U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

MSc

2.º CICLO

FCUP
2019

U.PORTO

Dissecting formin´s molecular mechanisms of action during embryonic cell division

Jaime Daniel Leite Pereira

FC

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

MSc

Dissecting formin´s molecular mechanisms of action during embryonic cell division

Jaime Daniel Leite Pereira

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Bioquímica

2019

MSc

Dissecting formin´s molecular mechanisms of action during embryonic cell division

Jaime Daniel Leite Pereira
Mestrado em Bioquímica
Departamento de Química e Bioquímica
2019

Orientadora
Ana Xavier de Carvalho, Group Leader
Instituto de Investigação e Inovação em Saúde (i3S)

Coorientador
Pedro Nuno Simões Rodrigues, Professor Associado
Instituto de Ciências Biomédicas Abel Salazar (ICBAS)

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto,_____/_____/_____

Autor

Jaime Daniel Leite Pereira

Rua do Campo Alegre, 4169-007, Porto, Portugal, up201708633@fc.up.pt

Orientadora

Ana Costa Xavier de Carvalho, Group Leader, i3S

R. Alfredo Allen, 4200-135 Porto, Portugal, anacarvalho@ibmc.up.pt

Coorientador

Pedro Nuno Simões Rodrigues, Professor Associado, ICBAS

R. Jorge de Viterbo Ferreira 228, 4050-313 Porto, Portugal, prodrigues@reit.up.pt

Acknowledgments

Após a finalização das atividades laboratoriais no âmbito do projeto de dissertação de mestrado em Bioquímica, gostaria de agradecer e deixar uma referência a todas as pessoas envolvidas no meu percurso académico e na realização deste meu objetivo pessoal.

Começo por prestar um agradecimento ao diretor do mestrado em bioquímica, Professor Doutor Paulo Correia de Sá pelo acolhimento, integração e ingresso no mestrado em bioquímica e às instituições: Faculdade de Ciências da Universidade do Porto (FCUP); ao Instituto de Ciências Biomédicas Abel Salazar (ICBAS) e ao Instituto de Investigação e Inovação em Saúde (i3S), onde decorreram as minhas atividades durante o mestrado.

Posteriormente, gostaria de agradecer à minha orientadora Ana Xavier de Carvalho, por me conceder a oportunidade de incorporação no seu grupo de investigação, que me permitiu poder evoluir tecnicamente como cientista, bem como por todos os seus conselhos, apoio, disponibilidade e ensinamentos transmitidos ao longo deste projeto de dissertação. Do mesmo modo, transmito também o meu agradecimento à minha supervisora de laboratório Elaine Chan, pela sua orientação e ensinamento de técnicas laboratoriais, pela sua paciência, amizade, companheirismo, por me oferecer o seu apoio durante os momentos difíceis, por todo o seu empenho e dedicação para me transmitir os seus conhecimentos e exigir de mim sempre a minha melhor versão.

Adicionalmente, agradeço às restantes pessoas que constituem o grupo Cytoskeletal Dynamics e aos membros do grupo Cell Division Mechanisms e Tiago Dantas Lab, por me receberem de braços abertos, estarem sempre disponíveis para me ajudar a ultrapassar as minhas dificuldades, por terem sempre uma palavra amiga nos momentos difíceis e usarem toda a sua experiência para me indicar sempre as melhores decisões a tomar. Foi um prazer compartilhar todos os momentos convosco e poder estar rodeado de um ambiente cheio de um positivismo contagiante.

Gostaria ainda de agradecer aos meus amigos que conheci na Residência Universitária do Campo Alegre: Cláudia Antunes, Diogo Rodrigues, Rodrigo Silva, Marcelo Loureiro, Sara Mendes, Cláudio Graça e Diogo Ferraz, por todas as experiências e histórias que compartilhamos e pela amizade que desenvolvemos e a todos os meus colegas de curso, com uma palavra de apreço para os meus amigos Flávio Costa, Rita Fonseca, Alexandre Pinto e Karenina Santos.

Por fim, agradeço, aos meus pais, Jaime e Maria José pelos sacrifícios que

fazem diariamente para me proporcionarem um futuro melhor, pelo carinho e amor que sempre me demonstraram, pela importância que tiveram na minha educação e formação como pessoa e à minha irmã Sónia Pereira por todo o seu apoio e por ser um modelo para mim pela sua capacidade de trabalho e dedicação e por todos os seus ensinamentos e que aprendi com ela.

Muito obrigado a todos os que contribuíram de alguma forma no meu percurso académico e para o meu bem-estar e satisfação pessoal. Um bem hajam!!!

Resumo

Nas células eucarióticas, a actina desempenha um papel essencial devido à sua participação em diversos processos celulares, entre os quais, a citocinese. A citocinese envolve a formação e posterior constrição de um anel de actomiosina que divide a célula mãe em duas células-filhas. A ação de forminas Diaphanous é essencial para a elongação de filamentos de actina não ramificados que compõem o anel contrátil. Os mecanismos subjacentes a esta regulação espaço-temporal não são totalmente conhecidos. Apesar de alguns mecanismos terem sido propostos, o único mecanismo bem caracterizado é a libertação da autoinibição das forminas por RhoA ligado a GTP. Sabe-se que a citocinese nas células animais é completamente dependente da ativação da RhoA mas em leveduras, a ativação de forminas pela ligação a Rho não é biologicamente relevante. O estudo da regulação de forminas é essencial para entender a citocinese, especialmente porque a sua desregulação tem sido associada a várias doenças incluindo cancro. O principal objetivo do meu projeto, é investigar se a ativação por RhoA (RHO-1 em *C. elegans*) é o principal mecanismo para a regulação de forminas durante a citocinese embrionária em *C. elegans, in vivo.*

Existem 6 genes de forminas em *C. elegans,* mas apenas CYK-1 foi demonstrado ser necessário para a integridade da estrutura do anel contrátil. Assim, nós propusemo-nos a realizar um estudo da estrutura-função de CYK-1 em embriões de *C. elegans.* Para estudar a regulação de CYK-1 *in vivo,* nós geramos com sucesso estirpes com a mutação CYK-1(V279D) e uma versão truncada de CYK-1, CYK-1(Δ1250-1437), sem alguns elementos regulatórios do C-terminal, usando a técnica CRISPR/Cas9.

A mutação V279D é suposto impedir a ligação a RhoA. Este resíduo é altamente conservado entre espécies e foi demonstrado que a sua mutação causa uma forte deficiência na ligação de RhoA a forminas em células de mamíferos. Os animais homozigóticos de *C. elegans cyk-1(V279D)* são estéreis. Para examinar o impacto desta mutação na embriogénese, introduzimos um transgene recodificado de CYK-1::GFP^{re} em animais *cyk-1(V279D)* que pode ser especificamente deletado por RNAi. Quando CYK-1::GFP^{re} foi completamente deletado, embriões expressando apenas o mutante de CYK-1 deficiente para a ligação a Rho falharam a citocinese e não eram viáveis. A geração de animais expressando CYK-1(Δ1250-1437), é suposto impedir a formação da interação autoinibitória que mantem a formina numa configuração inativa, incapaz de elongar filamentos de actina. CYK-1(Δ1250-1437) é esperado constituir uma versão de CYK-1 constitutivamente ativa. Animais

homozigóticos *cyk-1(Δ1250-1437)* geraram embriões inviáveis. Para examinar o impacto desta mutação na embriogénese, uma vez mais, introduzimos um transgene recodificado de CYK-1::GFP^{re} em animais *cyk-1(Δ1250-1437)* que pode ser especificamente deletado por RNAi. Após completa deleção de CYK-1::GFP^{re}, embriões expressando apenas a versão truncada de CYK-1 falharam o completar da citocinese, durante a sua última etapa, a abscisão.

