Identification of Novel Regulators of Cytokinesis

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Abstract

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Cytokinesis describes the division of cytoplasm to produce two daughter cells, due to the formation and ingression of an actin-myosin contractile ring. The position and assembly of the contractile ring is directed by the central spindle during anaphase. The RhoGEF Ect2 localizes to the central spindle in a Cyk-4(MgcRacGAP)-dependent manner where it activates the GTPase RhoA to form the contractile ring in the central plane of the cell.

Anillin is a conserved protein that contains N-terminal actin and myosin binding domains as well as a C-terminal AHD (Anillin Homology Domain) region that binds to RhoA, Ect2 and Cyk4 and a PH (Pleckstrin Homology) domain that interacts with Septins (another cytoskeletal component) and membrane phospholipids. Anillin plays a crucial role in the localization of active RhoA and is an early marker for the location of the contractile ring in many different eukaryotic cells. Anillin's interaction with RhoA, contractile ring components, the central spindle and the overlaying membrane, suggest that Anillin is a global regulator of cytokinesis.

Although many of the core cytokinesis proteins have been identified, additional proteins likely regulate cytokinesis but have not been identified due to redundancy, or because they are also required for other cellular processes. To identify potential interactors of Anillin, and possibly of cytokinesis, a mass-spectrometry approach was used. A bacterially expressed C-terminal fragment of Anillin was used to pull down proteins from anaphase-synchronized cell lysates. The co-purified proteins were identified as GTPase regulators, cytoskeletal, mitotic,

signaling and vesicle proteins. Of these proteins, nine have homologues in *C. elegans*. Therefore, RNAi was performed in both wild-type and genetically sensitized (*mlc-4/qC1*) worms to determine if they cause embryonic lethality and could be required for cytokinesis. RNAi to two gene products in particular, *nol-1* and *fhod-2*, did not cause embryonic lethality in wild-type worms, but did cause extensive lethality in *mlc-4/qC1* worms. Based on the specificity of these interactions, the lethality could be due to cytokinesis defects and warrants further study. Therefore, this multi-disciplinary and multi-organism approach may have revealed novel cytokinesis regulators.

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List of Abbreviations

aa: amino acid

AHD: Anillin homology domain

AIR: aurora/lpl1 related kinase

ANI: Anillin

Cdk1: cell division cycle kinase

CED: Cell Death abnormality

CPC: chromosome passenger complex

CRML: capping ARp2/3 myosin linker

DEPC: diethylpyrocarbonate

DMEM: Dulbecco's Modified Eagle Medium

ds: double stranded

DTT: dithiothreitol

Ect2: epithelial cell transforming factor2

FBS: fetal bovine serum

FHOD: formin homology domain

GAP: GTPase activating protein

GDP: Guanosine diphosphate

GEF: guanine nucleotide exchange factor

GEX: gut on exterior

GTP: guanosine triphosphate

HIM: high incidence of males

INCENP: inner centromere protein

KIN: protein kinase

KLP: kinesin like protein

LET: lethal

MAPs: microtubule associated proteins

MBS: myosin binding subunit

MEL: maternal effect lethal

MKLP1: mitotic kinesin like protein

MLK: mixed lineage kinase

NGM: nematode growth medium

NOL: Nucleolar protein

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PH: Pleckstrin homology domain

PLK1: Polo-like kinase1

PRC1: protein regulator of cytokinesis1

PS: penicillin streptomycin

rMLC: regulatory myosin light chain

RNAi: RNA interference

ROCK: Rho dependent kinase

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser: serine

SPD: spindle defective

Thr: threonine

UNC: uncoordinated

ZEN: zygotic epidermal enclosure defective

ZYG: zygotic defective

1. Introduction

1.1 The Cell Cycle

Cell division is crucial for the development and function of all organisms and occurs through a highly regulated series of events, collectively called the cell cycle. While one part of the cycle, *S* phase, involves the duplication of cellular components such as cytoplasmic organelles and chromosomes, *M* phase is devoted to the equal distribution of those components into a pair of daughter cells. [(1)]

M phase, also referred to as mitosis, involves both nuclear division and cytokinesis. The main goal of mitosis is to equally partition duplicated chromosomes into the new daughter cells. In early mitosis, replicated chromosomes undergo condensation. After the nuclear envelope breaks down, microtubules interact with sister chromatids and attach them to the mitotic spindle. By the midpoint of mitosis, sister chromatids converge toward the center of the spindle. Sister-chromatid separation then takes place due to the destruction of cohesin and each sister chromatid moves toward its respective pole. By the end of mitosis, chromatids arrive at the opposite poles and new membrane is formed around the decondensing chromatids to form the daughter nuclei. Another important part of mitosis is to divide the cytoplasmic contents of the cell by cytokinesis. This process is directed by the mitotic spindle and is accomplished by the formation of an actomyosin ring that contracts to physically separate the two daughter cells. [(1)]

1.2. Cytokinesis

Although major differences in cytokinesis exist among different organisms, the main events of this process are universal. Cytokinesis has been studied in various systems including vertebrates, several species of marine invertebrates, *Drosophila*, *Caenorhabditis elegans*, yeast, *Dictyostelium* and different plants. In animal cells, the mitotic spindle dictates the position of the cleavage furrow, an invagination that forms at the cortex and plasma membrane. Furrow ingression occurs due to the cortical assembly and contraction of actin and myosin at the division site and works in concert with the fusion of membrane vesicles with the ingressing plasma membrane. [2, 3] In contrast, plants do not make an actomyosin contractile ring and cytokinesis mainly occurs through targeted secretion to the cell division site. [2, 4]

Yeast shares some similarities with animal cytokinesis. The budding yeast *Saccharomyces cerevisiae* requires an actomyosin contractile ring for cytokinesis. This ring forms at the bud neck (division site) and deletion of *myo 1*, the single nonmuscle myosin II gene, results in cytokinesis failure. [5] However, unlike animal cells, the division site is determined in earlier stages of the cell cycle, before formation of the mitotic spindle. Furthermore, a new cell wall is deposited at the septum, which forms behind the constricting ring. [2, 5] In the fission yeast, *Schizosaccharomyces pombe*, an actomyosin ring assembles at the division site on entry to mitosis and constricts to divide the cells by binary fission [2, 5]. Unlike animal cells and similar to *S. cerevisiae*, *S. pombe* synthesizes a division septum behind the constricting ring and develops a new cell wall between the two daughter cells. [2]

1.3 Cytokinesis in Animal Cells

In animal cells, cytokinesis can be divided into four stages: determining the division plane; cleavage furrow formation and ingression; midbody formation; and cell separation (abscission). The mitotic spindle, comprised of both astral and central spindle microtubules, provides signals to determine the future division plane and initiate cytokinesis (Figure 1.1). An actomyosin contractile ring forms during anaphase and ingresses during telophase to form an invaginating furrow through association with the plasma membrane. As the furrow ingresses, the central spindle compacts until it forms a structure called the midbody, which maintains a connection between the two daughter cells (Figure 1.1). This structure provides a platform for vesicle delivery and is the site for abscission of the two daughter cells. [3 -8]

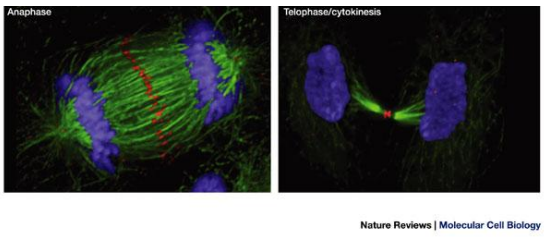

Figure1.1. Microscopic image of a dividing cell, red: centromere protein E- a marker for kinetochores, the central spindle, and midbody,green: microtubule, blue: chromosomes. Note: Figure is adapted from Carmena et al [6]

1.3.1 Mitotic Spindle

The organization of microtubules is crucial for performing the various steps of mitosis such as chromosome segregation and cytokinesis. [8, 9] A bipolar mitotic spindle aligns chromosomes in metaphase through the attachment of sister chromatids with kinetochore fibres, which then mediates their separation during anaphase. Astral and central spindle microtubules are key structural features of the anaphase mitotic spindle that work together to determine the cleavage plane and ensure that the daughter cells inherit the appropriate chromosome content. [9, 10]

The astral microtubules are radial arrays of centrosome-nucleated microtubules whereas central spindle microtubules are antiparallel bundled nonkinetochore microtubules, which form between the separating chromatids during anaphase (Figure 1.1). [10, 11] The cleavage plane is determined by a combination of inhibitory cues from the astral microtubules and stimulatory cues from the central spindle. Early experiments performed by Rappaport used echinoderm embryos to show that a new furrow always formed in a plane that bisects the mitotic spindle and more recent studies showed that both sets of microtubules work together to establish the division plane.[10-14] A mutation in *zyg-9* causes the mitotic spindle in the early *C. elegans* embryo to be extremely asymmetric and positions the central spindle close to the cortex in the posterior and the astral microtubules radiate out toward the anterior cortex. In these embryos, one cleavage furrow forms over the central spindle and a second furrow forms in the anterior, in a region of low microtubule density.[15] From this and many other experiments, it was concluded that astral microtubules prevent the cortical accumulation of contractile ring proteins, and because these microtubules are denser in the poles, this ensures that the

contractile ring forms in the centre of the cell where the density of astral microtubules is the lowest.[10-15] In addition, the central spindle provides a stimulatory cue for cytokinesis, since a highly conserved complex of central spindle proteins localizes and activates the GEF Ect2, the upstream regulator for RhoA, to the central spindle to form the contractile ring in the central plane of the cell. [9, 12-15, 19] Therefore, astral microtubules and the central spindle function redundantly in metazoans to ensure the correct timing and placement of the contractile ring. [9-15]

