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Studying the Central Spindle-Associated Rho Guanine Nucleotide Exchange Factor ECT2 during Meiotic Cytokinesis

By

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Student Number 4402989

Thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of Master of Science

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Abstract

Polar body formation has been of great interest both as a fundamental process in sexual reproduction and as an extreme example of asymmetric cell division (cytokinesis) in cell biology. The guanine nucleotide exchange factor ECT2 regulates cytokinesis in all metazoans analyzed to date. Although it has been known that ECT2 is required for the localization of active RhoA to the equatorial cortex, its role in vertebrate polar body emission is not explored yet. It is believed that ECT2 is the GEF for RhoA during mitotic cytokinesis. However, there is no direct evidence supporting co-localization of ECT2 with Rho contractile ring, at least during the early stage of cytokinesis. To investigate this hypothesis, we employed fluorescence confocal microscopy to reveal for the first time that ECT2 never overlaps with the active RhoA zone in the oocytes during polar body emission. These results suggest that, although ECT2 function is required for Rho contractile ring formation and constriction, ECT2 is unlikely the direct activator of RhoA during polar body emission. More importantly, our time-lapse imaging experiments with ECT2 and active Rho reveals an exciting mechanism by which Cdc42, a small Rho GTPase, regulates polar body emission: during Rho contractile ring constriction, Cdc42 promotes membrane protrusion to "pull" one spindle pole and the associated polar body chromosomes into the forming polar body enclosure.

Acknowledgement

I would like to start by thanking Allah (God) for all his blessings and for making all things possible.

I would like to acknowledge and extend my heartfelt gratitude to those who have made the completion of this thesis possible.

First and foremost, my professor and supervisor, Dr. Johne Liu, for his guidance, patience, leadership and many valuable lessons.

Dr. Chunqi ma, for all her help, understanding, and assistance.

All members of Dr. Liu's lab, for their assistance and friendship. And Shadia for her relentless patience and love.

My advisory committee members (Dr. Adam Rudner and Dr. Johnny Ngsee) for their valuable comments and counsel throughout my research.

A very special gratitude to my parents, especially my dad, who had trust in me, and made me believe in myself, and pushed me to continuing this journey; I hope you are proud Baba.

Last but not least, thank you Yamen, my husband, for putting up with me, giving me the strength to go on, reading my thesis, and helping me at every step of the way with your love, understanding and support.

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List of Abbreviation

fiM	Micromolar
APC	Anaphase Promoting Complex
BRCT	Breast cancer 1 tandem repeat
CDC42	Cell Division Cycle 42
CDK	Cyclin Dependent Kinase
Cdkl	Cyclin dependent kinase 1
CSF	Cytostatic Factor
DH	Dbl homology
ECT2	Epithelial Cell Transforming sequence
F-actin	Filamentous actin
GAPs	GTPase activating proteins
GDIs	guanine nucleotide-dissociation inhibitors
GEFs	guanine nucleotide-exchange factors
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTPase	Guanosine triphosphatase
GV	Germinal vesicle
GVBD	Germinal Vesicle Breakdown
IU	International units
M	Molar
MAPK	Mitogen-activated protein kinase

MBS	Myosin-binding subunit
MI	Meiosis one
Mil	Meiosis two
mM	Millimolar
MPF	M phase promoting factor
PH	Pleckstrin homology
Plkl	polo-like kinase **1**
PMSG	Pregnant Mare Serum Gonadotropin
RFP	Red fluorescent protein
Rho	Ras homologous
rMlc	Regulatory Myosin light chain
ROK	Rho-dependent kinase
siRNA	Small interfering RNA
WASP	Wiskott-Aldrich syndrome protein
XECT2	Xenopus ECT2

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1. Introduction

1.1. Meiosis in *Xenopus laevis* Oocytes:

The study of cell division has always been a great interest in the field of biology. There are numerous different reasons that attract biologists to this field. Recently stem cells have been a major area of research due to their ability to proliferate and differentiate into any cell line. Also attracting biologists to study cell division is due to cancer, which results from excessive and uncontrolled cell division. In addition, development of all organisms requires proliferation and cell division.

One important model system for the study of cell division and cell cycle regulations are oocytes of *Xenopus laevis* (South African clawed toad). The frog model has become one of the top choices for the study of oocyte maturation, as the oocytes are easy to maintain and numerous in number from each frog. As well, frog produces very large eggs such that microinjections, extract preparations and developmental analysis are relatively easy to undertake.

Prior to describing the details of cell cycle regulation, it is of value to review stages of cell cycle. A cell cycle is the series of events that take place in a cell leading to its division and replication. The cell cycle is represented by four discrete phases: G1 phase (Gap or Growth), S phase (Synthesis), G2 phase and M phase (Mitosis). Briefly, during G1 phase the cell grows and increases in size while synthesizing various enzymes that are required for the S phase. In S phase DNA synthesis begins and at the end of this phase all the chromosomes are duplicated and each chromosome will have two sister chromatids. Following this is the G2 phase where the cell continues growing and synthesizes more proteins that are required for the M phase. In the M phase, sister chromatids separate into two daughter cells.

Cells undergo this type of cell cycle, mitosis, to produce daughter cells that are identical to their mother cell. But in order to give rise to gametes (eggs or sperm) cells undergo a different type of cell cycle known as meiosis. The meiotic cycle is characterized by two consecutive M phases, meiosis I (MI) and meiosis II (Mil) without an intervening S phase, producing cells that have half the number of chromosomes (haploid). During MI, homologous chromosomes (those from different parents) are segregated. Then at Mil, sister chromatids segregate resulting in the formation of up to four haploid gametes.

During cell division, the chromosomes are segregated to the daughter cells by a bipolar spindle. At pro-metaphase, the spindle's microtubules attach to the kinetochores (a protein structure on chromosomes where the spindle fibers attach during division to pull the chromosomes apart) and when both sister chromatids' (or sister homologues) kinetochores attach to the spindle, the chromosome become bioriented and congress to the central spindle. The mechanism of spindle assembly differs between meiotic and mitotic systems. It can even differ between male and female gametes of the same species. But there are main features for the spindle, shared by all eukaryotes. This is the two spindle poles, which are responsible for the microtubules' polarity (the minus ends at the poles and the plus ends at the spindle equator). As well, since microtubules are polar with a plus end (fast growing) and a minus end (slow growing), another common feature for the spindle in all cell types is that the chromosomes are stabilized on the microtubules' plus ends via their kinetochores (Waters and Salmon, 1997).

There are several features that are known to differentiate the meiotic spindles of *Xenopus* oocytes from typical mitotic spindles in other animal cells. One main feature is that the meiotic spindle in *Xenopus* oocytes (as well in other vertebrate oocytes) lack centrioles, which are involved in arranging microtubules in the cytoplasm and the organization of the spindle.

There are four main stages for the assembly of the meiotic spindles during the maturation of *Xenopus* oocytes: (1) Formation of a compact aggregate of microtubules associated with the condensed meiotic chromosomes; (2) Reorganization of the microtubules-chromosome aggregate and formation of a short bipolar spindle; (3) Rapid elongation of the spindle during prometaphase, in an orientation transverse to the oocyte animal-vegetal axis; (4) Rotation of the spindle into alignment with the oocyte axis (Gard, 1992).

At the meiosis-I-meiosis-II transition, cyclin dependent kinases (CDKs) (protein kinases that require associated cyclin proteins for activity) play a crucial role. Low CDK activity triggers meiotic spindle disassembly. On the other hand, high CDK activity will prevent the formation of pre-replicative complexes that forms at the origin of replication during the initiation step of DNA replication (Furuno et al., 1994). Work in frog oocytes has shown that an intermediate level of CDK activity exists between meiosis I and meiosis II, and that this is necessary to prevent DNA replication while allowing exit of meiosis I (L.Marston and Amon, 2004).

While the CDK activity is low at the G2 meiotic phase, chiasmata (cross-over linkages between homologous chromosomes as a result of meiotic recombination) take place to ensure that the chromosomes will align well on the first meiotic spindle and then the homologous chromosomes will separate during MI. The two sister chromatids are linked to the same pole of the first meiotic spindle's microtubules by kinetochore proteins.

Another key player in the homologous chromosome separation in MI is the anaphase promoting complex (APC). APC is a ubiquitin ligase which (together with a ubiquitin-conjugating enzyme) will attach ubiquitin peptides to a substrate protein. These ubiquitilated proteins are recognized by the 26S proteasome and are subsequently degraded. During MI an

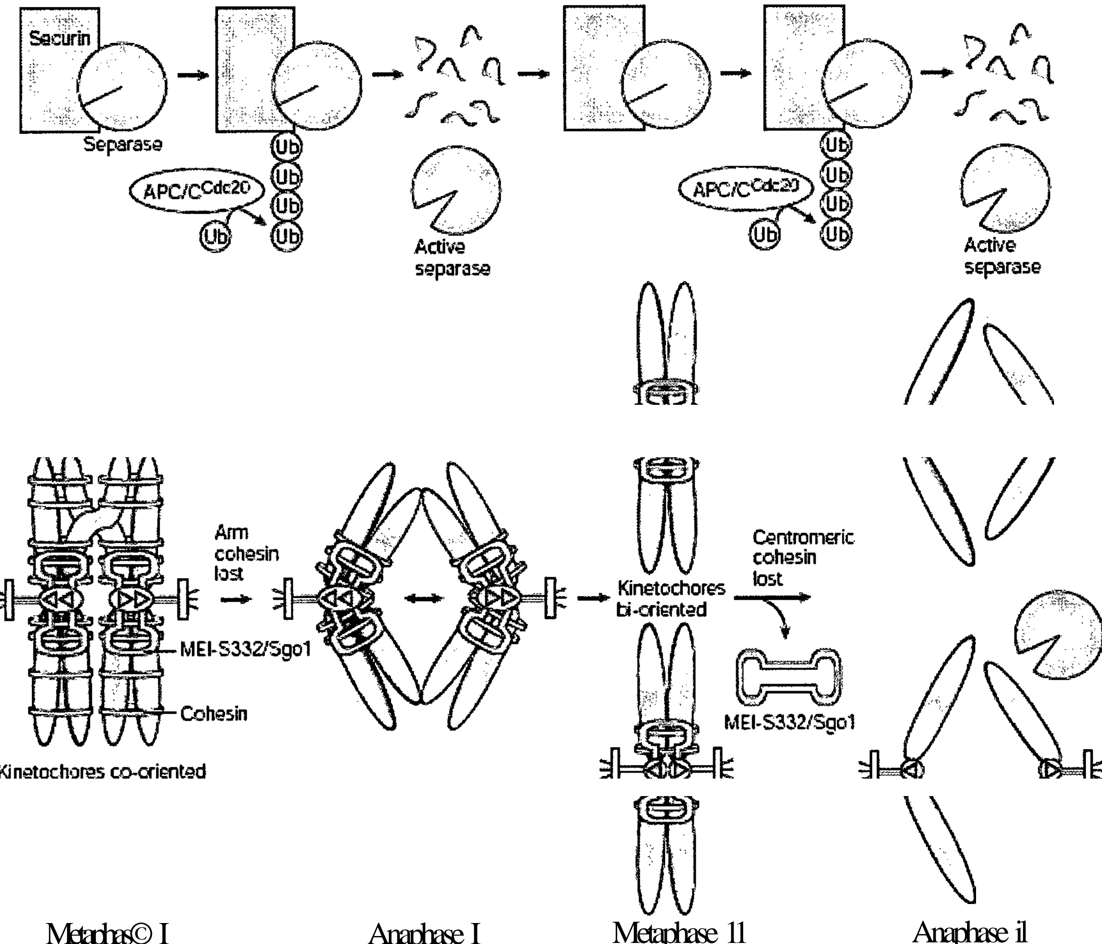
enzyme known as separase is important for the homologous chromosome separation. However it is kept inactive until the onset of anaphase through the binding of another enzyme called securin. This is where the APC comes into play. APC will liberate separase by ubiquitylation dependent degradation of its securing, which in turn triggers the separation of homologous chromosomes to opposite poles of the meiosis I spindle, and the emission of the first polar body. To permit the entry into Mil, CDK activity will rise again. Mature eggs are arrested at metaphase II with the two sister chromatids attached to microtubules from opposite poles. Fertilization breaks this second meiotic arrest (the first one at meiotic prophase) resulting in the separation of sister chromatids and the emission of a second polar body. (Figure. 1.) (L.Marston and Amon, 2004).

It has been well established that M phase in both meiotic and mitotic cycles is regulated by maturation or M phase promoting factor (MPF) (Iwabuchi et al., 2000). At the G2/M boundary of the first meiotic division (MI) the *Xenopus* oocyte will have its first arrest, where it has an intact Germinal Vesicle (nuclear envelope). An oocyte arrested at this state is termed a GV oocyte. During this phase, it contains a pool of pre-MPF (Figure.2.), a protein kinase composed of cyclin-dependent protein kinase 1 (Cdkl) and a B-type cyclin. Hormonal stimulation with progesterone induces the meiotic maturation and the exit of this arrest by triggering the conversion of pre-MPF to MPF, and the activation of a number of other protein kinases that play different roles in cell cycle and oocyte maturation like c-*mos,* Polo and Eg-2/Aurora (Hochegger et al., 2001).

Figure 1: A model for meiotic chromosome segregation

Three factors contribute to the segregation of homologues at meiosis I. First, homologues (shown in red and blue) are linked by at least one chiasma. Second, kinetochores attach to microtubules that emanate from the same pole. Third, sister chromatids are held together by cohesin rings (yellow). MEI-S332/Sgol (green) is shown around the centromere. During metaphase I, chromosomes are aligned ready for segregation, but separase (pink) is kept inactive by securin (purple). At the onset of anaphase I, APC/CCdc20 (red) becomes active and ubiquitinates securin, thereby targeting it for destruction. Active separase now cleaves the Rec8 subunit of cohesin on the chromosome arms, which triggers the separation of homologues to opposite poles of the meiosis-I spindle. Centromeric Rec8 is protected from cleavage, perhaps due to the presence of MEI-S332/Sgol.

Adapted from Marston and Amon, 2004 with permission.



Securin
Separase
APC/C^{Cdc20}
Ub
Active separase
APC/C^{Cdc20}
Ub
Active separase
Arm cohesin lost
MEI-S332/Sgo1
Cohesin
Kinetochores co-oriented
Kinetochores bi-oriented
Centromeric cohesin lost
MEI-S332/Sgo1
Metaphas© I
Anaphase I
Metaphase 11
Anaphase il

Figure 2: Frog Oocyte Maturation

A). Schematic view of oocyte maturation in *Xenopus laevis.* A fully grown oocyte is arrested at prophase I of the meiotic cell cycle. After hormone stimulation, the oocyte undergoes GVBD, resumes meiosis I, produces the first polar body, and arrests again at metaphase of meiosis II. Fertilization will release this meiosis II arrest and the zygote will then start mitosis and embryogenesis.

B). Light microscope picture of several fully-grown oocytes. On the left are oocytes before hormone stimulation. They show intact germinal vesicles (GV). The darker side of each oocyte is the animal hemisphere, and the lighter side is the vegetal hemisphere. On the right are hormone-stimulated oocytes. Their germinal vesicles are broken down (GVBD) and can be easily distinguished with a white spot on their animal hemisphere.

Modified from (Ferrell, 1999) with permission.

a)

I by
: pfo^vcltfoi^.