Todos estes resultados, sugerem fortemente que Rho-1 ativa se liga diretamente a CYK-1, regulando a sua atividade durante a citocinese e o desenvolvimento embrionário em *C. elegans.* Este estudo indica ainda que CYK-1 necessita de ser inativada durante as últimas etapas da citocinese, presumivelmente durante a abscisão, de modo a completar com sucesso a citocinese.

Abstract

In eukaryotic cells, the actin cytoskeleton plays an essential role in driving diverse cellular processes, including cytokinesis. Cytokinesis involves the assembly and subsequent constriction of a contractile actomyosin ring that pinches the mother cell into two daughter cells at the end of mitosis. The action of Diaphanous formins is essential for the elongation of non-branched actin filaments that compose the contractile ring and this formin activity has to be highly regulated in space and time. The mechanisms underlying this spatiotemporal regulation of formin activity are only poorly understood. Although several mechanisms have been proposed to regulate formin activity, the only well-characterized mechanism is RhoA-dependent release of formin autoinhibition. Cytokinesis in mammalian tissue culture cells is known to depend on RhoA activation but limited *in vivo* studies exist. In fission yeast, for instance, formin activation by Rho binding is not biologically relevant. As alternative mechanisms for formin activation have been proposed, the study of formin´s regulation *in vivo* is crucial to understand cytokinesis, especially because its deregulation has been linked to various diseases including cancer.

The main goal of my project is to investigate whether activation by RhoA (RHO-1 in *C. elegans*) is the main mechanism for formin regulation during *C. elegans* embryonic cytokinesis.

There are six formin genes in *C. elegans*, but only CYK-1 has been shown to be required for furrow initiation and contractile ring structure integrity. Thus, we proposed to perform a structure-function analysis of CYK-1 in *C. elegans* early embryos. To study the regulation of CYK-1 *in vivo*, we successfully generated strains expressing a RhoA-binding deficient mutant, CYK-1(V279D), and a truncated version of CYK-1 missing C-terminal regulatory elements of the protein, CYK-1(Δ1250-1437), by directly editing the *C. elegans* genome using the CRISPR/Cas9 technique. Homozygous *cyk-1(V279D)* animals were sterile. To examine the impact of this mutation on embryogenesis, we introduced a transgene-reencoded wild-type CYK-1::GFP^{re} into *cyk-1(V279D)* animals that could be specifically depleted by RNAi. When CYK-1::GFP^{re} was fully depleted, embryos only expressing RhoA-binding deficient CYK-1 mutant failed cytokinesis and were not viable. Animals expressing CYK-1(Δ1250-1437), a version of CYK-1 that is expected to be constitutively active, layed unviable embryos. To examine the impact of this mutation on embryogenesis, we also introduced in these animals a transgene-reencoded wild-type CYK-1::GFP^{re} that could be specifically

depleted by RNAi. After full depletion of CYK-1::GFP^{re}, embryos only expressing CYK-1 (Δ1250-1437) failed to complete cytokinesis during its last step, abscission.

Taken together, our results strongly suggest that active RHO-1 directly binds to CYK-1 regulating its activity during cytokinesis and embryonic development in *C. elegans*. Also this study indicates that CYK-1 needs to be inactivated during the last steps of cytokinesis, presumably during abscission, in order to successfully complete cytokinesis.

KeyWords

Cytokinesis, formins, actomyosin contractile ring, *C. elegans,* CYK-1; CRISPR/Cas9, Rho-dependent formin activation

Index

List of Tables and Figures

Abbreviation list

BWM - Body Wall Muscle
***C. elegans* -** *Caenorhabditis Elegans*
CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats
DAD - Diaphanous Autoregulatory Domain
DD - Dimerization Domain
DIC - Differential interference contrast
DID - Diaphanous Inhibitory Domain
DNA - Deoxyribonucleic Acid
DRFs - Diaphanous Related Formins
FH1 - Formin Homology domain 1
FH2 - Formin homology domain 2
FH3 - Formin homology domain 3
Fli-I - Flightless-I
GAP - GTPase Activating Proteins
GBD - GTPase Binding Domain
GDP - Guanosine Diphosphate
GEF - Guanine nucleotide Exchange Factor
GFP - Green Fluorescent Protein
GTP - Guanosine triphosphate
LB - Luria Broth medium
MLCK - Myosin light chain kinase
MosSCI - Mos Single Copy Insertion
NGM - Nematode Growth Medium
NHEJ - Non-homologous End Joining
NMY-2 - Non-muscle myosin 2
PAM - Protospacer Adjacent Motif
PCR - Polymerase Chain Reaction
PH - Pleckstrin homology domain
PTEN - Phosphatase and tensin homolog
RNA - Ribonucleic acid
RNAi - RNA interference
ROCK - Rho-associated Kinase
sgRNA - single-guide RNA
SH3 - SRC Homology domain 3
SIN - Septation initiation network
***S. pombe* -** Schizosaccharomyces pombe

Introduction

1- Importance of cell cycle to homeostasis in multicellular organisms

Since the establishment of the cell as a concept by the mid-nineteenth century, the study of the cell division has become crucial to understand the basis of growth and development of eukaryotic organisms that ensure the continuity of life across generations (Nurse 2000). Cell division is the last step of the cell cycle. Indeed, the goal of the cell cycle is to provide proper duplication of the genome and complete cell division by separation of duplicated DNA, cytoplasm and organelles into two individual daughter cells. Cell division is of major importance during embryonic development to allow the formation of different tissues and organs. In adults, most somatic cells are maintained in a nonproliferative, quiescent G0 phase. Cells in G0 are metabolically active and may be stimulated to initiate cell cycle in situations of injury or disease (Figel and Fenstermaker 2018; Salomoni and Calegari 2010).

In eukaryotes, the cell cycle is composed of two major and distinct phases: interphase and mitosis (or M phase). During interphase, the cell is under a state of development that promotes their growth, the replication of DNA and repair of DNA. This phase is the longest phase of the cell cycle and it is divided into G1, S, and G2 stages. The G1 and G2 stages constitute gap stages that occur before and after DNA replication, respectively. During G1, the cell synthesizes mRNA, enzymes, and nutrients that are essential for their development and preparation for DNA, which occurs in phase S. G2 is characterized by rapid cell growth and protein synthesis that garnishes the cell with resources for mitosis (M phase) (Vermeulen, Van Bockstaele, and Berneman 2003). During mitosis, the separation of the duplicated genome occurs in five stages: prophase, prometaphase, metaphase, anaphase, and telophase. Cell division only completes with the physical separation of the cytoplasm and organelles, in a process designated cytokinesis (Figel and Fenstermaker 2018).

2 – Cytokinesis

The last step of cell division, designated cytokinesis, is responsible for the physical separation of the mother cell into two daughter cells. Failure of cytokinesis suppressed the success of previous mitotic events and leads the cell to progress into

defective mitosis and chromosomal instability. This can lead to the development of several diseases. Thus, the dysregulation of cytokinesis has been related to the appearance of many diseases, including cancer, blood disorders, female infertility, lowe syndrome, and age-related macular degeneration (Lacroix and Maddox 2012).

In animal cells, the process of cytokinesis is initiated during anaphase with the reorganization of the mitotic spindle or de novo polymerization of microtubules to form a structure at the central region of the cell constituted by two parallel arrays of interpolar microtubules known as the central spindle. The central spindle along with the mitotic spindle are essential components for the establishment of the position of the division plane between the two masses of segregated chromosomes, which is crucial to prevent chromosome loss. During the occurrence of this event, the central spindle induces the localization and activation of the small GTPase RhoA in the equatorial region of the cell that will trigger the assembly of an actomyosin contractile ring. This ring is essentially composed of filamentous actin (F-actin), the non-muscle motor protein myosin II, along with other structural and regulatory proteins. Ring contraction causes the ingression of the plasma membrane leading to the formation of a cytokinetic furrow (Miller 2011), until the cytokinetic furrow overlaps with microtubules from the central spindle originating a structure designated intracellular bridge (Douglas and Mishima 2010). This structure connects the two daughter cells at the end of cytokinesis and contains a specific region in its center formed by a dense organization of antiparallel microtubules derived from the central spindle, the mid-body. After this region is formed, occurs the assembly of the machinery of abscission responsible for the physical separation of one mother cell into two daughter cells through procedures that include the removal of cytoskeletal structures from the intercellular bridge, constriction of the cell cortex and plasma membrane fission (Figure 1) (Mierzwa and Gerlich 2014).