Central spindle assembly is driven by MAPs (Microtubule Associated Proteins), kinesin motor proteins and mitotic kinases. The core regulators of central spindle assembly are PRC1, the centralspindlin complex and the chromosome passenger complex (CPC). [9] PRC1 is a highly conserved microtubule bundling protein, which is essential for central spindle formation in yeast, *Drosophila*, *C. elegans* and mammalian cells. [9] Despite its crucial role in central spindle formation, PRC1 is not essential for contractile ring formation and ingression in *C. elegans* and mammalian cells. The centralspindlin complex is comprised of a heterotetramer of MKLP1 (a kinesin 6 motor protein) and Cyk-4 (MgcRacGAP with GAP activity toward Rho GTPases) and promotes central spindle microtubule bundling. Cyk-4 recruits Ect2 to the central spindle, which is crucial for the ability of Ect2 to activate RhoA, and is essential for contractile ring formation in *C. elegans*, *Drosophila* and mammalian cells.[9,16] The CPC is composed of INCENP (Inner CENtromere Protein), survivin and borealin, as well as AuroraB kinase which functions as a catalytic subunit. The CPC are named for their switch in localization from kinetochores to the central spindle during anaphase. The CPC acts in parallel to the

centralspindlin complex for central spindle formation, and is not essential for contractile ring formation in *C. elegans*, *Drosophila* or mammalian cells. [1, 9, 16]

Given its significant role in cytokinesis, the timing of central spindle assembly is important. The central spindle forms in anaphase as Cdk1 levels decrease. High levels of Cdk1 activity inhibit many critical central spindle components during metaphase. For instance, phosphorylation of MKLP1 by Cdk1 prevents the centralspindlin protein complex from assembling and alters its interaction with microtubules. [9] Moreover, Cdk1 phosphorylation of PRC1 reduces both its binding efficiency to the spindle and recruitment of Plk1 (Polo-like Kinase1), which localizes to the central spindle during anaphase. [9] In addition, Cdk1 phosphorylation of Ect2, the GEF for RhoA (see below), keeps it in an inactive conformation and prevents its interaction with Cyk-4 (MgcRacGAP). [19] The importance of Cdk1 in regulating the timing of anaphase onset and cytokinesis is demonstrated by experiments in many different animal cells where inhibition of Cdk1 is sufficient to drive mitotic exit and cytokinesis. [3] Other kinases, including Plk1, are also important regulators of cytokinesis. Plk1 phosphorylates Cyk-4, which is essential for its interaction with Ect2, and disruption of Plk1 activity during anaphase blocks the formation of the contractile ring in human cells. Therefore, Plk1 activity is critical for activation of Rho pathway and contractile ring assembly. Aurora B kinase also contributes to cytokinesis, but acts later than Plk1 by phosphorylating MKLP1 during late anaphase, which is essential for its role in abscission. [9, 16-19]

1.3.2. Rho Pathway

The Rho family of small GTPases is a distinct family in the superfamily of Ras-related small GTPases that exists in all eukaryotes. Almost one percent of the human genome encodes proteins that regulate or are regulated by Rho GTPases, commonly known as Rho, Rac and Cdc42. [20, 21] They are critical for cytoskeletal dynamics and control various cellular processes such as morphogenesis, cell polarity, migration, vesicle trafficking and cytokinesis. [20, 21] The Rho subfamily has three isoforms; RhoA, RhoB and RhoC, which are highly homologous, and RhoB and RhoC null mice are viable without any major developmental effects. [20, 21] However, RhoA may have a distinct function, since cells fail cytokinesis after RhoA depletion, suggesting that RhoA (and not RhoB and/or RhoC) is a key regulator of cytokinesis. [21, 22]

Rho family members cycle between active GTP-bound and inactive GDP-bound states. This process is regulated by GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins). GEFs mediate the exchange of GDP for GTP to activate Rho proteins, while GAPs inactivate them by stimulating intrinsic GTPase activity to generate GDP. GTP binding induces conformational changes, which increases affinity for their effectors. Members of the formin family, Rho-dependent kinase (ROCK) and citron kinase are RhoA effectors that are required for furrow formation, furrow ingression and furrow completion, respectively. [21]

F-actin is an essential part of the contractile ring and its proper assembly is crucial for contractile ring formation. Formins control actin dynamics both *in vitro* and *in vivo* and increase actin filament assembly by regulating filament nucleation rate, filament

elongation rate and barbed-end capping. [22] After binding to active RhoA, formin undergoes a conformational change to interact with profilin and actin by its FH1 and FH2 formin-homology domains, respectively. [23, 24] These domains dimerize to generate a ring-like molecule that is required for adding additional actin subunits to the growing filament. [22-24]

The second major component of the contractile ring, myosin II, is activated by the RhoA effector ROCK (Rho-dependent kinase), and is important for furrow formation and ingression. Myosin II contains a pair of regulatory light chains (rMLC) and phosphorylation of rMLC at the highly conserved Ser19 residue leads to filament formation and activation of the actin-dependent ATPase activity of the motor domain.[25] ROCK phosphorylates rMLC at Ser19, to activate myosin. In addition, ROCK can increase myosin activity by phosphorylation of the myosin-binding subunit (MBS) of myosin phosphatase, an inhibitor of myosin. [25] Depletion of this phosphatase can promote furrow ingression and minimize the defects caused by ROCK mutations indicating an antagonistic role for MBS and ROCK in myosin regulation (Figure 1.2). [22, 25]

RhoA also regulates the activity of citron kinase (citron K) to mediate the end stages of cytokinesis. Citron K accumulates in the cleavage furrow in dividing human and *Drosophila* cells in a RhoA-dependent manner and over-expression of Citron K deletion mutants results in cytokinesis defects in cultured mammalian cells. [22, 26] Citron K may regulate cytokinesis by stably maintaining contractile ring components at the midbody. Citron K can also di-phosphorylate rMLC at Thr 18 and Ser 19 *in vitro*, which leads to

differential localization of myosin II at the furrow, notably during late stages of cytokinesis (Figure.1.2). [22, 26]

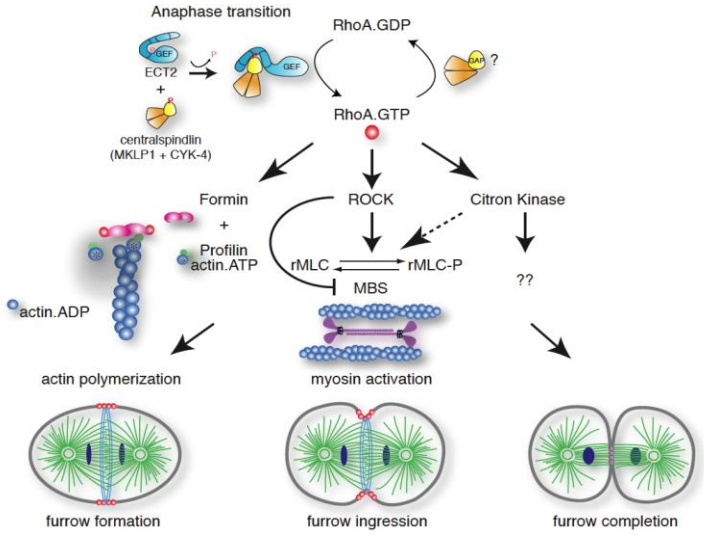

Figure 1.2. A schematic of RhoA pathway and its downstream effectors during cytokinesis.

Note: Figure is adapted from Piekny, *et al* [22]

RhoA activity is downregulated during the late stages of cell division, after the contractile ring has fully ingressed. Multiple mechanisms likely cooperate to switch off RhoA. One mechanism involves inactivating Ect2 through its sequestration in the nucleus in late anaphase. [22] Alternatively, or in addition, the GAP activity of Cyk-4, or another RhoGAP (*eg.* p190GAP) could downregulate RhoA. [22, 27]

1.3.3 Contractile ring

Cytokinesis is driven by the ingression of an actomyosin contractile ring, which forms in anaphase in a discrete cortical location that is determined by the mitotic spindle. Actin filaments within the contractile ring have opposite polarities and are oriented in parallel

to the cleavage furrow, which is closely juxtaposed to the plasma membrane. [9, 28] Myosin II is the main force generator of cytokinesis and constricts the contractile ring using the energy of ATP hydrolysis to translocate actin filaments within the ring. [28, 30] The organization of actin and myosin filaments suggests that constriction occurs by a mechanism similar to the contraction of muscle sarcomeres. Septins are another important filament system in the contractile ring. In budding yeast these GTPases are the first proteins to localize to the bud neck and are thought to act as a diffusion barrier to restrict the movement of molecules in and out of the bud neck. They also localize to the cleavage furrow in metazoans, but their role in cytokinesis is not well understood. [2, 3, 9, 10, 28-30]

1.3.4 Anillin

Anillin is a conserved protein, which is highly concentrated in the cleavage furrow and interacts with cytoskeletal components and their regulators during cytokinesis. Anillin is composed of N-terminal actin and myosin binding domains, an AHD (Anillin Homology Domain) that may interact with RhoA, Ect2 and Cyk-4, and a PH (Pleckstrin Homology) domain that possibly interacts with septins and the plasma membrane (Figure 1.3A). The C-terminal domains are crucial for Anillin's function and localization in *Drosophila* and mammalian cells (Figure 1.3B). [31, 32]