GVBD

OOSEHESIS

MAfUfATIGN

EMBfi YOGEMES&

pmptasa I

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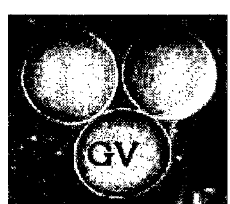
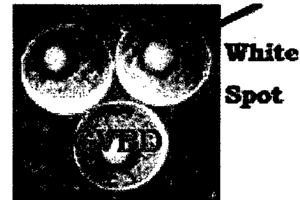
b)





After the exit of this arrest, the oocyte will resume MI and its germinal vesicle will break down (GVBD), this is where the MPF activity declines partially and transiently due to the APC complex and polyubiquitination mediated proteolysis (degradation by cellular enzymes called proteases) of its regulatory subunit: cyclin B. This partial degradation is essential to inhibit DNA replication between MI and Mil and the reformation of the nuclear envelope. Then, at the onset of Mil, cyclin B is synthesized and this results in the increase of MPF levels again and the progression from MI to MIL A second cell cycle arrest is subsequently established at MIL This arrest is maintained by a MAP kinase dependent activity named cytostatic factor (CSF) (Iwabuchi et al., 2000). Oocytes remain arrested at this stage with high levels of MPF until fertilization triggers the activation of the APC, permitting sister chromatid separation and destruction of B-type cyclins (Hochegger et al., 2001).

During oogenesis (The process of oocyte formation and development) the oocytes arrest in prophase after having their genome replicated once in S phase. The long period of meiotic prophase I arrest will allow the oocytes to store enough components for early embryogenesis. Depending on their size during prophase, oocytes are classified into six stages. A Stage VI *Xenopus* oocyte (Figure.2.) is a tremendously large cell. Its diameter is about 1.3 mm, and its intracellular volume is 1 |xl. A single oocyte contains about 25 (j,g of cytoplasmic protein, with a large nucleus (germinal vesicle) of 300-400 nm in diameter and residing in the darkly pigmented animal hemisphere which composes half of the oocyte while the other half is called the vegetal hemisphere (Ferrell, 1999).

Having discussing the main aspects of meiosis during *Xenopus* oocyte maturation, focus can now be shifted to the last step in the meiotic cycle, known as cytokinesis.

1.1.1 Cytokinesis in Animal Cells:

Despite all the studies that have been taking place for more than a hundred years on cytokinesis, this process remains a source of fascination for cell biologists. Not only does the cell produce two entities from one, it also positions the division plane so that each daughter cell receives a full complement of chromosomes and other essential organelles (Glotzer, 1997). The study of cytokinesis has been handicapped for years by the difficulty of imaging regulatory proteins during this process. The combination of new molecular techniques and imaging approaches in recent years has finally shed some new light on this challenging problem.

Although the end result of cytokinesis (two cells) is the same in all cell types, there are many different ways that cell division can occur; plants and fission yeast cells build a septum (a new cell wall formed by the fusion of membrane vesicles with the plasma membrane near the inner edge of the cleavage furrow) in the centre of the cell, budding yeast divide by budding a smaller cell from a larger one followed by cleaving off the bud, and animal cells divide by forming a central constriction that pinches the cell into two daughters. The focus in this thesis will be on animal cells, since this is where our model, the frog, fits in (Glotzer, 1997).

In animal cells, cytokinesis can be subdivided into five sub-processes: cleavage plane specification, contractile ring assembly, furrow ingression, midbody formation, and cell separation (Figure.3.). During the phase preceding cytokinesis, anaphase, the spindle specifies the site at which the cleavage furrow will form. A contractile ring, known as the actomyosin ring (because it contains mainly F-actin and myosin) assembles at the cleavage furrow site, then this ring ingresses or contracts, furrowing the overlying plasma membrane. This ingression furrow constricts the components of the spindle's midzone into a transient structure called the midbody,

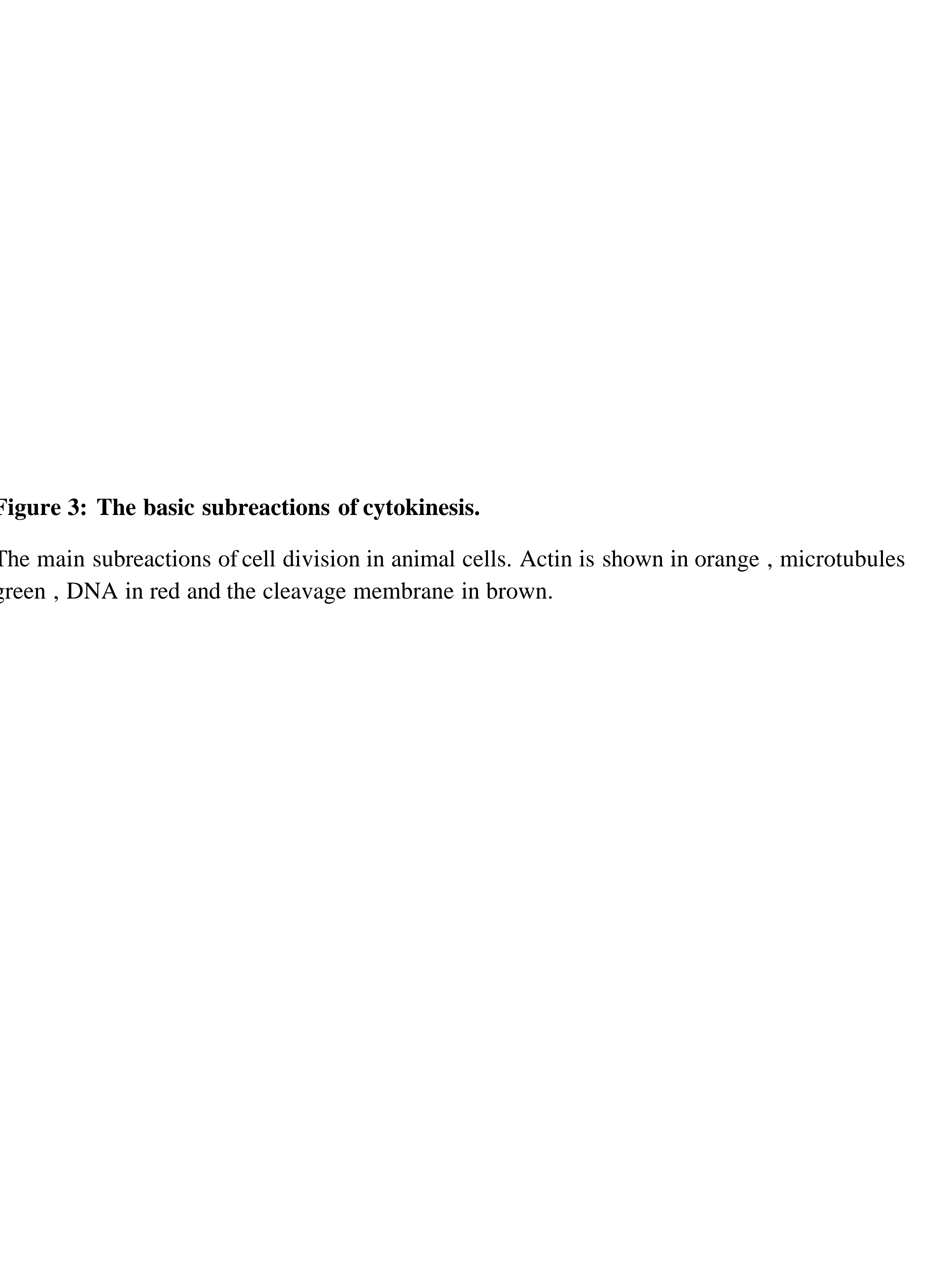
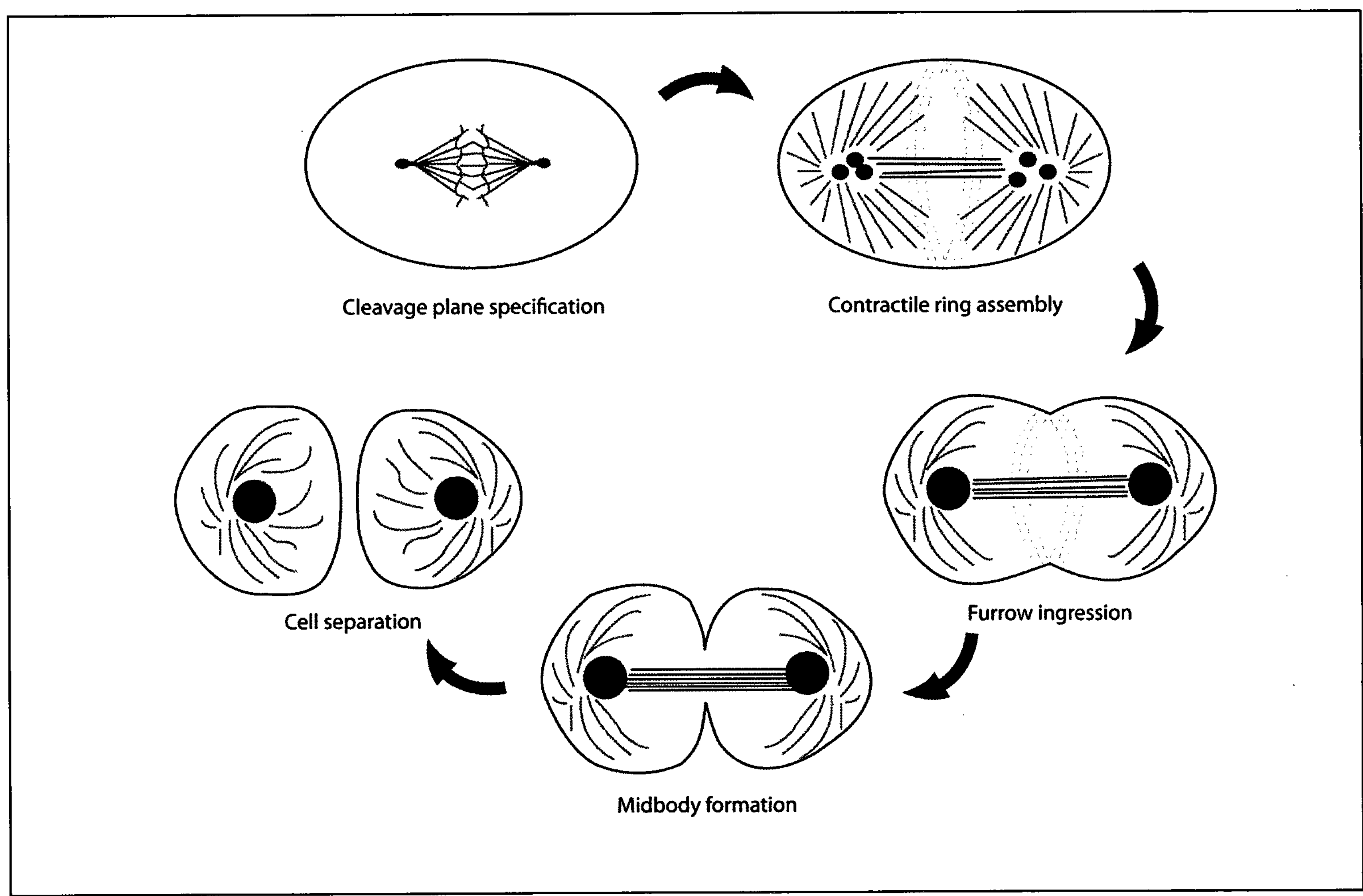


Figure 3: The basic subreactions of cytokinesis.

The main subreactions of cell division in animal cells. Actin is shown in orange , microtubules green , DNA in red and the cleavage membrane in brown.


Cleavage plane specification
Contractile ring assembly
Furrow ingression
Midbody formation
Cell separation

which forms when the furrow reaches the remnants of the spindle. Finally, the membrane that surrounds the two emerging cells is divided so that the cells may separate (Guertin et al., 2002).

In addition to the cytokinesis process just described, there are also other types of non-classical cytokinesis in animal cells; one is where cytokinesis is only partial, permitting nutrients and metabolites exchange between cells, another is called syncytial, where cells will have more than one nucleus, for example, binucleate cells in the liver. Our main focus in this study is to seek out the factors that govern the asymmetric form of cytokinesis which is represented by the first polar body formation in *Xenopus laevis* oocytes.

1.1.1.1 Polar Body Formation

Polar body formation is of interest both as a fundamental process in sexual reproduction and as an extreme example of unequal cytokinesis (asymmetric cell division) in cell biology. It occurs during female meiosis to ensure that most of the maternal nutrients are retained within the oocyte. This results in the formation of daughter cells of different sizes: the large oocyte and the small polar body. Polar body formation has always been an area of great interest in research. The identification of the major factors which control this process can open the way for the characterization of the molecular mechanisms involved in the control of the female meiotic divisions in mammals.

As it was mentioned earlier, there are two successive M phases without an intermediate S phase during meiosis to produce haploid gametes. The first meiotic division is a unique reductional event that ensures homologous chromosomes are segregated while the cohesion between sister chromatids is maintained. During the second meiotic division the sister

chromatids are segregated as in a mitotic division. In the first meiotic division, asymmetric cell division occurs once the metaphase I spindle assumes a highly asymmetric position with one pole anchored to the animal pole cortex. This triggers the metaphase-anaphase transition and the first polar body is extruded (Figure.4). It is only after sperm binding that meiosis II will resume, resulting in the second asymmetric cell division and extrusion of the second polar body (Maro and Verlhac, 2002).

The well-characterized mechanisms that regulate cytokinesis and the formation of the polar body fall into five broad categories: (1) components of the central spindle, (2) RhoA and its regulators and direct effectors, (3) nonmuscle myosin II, (4) actin and direct regulators of its assembly into filaments, and (5) factors required for trafficking and fusion of membrane vesicles (Glotzer, 2005). We will focus on the first four categories below as it pertains to our research, starting with RhoA and its family, as it is heavily involved in the three other categories to be discussed.