During cytokinesis, the event that constitutes the major driving force to allow the ingression of the cleavage furrow is the assembly and contraction of the actomyosin contractile ring, so it is important to understand the mechanisms that are behind its assembly to ensure that cytokinesis occurs. Besides F-actin and non-muscle myosin II, other proteins are required to play functional roles in the contractile ring. Within these proteins, stands out the presence of ADF/cofilin, septins and anillin (Miller 2011). Cofilin is important to regulate the actomyosin dynamics in the ring, due to its ability to sever F-actin and thereby contributing to F-actin dynamics, and for controlling the access of myosin II to F-actin (Wiggan et al. 2012). Septins are GTPases that cooperate with anillin to organize the assembly of the contractile ring (Bridges and Gladfelter 2015). Anillin acts as a scaffold protein that binds to F-actin, non-muscle

myosin II and septins, contributing to the organization and recruitment of these components to the ring, and to the anchorage of the ring to the plasma membrane (Bridges and Gladfelter 2015; Kechad et al. 2012).

Two pathways that involve RhoA are essential to promote actin and myosin II assembly during the formation of the contractile ring. RhoA is a small GTPase that can be maintained into two different configurations: an active (GTP–bound state) and an inactive (GDP-bound conformation). The transition between these two different conformations is regulated by activators and inhibitors. Activators known as guanine nucleotide exchange factors (GEFs) promote the exchange of GDP to GTP and trigger the activation and targeting of RhoA to the plasma membrane while the inhibitors designated GTPase activating proteins (GAPs) stimulate their GTPase activity (Piekny, Werner, and Glotzer 2005).

Ect2, a RhoA GEF, localizes in the equatorial region and promotes RhoA activation. When RhoA is activated it has the ability to interact with Rho-associated Kinase (ROCK) and with members of the formin family proteins in two distinct and parallel pathways. In response to RhoA-GTP binding, formins become active and functional having the ability to nucleate the formation of unbranched actin filaments (Chircop 2014).

On the other hand, the interaction of RhoA with ROCK stimulates this kinase, which along with other kinases, the citron kinase and the myosin light chain kinase (MLCK), phosphorylate several residues on the myosin regulatory light chain. This phosphorylation regulates the activity of myosin II inducing the interaction with actin and the activation of the myosin ATPase to promote contraction (Figure 2) (Amano et al. 1996).

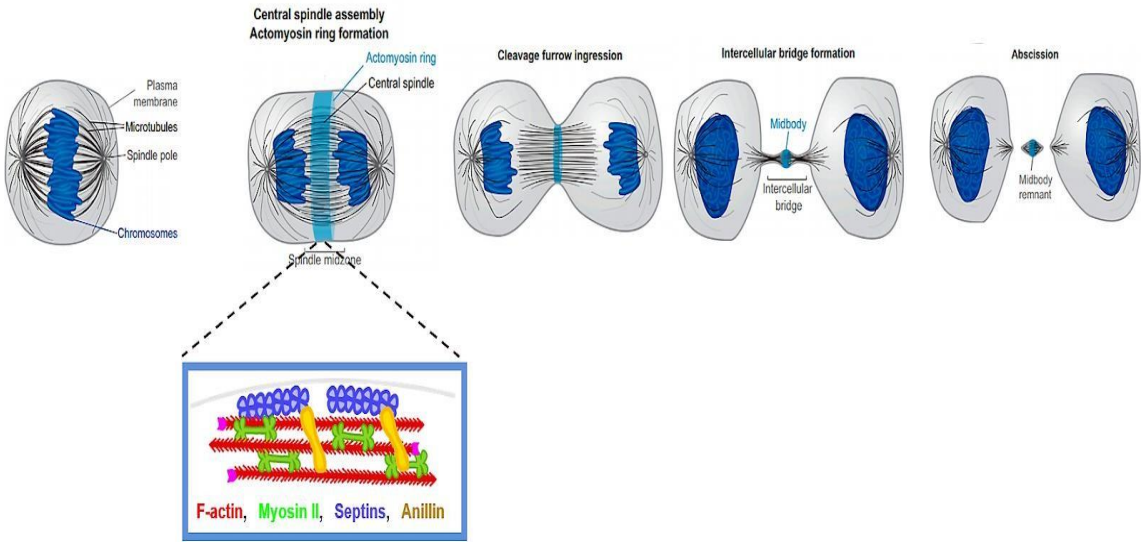

Figure 1 - Overview of animal Cytokinesis. Animal cytokinesis begins after anaphase onset after separation of sister chromatids to opposite spindle poles. Microtubules of mitotic spindle reorganize and form central spindle.The central

spindle signals to the cell cortex to localize and activate Rho GTPase at the cell equator that promotes the assembly of a contractile ring at the equator position composed by linear actin filaments (red) that is a component of the cytoskeleton and other proteins like non-muscle myosin II, a motor protein (green) that interacts with actin and it's able to hydrolyze ATP that allows myosin protein slides actin filaments during contraction. Anillin (yellow) and septin are other contractile ring components that bind F-actin and myosin II to the membrane. The contraction of the contractile ring will lead to the formation of an intracellular bridge that contains bundles of antiparallel microtubules derived from the central spindle, which at its center forms a dense structure termed midbody that directs abscission of a single cell into two cells (Adapted from Mierzwa et al, 2014).

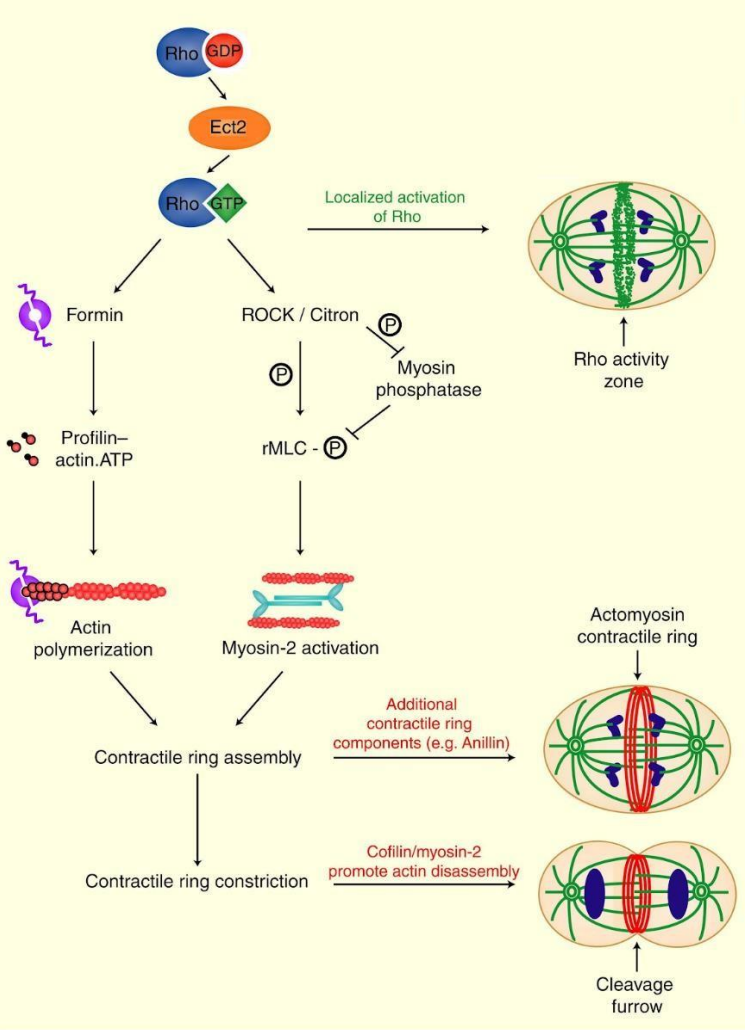

Figure 2 - Schematic representation of the recruitment and organization of cytokinetic machinery to the formation of the contractile ring during cytokinesis. To assemble the contractile ring, Rho is activated at the equatorial cortex by ECT2-GEF. Active Rho activates the downstream protein, formin, to nucleate linear actin filaments at the equator. In a parallel pathway, active Rho also has the ability to promote myosin II assembly by activating ROCK and citron kinases that directly phosphorylate the myosin light chain leading to the activation of its motor activity. Finally, actin filaments and myosin II along with additional components form a contractile ring that is the major driving force of cytokinesis (Adapted from Miller,2011).