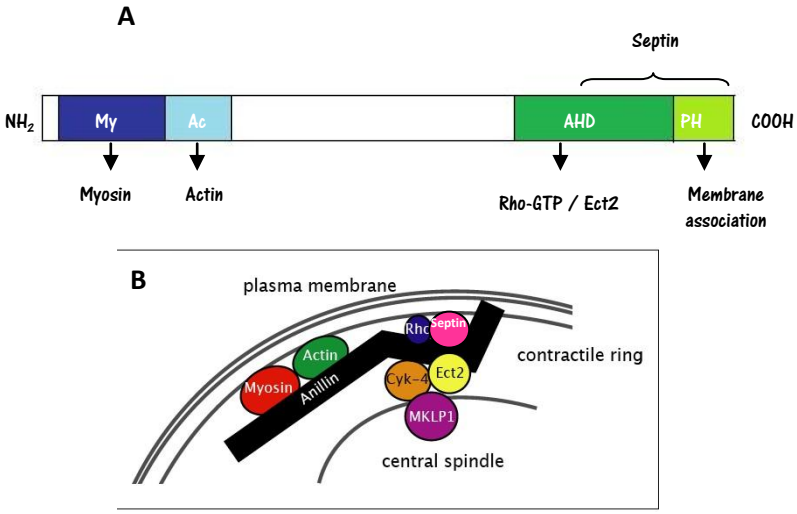

Figure 1.3. A schematic of A) Anillin multidomains, B) Anillin and its interacting partners.

Anillin was first isolated from *Drosophila* embryo extracts by its ability to bind F-actin. Anillin binds specifically to F-actin (through region 258-340 aa) and is able to bundle actin filaments *in vitro*. Recently, human Anillin was shown to interact with Formin through another region in its N-terminus (1-91 aa). It was proposed that this interaction helps to stabilize F-actin in the division plane, and could promote F-actin filament formation. [35] Anillin can also interact with active non-muscle myosin II (142–250 aa). Localization of both actin and myosin to the equatorial cortex occurs independently of Anillin (and vice versa), although their stable localization at the furrow requires Anillin. [32-34, 35] Depletion of *Drosophila* and human Anillin in spermatocytes and cultured cells causes actin and myosin to leave the cell equator, resulting in cortical oscillations and failed cytokinesis. [32, 34]

Human Anillin interacts with RhoA via its AHD (608-940 aa), which shares homology with Rhotekin, a RhoA-GTP binding protein. This region is crucial for RhoA localization *in vivo*, and can bind directly to RhoA *in vitro*. Depletion of *Drosophila* Anillin also reduces equatorial Rho in both S2 cells and spermatocytes. [31, 32] Human Anillin may also interact with Ect2, by which it ensures the stable localization or activation of RhoA in the cleavage plane. [36] However, the temporal regulation of these interactions is not known. [33] In *Drosophila*, RacGAP50C (Cyk-4) interacts with the AHD of Anillin *in vivo* and *in vitro*. [37] The central spindle does not extend to the cortex in Anillin-depleted cells in larval brains, suggesting that Anillin may have a role in localizing peripherally-associated microtubules. [31, 32] Anchoring these microtubules at the cortex may be important for establishing the division plane. They would ensure that Ect2 generates a discrete zone of active RhoA in the overlying cortex to form the contractile ring. It is not known if Anillin directly interacts with Cyk-4 in other organisms, but work from our lab suggests that in human cells, this microtubule anchoring function for Anillin is carried out by its potential interaction with Ect2. [32, 36- 38]

Anillin could possibly interact with septins, which are implicated in various cellular processes such as cytokinesis, vesicle trafficking and exocytosis in mammalian cells. In humans, 14 septins have been identified, several of which localize to the cleavage furrow in an anillin-dependent manner and are involved in cytokinesis. However, since there are a large number of septin proteins, which typically heteroligomerize, their specific roles in cytokinesis have remained elusive. [32, 38]

Given its many potential interactions with cytoskeletal components and their regulators, Anillin appears to be a global regulator of cytokinesis. [32]

1.4 *Caenorhabditis elegans*

1.4.1 *C. elegans* as a Model Organism

The nematode *C. elegans* has become an ideal model organism due to its anatomic and genetic simplicity. The small size (~ 1mm long adults, of ~1000 somatic cells), short generation time (2 days at 25 °C), rapid proliferation, large brood size (200-300 progeny), hermaphrodite sexual system, transparency, and susceptibility to RNAi make *C. elegans* a perfect model organism for the genetic and phenotypic analyses of different developmental processes. [39, 40] The complete genome sequence of *C. elegans* (*C. elegans* Sequencing Consortium, 1998) shows that it has about 20,000 genes, 60% of which are homologous to genes found in other organisms including humans. [39, 40] Therefore, in spite of its structural simplicity, *C. elegans* contains many genes that are also found in other eukaryotes, indicating conserved molecular, cellular and developmental mechanisms. [39, 40]

1.4.2 *C. elegans* Life Cycle

The life cycle of *C. elegans* occurs with such high conformity that any perturbations are easy to visualize. They reach adulthood within 5.5 days at 15°C, 3.5 days at 20°C and 2.5 days at 25°C. Embryogenesis is comprised of both proliferation and morphogenesis, during which the ovoid embryo is transformed into a vermiform worm consisting of 558 cells. [40, 41] Gastrulation, dorsal intercalation, ventral enclosure and elongation occur during morphogenesis where cells undergo migration and shape changes to form a worm with fully differentiated tissues and organs. [39- 41] Upon completion of embryogenesis, postembryonic development is initiated by feeding after hatching. [41] *C. elegans* adopts

four larval stages throughout its life cycle (Fig.1.4) and each larval stage ends in a molt. It can also go through an alternative form, known as dauer larva in the absence of food. They remain viable for months and reenter the L4 stage, upon administration of food, which is useful for synchronizing worm populations (Figure 1.4). [41-43]

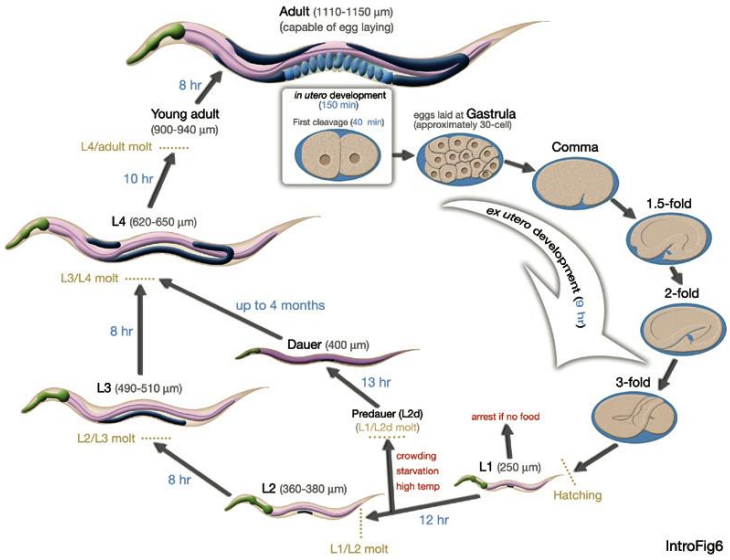

Fig.ure 1.4 *C. elegans* life cycle at 20°C; embryogenesis, L1-L4 and dauer larval stages.

Note: Figure is taken from http://www.wormatlas.org/ver1/handbook/fig.s/IntroFIG6.jpg ,Consulted 10/07/2010

1.4.3 Cytokinesis in *C.elegans*

Many genes that are essential for cytokinesis in the early *C. elegans* embryo have been identified due to the spatial and temporal fidelity of the first cell division. In addition, its syncytial gonad makes *C. elegans* amenable to RNAi, to study the function of specific genes in cytokinesis. Introduction of double stranded (ds) RNA into the gonad leads to the rapid destruction of target mRNA, such that newly formed oocytes are depleted of both the mRNA and corresponding protein. [44] Also, the large size of the newly fertilized egg makes it easy to identify phenotypes in early embryos using microscopic methods. [43]

As in other metazoans, *cdk-1* (previously known as *ncc-1*), the functional orthologue of mammalian Cdk1, is crucial for entry into mitosis and must be downregulated for mitotic exit. Also, centrosomally-derived microtubule asters and the central spindle position and form the actomyosin-based contractile ring. [45, 46]

Components of the microtubule and actomyosin cytoskeletons with roles in *C. elegans* cytokinesis can be divided into several groups. The first group is comprised of proteins such as RHO-1 and its downstream effectors CYK-1/Formin (functions with PFN-1/profilin for actin polymerization) and LET-502/ROCK (functions with MEL-11 to regulate MLC-4/rMLC phosphorylation), which are critical for contractile ring formation and contractility. [41] Another class of proteins includes centralspindlin (comprised of the kinesin ZEN-4/MKLP1 and CYK-4/MgcRacGAP) and the Chromosomal Passenger Complex (CPC; containing AIR-2/Aurora B kinase), which are essential for formation of the spindle midzone. [13, 44] Another group contains SPD-1/PRC1, which is also critical for forming the spindle midzone. In addition, Dynamin, RAB-11 and Syntaxin-4 are required for membrane fusion during abscission. [44]

C. elegans has three Anillin-like proteins, one of which, ANI-1, is required for contractile events in the early embryo. However its role in cytokinesis is still not fully understood. ANI-1 accumulates at the contractile ring and is required for the localization of UNC-59/UNC-61 (septin) to the furrow. ANI-1 is required for asymmetric furrow ingression and enhances cytokinesis phenotypes in combination with other cytokinetic regulators. It likely functions as a molecular component of both the central spindle pathway and the astral pathway, which dictates the division plane by an inhibitory mechanism, and functions redundantly in both processes. [30, 31, 44]

Although many genes that are essential for cytokinesis have been identified, there are many others that contribute to this process and have remained elusive due to their roles in other cellular processes (making their roles in cytokinesis difficult to distinguish), or due to their redundancy. To identify additional, novel regulators of cytokinesis, we took an approach that combines the power of both mammalian and *C. elegans* systems. We pulled down proteins from synchronized human cell lysates using the C-terminus of human Anillin and identified them by mass spectrometry. We then identified putative homologues in *C. elegans* and initiated a secondary screen in *C. elegans* to determine if any of these genes have cytokinesis phenotypes alone or in combination with other cytokinetic regulators.