1.2. Rho GTPases:

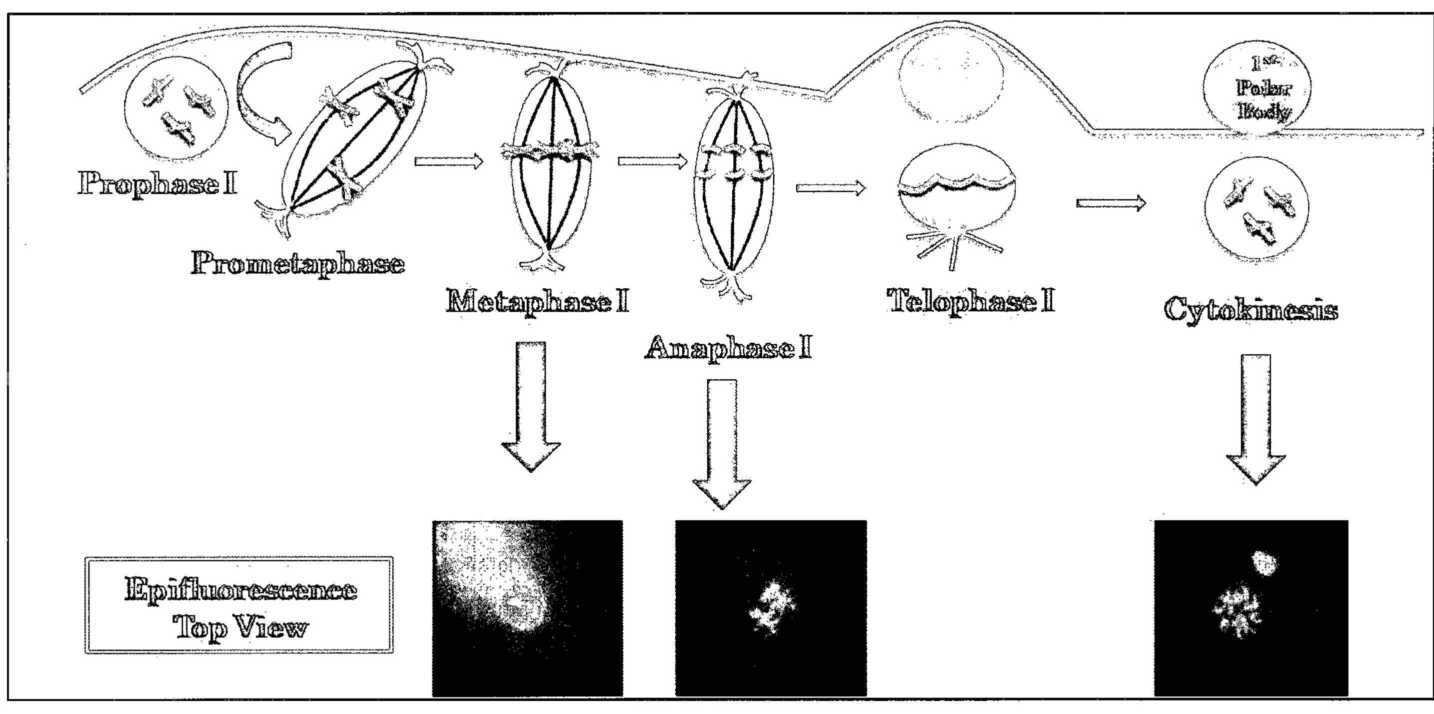
Rho GTPases belong to the Ras superfamily. Ras proteins are guanine nucleotide-binding proteins that play fundamental roles in the control of cell growth and are among the most intensively studied proteins of the past decade. The Ras superfamily consists of small (—25 kDa) monomeric guanosine triphosphatase **(GTPases).** This superfamily has 154 mammalian members that include Ras, Rho, Arf, Rab and Ran subfamilies. The Ras subfamily is generally responsible for cell proliferation, Rho for cell morphology, Ran for nuclear transport and Rab and Arf for vesicle transport (Wennerberg et al., 2005). All these small GTPases share a common biochemical mechanism and act as binary molecular switches; they cycle between active GTP-bound and inactive GDP-bound states.

Figure 4: First Polar Body formation in Frog Oocytes

Schematic view of the first polar body formation in *Xenopus* oocytes. The spindle rotates and aligns perpendicularly to the cell cortex with one pole attached, then the chromosomes align at the metaphase I spindle preparing to separate at anaphase, and releasing one set of chromosomes into the first polar body after cytokinesis.

Spindle (red), Chromosomes (green), Plasma membrane (brown).

The lower panel shows chromosomal images in *Xenopus* oocyte, as revealed by fluorescent microscopy. The metaphase I chromosomes appear like a flower from the top view, then the two sets of chromosomes starts separating and finally the emitted polar body appears as a bright dot beside the metaphase II chromosomes.


1st
Polar
Body
Prophase I
Prometaphase
Metaphase I
Anaphase I
Telophase I
Cytokinesis
Epifluorescence
Top View

The GTP-bound forms complex with and activate multiple downstream effector target molecules to stimulate cytoplasmic signalling cascades. Subsequently, an intrinsic GTP hydrolysis activity regenerates the GDP-bound form terminating the active state (Vetter and Wittinghofer, 2001).

Ras homologous family proteins **(Rho)** GTPases are members of the Ras superfamily of monomeric 20-30 kDa GTP-binding proteins. Their activity is controlled by three groups of regulatory proteins: guanine nucleotide-dissociation inhibitors **(GDIs),** which interact only with the GDP-bound state and inhibit nucleotide dissociation and control cycling of Rho GTPases between membrane and cytosol. The second group is guanine nucleotide-exchange factors **(GEFs),** which bind independently of the nucleotide-bound state and accelerate the exchange reaction of bound GDP for GTP. And the last group is the GTPase activating proteins **(GAPs),** which stimulate GTP hydrolysis, leading to inactivation and the return of the GTPase to GDP-bound state (Piekny et al., 2005).

Understanding the biochemical basis of the GTPases' function had a great impact on research. This understanding led to the development of dominant negative (nucleotide exchange-defective) mutants (a dominant negative mutation is that whose gene product adversely affects the normal wild-type gene product within the same cell). The introduction of such mutants into diverse experimental systems allows for either overactivation (constitutively active) or functional deletion (dominant negative) of a specific GTPase (Heo and Meyer, 2003).

The information currently available from the human genome sequence project has suggested the presence of a total of 22 Rho family members. The RhoGTPases can be divided into six groups: Rho (RhoA, RhoB, RhoC), Rac (Racl, Rac2, Rac3, RhoG), Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1), Rnd (Rndl, Rnd2, Rnd3/RhoE), RhoBTB (RhoBTBl and RhoBTB2) and Miro (Miro-1 and Miro-2). This increase in the number of players certainly adds

to the current complex picture of the signalling pathways that involve Rho GTPases (Wennerberg K, 2004).

The Rho GTPases act as molecular switches that regulate variety of processes in the cell such as lipid metabolism, microtubules and actin-based structures, epithelial cell-junctions, cell cycle and apoptosis regulatory proteins, and as transcription factors. They also play a critical role in processes such as gastrulation in embryonic development, immune and inflammatory responses, differentiation, wound healing, and tissue morphogenesis (Pulgar et al., 2005).
Among this big family of Rho members, this thesis will focus on RhoA and Cdc42 .

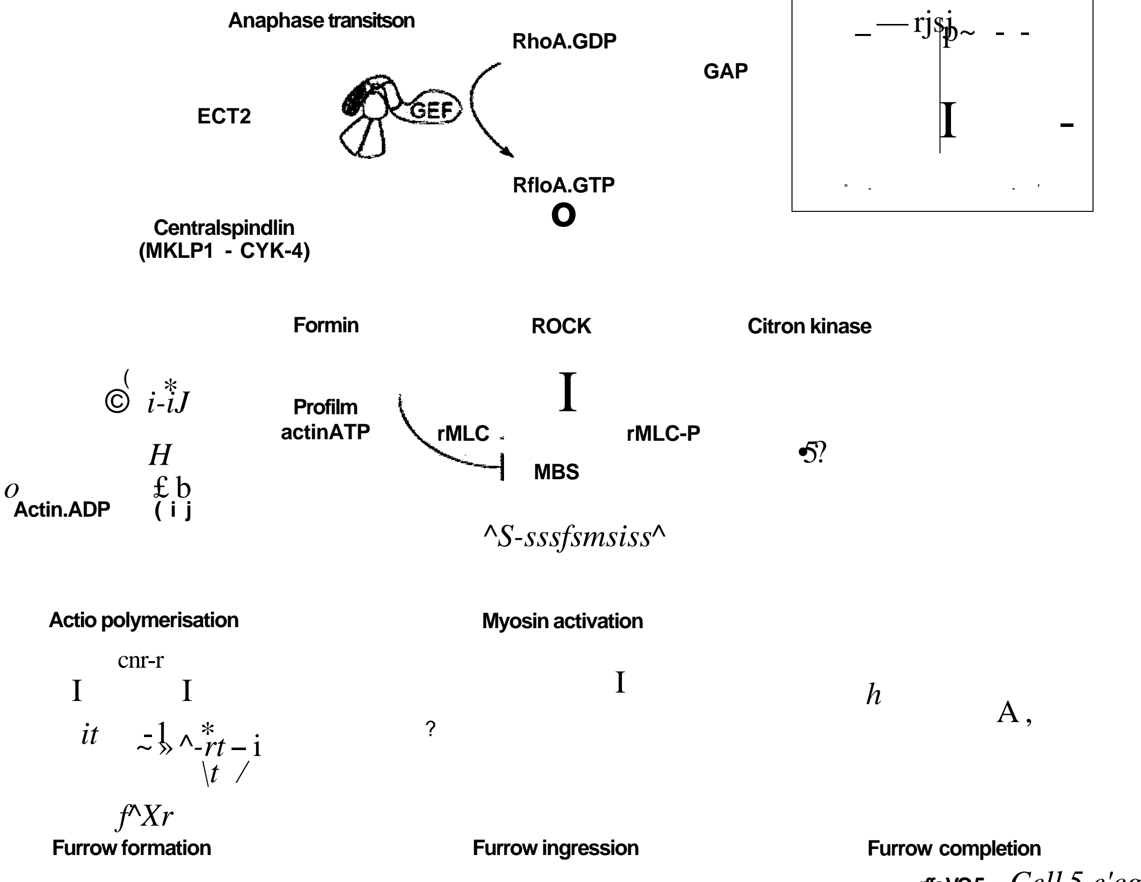
1.2.1 RhoA: The Key Switch for Cytokinesis:

Activated RhoA coordinates cytokinesis by binding and regulating specific protein effectors that profoundly affect the actomyosin contractile network (Figure.5.). The key RhoA effectors involved in cytokinesis are Rho-dependent kinase (ROCK, also known as ROK), Citron kinase (Citron K) and members of the formin family (formins are proteins that have been linked to regulation of the cytoskeleton and cell migration). RhoA's activation of cytokinesis occurs briefly as follows; formin promotes the polymerization of actin necessary for formation of the contractile ring. ROCK regulates myosin, which is a crucial component of the contractile ring that generates the force necessary for contractile ring ingression. Finally, RhoA activates Citron K, which is required in some cells for the late stages of cytokinesis after the furrow has ingressed to completion (Piekny et al., 2005).

Figure 5: RhoA activation and regulation

A schematic diagram of RhoA activation and its regulation of downstream effectors for the various stages of cytokinesis. Upon onset of anaphase, the RhoGEF ECT2 (aqua blue) is dephosphorylated, which allows it to bind to the centralspindlin component CYK-4 (CYK-4 yellow, MKLP1 orange). This complex results in the activation of RhoA, which accumulates at the furrow [inset at top right; arrows indicate positive stimulation from the central spindle, minus signs suggest inhibition by the astral microtubules (green)]. Active Rho activates several effectors. Formin (pink) regulates actin polymerization (blue) and formation of the contractile ring (light blue), ROCK phosphorylates rMLC and MBS to activate myosin (purple) for furrow ingression, and Citron K is required for the late stages of cytokinesis. The various stages of furrowing are shown in the bottom panel of cells.

Adapted from Piekny et al, 2005 with permission


Anaphase transitson
RhoA.GDP
GAP
ECT2
GEF
RfloA.GTP
Centralspindlin
(MKLP1 - CYK-4)
Formin
ROCK
Citron kinase
Profilm
actinATP
rMLC
rMLC-P
MBS
Actin.ADP
Actio polymerisation
Myosin activation
Furrow formation
Furrow ingression
Furrow completion

Cleavage furrow formation results from the local activation of RhoA, which promote key aspects of contractile ring assembly. There is convincing evidence that active RhoA is required for furrow formation, ingression and stabilization. Experiments have also shown that active RhoA concentrates at the site of furrow formation. Importantly, RhoA accumulates at this site before furrow ingression, and this accumulation is independent of actin and myosin II (Bement et al., 2005). Discrete localization of RhoA to the cleavage furrow was first reported in early sea urchin embryos (Nishimura et al., 1998).

A study conducted by Bement et al in 2005 has shown that active RhoA forms a narrow zone at the site where the contractile ring will form. Moreover, live cell imaging of active RhoA in this study showed that a discrete, well defined zone of RhoA activity is critical for successful cytokinesis. When the zone is diffuse using actin toxins (Cytochalasin D or Latrunculin B), the furrowing is initiated, but cleavage fails. On the other hand, when microtubules toxin was used (Nocodazole), both the zone formation and the furrowing were prevented. This led to conclude that microtubules are what specify the cleavage plane by directing the formation of a highly localized zone of RhoA activity (Bement et al., 2005).

Another study showed that in *Xenopus* embryos, the inhibition of endogenous Rho by botulinum ADP-ribosyltransferase C3 (one of the eight botulinum toxins produced by *Clostridium botulinum)* blocked cytokinesis (Kishi et al., 1993). In the same system, the *Xenopus* embryos, Rho is apparently important for assembly of actin filaments and proper constriction of the contractile ring (Drechsel et al., 1997).

1.2.2 RhoA; A Key Regulator in Contractile Ring Assembly:

Although there are different mechanisms in which a division site is specified in yeast and animal cells, they both divide through the use of an actomyosin contractile ring. As it was mentioned earlier, the contractile ring is also known as the actomyosin ring as its main components are F-actin (filamentous long polymers of globular actin) and myosin II. This ring is a transient bipolar array of actin filaments with their ends attached to the plasma membrane. The interaction of these actin filaments with bipolar myosin II applies tension to the membrane, causing it to constrict, releasing two cells (Satterwhite and Pollard, 1992). RhoA activates both actin polymerization and myosin II phosphorylation during cytokinesis. Myosin II consists of a parallel dimer of two heavy chains, each bound to a light chain and a regulatory light chain (rMlc). In animal cells, phosphorylation of the rMlc is what releases myosin II from its inactive state. This is where the role of RhoA comes in: RhoA activates ROCK which will in turn phosphorylates rMlc to promote the interaction of myosin II with actin filaments (Kosako et al., **2000).**

RhoA is involved in activating actin polymerization by regulating formin proteins that have a critical role in actin filament nucleation and elongation. The activity of myosin is dormant until it interacts with the actin filaments. Contraction of actin filaments involves myosin-mediated sliding of antiparallel actin filaments. These filaments move relative to one another, as motor domains at the opposite ends of bipolar myosin II complexes walk toward the plus ends of adjacent actin filaments (Romero et al., 2004).

1.2.3 Cell Division Cycle 42. fCdc42) an Essential Rho-Type GTPase:

Cell Division Cycle 42 (Cdc42), a small GTPase of the Rho-subfamily, regulates signaling pathways that control diverse cellular functions. Cdc42 regulates the dynamic organization of the cytoskeleton and membrane trafficking for physiologic processes such as cell proliferation, motility, polarity, cytokinesis and growth (Etienne-Manneville, 2004). The Cdc42 family of proteins currently has 11 members ranging in size from 190 to 192 amino acids (Johnson, 1999). Numerous studies has shown that the Cdc42 GTPase plays a vital role in regulating the signal transduction pathways that control the generation and maintenance of cell polarity in many eukaryotic cell types (Etienne-Manneville, 2004). On the other hand, the deregulation of Cdc42 is found in several pathogenic processes such as cancer, cardiovascular diseases, and neuronal degenerative diseases (Sinha and Yang, 2008).