3 - Formins

3.1 Domain organization of formins

Formins are a family of large multidomain proteins very well characterized with their size varying between 120 and 220 kDa, that were initially identified in flies, mice, and yeast. The term “formin” was applied for the first time in 1990 to designate protein products of the limb deformity gene that were suggested to participate in the formation of organ systems (Chesarone, DuPage, and Goode 2009; Schönichen and Geyer 2010). It was observed that mutations in those genes cause defects in cytokinesis, polarity, and morphogenesis of tissues and cells (Breitsprecher and Goode 2013). Formins are conserved among eukaryotes and participate in diverse cellular processes. Besides the capacity of reorganizing the actin and microtubule cytoskeletons, formins also play crucial roles in cell polarity, cytokinesis, vesicular trafficking, the formation of adherens junctions, embryonic development and signaling to the nucleus (R. Liu et al. 2010).

Bioinformatic studies show that different eukaryotic species have multiple genes that encode for formins. The yeast *S. cerevisiae* and *S. pombe* have 2 and 3 formins respectively; the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* contain 6 formins each, while mammals have 15 formins (Goode and Eck 2007). Most of the formins are characterized by two regions of sequence homology termed formin homology domain 1 (FH1) and formin homology domain 2 (FH2) (Figure 3). Both are essential for the nucleation and elongation of linear F-actin (Breitsprecher and Goode 2013; Frazier and Field 1997). Another formin homology domain (FH3) was identified in the N-terminus of a formin isoform Fus 1, present in fission yeast *S. pombe*. However, there is no evidence of the presence of this domain in other formins, which leads this to be considered the less conserved domain of the formin homology domains (Petersen et al. 1998; Wallar and Alberts 2003).

The FH2 domain is the best conserved domain of formins among species. The flanking regions of this domain vary between isoforms, which leads to different isoforms of formins to have different roles in cellular processes and different types of regulatory mechanisms (Schönichen and Geyer 2010).

The FH1 domain is also very conserved among formins of different organisms and the only formin that is known to lack this domain is the isoform ForC of *Dictyostelium discoideum* (Breitsprecher and Goode 2013; Kitayama and Uyeda 2003). FH1 domain is predicted to be rope-like and its major structural characteristic is the presence of multiple polyproline stretches that form rigid type II polyproline helices that can bind to profilin. Profilin is usually associated with actin monomers and is

responsible to suppress the inefficient process of spontaneous nucleation of actin (Courtemanche 2018; Chesarone and Goode 2009). FH1 is able to recruit profilin associated with actin. Besides the interaction of the proline-rich regions of FH1 with profilin-actin complexes, these regions also have the capacity to interact with diverse SH3 and WW2 domains of proteins that participate in signal transduction. In the structure of the FH1 domain, between polyproline stretches, there are regions that are not conserved and are predicted to be disordered, conferring flexibility to FH1 domains. This flexibility is essential for the function of this domain which is based on the delivery of profilin-actin complexes to the FH2 domain, to increase the rates of elongation and actin nucleation. The FH1 domains of most formins have similarities in structure and organization, which means that the delivery of profilin-actin complexes to the FH2 domain occurs through a general mechanism (Chesarone, DuPage, and Goode 2009; Higgs 2005; Courtemanche and Pollard 2012; Courtemanche 2018). Studies *in vitro* with the two formin isoforms present in budding yeast, Bni1p and Bnr1p, first demonstrated the role of the FH2 domain noting that constructs with only FH1 and FH2 domains were able to nucleate actin filaments through the association with growing barbed ends of actin filaments. This association also functions as processive capping in which the FH2 domain remains associated with the filament barbed end, for rapid addition of actin monomers. The deletion of the FH2 domain abolished nucleating activity, while the deletion of the FH1 domain diminished the nucleation activity demonstrating that the FH2 domain constitutes the minimal and sufficient domain that is necessary for the nucleation of actin filaments (Pruyne et al. 2002; Sagot, Klee, and Pellman 2002). A later study with similar constructs of the formin cdc12p in fission yeast demonstrated similar results that led to the conclusion that this formin had similar mechanisms of action (Kovar et al. 2003). After these studies in yeast, FH2 and FH1-FH2 fragments from a variety of formins of different organisms were characterized and it was confirmed that in general, the FH2 domain is the domain responsible for actin polymerization (Goode and Eck 2007). The crystallographic structure of the FH2 domain of yeast Bni1p and mammalian mDia1, DAAM and FMNL3 formins were solved and revealed that this domain dimerizes forming a tethered ring-shaped dimer composed of two rod-shaped subunits that are connected by a flexible linker (Xu et al. 2004; Lu et al. 2007; Thompson et al. 2013; Shimada et al. 2004).

A crystallographic structure of the yeast Bni1p FH2 domain in complex with actin shows that each subunit of the FH2 domain has two different binding sites to actin monomers. A model proposes that the nucleation of actin occurs through the FH2 domain either by stabilization of an actin dimer formed by spontaneous nucleation or by the sequential binding of two actin monomers (Otomo, Tomchick, et al. 2005). The

generated model was consistent with *in vitro* studies of fragments of FH1-FH2 domains of the Bnr1p formin that additionally indicate that filament nucleation by the FH2 domain is inefficient and the nucleation rate increases with the presence of profilin-actin complexes (Pring et al. 2003).

This suggests that it is the FH1 domain, which has the ability to bind profilin-actin, that donates these complexes to FH2, which in turn promotes the nucleation and elongation of non-branched actin filament.

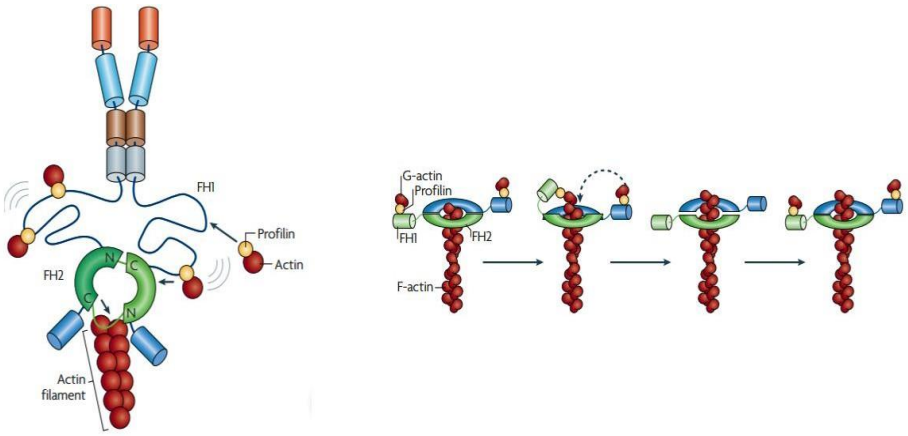

Figure 3 - Mechanism of nucleation and elongation of linear actin filaments through the conserved FH1 and FH2 domain of formins. Two formin molecules dimerize and associate to the barbed end of actin filaments to promote their rapid elongation (left). The FH1 domain of formin binds profilin-actin complexes and deliver actin subunits to the dimeric head-to-tail doughnut-shaped FH2 domain to nucleate and elongate unbranched linear actin filaments (right) (Adapted from Chesarone et al, 2009 and Campellone et al, 2010).