2. Materials and Methods

2.1 Plasmid Construct

For the pulldown assay pGEX-Anillin C-term was used as the bait construct (Table 2.1)

Plasmid construct	Fusion	Epitope	Bacterial selection	Source
pGEX-Anillin C-term	GST-Anillin C-term (608-1087)	GST	ampicillin	A. Piekny

Table 2.1 Plasmid construct used in this study

2.2 Tissue Culture and Preparation of Cell Lysates

Hela cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent), 2mM L-Glutamine and 100 U penicillin/ 0.1mg/ml streptomycin (PS; Wisent) and kept in a humidified 5% CO_2 incubator at 37°C. Hela cells were synchronized with two thymidine (2.5mM, Bioshop) blocks (arrests cells in S phase), each for 14-16 hours. Seven hours after releasing cells from the second thymidine block, they were treated with 1µM nocodazole (Sigma-Aldrich)(40ng/ml; arrests cells in metaphase) for 3 hours, then released for 45 minutes to ensure mitotic exit and lysed when the majority of cells were in anaphase.[18] Cells at ~80-100% confluency from 15ml dishes were lysed in 1 ml of lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 3mM $MgCl_2$, 1% Triton-X100, 20mM NaF, 40mM β glycerophosphate, 1mM DTT) with protease inhibitor (Roche). [19] The lysates were

immediately placed on ice, and then centrifuged at maximum speed at 4^{o}C for 4 minutes. The supernatant was collected and added to ~20ug of purified Anillin protein for the pulldown assay (~7mg of total protein was used per pulldown).

2.3 Purification of Recombinant Proteins and Pull down Assay

2.3.1 Purification of GST-tagged Protein

A modified protocol for purifying GST-tagged proteins by Amersham Biosciences (Bulk and RediPack GST Purification Modules) was used to purify GST-Anillin C-term (608-1087). BL21 cells containing pGEX-Anillin C-term were grown at 37°C for 14–16 hours, aerated at 290 rpm. The overnight culture was diluted and grown to an OD_{600} of 0.3-0.7 (2-4 hours) at 37°C and gene expression was induced by the addition of 1mM IPTG (Bio Basic) with shaking at 290 rpm at 25°C for 4-5 hours. Cells were then pelleted by centrifugation at 4000xg for 20 minutes at 4°C. Pellets were then washed by resuspending them in 30ml cold 1XPBS (Phosphate Buffer Saline; 150mM NaCl, 3mM KCl, 10mM $Na_2HPO_4$and 2mM KH_2PO_4) and re-centrifuging them. After a second wash, the PBS was decanted from the pellets, which were then frozen at -80°C for future use.

To purify protein, frozen pellets were resuspended in 20ml of lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 3mM $MgCl_2$, 1% Triton-X100, 1mM DTT), supplemented with protease inhibitors 10µg/ml leupeptin (Bioshop), 10µg/ml pepstatin (Bioshop) and 1mM PMSF. 5mM lysozyme was added to the resuspended pellet and incubated on ice for 30 minutes, then sonicated three times, each time for 30 seconds by a series of non-consecutive 1 second pulses. Lysates were then centrifuged for 20,000xg for 30 minutes

at 4°C and the supernatant was incubated with 1 ml of pre-equilibrated Glutathione Sepharose (GE) on a nutator at 4°C for 3-4 hours. Beads were then washed three times with 10ml of lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 1% Triton-X100, 1mM DTT). Beads were analysed by SDS-PAGE to check their purity and their concentration was determined using ImageJ software to compare pixel densities with a standard protein (BSA) of known concentration (1mg/ml).

2.3.2 Pull down Assay

To pull down potential interactors of Anillin, ~7mg of anaphase synchronized cell lysates was incubated with ~20µg of purified GST-Anillin C-term at 4°C, overnight. GST-bound Glutathione Sepharose (GE) was also incubated with ~7mg of anaphase synchronized cell lysates as a control.

Pulldowns were fractionated by SDS-PAGE and stained for one hour with Bio-safe Coomassie (Bio-Rad) as per standard protocol. Proteins within the range of 80 to 200 KDa were excised and gel purified. In-gel digestions were performed according to a protocol kindly provided by Dr. Heng Jiang (Dr. Vincent Martin Lab, Concordia University). Gel pieces were cut into squares of 1mm × 1mm, placed into microfuge tubes, then 300µl of 10mM DTT, 50mM ammonium bicarbonate was added to the samples and incubated at room temperature for 30 minutes. The above solution was discarded and 300µl of 50mM iodoacetamide and 50mM ammonium bicarbonate was added to the gel pieces and incubated as above (protected from light). After removing the solution, 300 µl of 50mM ammonium bicarbonate was added to the samples and incubated for 15 minutes at room temperature. Following this step, 300 µl of 25mM

ammonium bicarbonate and 5% acetonitrile was added to the samples and incubated as above. The solution was discarded and 300µl of 25mM ammonium bicarbonate and 50% acetonitrile (Burdik & Jackson, kindly provided by Dr. Vincent Martin's lab, Concordia University) was added to each tube and incubated for 30 minutes at room temperature (this step was repeated). After removing this solution, 300µl of 100% acetonitrile was added to the samples and incubated for 10 minutes at room temperature. The acetonitrile was discarded and gel pieces were dried using a SpeedVac for 10-20 minutes. Following this treatment, 50µl of trypsin solution (20µg of trypsin (Wisent) in 2.0ml of 25mM ammonium bicarbonate) was added to each tube and samples were incubated overnight at 30°C. The following day, a four-fold volume of trypsin solution with 0.5% formic acid and 60% acetonitrile was added to the samples and incubated for 30 minutes at room temperature. The solution was then collected and the above step was repeated three times. Samples were collected and dried in a SpeedVac at medium temperature overnight and sent for mass spectrometry. Peptide identification was performed by Dr. Heng Jiang (Dr. Vincent Martin Lab, Concordia University) using a Thermo Scientific linear ion-trap mass spectrometer equipped with an electrospray ionization source. The resulting spectra were searched against a human database downloaded from NCBI using SEQUEST from Bioworks 3.3.1. In addition, gels were brought to the McGill Mass Spectrometry Core Facility where proteins were excised from the gels, purified and analyzed by a Bruker ESI ion-trap machine on site. MASCOT software was used to search the resulting spectra. All spectra were compared against those collected from the control and only proteins unique to the Anillin pulldown were considered for further study. Furthermore, only spectra with coverage higher than 5% were considered.

2.4 *C. elegans* Strains and Genetics

2.4.1 Nematode Strains

Bristol N2 and *mlc-4 (or 253)/qCI* mutant worms were used as the wild-type and mutant strains, respectively. Worms were maintained on NGM plates (3g NaCl, 2.5g Bactopeptone, 17g Agar) supplemented with, 1ml $CaCl_2$ (1M), 1ml $MgSo_4$ (1M), 1ml cholesterol in ethanol (5mg/ml) and 25 ml PPB pH 6 (98g KH_2PO_4 and 48g K_2HPO_4) seeded with the E. Coli strain OP50 (CGC – *Caenorhabditis* Genetics Center, University of Minnesota) and kept at 15°C. [47]

2.4.2 RNA Isolation and cDNA Synthesis

Total RNA was isolated from worms using Trizol (Invitrogen). Adult hermaphrodite worms were washed from a highly populated (but not starved) 6cm plate with 1ml DEPC (Sigma-Aldrich)-treated water and transferred to a microfuge tube. The tube was centrifuged at 4000xg for 1 minute, then the supernatant was discarded and 1ml Trizol (Invitrogen) was added. The tube was vortexed and incubated at room temperature for 20 minutes, after which it was centrifuged at 14000xg for 10 minutes at 4°C. The supernatant was transferred to a fresh, RNase free tube and 200µl of chloroform was added. The tube was vortexed for 15 seconds, incubated at room temperature for 3 minutes, then centrifuged at 12000xg for 15 minutes at 4°C. Following this step, the supernatant was transferred to a fresh, RNase free tube and 500µl of isopropanol was added. It was then incubated at room temperature for 10 minutes and centrifuged at 7500xg for 5 minutes at 4°C. The supernatant was removed and the pellet was air dried for 10 minutes, then dissolved in 25µl of DEPC-treated water.

cDNA was synthesized using SuperScript^{TM} III First Strand Synthesis kit (Invitrogen). Briefly, 1µl of 50µM oligo dT, 1X 10mM dNTP mix and 5µg of total RNA was mixed in an RNase-free microcentrifuge tube (final volume 10µl), incubated at 65°C for 5 minutes, then placed on ice for at least 1 minute. 10µl of cDNA synthesis mix (2X reaction buffer, 10mM $MgCl_2$, 0.02M DTT, 4 units RNAseOUT, 20 units Superscript^{TM}III RT) was added to the tube and incubated at 50°C for 50 minutes. The reaction was then terminated by incubating the tube at 85°C for 5 minutes, then chilling it on ice. 1µl of RNaseH was added and incubated for 20 minutes at 37°C, after which it was stored at -20°C.