Activation of Cdc42 in response to upstream signaling is catalyzed by GEFs, which convert Cdc42 from an inactive GDP-bound form to the active GTP-bound form (Sinha and Yang, 2008). As more and more Cdc42 GEFs and effectors are identified, a major challenge is the understanding of Cdc42 signaling specificity. Cdc42 was the first signaling protein to be shown to induce filopodia (cytoplasmic projections, which extend from the leading edge of migrating cells, and contain actin filaments cross-linked into bundles by actin-binding proteins) in fibroblasts and *Drosophila* epithelial cells (Small et al., 2002). There have been several potential pathways suggested for the transduction of signals from active Cdc42 to actin polymerization to create filopodia. The development of filopodia is somewhat similar to the development of polar bodies, in the sense that they both involve membrane protrusion. It will become clear that Cdc42 also has a significant role in polar body emission, as revealed by work in our laboratoiy.

Our laboratory has recently revealed that activation of Cdc42 is required for cytokinesis during first polar body emission in *Xenopus* oocytes. Cdc42 is first activated at the spindle pole-cortex contact site at the start of cytokinesis. Then, the activity zone of Cdc42 defines the inner boundary of a ring of RhoA activity that patterns the cytokinetic contractile ring (Ma et al., 2006). My thesis research project focused on functional characterization of a Rho guanine nucleotide exchange factor, ECT2, in regulating Rho contractile ring and in regulating Rho-Cdc42 interaction during polar body emission.

1.3. Epithelial Cell Transforming Sequence, ECT2:

As mentioned earlier, RhoA is activated by GEFs which bind independently of the nucleotide-bound state and accelerate the exchange reaction of bound GDP for GTP. GEFs work immediately upstream of Rho proteins to provide a direct link between Rho activation and cell-surface receptors. In most cases, for nucleotide exchange to happen, Rho GEFs interacts with some rigid regions of the Rho GTPase which causes remodeling of this region. This alteration in the GTPase encourages the dissociation of GDP and Mg^{2+} and activation of Rho (Rossman et al., 2005). There are large numbers of proteins containing RhoGEF domains and have diverse biological functions. One such RhoGEF is Epithelial Cell Transforming sequence (ECT2), which was originally identified as a proto-oncogene (Miki et al., 1993).

ECT2 expression is induced in S phase and reaches its highest level of expression in G2 and M phases (Sakata et al., 2000). ECT2 is phosphorylated in G2 and M phases by Cdkl and polo-like kinase 1 (Plkl), and this phosphorylation is required for the exchange activity of ECT2 (Niiya et al., 2006). ECT2 localizes to the nucleus in interphase and disperses to the entire cell after nuclear membrane breakdown. Subsequently, ECT2 is concentrated on mitotic spindles during mitosis and then localized to the midbody during cytokinesis.

ECT2 regulates cytokinesis in many organisms ranging from flies to humans. It is a critical RhoGEF for cytokinesis in all metazoans analyzed to date and accordingly it is required for the localization of active RhoA to the equatorial cortex. It has been shown that overexpression of the NH2 terminus of ECT2, lacking the GEF domain, created a dominant negative effect and inhibited the completion of cytokinesis in mammalian cells (Ytice et al., 2005). Loss of function studies confirmed the requirement for ECT2 in cytokinesis. For example, in *Drosophila melanogaster,* the orthologue of ECT2, Pebble, is required for cytokinesis

(Prokopenko et al., 1999). Another example is the *Caenorhabditis elegans* orthologue, LET-21 (Dechant and Glotzer, 2003), which is required for formation of a cleavage furrow. As well, depletion of ECT2 in human cells prevents cytokinesis (Kim et al., 2005). In addition, a study by Tatsumoto et al has shown that ECT2 is a critical regulator of spindle assembly in *Xenopus* egg extract (Takashi Tatsumoto, 2003). ECT2 is an excellent candidate molecule to integrate the spatial and temporal information that directs establishment of the contractile ring. In vertebrate cells, ECT2 localizes to the central spindle and implicated in the spatial control of cytokinesis (Yiice et al., 2005).

The *Xenopus* cDNA of ECT2 contains a long open reading frame of 910 amino acids with a predicted molecular mass of 103 kDa. The amino-acid sequence is very similar to that of human ECT2, sharing 77% identity (Takashi Tatsumoto, 2003). *Xenopus* ECT2 (XECT2) contains a Dbl (diffuse B-cell lymphoma) homology (DH) which encodes the GEF catalytic domain of the Rho family small GTP-binding proteins. Adjacent to the DH domain, the XECT2 also has a pleckstrin homology (PH) domain in the central region (Figure.6). The combination of DH and PH domains is a common motif of GEFs for the Rho GTPases. The amino-terminal half of XECT2 contains a domain related to the fission yeast cell cycle regulator Cut5. The Cut5-related domain consists of two tandem repeats of the Breast Cancer Terminal (BRCT) motif, which is widespread in a number of molecules involved in cell cycle checkpoint and DNA repair (Takashi Tatsumoto, 2003).

Although ECT2 appears to act as a GEF with all three Rho-type small GTPases in vitro (RhoA, Cdc42 and Rac), recent studies suggest that RhoA is the primary target of ECT2 in vivo

Figure 6: Schematic representation of ECT2 protein

N: N-terminal. **XRCC1:** human repair protein. **CLB6** :Cyclin B6 homology, it encodes a B-type cyclin which promotes the transition from G1 to S phase **Cut5/Rad4:** Cut (cell untimely torn) and Rad (Radiation) are identical, they are both required for the onset of S phase and the restraint of M phase before the completion of S phase. **BRCT1/2** : BRCA1 C-terminal. **S:** small central domain. **DH:** Dbl homology (diffuse B-cell lymphoma), it encodes the GEF catalytic activity of the small GTP-binding proteins. **PH** : pleckstrin homology, which catalyzes guanine nucleotide exchange on the Rho family of small GTPases. **C:** C-terminal

We used the N-terminal 1-390 amino acids of *Xenopus* ECT2; it contained the domain required for localization but lacked the catalytic domain (PH) and this induces a dominant negative phenotype.

Modified from Saito et al, 2003.

M In	XRCC1	CLB6	Rad4/Cut5 BRCT1 | 8RGT2	S	DH	PH	P®S

1 390 910

(Birkenfeld et al., 2007). ECT2 has been implicated as a centralspindlin-associated RhoA GEF which is involved in controlling the cytokinetic contractile ring. Centralspindlin is a ~300kDA tetramer containing two molecules: a Rho GTPase activating protein (CYK4), and a kinesin-6 motor (MKLP1) (Mishima et al., 2002). This centralspindlin complex localizes at the tips of astral microtubules towards the equator and at central spindle microtubules. This complex then recruits ECT2 at the equatorial cortex and central spindle microtubules (Nishimura and Yonemura, 2005).

Over the past few years there have been remarkable advances in cell biology and genetics that have answered many questions on how cells divide and thus have increased our understanding of the molecular basis for cytokinesis. But this understanding of the structural and regulatory machinery necessary to make two cells from one yet incomplete. This thesis attempts to add more information to the understanding of this complicated process.

1.4 Research Objectives:

Research in our laboratory has demonstrated that activation of the small guanine nucleotide binding protein Cdc42 is required for cytokinesis during first polar body emission in *Xenopus* oocytes. Cdc42 is first activated at the spindle pole-cortex contact site at the start of cytokinesis. The activity zone of Cdc42 defines the inner boundary of a ring of Rho A activity; the latter patterns the cytokinetic contractile ring (Ma et al., 2006). In this study I aimed to explore this unique form of asymmetric cytokinesis to determine a possible role for the Rho family GEF, ECT2, in regulating RhoA and Cdc42.

Based on studies of ECT2 in mitotic cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999), I hypothesized that ECT2 co-localizes with active RhoA during polar body emission and that ECT2 is required for RhoA contractile ring formation. Thus, my overall objective was to determine the dynamic localization of ECT2 during polar body emission and the functional consequences of inhibiting ECT2 during polar body emission.

2. Materials and Methods

2.1 Chemicals

Sytox Green was purchased from Molecular Probes. Collagenase type I, Pregnant Mare Serum Gonadotropin (PMSG) and trypsin inhibitor.were purchased from Sigma. Gentimycin, the antbiotic, was purchased from Gibco, Invitrogen corp.

2.2. Animal and Oocyte Manipulation

Sexually mature *Xenopus laevis* females were purchased from NASCO and maintained between 18-20°C. Frogs were primed with 50 units of Pregnant Mare Serum Gonadotropin (PMSG), and then sacrificed three to ten days later. Ovarian fragments were removed surgically under hypothermia, then torn into pieces containing about fifty oocytes and incubated in medium OR2 (83mM NaCl, 2.5mM KC1, ImM $CaCl_2$, ImM $MgCl_2$, ImM Na_2HP0_4 and 5 mM HEPES pH 7.8) lacking the $CaCl_2$ but containing collagenase (1.5 mg per ml) and trypsin inhibitor (lmg/ml). The released oocytes were washed in OR2 and selected based on size and appearance. We used stage VI oocytes (fully grown) for all our experiments (Smith LD, 1991). The medium used for oocyte incubation experiments was OR2 medium supplemented with Gentamicin (1 mg/ml).

2.3. Plasmid DNA Structure and mRNA Synthesis

Probes were either generated at Bement lab or by Dr. Chunqi Ma in our laboratory (Zhang et al., 2008). Full-length Xenopus ECT2 cDNA, ECT2-3GFP and ECT2-RFP were described by Sokac et al. As well as GFP-wGBD and eGFP-rGBD (Sokac et al., 2003).

Dominant-negative mutant of Cdc42 (Cdc42N17) has been described in Ma et al (Ma et al., 2003). All mRNAs were dissolved in water to 1 mg per ml. Typically, 10 nl was injected per oocyte. mRNAs coding for fluorescent probes often required concentration calibration for optimum fluorescence quality, since fluorescence is subject to temperature and other environmental effects and it is an extremely sensitive measurement.

2.4. Chromosome DNA Imaging in Whole Frog *Oocyte*

Oocytes were fixed in methanol for 30 minutes, rehydrated in 50% (v/v) methanol/OR2 for 2 minutes, and then transferred to OR2 containing fluorescence DNA-binding dye (Sytox Green used at 1:10000 dilution according to the manufacturer) for 20 minutes. Sytox Green is a high-affinity nucleic acid stain with three positive charges that is completely excluded from live eukaryotic and prokaryotic cells; it easily penetrates cells with compromised plasma membranes and yet does not cross the membranes of live cells. Its Approximate fluorescence excitation/emission maxima: 504/523 nm, bound to DNA (Roth et al., 1997). The meiotic spindle is right beneath the GVBD spot and was viewed under a dissecting fluorescence microscope (Leica, MZ FLIII), equipped with Qimaging Retiga 1300 digital camera (W. Nuhsbaum Inc) and Openlab 3.0.4 software (Improvision Inc.). An Ebq-100 mercury burner provides the excitation light source. We used a GFP filter set to visualize Sytox Green fluorophore.

2.5. Microinjection of mRNA or Other Reagents

Oocyte injection of mRNA or other reagents was conducted in calcium-free OR2 medium and usually 20nl of liquid was injected into each oocyte. The PLI-100 PICO-INJECTOR was used (Warner Instruments, USA) which allows small liquid volumes to be delivered

precisely through micropipettes by applying a regulated pressure for a digitally set period of time. Attached to this injector is the needle which was pulled using P-97 Flaming/Brown Micropipette Puller (Sutter Instruments, USA). GV oocytes were adjusted individually to make sure its animal pole appeared upmost, then the solution was injected after the tip of the glass needled pierced into the area separating the animal pole from the vegetal pole.

2.6. Probes Used for Injection and Confocal Visualization

To visualize the chromosomes we employed fluorescently conjugated antibodies against *Xenopus* Aurora B which recognizes endogenous aurora B kinase, an inner centromere protein functioning in the attachment of the mitotic spindle to the centromere. These antibodies were generated by Dr. Chnuqi Ma in our laboratory (Zhang et al., 2008).

To visualize active RhoA, the RhoA-binding domain of rhotekin (rGBD) was fused to eGFP resulting in a fluorescent probe (eGFP-rGBD) that binds specifically to active (GTP bound) RhoA (Benink and Bement, 2005). Similarly, for active Cdc42 visualization, the GTPase-binding domain of WASP (Wiskott-Aldrich syndrome protein) or wGBD was fused to eGFP or RFP, generating green or red fluorescent probes specifically for active Cdc42 (Sokac et al., 2003).

2.7. Time-Lapse Confocal Fluorescence Imaging of Xenopus Oocytes

To analyze polar body formation in the big Xenopus oocyte, in our laboratory we use laser-scanning confocal microscopy. This technology makes it possible to study and analyze live oocytes despite their enormous size and volume. Besides, most of what we are interested in during polar body formations takes place near the cell surface (Figure.7a.) (Hausen and

Riebesell, 1991). Time lapse confocal imaging (also called four dimensional (4D) imaging) uses a stack of images for each time point in a time-lapse series (Figure.7b.) (Bement et al., 2003).

To apply this time lapse confocal imaging, oocytes were typically injected with mRNA and fluorephorescence probes the day before imaging experiments and incubated overnight in OR2 at 20°C. The injected oocytes were placed in OR2 containing 1 |iM progesterone in the morning and monitored for GVBD (the appearance of the white maturation spot which results from rearrangement of cortical pigment granules, and appears at the oocyte's animal pole). Occasionally, we would add progesterone the night of the injection (with at least 6 hr between mRNA injection and the addition of progesterone), in which case we would place the oocytes at 16°C following the addition of progesterone. Oocytes were transferred to 20°C in the morning and monitored for GVBD. This manoeuvre (16°C overnight) often saved hours in the morning before the first oocyte is identified as GVBD. GVBD oocytes were individually transferred to fresh OR2 and further incubated until imaging time. Although individual oocytes vary, often considerably, in the timing of GVBD following the addition of progesterone, we found that they are remarkably synchronized from the first appearance of the GVBD spot to first polar body emission (Ma et al., 2006).

Typically, 120 min after the first appearance of GVBD, the oocyte initiates anaphase I (chromosome separation). Within a few minutes, cytokinesis starts, evident by the activation of Cdc42 and RhoA (contractile ring). The constriction of the contractile ring takes less than 10 min, so we typically started imaging 100- 120 min after the appearance of the GVBD spot. Time zero (00:00) corresponds to the start of the imaging experiments. Oocytes were imaged with a 60X oil objective on a Zeiss Axiovert with a BioRad 1024 laser scanning confocal imaging system. Time-lapse image series (Bement, 2003) were collected at various time intervals (30 s to 5 min depending on the experiments). Each time point volume comprised 15-30 image planes 1-

3 mm apart. Image series were 3D-rendered using Volocity Presentation Software (Improvision). This software enabled us to import image sequences from the confocal microscope and then integrate (volume rendering) and display 3D slices at selected time points.

Snapshots were taken at the indicated time points, either top view or side view, of these 3D series. Each of these imaging experiments was performed at least three times. Each experiment used a different frog, with multiple oocytes being imaged on the same day.