3.2 Diaphanous Formins and their regulation by Rho-GTP

Formins can be classified into two different subgroups: formin homology proteins, also designated as non diaphanous related formins and Diaphanous related formins. The diaphanous related formins (DRFs) are the best-characterized formins and besides the presence of FH1 and FH2 domains in the C-terminus, they also contain domains that are characteristic of this subgroup. In the N-terminus, this type of formins contains a GTPase-binding domain (GBD), a Dia inhibitory domain (DID) and a dimerization domain (DD) followed by a coiled-coil domain (CC). In the C-terminus, they have the FH1 and FH2 domains and a Diaphanous autoregulatory domain (DAD) (Bogdan, Schultz, and Grosshans 2013). Non diaphanous related formins are diverse and composed of several alternative domains. In the N-terminus, this group of formins can contain GTPase binding domains, PDZ domains, Pleckstrin domains (PH) or PTEN domains that regulate the activity of these formins (Pruyne 2016).

In mammals, there are 15 formins that are subdivided into seven different

subfamilies based on the protein domains they have. These subfamilies of formins are designated as: Dia (Diaphanous), FRL (formin-related proteins in leukocytes), DAAM (Dishevelled-associated activators of morphogenesis), FHOD (formin-homology domain proteins), FMN (Formin), Delphilin, and INF (inverted-formin). Four of the seven mammalians subfamilies, mDia, Daam, FMNL and FHOD are DRFs with similar domain organization (Figure 4) (Kühn and Geyer 2014). The mammalian DRFS are mainly found in a resting state maintained by autoinhibition that is dependent on the intramolecular binding between the N-terminal DID domain and the C-terminal DAD domain. This intramolecular binding keeps the formin in a closed conformation inhibiting the polymerization of linear actin filaments. The intramolecular binding is disrupted after the binding of GTP-Rho to the N-terminal GBD, promoting the change to an open conformation that allows the polymerization of linear actin filaments (Alberts 2001; Schönichen et al. 2006; Vaillant et al. 2008; Li and Higgs 2005; W. Liu et al. 2008).

The formin mDia1, one isoform of the mDia family, constitutes the formin where this process is best characterized (Lammers et al. 2005; Otomo, Otomo, et al. 2005; Rose et al. 2005). The formin mDia1 was shown to interact with Rho A, B and C but only Rho A is specific to bind to the GBD. The interaction between DID-DAD occurs through the central portion (GVMDxLLEALQS) of DAD, which forms an amphipathic helix that binds to the concave surface of DID through several hydrophobic and hydrogen bonds (Nezami, Poy, and Eck 2006). To disrupt this interaction, it is necessary that RhoA induces the displacement of DAD. In mDia1, RhoA interacts with GBD and DID through numerous electrostatic interactions. The switch region I is able to interact with GBD, while the switch II region can interact with both GBD and DID. It was demonstrated that DAD binding sites in DID partially overlap with Rho interaction sites, which indicates that RhoA competes with DAD for the binding to DID. *In vitro* assay, RhoA-GTP seems to only partially dissociate the binding between DID and DAD, which suggests that other mechanisms or proteins can be involved in the activation of Diaphanous related formins (Figure 5) (Rose et al. 2005; Otomo, Otomo, et al. 2005).

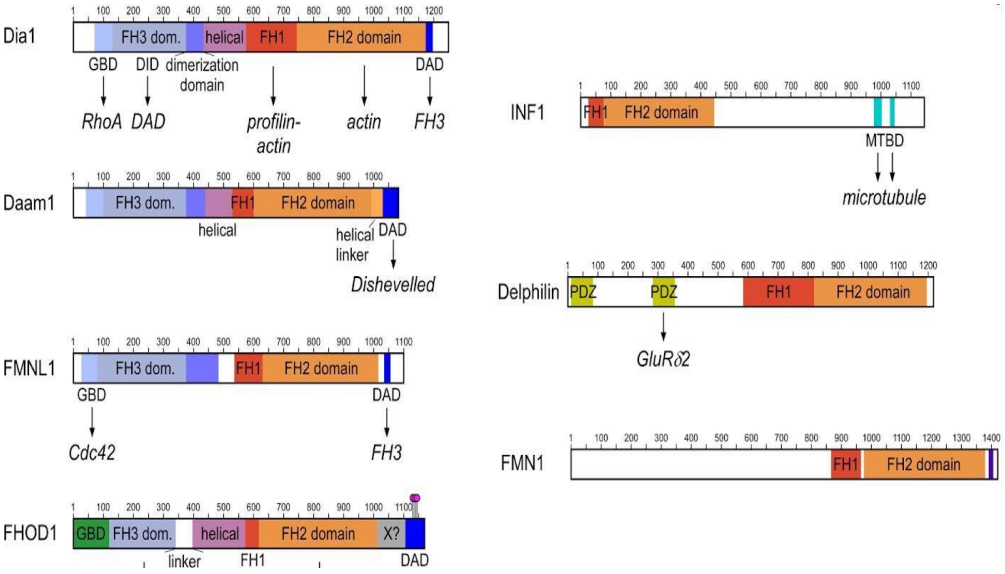

Figure 4 - Schematic representation of the architecture of families of mammalian formins. Mammalians DRFs: mDia, Daam, FMNL and FHOD besides the FH1 and FH2 characteristics of all types of formins contain additional domains. In N-terminus, the regulatory region of mammalian DRFs consists of GTPase-binding domain (GBD) that is important to bind Rho family GTPases; a Diaphanous Inhibitory Domain (DID) that contains tandem armadillo repeats; a Dimerization Domain followed by a coiled-coil domain that are responsible for the dimerization of the protein. In C-terminus, mammalian DRFs contained the FH1 and FH2 domains responsible for nucleation and elongation of linear actin filaments, and the DAD domain for regulation (left). Non DRFs: INF, Delphillin and FMN, besides FH1 and FH2, are composed of alternative domains. In the N-terminus, this group of formins can contain GTPase binding domains, PDZ domains, Pleckstrin domains (PH) or PTEN domains that regulate the activity of these formins (right) (Adapted from Schönichen et al, 2010).