2.4.3 Double Stranded (ds) RNA Synthesis

DNA templates for dsRNA synthesis were prepared using the *C.elegans* cDNA library synthesized as described in 2.4.2 and primers designed to contain the T7 RNA polymerase promoter at their 5' ends (Table 2.2). PCR products were obtained using different annealing temperatures (45-50°C for 30 to 45 seconds) and mixes with 1X buffer, 200µM dNTP, 50ng template, 0.05µM each of forward and reverse primers and 1.25U Taq DNA polymerase. All reactions were run on 1% agarose gels by electrophoresis to determine their size, purity. Concentration was determined using a spectrophotometer. Bands of the appropriate size were excised and gel purified as per manufacturer's instructions (Qiagen).

Oligonucleotide	Sequence(5'-3')
rho-1-F	TAATACGACTCACTATAGGGAGAATGGCTGCGATTAGAAAGAAG
rho-1-R	TAATACGACTCACTATAGGGAGACAAAATCATGCACTTGCTCTTC
gex-2-F	TAATACGACTCACTATAGGGAGAAGATAATCGAGAATGTCCTGA
gex-2-R	TAATACGACTCACTATAGGGAGATCGGTCATCGAATCTGAGAGC
crml-1-F	TAATACGACTCACTATAGGGAGAATATCTGGAATCATCAATCTGG
crml-1-R	TAATACGACTCACTATAGGGAGAAGAAGAACTTTATTGATCACATGC
nol-1-F	TAATACGACTCACTATAGGGAGACGGCCTGGAAGGTCTCAGCTTCCC
nol-1-R	TAATACGACTCACTATAGGGAGACGTTTAACCTTGAGCGAGTTGGCT
klp-12-F	TAATACGACTCACTATAGGGAGACTGAACGAGATCGAGTCTTGAA
klp-12-R	TAATACGACTCACTATAGGGAGACACCATTACAGCATGCCGTTC
mlk-1-F	TAATACGACTCACTATAGGGAGATCGACAATGCCGATGAGGTA
mlk-1-R	TAATACGACTCACTATAGGGAGATGGTATGTAGTCGTCACAACC
fhod-2-F	TAATACGACTCACTATAGGGAGAATCACCGTAATTGACCCGAGA
fhod-2-R	TAATACGACTCACTATAGGGAGATCTGCAGTAGCCTTCTCGATG
ced-5-F	TAATACGACTCACTATAGGGAGACGTACGCCTCGGACATCATG
ced-5-R	TAATACGACTCACTATAGGGAGACGAAGTCTCACGATCATGCCA
him-1-F	TAATACGACTCACTATAGGGAGAATCGCTGCGGTTCATCAGGAG
him-1-R	TAATACGACTCACTATAGGGAGAATTGCCTTGGCAGCAGTCTCT
kin-25-F	TAATACGACTCACTATAGGGAGAGACCGCCATTCGAATATGCC
kin-25-R	TAATACGACTCACTATAGGGAGATGTGGCAGATGGCAGACGAGT

Table 2.2 Oligonucleotides used in this study

Double stranded RNA synthesis was performed as described in the MEGAscript^{R} kit instruction manual (Ambion). Briefly, 0.5µg of gel-purified PCR product was added to a reaction mixture containing 1 X buffer, 1 X enzyme reaction mix, 7.5mM each of ATP, GTP, CTP and UTP solutions, and incubated at 37°C for 4 hours. 0.1 U TURBO DNase was then added to the mixture and incubated at 37°C for 15 minutes. To precipitate dsRNA, 3M LiCl was added to the reaction mix and chilled at -20°C, 2-4 hours. RNA was then pelleted by centrifugation at 4°C for 15 minutes at maximum speed. The supernatant was discarded and the pellet was washed with 1ml of cold 70% ethanol and centrifuged as above. The ethanol was removed and the RNA was resuspended in 15µl of nuclease free water and stored at -80°C for future use. All dsRNA was checked by agarose gel electrophoresis to check their purity and concentration was determined using a spectrophotometer.

2.4.4 RNAi by Soaking

5-10 L4 N2 and *mlc-4 (or 253)/qCI* mutant larvae were picked and transferred to an NGM plate without OP50. The worms on the plate were placed into a microfuge tube by washing the plate with 1 X soaking buffer (0.25X M9 (Mg^{2+} free), 3 mM spermidine (SIGMA), 0.05% gelatin (kindly provided by Dr. Zerges's lab, Concordia University) and transferring the liquid to the tube using a Pasteur pipette. The tube was centrifuged at 400xg for 30 seconds, then the majority of buffer was removed leaving the worms in ~20 µl. 1-2 µg of dsRNA was then added to the buffer, gently mixed and the worms were incubated at 20°C, overnight. The next day, the worms were transferred to new NGM plates with OP50 (1 worm per plate) and incubated at 20°C for 24 hours, these are plate 1 (P1). The following day, the mothers were transferred to new NGM plates with OP50 and incubated as above, these are plate 2 (P2). The P1 plates were then checked to count the number of eggs the worms laid. The next day, P1 plates were checked to count the number of eggs that hatched. Mothers on P2 plates were transferred to new NGM plates with OP50 (P3 plates) and checked to count the number of eggs. These plates were analyzed the following day to check hatching rates. [48]

3. Results

3.1 Identification of Potential Interactors of Anillin

3.1.1 Pull down Assay

Anillin is a conserved protein that is required for cytokinesis and localizes to the cleavage furrow. [29] Previous studies have shown that the N-terminus of Anillin interacts with actin and myosin, two major components of the cytoskeleton that form the contractile ring, while the C-terminus of Anillin may interact with their upstream regulators, RhoA, Ect2 and Cyk-4 in addition to septins and the plasma membrane. [7, 19, 36] Since the C-terminus of Anillin may interact with the upstream components of the core cytokinesis machinery, other interactors of this region could similarly regulate cytokinesis. Some of these proteins could be novel and have redundant functions for cytokinesis, precluding their identification from reverse genetic screens, or be required for other cytoskeletal events.

A mass-spectrometry approach was used to identify interactors of the C-terminus of human Anillin from pull down assays using lysates from synchronized human cells. Recombinant GST tagged Anillin (C-term) was bacterially expressed and purified, and incubated with lysates collected from anaphase-synchronized Hela cells (Figure 3.1). Recombinant GST was used as a control, and also was incubated with lysates (Figure 3.1). Several proteins in the range of 80 to 200 KDa were enriched in the Anillin pull down and were excised; gel purified and sent for analysis by mass spectrometry. Proteins

below this range were likely break down products of Anillin, based on Western blotting using anti-Anillin antibodies (data not shown).

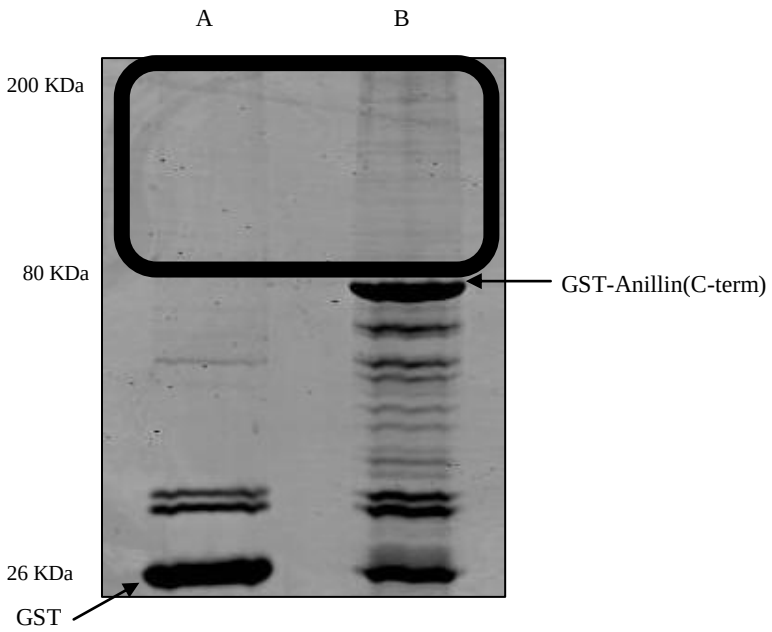

Figure 3.1 Pull down assay to probe potential interactors of Anillin. Coomassie stained 12% SDS polyacrylamide gel. A) GST-bound Gluthatione Sepharose. B) GST tagged C-terminus of Anillin. Black box indicates proteins within the range of 80 to 200 KDa, which were excised and gel purified.

3.1.2 Mass spectrometry

Anillin is likely a global regulator of cytokinesis and its interactors could fall into several categories based on predicted functions. Some could be upstream signaling proteins or regulators of Rho GTPases (*i.e.* GAPs and GEFs) that modify the actin-myosin cytoskeleton. Others could be components of the mitotic machinery (*i.e.* microtubule associated proteins), while some could modulate the plasma membrane (*i.e.* trafficking). Indeed, mass spectrometry results (Table 3.1) indicated that the co-purified proteins are signaling proteins,

GTPase regulators, cytoskeletal components, mitotic proteins and modulators of the plasma membrane. The identification of these many different types of proteins supports the hypothesis that Anillin is a global regulator of cytokinesis.