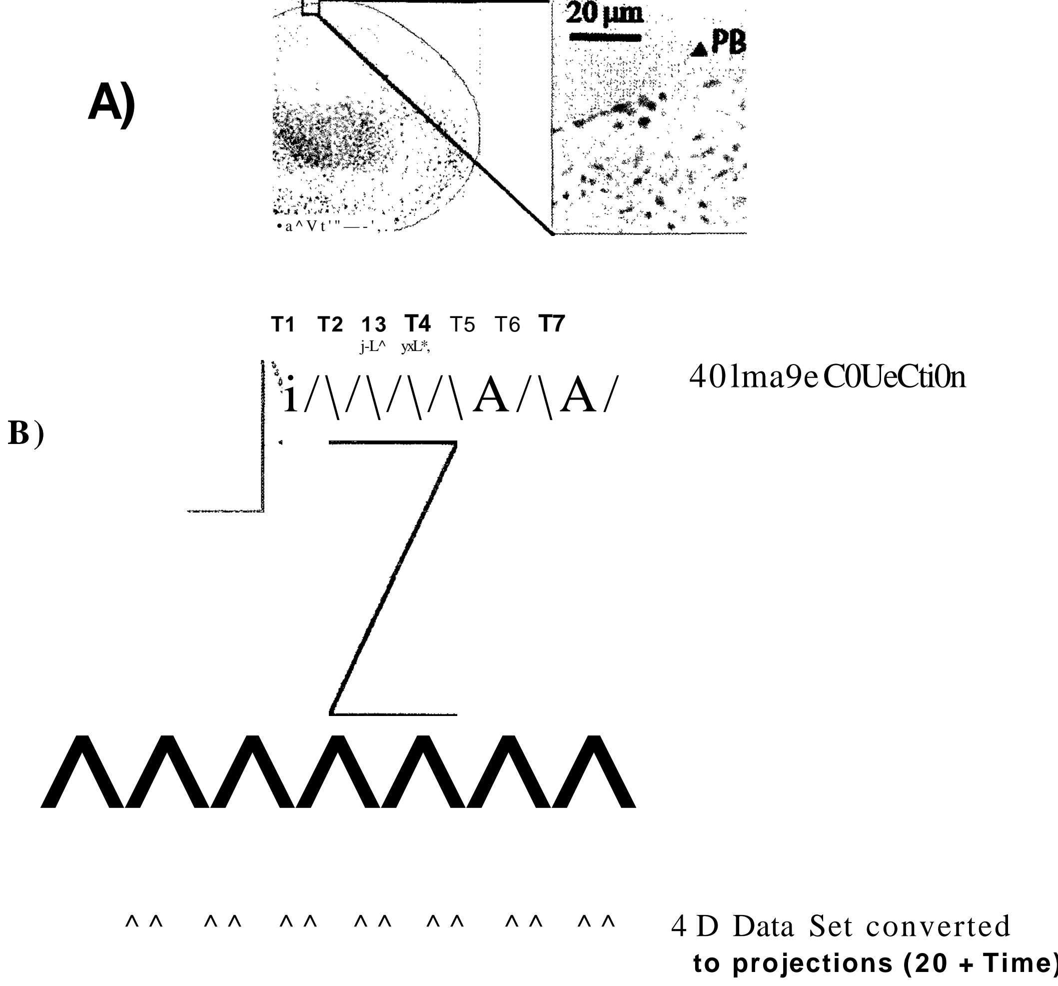
Figure 7: Basic principle of confocal imaging in *Xenopus* oocytes

A). This figure shows where our confocal imaging focused in an oocyte; most of what we are interested in during polar body formations takes place near the cell surface (the small square inset).

Modified from Hausen and Rebesell, 1991.

B).This figure shows the basic principles of 4D imaging; the top part shows a *Xenopus* oocyte mounted for imaging. The small arrows to the right indicate movements of the objective lens through the sample to generate optical sections. The large arrows indicate the return of the objective to the original focal plane. T1, T2, etc. indicate the individual time points. The lower part of the figure shows a representation of the information obtained from each time point and how it can be processed.

Adapted from Bement et al, 2003 with permission.


A)
20 µm
PB
B)
T1 T2 13 T4 T5 T6 T7
401ma9e C0UeCti0n
4 D Data Set converted
to projections (20 + Time)
4D Data Set converted
to volumes (3D + Time)

3. Results

3.1 Functional Interaction between Cdc42, RhoA and ECT2

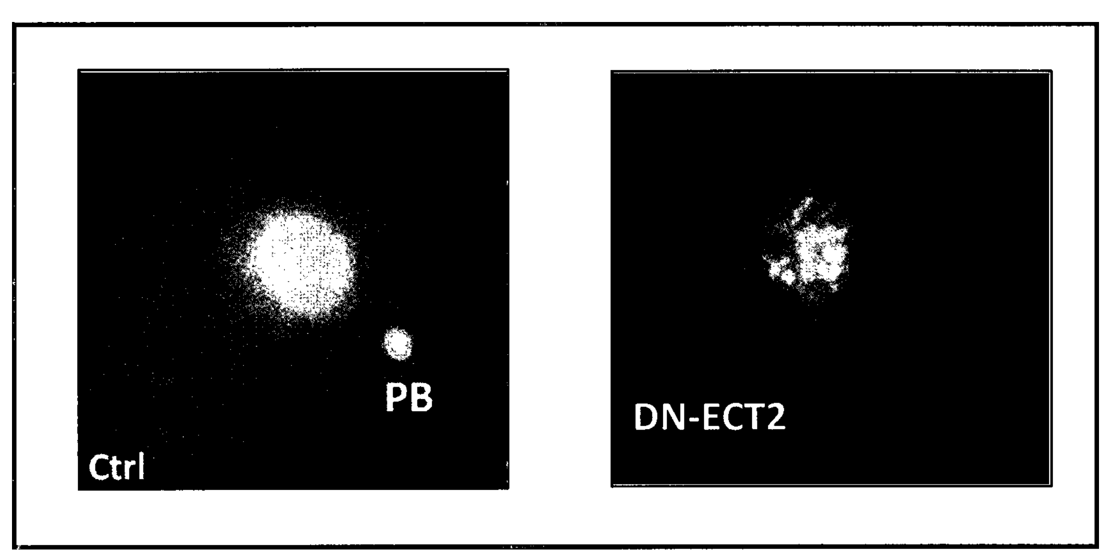
In the Tatsumoto study, it was observed that ECT2 is involved in the regulation of cell division, specifically at the cytokinetic contractile ring. Thus ECT2 has been considered an important link between the cell cycle machinery and Rho signaling pathways (Tatsumoto et al., 1999). The Tatsumoto study also concluded that human ECT2 catalyzes guanine nucleotide exchange on Rho proteins. Furthermore, ECT2 inhibition by either an ECT2 dominant negative mutant or microinjection of anti-ECT2 antibody specifically blocks the completion of cytokinesis, resulting in multinucleated cells. However, it is not clear what role, if any, this GEF (i.e. ECT2) plays during polar body emissions in vertebrate oocytes.

In order to clarify the ECT2 role, our laboratory employed a dominant negative construct of ECT2, namely mRNA encoding the N-terminal 390 amino acids of *Xenopus* ECT2 (Miki et al., 1993). The N-terminal region contains the domain required for localization but lacks the catalytic domain, and this induces a dominant negative phenotype. When this dominant negative ECT2 (DN-ECT2) was injected into the oocytes, polar body emission was inhibited (87/94 failed, compared with 0/50 in control oocytes) (Figure.8.).

Previous results from our laboratory demonstrated that endogenous Cdc42 is activated at the spindle pole-cortical contact site immediately prior to polar body formation (Ma et al., 2006). The cortical Cdc42 activity zone, which directly overlays the spindle pole, is bordered by a cortical RhoA activity zone. This RhoA activity zone is what defines the cytokinetic contractile furrow. Moreover, this study confirmed that as the RhoA ring contracted during cytokinesis, the

Figure 8: Chromosomal analysis of control and DN-ECT2 injected oocytes

Control oocytes and oocytes injected with DN-ECT2 mRNA were treated with progesterone and fixed 3-4 hours after GVBD for chromosome analyses. These oocytes were stained with Sytox Green and viewed under an epifluorescence microscope. The control oocytes showed a beautiful flower-like shape of their Mil chromosomes along with a bright dot representing the polar body. On the other hand, DN-ECT2 inhibited polar body formation.


PB
Ctrl
DN-ECT2

Cdc42 activity zone expanded, maintaining its complementary relationship with the RhoA ring (Ma et al., 2006).

In my current research, I also examined RhoA activity zone in oocytes injected with DN-ECT2 mRNA. When DN-ECT2 mRNA was injected into the oocytes (Figure.9.), it abolished the formation of the ring-like RhoA activity zone, probed with eGFP-rGBD. In figure 9, it can be observed from the control uninjected oocytes that the RhoA rings formed then constricted, and the local activation of RhoA resulted in the cleavage furrow formation. On the other hand, the DN-ECT2 injected oocyte had disorganized local RhoA activity and never formed a ring.

I also noted that DN-ECT2 however did not affect spindle assembly, spindle-cortex contact, or anaphase initiation. Thus, DN-ECT2 blocked cytokinesis by disrupting the contractile ring (Figure. 10). In DN-ECT2 injected oocytes, homologous chromosomes (as probed by fluorescent anti-aurora B) separated completely. The midzone (arrows) first appeared similar to that in control oocytes; this midzone usually appears only when chromosomes segregate. However, after segregation, the chromosomes in these injected oocytes re-congressed, since no polar body is formed. But in this figure when the chromosomes separated into two sets, the deeper chromosome set was difficult to see in some time points. The trace (circle) was the associated microtubule remnant that appeared more evident.

After establishing the dominant negative effects of DN-ECT2 on completion of cytokinesis, polar body formation and RhoA ring formation, I wanted to explore DN-ECT2's effects on Cdc42 activation. This was done by probing for active Cdc42 in oocyte using eGFP-wGBD and studying the Cdc42 activity in the presence of a dominant negative ECT2 mutant. Interestingly, DN-ECT2 diminished Cdc42 activation but did not eliminate it. I noted similar

Figure 9: Time-lapse images to visualize RhoA in control and DN-ECT2 injected oocytes.

A series from a 4D movie of a control oocyte (upper row) and that of an oocyte injected with DN-ECT2 mRNA (lower row). Both oocytes were also injected with eGFP-RGBD (green) and rhodamine tubulin (red). Scale bar is 15-20

In the control oocyte, the RhoA contractile ring was clearly visible (00:12 and 00:14). On the other hand, DN-ECT2 injection inhibited the activation of RhoA and thus no RhoA contractile ring was observed.

Control

00:00 00:09 00:10 00:12 00:14 00:15 00:16 00:16 00:20 00:53

DN-Ect2

00:00 00:06 00:09 00:12 00:15 00:18 00:21 00:24 00:30 00:46

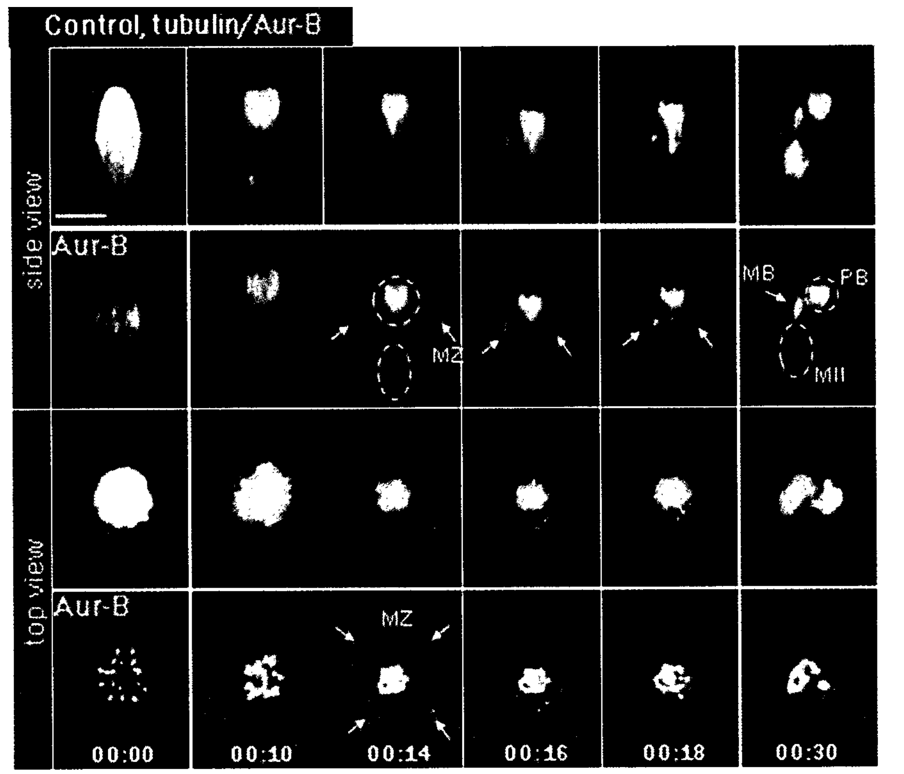
Figure 10: Time-lapse images to visualize AuroraB in control and DN-ECT2 injected oocytes

A). A series from a 4D movie of a control oocyte injected with Alexa 488 anti-Aur B (green) and rhodamine-tubulin (red). Clearly, the midzone (MZ) forms after anaphase. The midbody (MB) constricts at the middle at the end of telophase I, then the first polar body (PB) is formed. Scale bar is 15-20 |im.

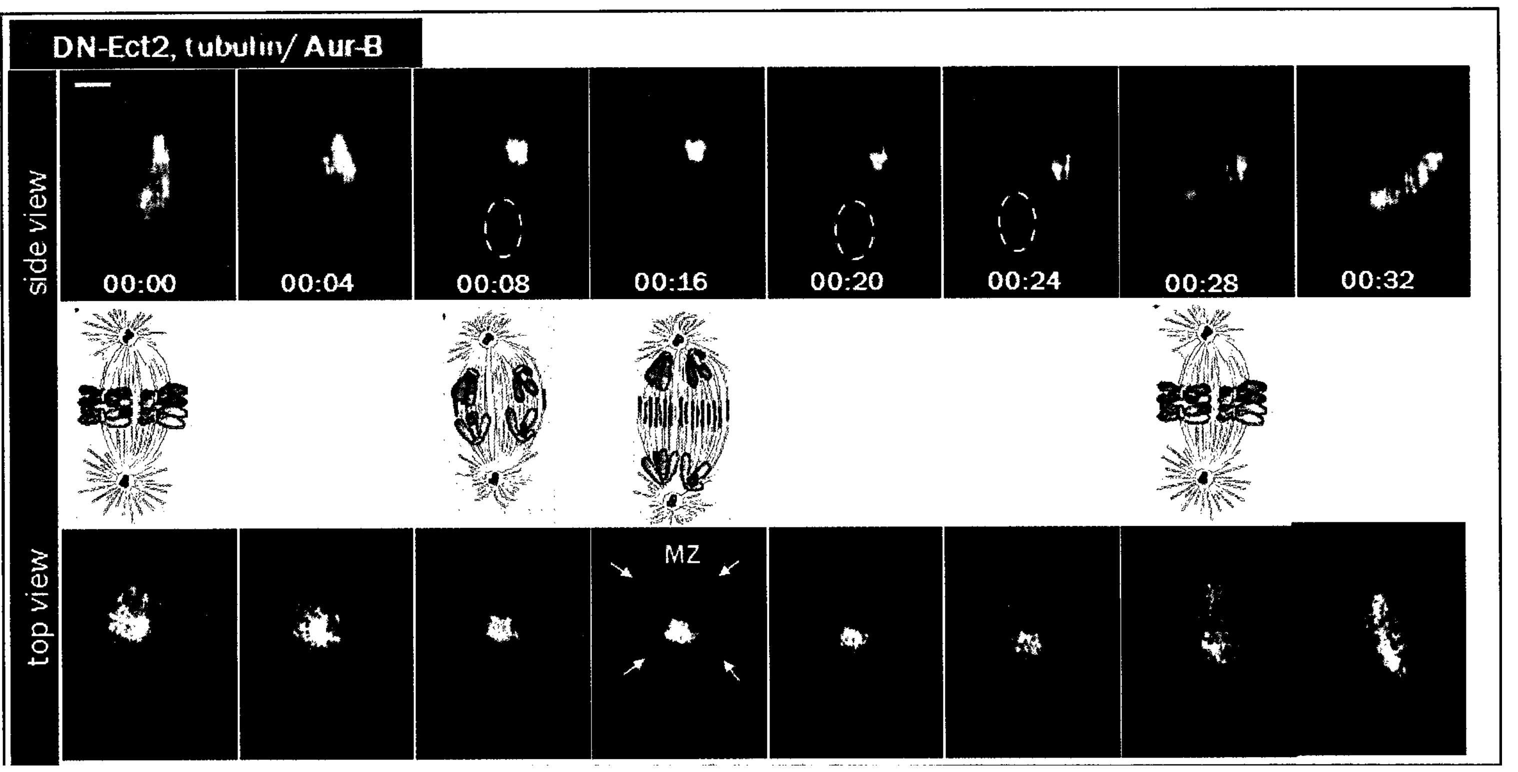
B). A series from 4D movie of oocyte injected with DN-ECT2 mRNA, as well as Alexa 488 anti-Aur B (green) and rhodamine-tubulin (red).