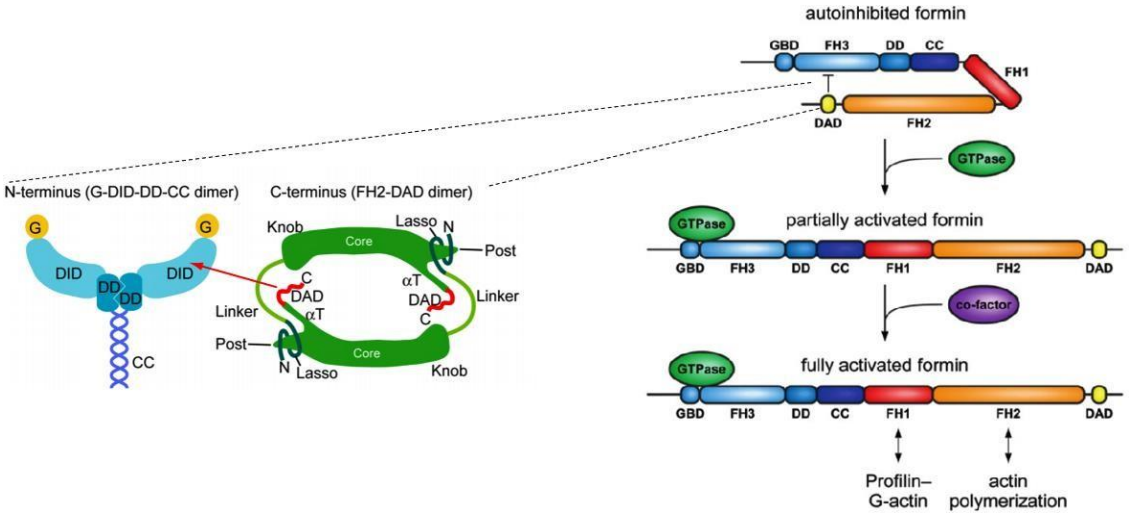

Figure 5 - Schematic representation of the regulatory mechanism of DRF mDia by active Rho GTPase. mDia1 is usually in a close conformation in a process of autoinhibition which is necessary for the formin not to be continuously nucleating and elongating actin filaments. The autoinhibition is assured by the intramolecular binding between N-terminal DID and C-terminal DAD that occurs due to a central portion GVMDxLLEALQSf of DAD which forms an amphipathic helix that binds to the concave surface of the DID domain through several hydrophobic contacts (left). To activate formin, active RhoA GTPase binds to the GTPase binding domain of formin releasing the interaction between DID and DAD domain. Other factors are reported to fully activate mDia1 that is placed in an open conformation

that allows the FH1 and FH2 domains to be available to nucleate actin linear filaments (right) (Adapted from Otomo et al,2010 and Kühn et al, 2014).

3.3 Other regulatory mechanisms of diaphanous formins

Although multiple evidences indicate that the binding of active Rho-GTPases is necessary to activate mDia formins, the mechanism of activation is not fully understood. It was demonstrated *in vitro* that nanomolar concentrations of active Rho are sufficient to disrupt the DID-DAD interaction, however increasing the concentration of active Rho only activated mDia1 partially, which suggests that other factors should cooperate with Rho GTPases to fully activate mDia formins (Rose et al. 2005; Li and Higgs 2003).

Other factors may include the Rho-associated protein kinase (ROCK), the protein flightless-1 (Fli-I) and anillin, which have all been demonstrated to help RhoA in activating DRFs in mammalian cells (S. Watanabe et al. 2010; Higashi et al. 2010; Staus, Taylor, and Mack 2011). ROCK and anillin have been suggested to interfere with the activation of mDia2. *In vitro* and *in vivo* kinase assays revealed that mDia2 is phosphorylated by ROCK in two conserved residues of threonine and serine localized near the DAD basic region. The phosphorylation of these residues prevents the basic region of DAD from interacting with DID, abolishing the autoinhibitory interaction within mDia2. Phosphorylation of these residues led to an increase of F-actin levels, which indicates that ROCK-dependent phosphorylation enhances RhoA during mDia2 activation (Staus, Taylor, and Mack 2011). Additionally, another study indicates that anillin also can assist mDia2 activation. An N-terminal region of anillin binds to the DID of mDia2. This binding is competitive with DAD and is required for the localization of mDia2 in the cleavage furrow during cytokinesis. As the depletion of anillin by RNAi caused cytokinesis failure and this process can be rescued when anillin full length is expressed is suggested that its interaction with DID domain can be supplementary to the role played by RhoA in mDia2 activation (S. Watanabe et al. 2010). Also, flightless-I (Fli-I), a gelsolin family protein, plays a role in fully activating mDia1 (Higashi et al. 2010). This protein competes with DID for binding to DAD of mDia1. *In vitro* assays evidenced that Fli-I binds to a conserved residue of leucine present in the DAD of mDia1, which prevents the DID-DAD interaction leading to an increase of F-actin levels in the presence of RhoA. This suggests that Fli-I contributes to RhoA-mediated activation of mDia1 (Higashi et al. 2010).

3.4 Diaphanous formins and cytokinesis

In mammalian cells, it is known that from all the mDia formins, the only isoform required for cytokinesis is mDia2. This formin localizes in the cleavage furrow during cytokinesis. Its depletion by RNAi causes an increase in binucleated cells that failed to assemble the contractile ring due to decreased levels of F-actin. Depletion of mDia1 or mDia3 did not interfere with cytokinesis (S. Watanabe et al. 2008). To check whether RhoA activation of mDia2 is important for cytokinesis, two strategies were used. First, the Ect2 GEF, which activates RhoA, was depleted by RNAi, which caused RhoA to be always inactive. In this situation, the localization of mDia2 was affected during cytokinesis. In control cells, RhoA and mDia2 localized at the cleavage furrow whereas in Ect2-depleted cells RhoA and mDia 2 localized at the interdigitating microtubules of the central spindle. This revealed that the localization of mDia2 at the cleavage furrow is dependent on active RhoA. In a second strategy, a mDia2 Rho-binding deficient mutant was generated (S. Watanabe et al. 2010). The construction of this mutant was based on previous *in vitro* studies that showed that the V161D mutation in mDia1's GBD decreases mDia1 affinity to RhoA without affecting the DID-DAD interaction, and abolishes the ability of FH2 domain to elongate actin filaments. This demonstrated that the interaction of RhoA with the GBD domain is necessary to disrupt the DID-DAD interaction and promote activation of mDia1 (Otomo, Otomo, et al. 2005; Seth, Otomo, and Rosen 2006). An analogous Rho-binding deficient mutant of mDia2 was generated: mDia2 (V180D). This mutant was confirmed *in vitro* to be deficient for Rho-binding, and as expected caused a similar effect to that of inactive RhoA (Ect2 RNAi), leading to mDia2 localization on the central spindle instead of the cleavage furrow and was unable to rescue cytokinesis failure caused by the depletion of mDia2 by RNAi. These experiments revealed that the binding of active RhoA to mDia2 is fundamental and indispensable for the localization and function of mDia2 in mammalian cells to promote proper cytokinesis in mammalian cells (S. Watanabe et al. 2010).

Although the importance of active Rho GTPases has been notorious to activate mammalian formins and hence to promote the assembly of the contractile ring during cytokinesis there is no evidence that a similar mechanism occurs in the fission yeast *S. pombe,* which is a popular model organism for cytokinesis studies. *S. pombe* cells are rod-shaped and, similarly to mammalian cells, utilize a medially-placed actin-and myosin-based contractile ring. A cell wall division septum is deposited behind the constricting ring, forming the new ends of each daughter cell. The *S. pombe* contractile ring forms from cortical precursor nodes that form at the cell equator. The anillin like protein Mid1 is the major upstream protein in the node-assembly pathway and is