Table 3.1 Preliminary mass spectrometry results

Accession number	Peptide
gi|7022746|	unnamed protein product [Homo sapiens]
gi|7581970|	epithelial microtubule-associated protein [Homo sapiens
gi|27477138|	zinc finger antiviral protein isoform 2 [Homo sapiens]
gi|189422|	Proliferating cell nuclear protein P120
gi|13514831|	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10 [Homo sapiens]
gi|55956788|	nucleolin [Homo sapiens]
gi|32189362|	PTPRF interacting protein alpha 3 [Homo sapiens]
gi|91979656|	immunoglobulin heavy chain variable region [Homo sapiens]
gi|2119645|	A36429 integrin beta-4 chain precursor
gi|193787632|	unnamed protein product [Homo sapiens]
gi|119619384|	connector enhancer of kinase suppressor of Ras 2, isoform CRA_c [Homo sapiens]
gi|119614361|	hCG1642699, isoform CRA_b [Homo sapiens]
gi|10047197|	KIAA1566 protein [Homo sapiens]
gi|30725047|	KIAA1708[Homo sapiens]
gi|18860916|	5'-3' exoribonuclease 2 [Homo sapiens]
gi|685120|	insulin activator factor; INSAF [Homo sapiens]
gi|224465158|	hypothetical protein LOC285941 [Homo sapiens]
gi|545357|	Brefeldin A-inhibited guanine nucleotide-exchange protein-1
gi|51476855|	hypothetical protein [Homo sapiens]
gi|3319956|	E1B-55kDa-associated protein [Homo sapiens]
gi|119622078|	hCG1994437 [Homo sapiens]
gi|10798804|	sperm antigen [Homo sapiens]
gi|14141152|	heterogeneous nuclear ribonucleoprotein M isoform a [Homo sapiens]
gi|8923040|	CDKN2A interacting protein [Homo sapiens]
gi|32483374|	nucleolar protein 5A [Homo sapiens]
gi|118498359|	ribosomal L1 domain containing 1 [Homo sapiens]
gi|12654399|	Guanine nucleotide binding protein-like 3 (nucleolar) [Homo sapiens]
gi|21732307|	RAB11FIP4RAB11family interacting protein4 [Homo sapiens]
gi|12697991|	KIAA1723 protein [Homo sapiens]
gi|16507237|	heat shock 70kDa protein 5 [Homo sapiens]
gi|34419635|	heat shock 70kDa protein 6 (HSP70B') [Homo sapiens]
gi|38488438|	immunoglobulin heavy chain V-D-J region [Homo sapiens]
gi|24234686|	heat shock 70kDa protein 8 isoform 2 [Homo sapiens]
gi|10435466|	unnamed protein product [Homo sapiens]

Table 3.1 Cont'd Preliminary mass spectrometry results

Accession number	Peptide
gi|27693901|	PLEKHA7 protein [Homo sapiens]
gi|51095055|	similar to Chain , Heat-Shock Cognate 70kd Protein (44kd Atpase N-Terminal Fragment) (E.C.3.6.1.3) Mutant With Asp 206 Replaced By Ser (D206s) [Homo sapiens]
gi|4758138|	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 [Homo sapiens]
gi|4757926|	RNA binding motif protein 39 isoform b [Homo sapiens]
gi|1679766|	IgM heavy chain variable region [Homo sapiens]
gi|19353264|	similar to disheveled associated activator of morphogenesis 2 [Homo sapiens]
gi|13676857|	heat shock 70kDa protein 2 [Homo sapiens]
gi|4502027|	albumin preproprotein [Homo sapiens]
gi|193786411|	unnamed protein product [Homo sapiens]
gi|42733592|	hypothetical protein LOC399693 [Homo sapiens]
gi|27463365|	a disintegrin-like and metalloprotease with thrombospondin type 1 motifs 9B [Homo sapiens]
gi|27501450|	axin 1 isoform a [Homo sapiens]
gi|22535296|	SMC1 structural maintenance of chromosomes 1-like2; mitosis specific chromosome segregation protein like protein beta[Homo sapiens]
gi|34533851|	CYFIP1 cytoplasmic FMR1 interacting protein1[Homo sapiens]
gi|2119276|	beta-tubulin - human (fragment)
gi|14389309|	tubulin alpha 6 [Homo sapiens]
gi|21361322|	tubulin, beta 4 [Homo sapiens]
gi|25149258|	Human mixed lineage kinase homolog [Homo sapiens]
gi|68077166|	DnaJ homology subfamily A member 5 isoform 2 [Homo sapiens]
gi|5031753|	heterogeneous nuclear ribonucleoprotein H1 [Homo sapiens]
gi|17538408|	cell death abnormality CED-5, cell corpse engulfment protein, similar to human major CRK-binding protein DOCK 180, dedicator of cytokinesis[Homo sapiens]
gi|4758012|	clathrin heavy chain, heavy polypeptide like2[Homo sapiens]
gi|4758032|	coatomer protein complex, subunit beta 2 (beta prime) [Homo sapiens]
gi|4758012|	tyrosine kinase, non-receptor, 1[Homo sapiens]
gi|9910376|	inner centromere protein antigens135/155kDa[Homo sapiens]
gi|13994268|	Ubiquitin-specific protease 26 [Homo sapiens]
gi|209512812|	capping ARp2/3 Myosin Linker[Homo sapiens]

Since the core cytokinesis machinery is well-conserved across eukaryotes, additional regulators and components of cytokinesis should also be well-conserved. Therefore, proteins

with the highest scores (*i.e.* greatest number of peptides, and not pulled down by GST alone) were examined for their conservation in *Caenorhabditis elegans*, a model organism that has been used extensively for cytokinesis studies (Table 3.2).

Table 3.2 proteins identified by mass spectrometry with homologs in *C. elegans*

Accession number(Human)	Human	*C. elegans*
GI 34533851	CYFIP1 cytoplasmic FMR1 interacting protein 1	Gut on Exterior (GEX-2)
GI 209512812	CARMIL (Capping ARp2/3 Myosin Linker)	CARMIL (Capping ARp2/3 Myosin Linker) (CRML-1)
GI 19353264	Similar to dishevelled associated activator of morphogenesis 2	Formin Homology Domain (FHOD-2)
GI 30725047	KIAA 1708	Kinesin like protein (KLP-12)
GI 22535296	SMC1 structural maintenance of chromosomes 1-like 2;Mitosis specific chromosome segregation protein like protein beta	High incidence of Males (increased X chromosome loss) (HIM-1)
GI 25149258	Human Mixed Lineage Kinase homolog (MLK-1)	Human MLK homolog (MLK-1)
GI 17538408	Cell Death abnormality CED-5, cell corpse engulfment protein, similar to human major CRK-binding protein DOCK 180, dedicator of cytokinesis	Cell Death abnormality (CED-5)
GI 4507611	Tyrosine kinase, non-receptor, 1	Protein Kinase (KIN 25)
GI 189422	Proliferting cell nuclear protein P120	Nucleolar protein (NOL-1)

These proteins (Table 3.2) have various functions in *C.elegans*. GEX-2 and CRML-1 are involved in cell migration. GEX-2 mediates Rac signaling necessary for cell migration while CRML-1 functions antagonistic to the Rac pathway and inhibits migration. [49, 50] FHOD-2 is required for cell polarization in asymmetric divisions and modulating cell migration in

C.elegans. [52] KLP-12 is a motor protein which is crucial for organizing chromosomes at the metaphase plate, and its depletion may result in embryonic lethality. [53] HIM-1 is also critical for embryonic viability and plays a role in mitotic chromosome condensation, sister chromatid cohesion and meiotic chromosome segregation. [44, 54] MLK-1 is a MAPK Kinase Kinase which functions in a JNK (c-Jun N-terminal Kinase) - like pathway in *C. elegans* and is essential for stress response to heavy metals. [55] CED-5 is one of the proteins involved in the removal of cell corpses generated by programmed cell death during the C. *elegans* life cycle. [56, 57] KIN-25 is a member of the Ack superfamily of protein kinases which may be required for regulation of vesicle dynamics. [58, 59] NOL-1 is a nucleolar protein whose function is elusive in *C.elegans.* [60]

3.2 RNAi based Screens to Identify Novel Regulators of Cytokinesis in *C. elegans*

C. elegans was chosen as a model to determine if the homologues of some of the genes identified from the mass spectrometry experiment (Table 3.2) are involved in cytokinesis. *C. elegans* has both anatomic and genetic simplicity, and is susceptible to RNAi. [39, 40] Furthermore, *C. elegans* contains many genes that are also found in other eukaryotes, indicating conserved molecular, cellular and developmental mechanisms. [39, 40] In particular, many of the proteins that form the core cytokinesis machinery are conserved in *C. elegans,* and their disruption by RNAi results in embryonic lethality due to cytokinesis defects. Therefore, to assess if a protein is essential for cytokinesis, after exposing *C. elegans* to dsRNA to reduce the levels of that protein, the embryos should display embryonic lethality due to cytokinesis defects. If the protein is redundant, then

performing dsRNA in a genetically sensitized background should cause synthetic embryonic lethality, or alleviation of phenotypes depending on the function of the protein. With these predictions in mind, RNAi was performed to determine if any of the proteins identified in Table 3.2 are required for cytokinesis.