The homologous chromosomes separated completely and the midzone Aurora B (arrows) appeared. However, chromosomes re-congressed as no polar body was formed in the DN-ECT2 injected oocytes. When the two sets of chromosomes separated, it was difficult to see the second set because its deep, but our clue was the associated microtubule remnant which was more evident (circle).

A)



B)



results in oocytes injected with a dominant negative form of Cdc42, Cdc42N17. This dominant negative mutant (cdc42N17) blocked first polar body formation by inhibiting cytokinesis without affecting asymmetric spindle positioning or chromosome separation (Ma et al., 2006). In these Cdc42N17 injected oocytes, Cdc42 activity diminished similar to what was noted in DN-ECT2 injected oocytes (Figure. 11.). From figure 11, it can be noted that DN-ECT2 diminished the Cdc42 activity zone, represented by a cap but there was no complete inhibition. This diminution was due to the inhibition of the polar body protrusion. Hence, when polar body formation is blocked, either by Cdc42N17 or DN-ECT2, no optimal activation of the Cdc42 cap can be observed.

3.2 Localization of ECT2 during Polar Body Emission

To determine the localization of ECT2 during polar body emission, a Green fluorescent protein-tagged *Xenopus* ECT2 (ECT2-3GFP) was employed. Prior to anaphase initiation, very little specific ECT2 signal was detected (Figure. 12). At anaphase, ECT2 became clearly concentrated to a ring between the two spindle poles during the first minute of imaging (00:01 min). In the next few minutes, the central spindle expanded laterally and attracted more ECT2. The ring then started to constrict (00:09) and closed in less than 10 min (00:17). As it was mentioned earlier in the introduction, studies in mitosis have shown that the ECT2 ring forms at the central spindle, then constricts at the midbody zone. This was consistent with our findings in meiosis, where ECT2 ring forms at the central spindle, then as the polar body was formed, the ring constricted at the midbody. Following this experiment, I wanted to authenticate our dominant negative ECT2; so I injected it into the oocytes along with ECT2-3GFP (Figure. 13.). It can be clearly observed that this mutant inhibited the ECT2 ring that forms in controls.

Figure 11: Time-lapse images to visualize Cdc42 in control, DN-ECT2 injected oocytes and Cdc42N17 injected oocytes

A series from a 4D movie of a control oocyte (top row), that of an oocyte injected with DN-ECT2 mRNA (middle row) and that of an oocyte injected with Cdc42N17 mRNA (bottom row). All oocytes were also injected with eGFP-wGBD (green) and rhodamine-tubulin (red). Scale bar is 15-20 pm.

In control oocytes, Cdc42 appeared like a cap on top of the spindle and its intensity increased until it covered the first polar body and then separated from the forming metaphase II spindle (00:30). In DN-ECT2 injected oocytes, the Cdc42 activity was diminished but not eliminated. Likewise, the Cdc42N17 injected oocytes showed only a diminished Cdc42 activity. An ideal Cdc42 activation cap was not observed in both DN-ECT2 and Cdc42N17 oocytes, since polar body formation was inhibited in both groups.

/Cdc42-GTP

control
00:00
00:04
00:06
00:08
00:10
00:12
00:14
00:18
00:22
00:30

DN-Ect2
00:00
00:04
00:06
00:08
00:10
00:14
00:24
00:26
00:32
00:54

Cdc42N17
00:00
00:04
00:08
00:10
00:12
00:16
00:20
00:26
00:32
00:34

Figure 12: Time-lapse images to visualize ECT2 localization in control oocytes

A series from a **4D** movie of a control oocyte injected with rhodamine-tubulin (red) and ECT2-3GFP mRNA (green). Scale bar is 15-20 pm.

Prior to anaphase initiation, very little specific ECT2 signal was detected. At anaphase, ECT2 became clearly concentrated to a ring between the two spindle poles. Then the ring started to constrict and closed in less than 10 minutes at the midbody.

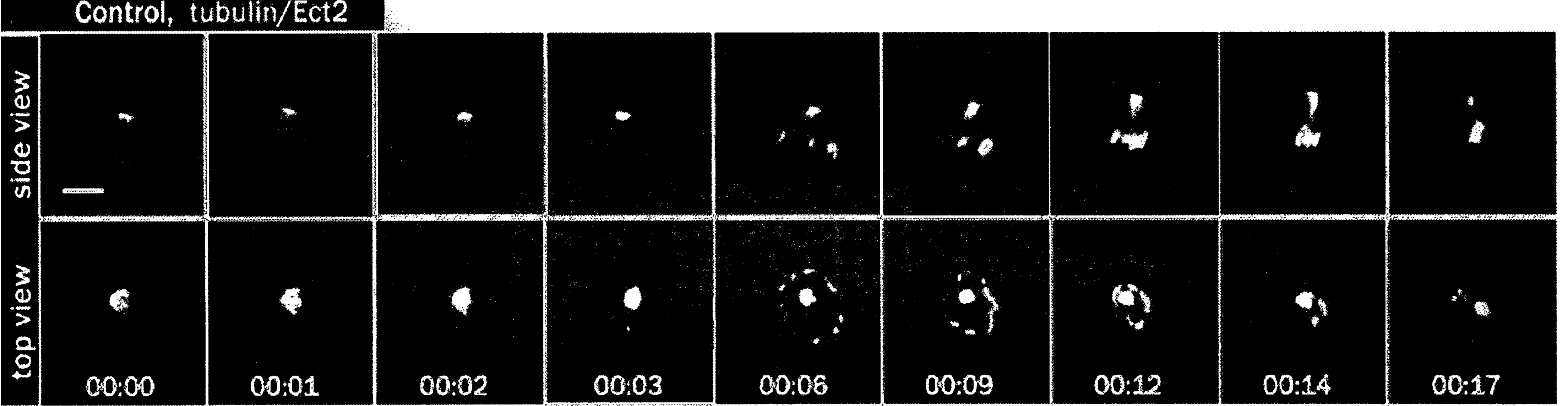

Control, tubulin/Ect2
side view
top view
00:00
00:01
00:02
00:03
00:06
00:09
00:12
00:14
00:17

Figure 12: Time-lapse images to visualize ECT2 inhibition in DN-ECT2 injected oocytes

A series from a 4D movie of a control oocyte injected with rhodamine-tubulin (red) and ECT2-3GFP mRNA (green) along with DN-ECT2. Scale bar is 15-20 pm.

As predicted, DN-ECT2 clearly inhibited central spindle localization of endogenous ECT2, i.e. DN-ECT2 inhibited endogenous ECT2.

DN-ECT2,

0:00

0:30

0:34

0:40

0:50

After establishing the localization of the ECT2 ring during meiosis in control oocytes, I wanted to observe how this ring localizes, in oocytes injected with different inhibitors. Starting with Cdc42N17 injected oocytes, I observed that the ECT2 ring also concentrated at the central spindle shortly after chromosome separation, just like in control oocytes. However, the ECT2 signal faded without constricting (Figure. 14; a total of 10 oocytes were imaged in three experiments and none contracted). In the absence of Cdc42 activity, there was no polar body formation, therefore there was no ECT2 ring constriction.

My next experiment was injecting the oocytes with xSecurindm, which is a D box mutant of *Xenopus* Securin. This mutant inhibits the destruction of securin, thus inhibiting separase activation and chromosomes separation. In these oocytes, the ECT2 ring was also formed at the central spindle in the beginning, but again, since no separation of chromosomes occurred, this ring never constricted and it faded away (Figure. 15.). The signal here was often less intense and more disorganized than those in oocytes injected with Cdc42N17.

To further investigate the properties of ECT2 localization during the polar body emissions, a third mutant was employed. The mutant was the truncated form of cyclin B1 (AN cyclin B1), which lacks the destruction box required for APC-targeted degradation. In these oocytes, ANcyclin Bl is not destroyed thus the exit from meiosis I is blocked. Furthermore, these injected oocyte don't emit polar bodies, and their transient inactivation of MPF is eliminated although they undergo normal GVBD (Gross et al., 2000). In these ANcyclin Bl injected oocytes, no specific ECT2 signal was seen (Figure. 16). The appearance of a slight signal was due to the changes in the anaphase-specific spindle (shortening of kinetochore microtubules) despite the absence of chromosome separation. From the three experiments above, the results indicated

Figure 14: Time-lapse images to visualize ECT2 localization in Cdc42N17 injected oocytes.

A series from 4D movie of an oocyte injected with Cdc42N17, together with rhodamine-tubulin (red) and ECT2-3GFP (green). Scale bar is 15-20 pm.

In oocytes injected with Cdc42N17, ECT2 also became concentrated at the central spindle (similar to control) when anaphase was initiated. However, we never observed constriction of the ECT2 ring in Cdc42N17 oocytes.

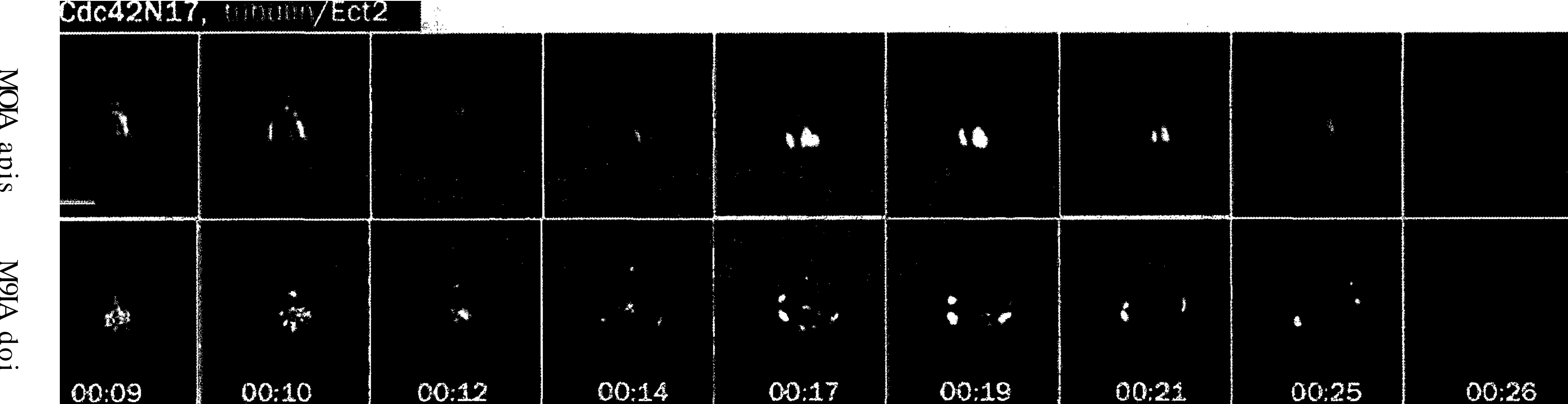
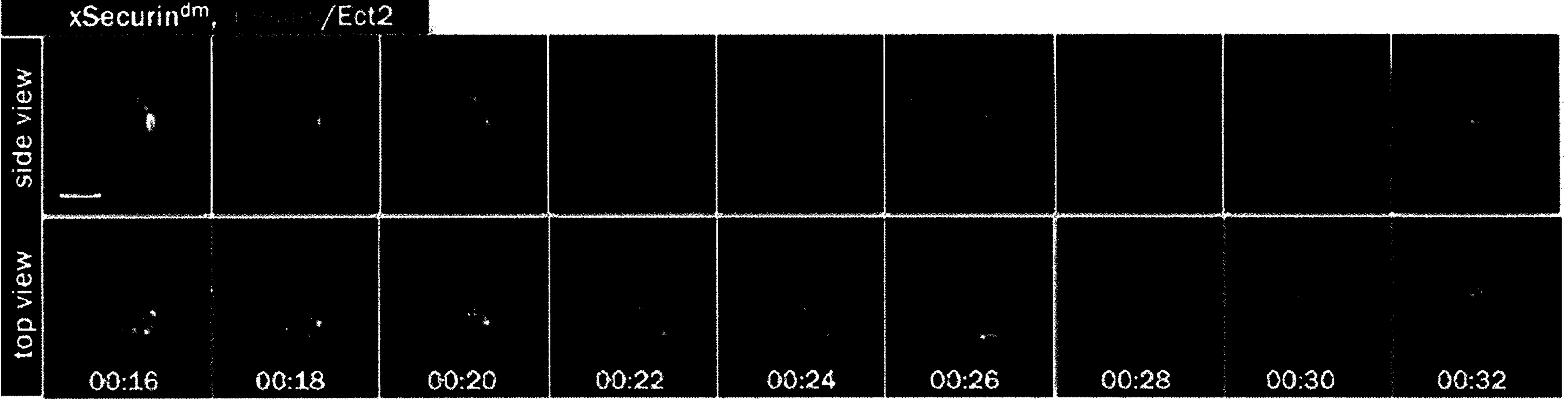

Cdc42N17, tubulin/Ect2
MOIA apis
M9IA doi
00:09
00:10
00:12
00:14
00:17
00:19
00:21
00:25
00:26

Figure 15: Time-lapse images to visualize ECT2 localization in x-Securin injected oocytes

A series from a 4D movie of an oocyte injected with xSecurin^{dm}, together with rhodamine-tubulin (red) and ECT2-3GFP (green). Scale bar is 15-20 pm.

In xSecurin^{dm} injected oocytes, chromosome separation is inhibited as a result of separase inhibition. In these oocytes, ECT2 signals appeared less intense and disorganized, then it faded quickly.