responsible for the recruitment of other contractile ring proteins and to determine the position of the division plane. The main proteins present in the cytokinesis nodes are the anillin like protein Mid1; the IQGAP protein Rng2, the myosin-II motor (heavy chain Myo2, essential light chain Cdc4, and regulatory light chain Rlc1), the F-BAR protein Cdc15 and the formin Cdc12 (Lee, Coffman, and Wu 2012). During ring assembly, Cdc12 has been shown to play an essential role in nucleation and elongation of actin filaments for contractile ring formation (Pelham and Chang 2002; Chang, Drubin, and Nurse 1997). One way to tightly regulate the formin Cdc12 activity is by multimerization. A domain in Cdc12 C-terminus mediates oligomerization to form puncta of different sizes on the cortex at interphase. At anaphase onset, the septation initiation network (SIN) becomes active to phosphorylate the four residues at the C-terminus of Cdc12 by the SIN kinase Sid2 that inhibits oligomerization. When this phosphorylation does not occur, Cdc12 and other contractile ring proteins cluster abnormally causing contractile ring disintegration and failure of cytokinesis. Thus, the phosphorylation by Sid2 constitutes a mechanism of regulation of the formin Cdc12 (Bohnert et al. 2013; Willet, McDonald, and Gould 2015). Interestingly, there is no evidence that Cdc12 is regulated by an autoinhibition mechanism, as mutations in most conserved residues of DID and DAD domains still allow for cytokinesis to complete with only subtle problems (Yonetani et al. 2008). Rho GTPases, the main regulators of mammalian DRFs, have also been suggested not to impact Cdc12 regulation (Martin et al. 2007). In fact, it was demonstrated that in *S. pombe* the main conserved Rho GTPases: Cdc42, Rho1, Rho3 and Rho4, are implicated in different processes that include septum formation, cell polarity and cell morphology. Cdc42 is responsible for the activation of formin For3 in a mechanism similar to that of mammalian formins, leading to the nucleation of actin cables that are important for polarized cell growth (Martin et al. 2007; Wei et al. 2016). Rho1 is essential for primary septum formation and must be inactive to allow for cell separation and along with Cdc42 and Rho4 regulates septum morphology (N. Wang et al. 2015). Rho3 and Rho4 participate in the delivery and secretion of specific cell wall glucanases required for septation and Rho4 regulates secondary septum formation (Nakano et al. 2003; H. Wang, Tang, and Balasubramanian 2003; Santos et al. 2005).

4 - *Caenorhabditis elegans*: A model organism to study developmental biology

Model organisms could be defined as non-human species that are studied to understand biological processes with the purpose of converting the data and theories generated into knowledge about other organisms that can be much more complex than the original models (Ankeny and Leonelli 2011).

C. elegans is a model organism proposed in 1963 by Sydney Brenner that is actively studied in laboratories worldwide for understanding questions of developmental biology, neurobiology and studying processes that go awry in human diseases, being an ideal system to tackle these problems. *C. elegans* is a small soil nematode with adults reaching 1 mm in length and exists primarily as a hermaphrodite (XX), although males (XO) can be generated in progeny with a low percentage of 0.1-0.2 % due to nondisjunction of the X chromosome during meiosis. Generation of males can be achieved through exposure of hermaphrodites to high temperatures for a short time. Males are important because through crossing with hermaphrodites, they allow the generation of progeny with different genetic composition. Through mating, due to competition between male sperm and hermaphrodite sperm a higher frequency of males (up to 50%) can be easily achieved.

C. elegans is a model organism with many advantages and benefits for eukaryotic genetic studies. First, it is easy to maintain *C. elegans* in a laboratory. Several features that contribute to this characteristic are their short life cycle and the small size. In the laboratory, worms usually grow on agar plates with a lawn of *E. coli* bacteria as a food source and usually maintained at 20 ºC. In food, they develop through 4 stages (L1-L4) until reaching adulthood (Figure 6). At 20 ºC, worms need approximately 3,5 days to develop from eggs to adults. When food is depleted and animals are maintained in a starved stage, worms in L2 stage progress into an alternative state of development designated dauer. Worms are able to survive for some months in this stage and can continue their normal development if they are transferred to food. The small size (0,25 mm young worms and 1 mm adults) ensures that worms can grow in a small space. Additional advantages for this to be a convenient model organism to keep in the lab are the predisposition of *C. elegans* strains to be frozen and revived when needed, the possibility of decontaminating adults and isolating eggs that are resistant to bleach solutions, and lastly a single self-fertilizing hermaphrodite can originate a large number of progeny (Corsi, Wightman, and Chalfie 2015; Tucker and Han 2010).

In addition to its easy maintenance, *C. elegans* exhibits other advantages that allow it to be a relevant model for eukaryotic biology studies. One fundamental inherent feature that contributes to its importance is transparency. *C. elegans* animals are transparent, thus enabling individual cells and subcellular details to be visualized using differential interference contrast (DIC) (Figure 7) (Porta-de-la-Riva et al. 2012). Also, it is possible to use fluorescent protein reporters to label cells or cellular structures and follow its behavior in living animals (Chalfie et al. 1994). Thus, development processes can be followed to monitor the impact of mutations that affect cell development *in vivo*. Lastly, the conservation of key genes, pathways and similarities with cellular and molecular processes between *C. elegans* and other organisms makes findings in *C. elegans* broadly relevant (*C. elegans* Sequencing Consortium 1998).

In the specific case of studying cell division processes, the one-cell *C. elegans* embryo constitutes a powerful system being relatively accessible to introduce precise genetic modifications and quantitative live-cell imaging (Hattersley et al. 2018). To perform these modifications, two processes are essential to promote mechanistic cell division studies. The first process is the RNAi mediated protein depletion (Min and Lee 2007). With this procedure, more than 95% of a specific protein can be deleted promoting a gradual depletion of mRNA that codifies for that specific protein and removal of preexisting protein by continued embryo production (Oegema and Hyman 2006). In addition, it is also possible to insert single copy transgenes, which enable expression of RNAi-resistant versions of essential genes at endogenous levels (Frøkjær-Jensen et al. 2008).

The second process is the CRISPR/Cas9 technique, a novel genome editing tool that has also been successfully applied to *C. elegans* enabling edition and manipulation of DNA in a rapid, accurate and cost-effective way (Dickinson and Goldstein 2016).

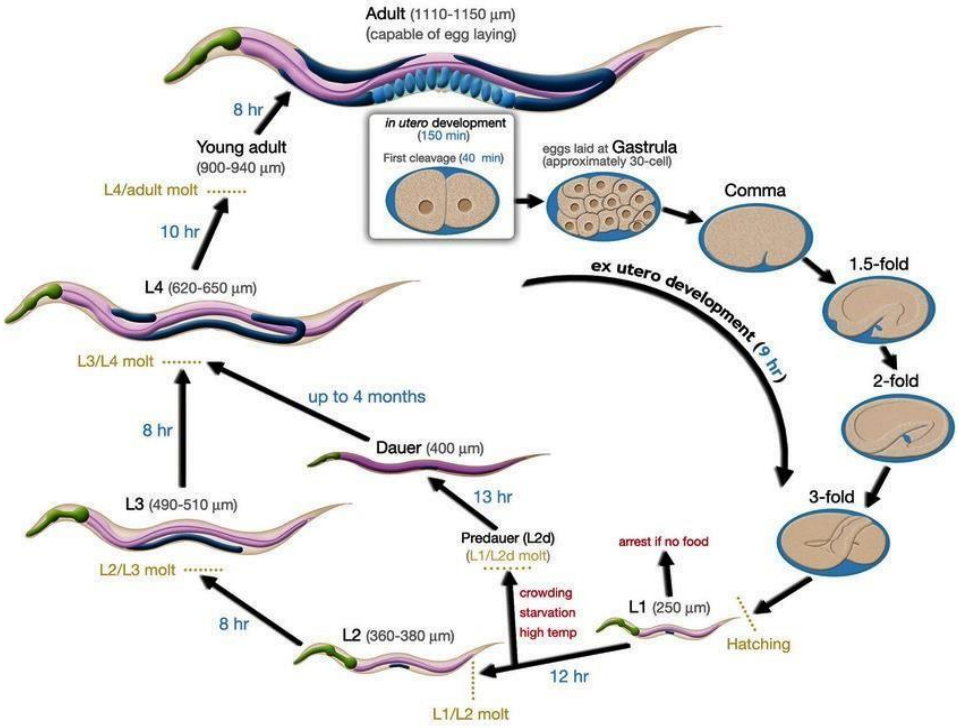

Figure 6 - *C. elegans* life cycle. After fertilization of a single oocyte, embryogenesis occurs over the next 13 h. Larval development consists of four stages (L1–L4) accompanied by a dramatic increase in size, followed by adulthood. During conditions of stress, including starvation and/or overcrowding, an alternative larval stage called the dauer state can be achieved. These larvae are capable of living for 3–6 months in a dormant state. During or after this period, if provided an environment with proper growing conditions to this worm, the larvae can development and continuing the normal life cycle to become fertile adults.

