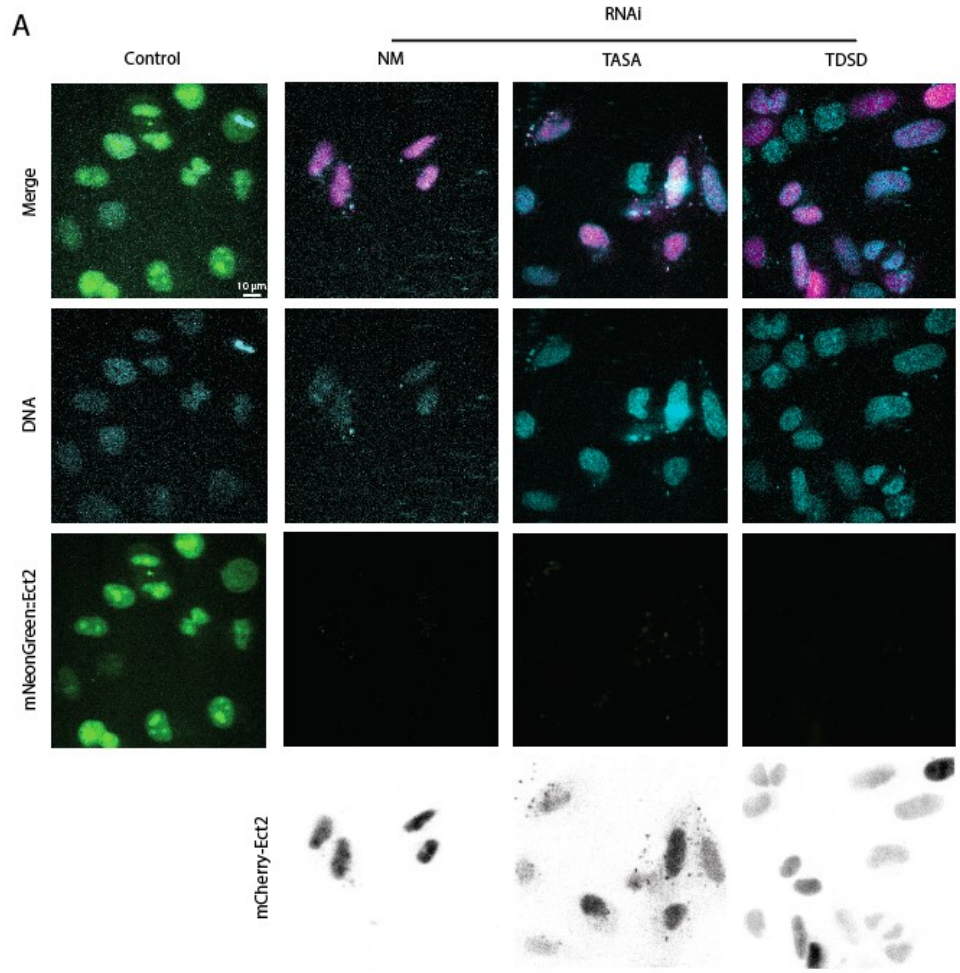


Figure 10 – The NLS of Ect2 is required for ring positioning and abscission. A) Images show time-lapse movies of HeLa cells where endogenous Ect2 is tagged with mNeonGreen (green) and chromatin is stained with Hoechst (blue). B) Images show time-lapse movies of HeLa cells depleted of endogenous Ect2 by RNAi and expressing RNAi-resistant mCherry-tagged non-mutant Ect2 (top panel) or NLS 3A mutant (middle and bottom panels). While the cell in the middle expressing the NLS 3A mutant failed to complete abscission with persistent Ect2 localization at the midbody, the furrow in the bottom cell oscillated, followed by regression. The times are shown in hours and minutes. The scale bar is 5 μm. The numbers to the right indicate the proportion of cells that showed that phenotype.

3.5 Cdk1 phosphorylation of the S-loop is not required for Ect2 function during cytokinesis

Previous studies have shown that there are two Cdk1 regulation sites in the S-loop NLS at T342 and S345, which could impact importin-binding (Suzuki et al., 2015; Yüce et al., 2005). Two mCherry-Ect2 mutants were designed to study the requirement for Cdk1 phosphorylation, a phosphodeficient mutant (TASA) where Threonine-342 and Serine-345 were mutated to Alanine and a phosphomimetic mutant (TDSD) where the same two residues were mutated to the negatively charged Aspartic Acid to mimic the charge of phosphorylation. These mutants were then used to conduct experiments as in 3.3 and 3.4.