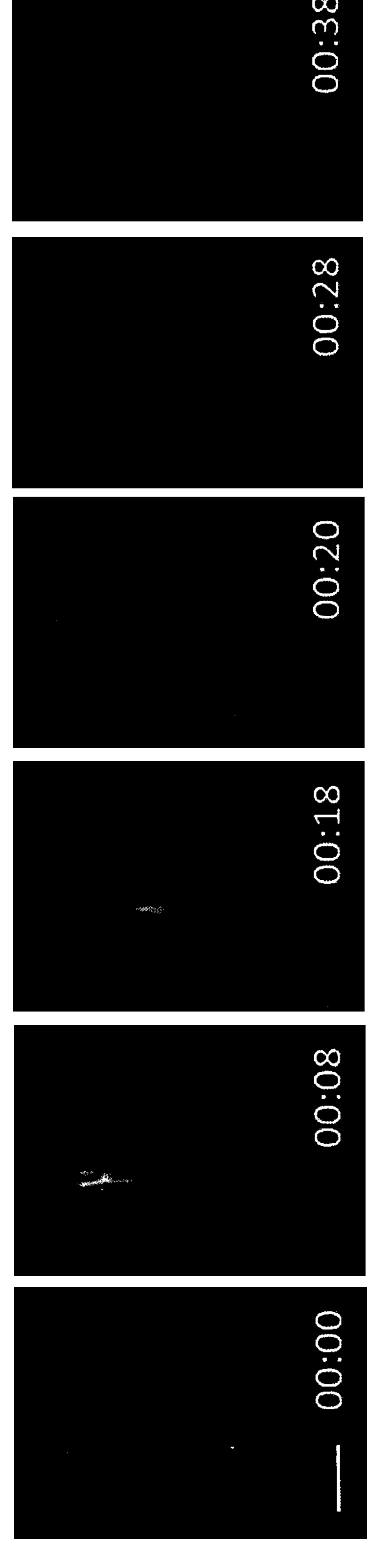


xSecurin dm, /Ect2
side view
top view
00:16
00:18
00:20
00:22
00:24
00:26
00:28
00:30
00:32

Figure 16: Time-lapse images to visualize ECT2 localization in AN-cyclinBl injected oocytes.

A series from a 4D movie of an oocyte injected with ANcyclin B1, together with rhodamine-tubulin (red) and ECT2-3GFP (green). Scale bar is 15-20 pm.

ANcyclinBl lacks the destruction box required for APC-targeted degradation. In these oocytes, cyclin Bl is not destroyed thus the exit from meiosis I is blocked. As a result of that, the oocytes attained no changes in their spindle. This was accompanied with a disorganized ECT2 signals that faded quickly since there was neither RhoA activation nor polar body formation.



M9|A apis

that the appearance of ECT2 signal at the central spindle is associated with spindle changes during anaphase and not with homologous chromosome separation.

As it was mentioned earlier, actin filaments control a variety of cellular processes. In this study, other researchers in our lab have shown that there is a remarkable overlap between the dynamic F-actin and Cdc42 (Zhang et al., 2008). This observation, lead to the potential conclusion that Cdc42 promotes rapid actin polymerization over the cortex of the protruding polar body. Accordingly, I wanted to explore the oocyte membrane curvature controlled by the actin filaments during polar body emission while monitoring the ECT2 signal. I employed a red fluorescence protein (RFP) tagged Utrophin based on the calponin homology domain of utrophin (Utr-CH), which binds F-actin without stabilizing it in vitro. This probe report the distribution of F-actin in the oocyte and distinguishes between stable and dynamic F-actin when used in combination with other F-actin probes (Burkel et al., 2007). Prior to polar body emission, the cortex was flat and uniformly labelled with RFP-Utr-CH (Figure 17). An F-actin activity that resembled a cap first appeared at the spindle pole-cortex contact site, reminiscent of the Cdc42 activity cap. This F-actin cap then started outpocketing, creating a bulge representing the protruding polar body. Within a few minutes, the F-actin cap enclosed, signifying the completion of polar body emission. The ECT2 ring during the process was formed underneath the pocketing and constricted as well at the end. On the other hand, in Cdc42-N17 oocytes, the ECT2 ring is disorganized and never constricts as mentioned earlier.

As one of the main objectives of my research project, I investigated if the ECT2 ring co-localizes with the RhoA ring, since ECT2 has been implicated as the GEF for RhoA during animal cell cytokinesis. Surprisingly, as it is shown in figure 18, these two rings never co-localized; each ring was formed on a different plane, and even when they did constrict the RhoA

ring appeared around the ECT2 ring, and never co-localized. These two rings though do constrict at the same time, but at different levels and they never overlap. ECT2 ring formed below the levels of the plasma membrane (Figure. 18.), then it moved toward the plasma membrane until it reached the constricting RhoA ring at the midbody. There it appeared that the active RhoA ring was wrapped around the ECT2 ring.

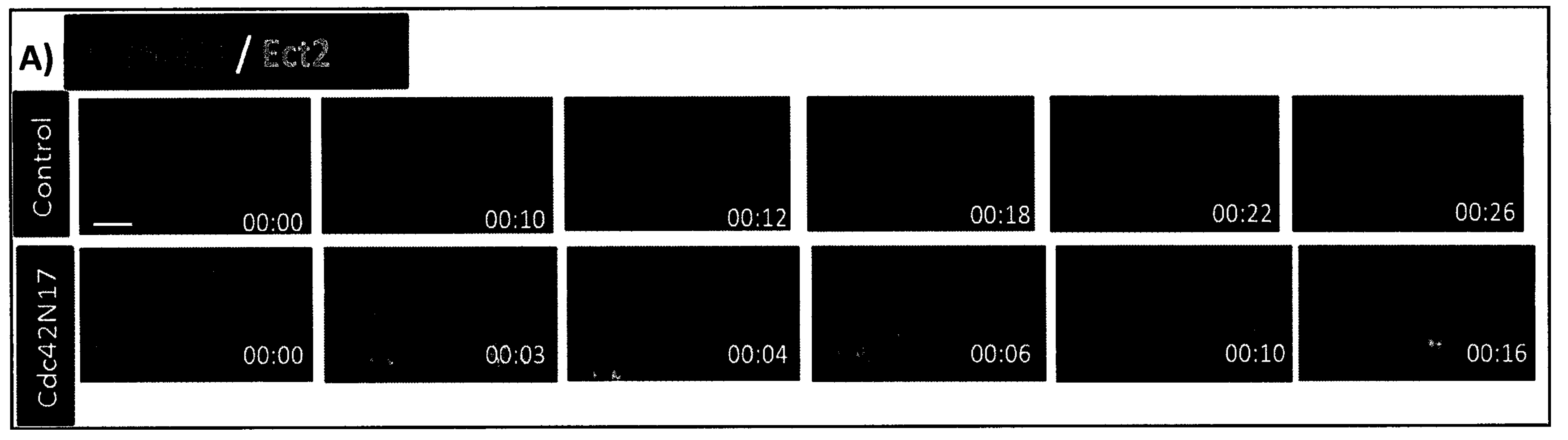
The above results suggested that RhoA contractile ring constricted at the level of the plasma membrane, while the ECT2 ring constricted at the central spindle. This constriction of the ECT2 ring was a result of the whole spindle apparatus being pulled into the polar body enclosure due to Cdc42-mediated membrane protrusion. This suggested that the ECT2 was not likely the direct GEF that activated RhoA during polar body emission.

Figure 17: Time-lapse images to visualize ECT2 localization in Utrophin-RFP injected oocytes.

A). A series from a 4D movie of a control oocyte (top row) and that of an oocyte injected with Cdc42-N17 (bottom row). Both oocytes were also injected with ECT2-3GFP (green) and RFP-Utrophin (red). Scale bar is 15-20 pm.

B). Same series as the top row (control oocytes) but here its not a 3-D image, it is a slice section of the 4D movie.

RFP-Utrophin probe labels the cortical F-actin which enabled us to demonstrate membrane curvature during polar body emissions in control oocytes. Prior to polar body emission, the cortex was flat and uniformly labeled with Utrophin and there was little ECT2 signal there. An F-actin cap then first appeared at the spindle pole-cortex contact site and the ECT2 ring started forming at the central spindle. Afterwards, the F-actin cap started outpocketing, creating a bulge representing the protruding polar body. Within a few minutes, the F-actin enclosed, signifying the completion of polar body emission.


A)
/ Ect2
Control
Cdc42N17
00:00
00:10
00:12
00:18
00:22
00:26
00:00
00:03
00:04
00:06
00:10
00:16

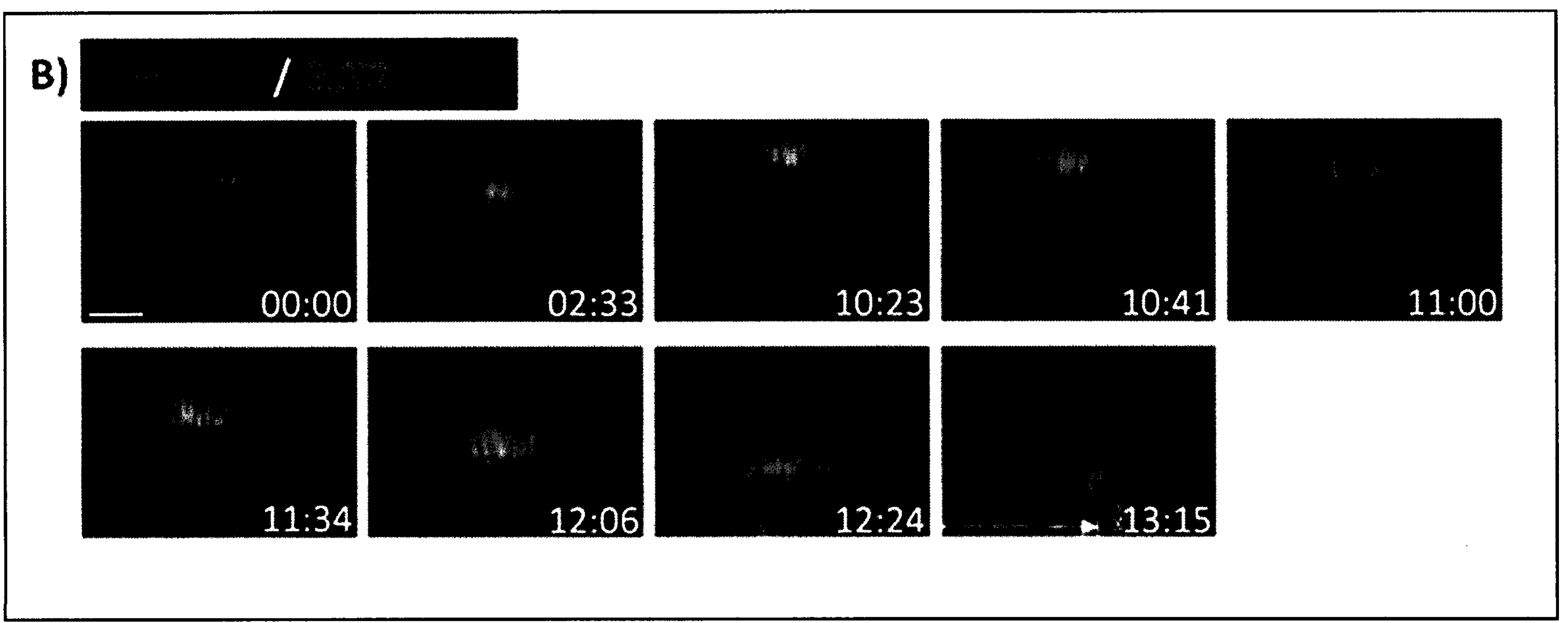

B)
00:00
02:33
10:23
10:41
11:00
11:34
12:06
12:24
13:15

Figure 18: Time-lapse images to visualize ECT2 and RhoA co-localization

A series from a 4D movie of an oocyte injected with a different probe for ECT2 (RFP-ECT2) which makes the ring appears red as opposed to green like in previous figures. The other probe used is eGFP-rGBD causes the Rho activity ring to appear green. Scale bar is 15-20 |um.

The ECT2 ring never overlapped with the Rho contractile ring. Both rings constricted at the same time but at different levels. The inset (top right) represents an interior view of the core ECT2 ring surrounded by the RhoA contractile ring at this time point. The dashed line marks the level of the plasma membrane (and RhoA contractile ring), and arrows indicate the levels of the ECT2 ring. This clearly shows different locations of each ring. The side view of this series is slightly tilted (see xyz coordinates) to show the relatively weak RFP-ECT2 signal at earlier time points

Control, Ect2/RhoA-GTP

top view

Ect2

side view

Ect2 00:00

00:02

00:04

00:06

00:08

00:09

00:10

00:11

00:14

3.3 XLfc, GEF-H1 Xenopus Homolog, a Potential Candidate as the Main GEF for RhoA

Since I found that ECT2 was unlikely the direct GEF for RhoA, I went on to look into other GEF candidates. A recent study has identified GEF-H1 as a candidate GEF to activate Rho during cytokinesis in HeLa cells (Birkenfeld et al., 2007). The Xenopus homolog of GEF-H1, known as XLfc (after the mouse homolog of GEF-H1, Lfc) is 981 amino acids long and of a molecular weight of 131 kD (Kwan and Kirschner, 2005). XLfc is a member of the Dbl family of Rho activators and contains the characteristics tandem arrangement of a Dbl homology (DH) and pleckstrin homology (PH) domain (Ren et al., 1998). XLfc is characterized as a microtubule binding Rho-GEF. It is inactive when bound to microtubules and becomes activated when microtubules are depolymerized, either as a result of inherent instability or after treatment with microtubule-depolymerizing drugs. Activated XLfc then promotes the binding of GTP to RhoA, resulting in the activation of RhoA, which in turn induces the upregulation of myosin II contractility and stress fibre assembly (Krendel et al., 2002).

The study by Birkenfeld et al has shown that GEF-H1 dephosphorylation occurred at the onset of cytokinesis, and was accompanied by GEF-H1 dependent GTP loading on RhoA (Birkenfeld et al., 2007). Also, this study revealed that GEF-H1 localized to cortical microtubules and the midbody region. Interestingly, depletion of ECT2 does not alter RhoA activation during telophase, but depletion of GEF-H1 through siRNA, lead to a strong reduction in the amount of activated RhoA during telophase. They concluded that GEF-H1 activates RhoA during mitotic cytokinesis under the control of mitotic kinases (Aurora A/B and Cdkl/Cyclin B).