3.2.1 Gene Amplification and dsRNA Synthesis

To study the proteins listed in Table 3.2 for possible roles in cytokinesis in *C. elegans*, an RNAi-based screen was performed. To screen the proteins listed in Table 3.2 *C. elegans* worms were exposed to fragments (~500-1500bp) of dsRNA generated from each gene of interest. To generate dsRNA for the genes listed in Table 3.2, first, a cDNA library was generated using mRNA collected from wild-type *C. elegans*. Total RNA was extracted and purified, and then PCR was used to amplify cDNA from the genes of interest (Figure 3.2A) using gene-specific primers. The T7 promoter was added to the 5' end of all primers for RNA synthesis *in vitro* using T7 RNA polymerase. Since complementary RNA was synthesized from both cDNA strands simultaneously, they hybridized to generate dsRNA. Fragment sizes were generated within the range of 500 to 1500 base pairs (Figure 3.2B) and were quantitated before use.

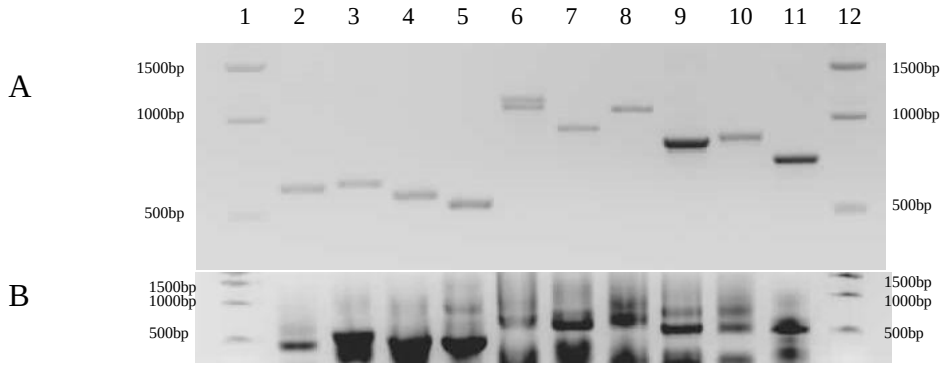

Figure 3.2 A) Agarose gel analysis of PCR products. B) Agarose gel analysis of dsRNA. 1) 1Kb ladder, 2) *rho-1*, 576bp, 3) *gex-2*, 597bp, 4 *crml-1*, 545bp 5*) nol-1*, 540bp, 6) *ced-5*, 1043bp, 7) *him-1*, 878bp, 8) *kin-25*, 1011bp, 9) *fhod-2*, 764bp, 10) *mlk-1*, 797bp, 11*) klp-12*, 695bp 12) 1Kb ladder

3.2.2 RNAi-based Screens

Both wild-type, and *mlc-4(or253)/qC1 C. elegans* strains were used in this study. There are three main methods of delivering dsRNA in *C. elegans*; feeding, soaking and injection, and the soaking method was used due to its high efficiency. L4-staged larva or young adults were incubated with dsRNA for the genes in Table 3.2 and analyzed for embryonic lethal phenotypes (as a negative control, N2 worms were soaked in buffer without RNA). Since some of these genes could be redundant, dsRNA was also performed using *mlc-4*/*qC1* worms, a strain that carries a mutation in the gene for nonmuscle myosin light chain, which is required for cytokinesis. This strain is heterozygous and is maternally rescued for the cytokinesis defect (as a negative control, *mlc-4/qC1* worms were soaked in buffer without RNA). RHO-1 is required for formation of the actin-myosin contractile ring and its knockdown by RNAi results in early

embryonic lethality and sterility. Therefore, dsRNA to *rho-1* was used in all of the experiments as a positive control.

The only dsRNA that displayed embryonic lethality with N2 worms was *him-1*. For each dsRNA treatment, three broods of multiple worms (the same mothers transferred twice to new plates) were checked to count the number of eggs the worms laid and the number of eggs that hatched. As stated above, *rho-1* knock down worms were used as a positive control to check the efficiency of RNAi. These worms typically laid 10-20 eggs per plate that failed to hatch (100% embryonic lethality; Table 3.3). N2 worms that were not treated with dsRNA, which were used as the negative control, typically laid 70-100 eggs per plate and all embryos hatched (0% embryonic lethality; Table 3.3). Worms exposed to *him-1* dsRNA showed 6-10% lethality in the first and second broods, respectively, and 30% lethality in the third brood (Table 3.3). Therefore, *him-1* is an excellent candidate for follow-up studies to determine if the embryonic lethal phenotype is due to cytokinesis defects.

Table 3.3 Wild-type (N2) treated with dsRNA as indicated.

	Bristol N_2								
	Brood 1			Brood 2			Brood 3		
gene	eggs	larvae	%embryonic lethality	eggs	larvae	%embryonic lethality	eggs	larvae	%embryonic lethality
rho-1	90	-	100%	73	-	100%	70	-	100%
gex-2	398	398	0%	305	305	0%	322	322	0%
crml-1	411	411	0%	377	377	0%	395	395	0%
nol-1	420	420	0%	398	398	0%	408	408	0%
him-1	315	296	6%	430	387	10%	309	216	30%
ced-5	386	386	0%	379	379	0%	383	383	0%
kin-25	432	432	0%	429	429	0%	417	417	0%
fhod-2	330	330	0%	346	346	0%	298	298	0%
Mlk-1	472	472	0%	463	463	0%	480	480	0%
Klp-12	466	466	0%	447	447	0%	456	456	0%
-	498	498	0%	502	502	0%	517	517	0%

The other candidates could still be required for cytokinesis in a redundant manner. Therefore, *mlc-4(or253)/qCI* mutant worms were treated with dsRNA to determine if they display embryonic lethality. This strain is maternally rescued for cytokinesis and does not have embryonic lethality, but produces *mlc-4/mlc-4* embryos with elongation defects due the requirement of *mlc-4* in morphogenesis, a later developmental process.

Several genes that did not display lethality with N2 worms showed synergistic lethality with *mlc-4/qC1*. *rho-1* RNAi showed 100% embryonic lethality similar to N2 and no

lethality was observed in the negative control (*mlc-4*/*qC1*). However, *him-1*, *nol-1* and *fhod-2* all showed higher embryonic lethality 30-42%, 38-48% and 31-42%, respectively (Table 3.4). Since *him-1* has extensive embryonic lethality in N2 worms, it is possible that the enhanced lethality in *mlc-4/qC1* worms is not significantly different. However, since *nol-1* and *fhod-2* both did not have lethality in N2 worms, these are good candidates for follow-up studies to determine if they have redundant roles in cytokinesis.

Table 3.4 *mlc4(or253)/qCI* mutant worms treated with dsRNA as indicated.

	mlc4/qCI mutant								
	Brood 1			Brood 2			Brood 3		
Gene	eggs	larvae	%embryonic lethality	eggs	larvae	%embryonic lethality	eggs	larvae	%embryonic lethality
rho-1	52	-	100%	37	-	100%	22	-	100%
gex-2	253	250	1%	230	230	0%	241	237	1.6%
crml-1	208	198	4.8%	211	203	3.8%	198	188	5%
nol-1	125	78	37.6%	117	63	46%	98	51	48%
him-1	103	60	41.7%	126	77	39%	109	76	30.2%
ced-5	256	256	0%	245	245	0%	244	244	0%
kin-25	237	237	0%	246	246	0%	215	215	0%
fhod-2	114	79	31%	97	56	42.2%	105	62	41%
Mlk-1	255	255	0%	275	275	0%	268	268	0%
Klp-12	207	207	0%	211	211	0%	205	205	0%
-	398	398	0%	401	401	0%	423	423	0%

4. Discussion

4.1 Identification of Novel Regulators of Cytokinesis

Cytokinesis describes the division of cytoplasm to produce two daughter cells, due to the formation and ingression of an actin-myosin contractile ring. The position and assembly of the contractile ring is directed by the mitotic spindle during anaphase. While proteins such as RhoA, its upstream regulators and downstream effectors have well-described roles in cytokinesis, there are likely many more proteins that contribute to cytokinesis by modulating the actin-myosin cytoskeleton, regulating the formation and positioning of the mitotic spindle and regulating the plasma membrane. Some of these proteins likely have not been previously described due to their pleiotropic roles in other processes, or due to redundancy. To further understand the regulation of cytokinesis, new players in this process were identified using a combined mass spectrometry and genetic approach that involves both mammalian cells and *C.elegans*.

The C-terminus of human Anillin was used to pulldown proteins from anaphase-synchronized human cell lysates. The proteins were then identified by mass spectrometry and their putative homologues in *C. elegans* were employed to perform a secondary RNAi-based screen in *C. elegans* to determine if any of these genes have cytokinesis phenotypes alone or in combination with a known cytokinetic regulator.

The proteins that were identified fell into categories based on predicted functions. Genes that could potentially modify the actin-myosin cytoskeleton, function as a signaling protein, or regulate the mitotic spindle were chosen for further analysis.

4.1.1 GEX-2/CRML-1

Several proteins that modulate the actin-myosin cytoskeleton are involved in the migration and fusion of cells, or cell shape changes that occur during *C.elegans* embryogenesis. GEX-2 accumulates at the cell boundaries and mediates Rac signaling necessary for epidermal cell fusion and migration. [49] CRML-1 also has a role in cell migration; however it has an inhibitory effect on cell and axon migration and acts in parallel to UNC-34, which is a member of Ena/VASP family of proteins that can bind to F-actin. Thus, CRML-1 inhibits migration through the Rac pathway. [50] Rac is a known regulator of the actin-myosin cytoskeleton for cell migration events and works antagonistically to RhoA. What is interesting about this is that Rac is predicted to be involved in cytokinesis, but its exact role remains elusive. [22, 51]

Based on GEX-2 function, it could function antagonistic to RHO-1, and possibly could cause embryonic lethality, but the cytokinesis defect could arise from a hyper-contracted or mispositioned ring. Interestingly, previous studies showed that RNAi to *rac* suppresses *cyk-4*, an upstream regulator of *rho-1* during cytokinesis. [22, 51] However, no lethality was observed in wild-type worms and RNAi treated *mlc-4/qC1* worms showed only slight enhancement (1.6%). One follow up study could be to look for suppression of *rho-1* lethality.