For the rescue assay, mNeonGreen::Ect2 HeLa cells were co-transfected with Ect2 siRNAs and either the TASA or TDSD phosphomutant, and live imaging was performed using the sweptfield confocal microscope. As indicated in 3.3, while 4.4% of control cells were binucleate, 67.0% were binucleate after Ect2 knockdown to <500 levels (vs. ~2000 for control), while 15.2% were binucleate after co-expression of RNAi-resistant mCherry-tagged non-mutant Ect2, where levels were between 300 and 2000. Of the cells co-transfected with Ect2 SiRNAs and mCherry-tagged TASA or TDSD phosphomutants, 21.7 and 23.7% were binucleate, respectively (Figure 11). These were not significantly different compared to those expressing the mCherry-tagged non-mutant Ect2, suggesting that neither of these phosphorylation sites are required for cytokinesis. In further support of this, cells co-expressing Ect2 siRNA and the phosphomutants were imaged as in 3.4. All cells co-expressing the TASA phosphomutant succeeded cytokinesis (n=7), and the majority of cells co-expressing the TDSD phosphomutant succeeded cytokinesis (n=7/8). Therefore, Cdk1 phosphorylation in the S-loop does not appear to be required for cytokinesis per se, although further experiments are needed to determine if there are more subtle changes in ring assembly or abscission.


A
RNAi
Control
NM
TASA
TDSD
Merge
DNA
mNeonGreen=Ect2
mCherry-Ect2
10 µm

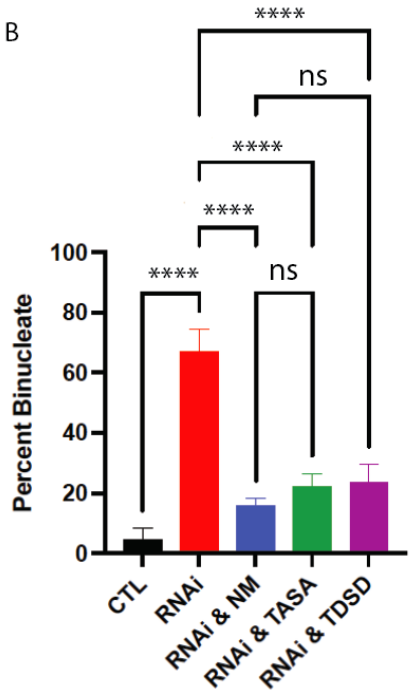

B
****
ns
****
****
****
ns
Percent Binucleate
100
80
60
40
20
0
CTL
RNAi
RNAi & NM
RNAi & TASA
RNAi & TDSD

Figure 11 – Cdk1 phosphorylation sites T342 & S345 are not required for the function of Ect2 in cytokinesis. A) Images show live HeLa cells with endogenous mNeonGreen::Ect2 (green) before and after Ect2 RNAi, and co-expressing mCherry-tagged non-mutant Ect2 (NM) or the phosphodeficient (TASA) or the phosphomimetic mutant (TDSD; red), with DNA in blue. **B)** A bar graph shows the percentage of binucleate cells indicating cytokinesis failure for the conditions as indicated with control cells in black, RNAi in red, mCherry-tagged non-mutant Ect2 in blue and mCherry-tagged TASA mutant Ect2 in green, and TDSD mutant Ect2 in purple. The *p* values shown were obtained using a one-way ANOVA with a Tukey Post Hoc test. The statistical significance is indicated as follows: $p > 0.05$ = ns (non significant), $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

Chapter 4. Discussion

We showed for the first time that the NLS in the S-loop of Ect2 is required for cytokinesis. We hypothesized that importin-binding could regulate Ect2 during earlier stages of cytokinesis for ring assembly, or to localize Ect2 to the newly forming daughter nuclei for abscission. We observed phenotypes consistent with both functions, which needs to be further characterized to determine if they reflect differences in Ect2 localization and/or different levels of knockdown. Interestingly, despite models persisting for years that the Cdk1 phosphorylation of Ect2 is required for its autoinhibition and regulation of function for cytokinesis (Mishima et al., 2004; Kurasawa et al., 2004; Hu et al., 2011; Suzuki et al., 2015), we observed no obvious cytokinesis phenotypes when mutating these sites to be phosphodeficient or phosphomimetic. Although preliminary, there could be very mild phenotypes that require further quantification. Regardless, the models need to be revised to account for other modes of increasing the accessibility of Cyk4 or affinity for Ect2 during anaphase.