We obtained the GFP-Xenopus GEF-H1 homolog, XLfc expression plasmid from Dr. Kwan and Dr. Kirschner (Kwan and Kirschner, 2005). Initially, I injected different dilutions of in vitro transcribed mRNA of eGFP- XLfc into oocytes to determine an

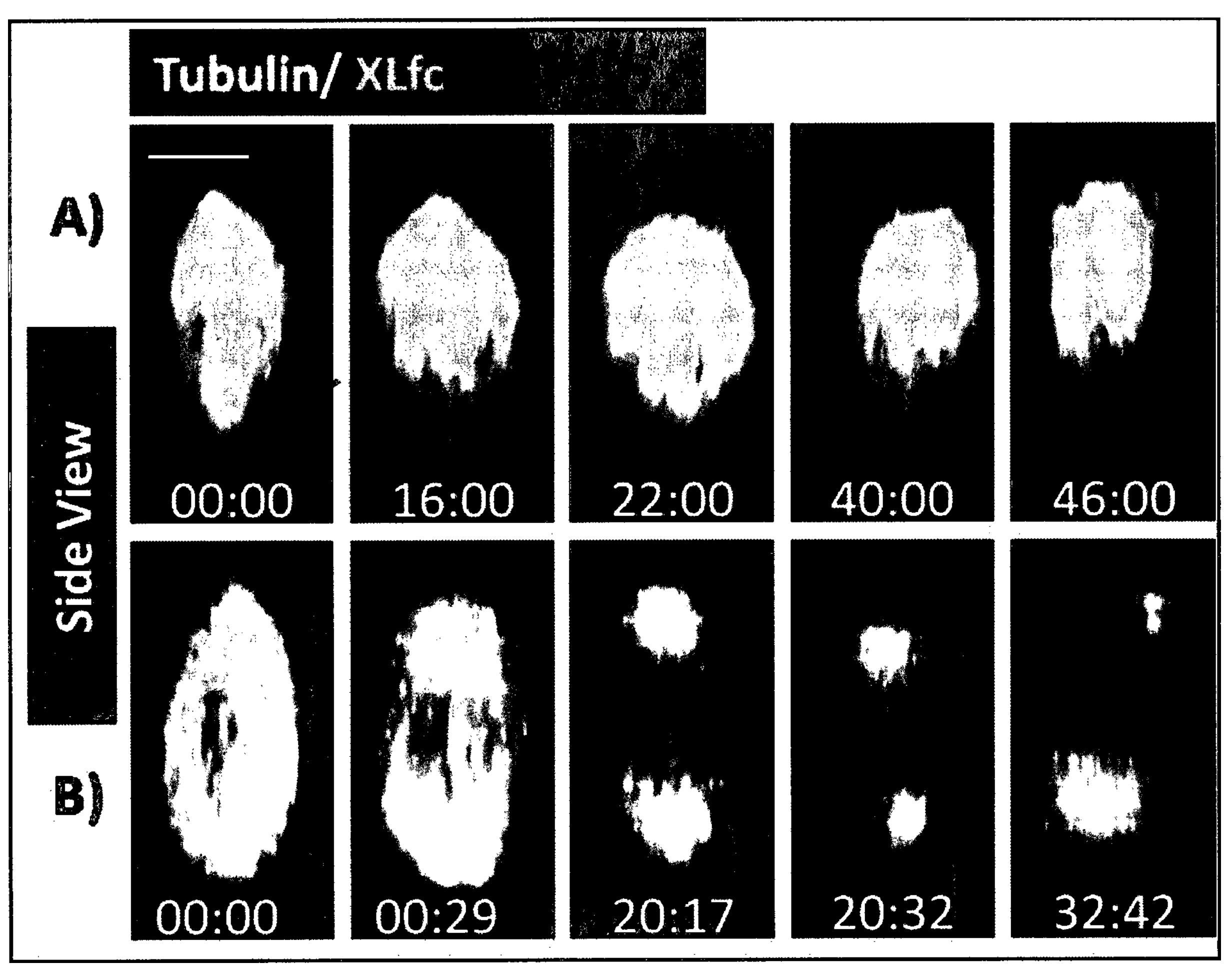
optimum mRNA concentration for 4D imaging experiments. After mRNA injection and treatment with progesterone, I observed chromosomes using Sytox Green fluorophore under UV to find the maximum concentration of mRNA that did not inhibit polar body formation, which was a 5X dilution of lmg/ml concentration of RNA. Using this information, I started to carry out live oocyte imaging experiments. Unfortunately, using this concentration did not give me consistent results. When specific XLfc signal was observed (see below), the oocytes appeared not to emit polar body (Figure 19.a.). On the other hand, oocytes that did emit the first polar body did not exhibit any specific XLfc signals (Figure 19.b.). Therefore, over-expression of XLfc, which was required to detect Xlfc signals under our experimental conditions, appeared to be damaging to the process of polar body formation.

My general observation was that the XLfc signal does not completely overlap with microtubules, as probed by rhodamine-tubulin. In particular, XLfc signals were absent from the spindle poles. One possible explanation could be that this GEF is associated with the plus ends microtubules, and this is why the signal is concentrated in the middle of the spindle. Further studies are required to determine whether XLfc is involved in polar body emission.

Figure 19: Time-lapse images to visualize XLfc

A).A series from a **4D** movie of an oocyte injected with eGFP- XLfc (green) and rhodamine-tubulin (red). An intense signal of XLfc can be observed, this signal is accompanied with polar body inhibition and no change in the spindle position. Scale bar is 15-20 µm.

B). Although similar amount of mRNA was injected into this oocyte, XLfc signals are not detectable. Perhaps as a result of reduced XLfc expression, polar body emission occurs normally in this oocyte.


Tubulin/ XLfc
A)
B)
Side View
00:00
16:00
22:00
40:00
46:00
00:00
00:29
20:17
20:32
32:42

4. Discussion

Although the structural elements of cytokinesis have been well documented, the precise signaling pathways and molecular mechanisms underlying this process are still not well understood. One key universal regulator of animal cell cytokinesis is the small GTPase Rho which is required to build a functional contractile ring. It is thought that ECT2 is the GEF for RhoA during mitotic cytokinesis. However, in all of the previous studies referenced in this thesis, the researchers localize RhoA using antibodies, hence they studied total RhoA, not specifically the active type, the way we did in our research. Nevertheless, there is no direct evidence supporting co-localization of ECT2 with Rho contractile ring, at least during the early stage of cytokinesis. In my research, I used DN-ECT2 which (Figure. 13.) abolished central spindle localization of endogenous ECT2. This assured us that indeed our DN-ECT2 inhibited endogenous ECT2. My results revealed, surprisingly, that ECT2 never overlaps with the active RhoA zone in the oocytes during polar body emission, an extreme form of asymmetric cytokinesis. These results suggested that, although ECT2 function is required for Rho contractile ring formation and constriction, ECT2 is unlikely the direct activator of RhoA during polar body emission.

However, given that ECT2 is required to activate RhoA, it might only co-localize with the inactive GDP form of RhoA, and once activated, RhoA-GTP separates from ECT2 and moves to the contractile ring. This might be true, and perhaps our imaging conditions were not rapid and/or of high resolution enough to capture the transient interaction between ECT2 and active RhoA. One way to prove the above is to probe for the inactive GDP form of RhoA. This can be achieved by probing for all Rho proteins (GDP or GTP), using Rho antibodies. After that, since we know where the active Rho proteins are using our eGFP-rGBD probe we can figure out

where Rho-GDP proteins are, because Rho proteins can only exist in two forms, GTP bound or GDP bound. Following this probing, it can be observed then if this inactive form of RhoA co-localizes with ECT2.

Another experiment that supports the notion that ECT2 is not the direct GEF for RhoA, is the one involving dominant negative Cdc42. When Cdc42N17 was injected into oocytes, the RhoA ring often constricted (roughly 50% of the time) (Zhang et al., 2008). However, in these Cdc42N17 injected oocytes, the ECT2 ring never constricted. Therefore there was no correlation between RhoA ring constriction and ECT2 ring constrictions, as might be expected if ECT2 functions as a direct GEF for RhoA.

The other GEF I tested was XLfc (GEF-H1 analog). It appeared as a good candidate for the activation of RhoA. Studies in mitosis show that this GEF works complementary to ECT2. ECT2 phosphorylation increases its catalytic activity at the onset of cytokinesis, while GEF-H1 is inhibited during the early stages of mitosis and is activated by de-phosphorylation at the onset of cytokinesis (Birkenfeld et al., 2007). These experiments suggested that the two GEFs have different but cooperative roles during cell division. ECT2 directs the localization of RhoA protein to the equatorial sites prior to cleavage furrow formation, whereas GEFH-1 participates in the GTP loading of RhoA after furrowing has been induced.

Still, my data on XLfc proved inconclusive. As I mentioned earlier, injecting too much of the XLfc probe in the oocytes, led to the inhibition of polar body emission. On the other hand, oocytes emitting the first polar body appeared to have received too little of the probe to be seen under our conditions. One explanation for that lies in the mechanism of which XLfc works. Others have shown that XLfc is a microtubule binding Rho-GEF, therefore the disassembly of microtubules results in the activation of XLfc which in turn activates Rho and induces the

formation of stress fibres (Kwan and Kirschner, 2005). On the other hand, the growth of microtubules is what activates the filopodia formation. Since filopodia is a membrane protrusion that resembles to some extent the polar body formation, then what activates it could activate polar body formation. One possible alternative is to carry out time course experiments to determine endogenous Xlfc protein localization in fixed oocytes at different time points after GVBD.

Even though ECT2 is not likely the direct GEF for RhoA, it is still required for RhoA contractile ring formation during polar body emission (Figure.9.). My data also confirms that ECT2 is required for proper Cdc42 activation (Figure. 11.), possibly through RhoA.

The results presented in this thesis, along with supplementary results obtained by others in our laboratory (Zhang et al., 2008), contributed to the working model illustrated in figure 19. In this model, the differential roles of Cdc42 and RhoA during first polar body emissions in *Xenopus* oocytes are represented. Cdc42 might somehow limit the boundaries of RhoA zone; when Cdc42N17 was injected, RhoA ring was still observed, but it appeared much more robust and spread out. Nevertheless, this expanded ring still constricted (Zhang et al., 2008). In contrast, ECT2 ring in these Cdc42N17 injected oocytes never constricted (Figure. 14.). Therefore, RhoA contractile ring can constrict in the absence of simultaneous ECT2 ring constriction but ECT2 ring can only constrict when RhoA ring constricts. The best way to explain these data is that ECT2 ring "constricts" as a result of the ring being pulled by a constricting RhoA contractile ring (Figure 20). On the other hand, when ECT2-DN was injected into the oocytes, RhoA ring formation was blocked and Cdc42 activation was diminished.

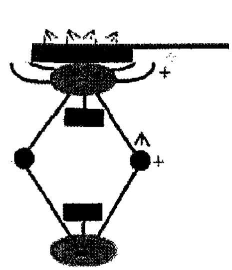
Even though ECT2 may not be the direct GEF for neither RhoA nor Cdc42 (because of the lack of co-localization with either), the function of ECT2 is clearly important for RhoA based

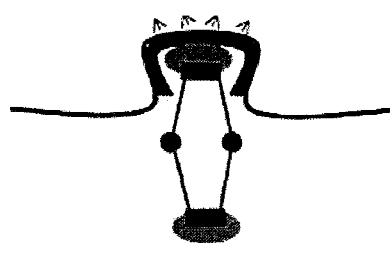
contractile ring formation, and for Cdc42 mediated membrane protrusion. Taken all together, we suggested the following scenario: during contractile ring formation, Cdc42 promotes membrane protrusion to "pull" one spindle pole and the associated polar body chromosomes into the forming polar body enclosure (Figure.20.). Pulling the spindle upward will facilitate the constriction of the ECT2 ring. In other words, while RhoA ring constricts intrinsically due to the presence of active myosin (see figure in Zhang et al), the apparent constriction of ECT2 ring is the result of the central spindle being pulled into a constricting RhoA. This pulling stops when the central spindle reaches the constricted contractile ring. At the end of the ring closure, one can still see that ECT2 core is wrapped by active RhoA signal (Figure. 18.).

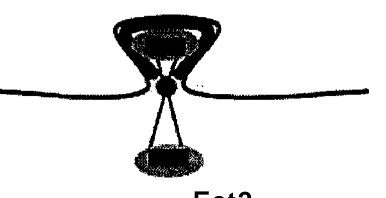
Principles described in this thesis may have wider implications in asymmetric cell division of other systems, perhaps not in all details. The cortical localization of RhoA to the cleavage furrow appears widely conserved and so is the constriction of ECT2 at the midbody zone. Although the basic machinery for cytokinesis is conserved, differences do exist in different cell types. Further studies on ECT2, RhoGTPases, and their effectors should clarify the signal transduction pathways involved in the control of cell division.

Figure 20: Our working model showing the differential roles of Cdc42 and RhoA during meiotic cytokinesis.

Cdc42 and RhoA may be activated by GEFs associated with the spindle pole (microtubule minus ends) and microtubule tips (plus ends), respectively. Cdc42 and RhoA may also functionally regulate each other (more details in the text). Cdc42 promotes membrane protrusion to "pull" one spindle pole and the associated polar body chromosomes into the forming polar body enclosure. Pulling the spindle upward will facilitate the constriction of the ECT2 rings while RhoA ring constricts independently. This pulling stops when the central spindle reaches the constricted contractile ring.







Ect2
RhoA-STP:
Cd C42-STP:
spindle poles:
Chromosomes:
Microtubules:
plasma membrane

Cdc42 : $_H$ RhoA

I | I

- Dynamic actIn polymerization to relax the cortex to facilitate polar body "out pocketing"
- Influence F-actin supply to the contractile ring
- Limit RhoA activity to focus contractile ring and direct constriction

- Recruit myosin II
- Recruit stable F-actin
- Template the contractile ring

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Curriculum Vilae:

- **Masters of Science in Biochemistry with specialization in Human and Molecular Genetics-2009**
- University of Ottawa, faculty of medicine, Ottawa, Canada.
- Thesis: Studying The Central Spindle-Associated Rho Guanine Nucleotide Exchange Factor ECT2 During Meiotic Cytokinesis.

- **Bachelor in Science with specialization in Genetic Engineering and Biotechnology-2005**
- Jordan University of Science and Technology, faculty of science, Irbid, Jordan.
- Graduated with highest Honor's and got a University appreciation award, top student in my faculty, 2003.

Experience

- Worked in the research laboratory of Dr. Johne X. Liu, Chronic Disease Unit, Departments of Obstetrics and Gynecology, Ottawa Health Research Institute, before I start my masters with him.
  Sep.06-Dec.06

  Research centers on studying the hormonal development of the mammalian oocyte and the proteins that governs its meiotic cytokinesis.

- Training at the Molecular Biology lab, Special Infectious Agent Unit, King Fahd Medical Research Centre, Jeddah, Saudi Arabia,
  Nov.05 - May.06. (Certified).

  Research focused on developing PCR techniques for pathological screening of different infectious diseases.

Skills

- Extensive knowledge in molecular biology, biochemistry, and biotechnology.
- Thoroughness: I ensure that the work is done correctly, and pay attention to detail.
- Judgment: I make appropriate logical and practical decisions.
- Self-sufficiency: I work from general instructions and use available resources before asking for help.

Focused on results: I balance work quality and objectives.
Effective interpersonal relations: establish and maintain professional working relationship.
Motivation: I can use personal resources and energies with determination to increase work satisfaction.
Dependability: I am reliable and punctual.
Experience of working in a multicultural environment.

Polymerase Chain Reaction (PCR); Conventional and Real-time
Confocal microscopy
Western Blots
Isolation and culture of mouse and frog (*.Xenopus laevis)* oocytes.
Gel electrophoresis.
DNA and RNA extractions.
Expression cloning.
DNA sequencing.

Yong Zhou, Chunqi Ma, Jennifer Karmouch, **Hadia Arabi Katbi** , and X. Johne Li u. An Anti-Apoptotic Role for Ornithine Decarboxylase during Oocyte Maturation. Molecular and Cellular Biology 29:1786-1795 (2009)
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Molecular Diagnostic Group Meeting, Jeddah-Saudi Arabia, 16 CME credit hours, Dec.05.
Real-Time Detection of MRSA, Practical Session by Cepheid, King Fahd Medical Research Centre, Jeddah, Saudi Arabia. Dec.05
In Vitro Fertilization training course, Consultative Center of Science and Technology, Jordan, 14 hours, Sep.03.
Training in basic Human Cytogenetic techniques training, Dr. Erfan and Bagedo Hospitals, Jeddah, Saudi Arabia, Sep.03.