CRML-1 could function antagonistically to GEX-2, and any cytokinesis defects could be similar to those observed for RHO-1, with failed contractile ring formation. However, embryonic lethality was not observed in N2 worms and RNAi to *mlc-4/qC1*worms resulted in a slight enhancement (5%). To further study CRML-1, RNAi could be performed in other sensitized strains such as *let502* or *mel-11* mutant worms.

4.1.2 FHOD-2

Signal transduction pathways regulate cell communication throughout development. One type of pathway is regulated by Wnt ligands, including the Wnt/β-catenin, Wnt/planar cell polarity (PCP) and Wnt/Calcium pathways. The activity of Dishevelled (Dsh) proteins partially determines which Wnt pathway is activated. Wnt signaling is crucial for embryonic and larval stages of development to determine cell fate, to polarize cells in asymmetric divisions and to control cell migration in *C. elegans*. Formin homology domain (FHOD-2) is one of the downstream regulators of Dsh in the PCP signaling pathway and contains domains that share homology with formins, well-studied effectors of RhoA and regulators of actin polymerization. [(52)] One possible role for FHOD-2 could be to similarly regulate F-actin during cytokinesis and if so, then its removal should cause cytokinesis defects and embryonic lethality, and should enhance myosin phenotypes. Although *fhod-2* RNAi alone did not cause any lethality, it did enhance *mlc-4/qC1*. Therefore, *fhod-2* may share redundant functions with other proteins, but is more essential for embryonic viability when the embryos have been sensitized by loss of myosin.

4.1.3 KLP-12

Kinesins are molecular motor proteins that move on microtubules and generate mechanical force by ATP hydrolysis. *C.elegans* has 21 kinesins, among which KLP-15, KLP-16 and KLP-17 are unique to *C.elegans*. According to their motor domain sequence, *C. elegans* kinesins are divided into different groups one of which is the chemokinesin group that includes *C.elegans* KLP-12. It is highly expressed in embryonic and postembryonic development. RNAi mediated depletion of *klp-12* causes abnormal spindle organization and polyploid cells suggesting that it is essential for organizing chromosomes at the metaphase plate. [53] Some kinesins function in spindle assembly, and have additional roles in cytokinesis (*eg.* Prc1 and MKLP1). In this study, RNAi of *klp-1* by soaking did not cause any embryonic lethality in N2 worms or in combination with *mlc-4/qC1*. This suggests that the RNAi soaking method for this particular gene is not efficient and an alternate method should be used for further study (such as feeding or injection).

4.1.4 HIM-1

Structural maintenance of chromosomes (SMC) complexes are required for various chromosome dynamics such as X-chromosome dosage compensation in *C.elegans*, mitotic chromosome condensation, sister chromatid cohesion and meiotic chromosome segregation. HIM-1, the *C. elegans* homolog of SMC-1, functions in the mitotic germline and a reduced number of germ cells and abnormal DNA morphology are observed in *him-1* mutants. HIM-1 localizes to interphase chromosomes and is required for embryonic viability. [44, 54] Embryonic lethality was observed with N2 and *mlc-4/qC1*

worms, consistent with previous studies. However, since HIM-1 has known early meiotic and mitotic functions, further studies are needed to unravel any possible roles for HIM-1 in cytokinesis.

4.1.5 MLK-1

Mitogen activated protein kinase (MAPK) signal transduction pathways are well conserved among eukaryotes throughout evolution and transduce signals in response to various extracellular signals. Each pathway is comprised of three groups of protein kinases: MAPK, MAPKK (MAPK Kinase) and MAPKKK (MAPK Kinase Kinase). One of these pathways is called the JNK (c-Jun N-terminal Kinase) pathway and is required for stress response and immune signaling in mammals. *C. elegans* also has a JNK-like MAPK pathway that mediates stress response to heavy metals and is composed of MLK-1 MAPKKK, MEK-1 MAPKK and KGB-1 MAPK. [55] The JNK pathway also cross-talks with the Rho pathway during *Drosophila* development, to mediate cell migration and cell shape changes for dorsal closure to define the dorsal ectoderm. [55] Therefore, MLK-1 is a good candidate for Rho-mediated events in *C. elegans*, including cytokinesis. However, no embryonic lethality was observed by *mlk-1* RNAi in N2 or *mlc-4/qC1* worms. Therefore, if there is a role for *mlk-1* in cytokinesis, it could be redundant and further analysis is required.

4.1.6 CED-5

The engulfment process is used to remove cell corpses generated by programmed cell death throughout *C. elegans* development. Two parallel and partially redundant pathways mediate this process. One pathway is comprised of *ced-1, ced-6* and *ced-7* genes and the

other consists of *ced-2*, *ced-5*, *ced-10* and *ced-12*. CED-2, CED-5 and CED-12 form a ternary signaling complex in response to upstream engulfment signals to activate CED-10, a homolog of human Rac. Active CED-10 is required for the rearrangement of cytoskeleton necessary for the cell corpse engulfment process. [56, 57] Since Rac typically functions antagonistically to RhoA, any functions for *ced-5* in cytokinesis could be different from *rho-1*, and as described for *gex-2*, its depletion could cause hyper-contracted or mispositioned contractile rings. No embryonic lethality was observed for *ced-5* RNAi in N2 or *mlc-4/qC1 worms*. However, a better way to further analyze this protein will be to examine its suppression of RHO-1 pathway members.

4.1.7 KIN-25

Protein kinases modify the activity, location and affinities of various proteins in the cell and mediate signal transduction. KIN-25, a member of the Ack superfamily of cytoplasmic tyrosine kinases, is a non-receptor tyrosine kinase that may be involved in the regulation of vesicle dynamics. However, its exact function is not known. [58, 59] Based on this limited knowledge of KIN-25, it was not clear how its depletion would affect cytokinesis. However, phosphorylation is essential for the regulation of many cytokinesis proteins, yet few kinases and phosphatases have been identified. Therefore, if *kin-25* RNAi did cause embryonic lethality, then this would be an interesting candidate for follow-up studies. Unfortunately, depletion of *kin-25* did not cause any embryonic lethality in N2 or *mlc-4/qC1 worms*. This suggests that if KIN-25 is required for cytokinesis, it is redundant.

4.1.8 NOL-1

Protein p120 is a nucleolar protein, which is expressed throughout the cell cycle and reaches its highest expression level in S-phase. Its expression is also increased in most human malignant tumor cells. Many mitotic proteins are also upregulated in tumors and support a mitotic function for p120. NOL-1 is the homolog of p120 in *C. elegans*, however its function is not known. [60] If *nol-1* is required for cytokinesis, then its depletion should cause embryonic lethality. Interestingly, although no embryonic lethality was observed in N2 worms, *nol-1* RNAi enhanced *mlc-4/qC1* worms. This type of genetic interaction warrants further analysis.

4.1.9 Summary

Using a mass spectrometry approach, interactors of the C-terminus of human Anillin and their putative homologues were identified. Using RNAi in *C. elegans*, these genes were screened for potential roles in cytokinesis based on embryonic lethal phenotypes. Furthermore, due to possible redundancy, a strain that carries a mutation in one of the key players of cytokinesis, *mlc-4* was used to sensitize the screen. In total, RNAi was performed to 9 genes (Table 3.2) in both wild-type and *mlc-4 (or253)/qCI* mutant strains and analyzed for embryonic lethality. In wild-type worms, the only gene that resulted in embryonic lethality was *him-1*, and this lethality was not significantly enhanced in *mlc-4* mutants. Due to *him-1*'s role in sister chromatid segregation, the embryonic lethality observed with its depletion could arise from mitotic effects that are independent of cytokinesis. However, further studies need to be performed to determine if *him-1* has any roles in cytokinesis. Interestingly, both *fhod-2* and *nol-1* showed enhancement with *mlc-*

4/qC1, but did not display lethality in wild-type worms and are worth pursuing for further studies. FHOD-2 likely regulates the actin cytoskeleton and could be an interesting novel regulator of cytokinesis. NOL-1 similarly could also be a novel regulator, although its molecular function is less clear and would require more studies at both the molecular and genetic level.

4.2 Future Directions

This study was a rapid verification of potential regulators of cytokinesis during which we found three proteins, HIM-1, FHOD-2 and NOL-1 that showed embryonic lethality in wild-type worms, or in combination with *mlc-4/qC1* mutant worms. At this point it is not clear if they are specifically involved in cytokinesis and in depth studies are required to further characterize these candidates. For instance, *let-502* and *mel-11* mutant strains could be used for further genetic analyses. LET-502 is a RhoA effector required for furrow ingression, and hypomorphic alleles can be used to sensitize the genetic background to identify other, potentially redundant genes based on enhancement. MEL-11 functions antagonistically to LET-502 to regulate myosin contraction and genes that function in the LET-502 pathway should be suppressed by *mel-11*. [(44)] Follow-up studies could include assessing their localization during cell division using fluorescent tags in *C.elegans*, and with their homologues in human cells. The ability of these proteins, NOL-1, FHOD-2 and HIM-1, to physically interact with Anillin should be verified in both *C. elegans* and in human cells. Essentially, this study has opened the door for further studies on potentially novel regulators of cytokinesis.

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