Our findings demonstrate an important requirement for the nuclear localization of Ect2 for abscission. As described in the introduction, the midbody undergoes changes in its composition over time including the transition of Cyk4-Ect2 complexes to Cyk4-Fip3-Rab11 complexes. Based on the cytokinesis phenotypes observed with our NLS 3A mutant, it is possible that the persistence of Ect2 at the midbody could prevent the transition of complexes at the midbody required for cytokinesis (Simon et al., 2008). Without this transition and recruitment of new machinery, cytokinesis stalls and ultimately fails, which can be seen by the regression of the membrane at the furrow (Figure 10C). To provide evidence for this hypothesis requires localization studies of the midbody components required for later steps of abscission. For example, a future experiment will be to determine if Fip3-Rab11 cannot stably localize at the midbody in cells expressing the NLS

3A mutant. Furthermore, Ect2 could continually generate active RhoA at the midbody, which could prevent the loss of contractile ring components that is typically required for the ring-midbody transition.

Our data also suggests that the Ran pathway could regulate Ect2 during earlier stages of cytokinesis for ring assembly and/or positioning. Importin-binding regulates the recruitment of anillin to the equatorial cortex during anaphase, and is partially redundant with subsequent spindle-dependent pathways (Beaudet et al., 2017, 2020). We hypothesized that importin-binding could mediate an open conformation that has greater accessibility to Cyk4- or lipid-binding to generate active RhoA. The localization of the NLS 3A mutant appears to be less enriched at the equatorial cortex compared to non-mutant Ect2, but this observation is preliminary and requires quantification. The lower levels of Ect2 at the equatorial cortex could lead to lower levels of active RhoA and delayed or improper ring assembly. To determine this, we need to accurately measure the localization of ring components and determine if the generation of active RhoA is delayed using a biosensor. It is also possible that in HeLa cells, importin-binding is partially redundant with other modes of regulation and could cause more severe phenotypes in combination with perturbing other pathways. To test this model, we could also perturb the astral and/or central spindle pathway in combination with the NLS 3A mutant to determine if we see only earlier cytokinesis defects, instead of a mix of early and late phenotypes.

We also found that Cdk1 phosphorylation in the S-loop does not appear to strongly regulate the function of Ect2 for cytokinesis. Prior studies showed that mutating one of these sites caused a change in the transformation ability of Ect2, leading to a model where Cdk1 phosphorylation controls the autoinhibition of Ect2 (Niiya et al., 2005, 2006; Hara et al., 2006). However, subsequent cell-based assays failed to show a requirement for Cdk1 phosphorylation in the

regulation of Ect2, although these studies only mutated one site (Petronczki et al., 2007; Su et al., 2011; Matthews et al., 2012). More recently, Suzuki et al. (2015) showed that there are two Cdk1 sites that are close together and redundant. We mutated both sites to either be phosphodeficient or phosphomimetic and found that neither caused an obvious cytokinesis phenotype. However, we still need to adequately characterize cytokinesis in cells expressing the phosphorylation mutants, as there could be more subtle phenotypes such as delays in ring assembly or abscission. This suggests that either there are additional sites, or that Cdk1 regulation plays a less crucial role than what was originally hypothesized. Further, Cdk1 phosphorylation could function redundantly with other mechanisms to control Ect2 inhibition, and perturbing these other mechanisms could cause stronger defects. Further, Suzuki et al. (2015) proposed that Cdk1 phosphorylation prevents importin-binding, but we did not observe any nuclear localization defects with the phosphomimetic mutant, suggesting that this model needs to be revised.

While our new methods for studying Ect2 function is much more ideal and sophisticated compared to prior systems, there are still drawbacks. This includes the variability of mNeonGreen::Ect2 knockdown and mCherry:Ect2 over-expression in cells since siRNAs and the cDNA for the mCherry constructs are transfected as separate entities. Our lab recently designed a bicistronic vector that can co-express the RNAi-resistant tagged-Ect2 construct and a shRNA. This system would limit the variability in the levels of endogenous Ect2 knockdown and Ect2 construct expression since they would be co-expressed in the same cells. Moving forward, we will use this system for additional rescue experiments.

The work presented in this thesis, along with previous and current research conducted in our lab, provides new knowledge of Ect2 function for cytokinesis. Shining a light on the role of Ect2 in cytokinesis has revealed that it is not just involved in activating RhoA at the cortex for

assembly of the contractile ring, but also that its removal is required for abscission. Additionally, this work has demonstrated that the role of the Ran pathway in cytokinesis is not limited to positioning the ring for cytokinesis, but also to ensure its completion by controlling the redistribution of midbody components to the newly forming daughter nuclei. Future work aims to further define the model by which importin-binding regulates the function of Ect2 during cytokinesis and how/if Cdk1 phosphorylation plays any role in this model, and to uncover novel regulators of the pathway.

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