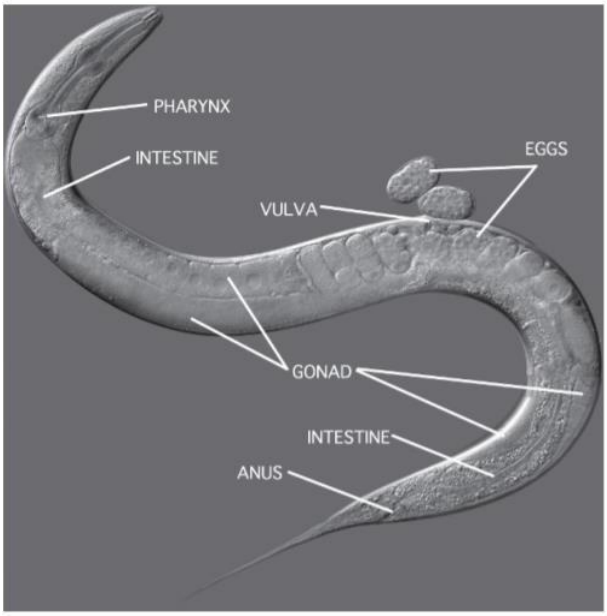

Figure 7 - Adult *C. elegans* hermaphrodite seen with DIC Due to being a transparent organism its easy to identify the major anatomic regions of the worms and follow individual cells and specific components during the occurrence of biological events *in vivo*.

5 - Generation of C. *elegans* mutants by CRISPR/Cas9 technique

The CRISPR (clustered regularly interspaced short palindromic repeats) and Cas (CRISPR-associated proteins) are mechanisms present in the adaptive immune system of bacteria to resist virus invasions. In this system, a specific region denominated CRISPR locus has a crucial role in this process. The CRISPR locus comprises a series of conserved short repeats and adjacent to this region a sequence leader A/T rich exists, which functions as a promoter element and several cas genes. After a virus introduces its genetic material into a bacterial cell, the CRISPR mechanism has the ability to integrate short fragments of viral DNA, known as protospacers, into the CRISPR locus between two adjacent repeat units. The short fragments of viral DNA are recognized due to a short sequence of conserved nucleotides that are part of its constitution named PAM (Protospacer Adjacent Motif) required for its acquisition in the CRISPR locus. Then, the CRISPR locus is transcribed, originating a pre CRISPR RNA (pre-crRNA) that is processed by different types of CRISPR/CAS which differ from each other by the presence of specific cas genes (Wiedenheft, Sternberg, and Doudna 2012; Bhaya, Davison, and Barrangou 2011). The CRISPR/CAS system is currently divided in two classes: class 1 that include types I, III and IV and class II that comprises types II, V and VI (Makarova, Wolf, and Koonin 2018; Makarova et al. 2015). The type II CRISPR/CAS9 is the system most well characterized and is recognized as a powerful system for genome editing in a variety of organisms, including *C. elegans* (Dickinson et al. 2013; Arribere et al. 2014; Ran et al. 2013).

In this type, is contained a trans-activating RNA (tracrRNA) that is complementary to the short repeat sequences of the pre-crRNA forming an RNA duplex. Posteriorly, the RNA duplex is cleaved by RNase III originating fragments of mature crRNA/tracrRNA hybrids. In case of a new invasion caused by the same virus, the hybrid crRNA/tracrRNA formed a complex with cas9, an endonuclease that constitutes the signature of the type II CRISPR/CAS system. The hybrid crRNA/tracrRNA guides the cas9 to recognize the PAM sequence in viral DNA. Next to the PAM, cas9 causes a double strand break in viral DNA through specific domains (Hidalgo-Cantabrana, Goh, and Barrangou 2019). The CRISPR/cas9 bacterial system can be adapted to *C. elegans* through the design of a single guide RNAs (sgRNA), that is complementary to the specific site desired to be cleaved and consisting a 20 nucleotides sequence identical to the desirable genome fused at its 3’ end to a PAM

motif specific for Cas9 – 5’ NGG 3’. Once cleaved, the double-strand break generated by Cas9 activity can be repaired by Nonhomologous end joining (NHEJ) if the goal is to generate deletions or small insertions that disrupt the gene’s function, or by Homology-directed recombinational repair that will incorporate a repair template harboring the designed changes (Chiu et al. 2013; Lemmens and Tijsterman 2011; Lo et al. 2013).

Aims of this study

Cell division is a fundamental process that constitutes the basis of growth and development of eukaryotic organisms and allow the continuity of life across generations. Cytokinesis is the last step of cell division and is critical for its success, as it causes changes in cell shape that are dependent on reorganization of cytoskeleton. Defects and dysregulation of this process can lead to several diseases. Successful cytokinesis relies on the assembly and activation of an actomyosin contractile ring in a spatially and temporally precise manner.

During the assembly of the contractile ring, formin proteins take on a crucial role being responsible for nucleating and elongating unbranched actin filaments and its dysregulation leads to cytokinesis failure. Although mechanisms of regulation of formins are well characterized in mammalians, this process is not totally known.

In fission yeast *S. pombe*, a relevant model system that is used to study basic principles of the cell and to understand biological pathways in more complex organisms like mammals, and in particular humans, a novel mechanism of regulation of formins that differ from the mechanism that occurs in mammalians was identified.

In *C. elegans*, other important model to study cell development, in which, several discoveries were achieved due to conservation of key genes, there is no knowledge about the regulatory mechanisms of formins during cytokinesis. Thus, this question needs to be studied and clarified. In this project, we aimed to study the regulatory mechanism of formin´s activity during embryonic cytokinesis *in vivo* using *C. elegans* as a model, through the following tasks:

1. Generation a version of formin that does not bind RhoA, the major activator of Diaphanous formins in other systems and characterize cytokinesis phenotype on the 1-cell embryo.

2. If regulation of CYK-1 is mediated by RhoA binding, generate a version of formin that is always active and characterize cytokinesis phenotype on the 1-cell embryo.

Material and Methods

The experimental work described in this thesis was conducted by myself and Fung Yi Chan. The immunoblots, live imaging, characterization of the CYK-1::GFP^{re} transgenic version and protein alignments that will be included in the Results section were conducted by Fung Yi Chan alone and therefore I am not including the methodology for those in this section.

C. elegans maintenance

C. elegans strains were maintained on Nematode growth medium (NGM) agar plates, in which *E. coli* OP50 strain is grown to be used as food source (Brenner 1974). OP50 is a uracil auxotroph that is not resistant to antibiotics and its growth requires uracil in the medium that limits their growth. This strain was initially obtained by streaking out some bacteria from a glycerol stock onto a Luria Broth (LB) agar plate [10 g/L Bacto-tryptone, 5 g/L Bacto-yeast, 5 g/L NaCl and 15 g/L agar, pH 7.5] that was left to grow overnight at 37ºC (Byerly, Cassada, and Russell 1976). A single colony was picked to be inoculated in LB liquid medium that grow overnight at 37ºC. The bacterial suspension was used to seed NGM plates.

The preparation of the NGM plates involved the sterilization of an NGM solution (3 g/L NaCl, 17 g/L agar and 2.5 g/L peptone) at 110 ºC for 30 minutes. This solution was cooled in a 55 ºC water bath for 15 minutes. After this step, 1 M $CaCl_2$, 5 mg/ml cholesterol in ethanol, 1M $MgSO_4$ and 1M potassium phosphate (pH 6) were added. Two distinct types of NGM seeded plates were used. Medium sized plates (60 mm diameter) were used for general worm maintenance while large plates (100 mm diameter) were used to maintain a large amount of worms that were posteriorly frozen at - 80ºC. The NGM solution was distributed into plates using sterile procedures through a peristaltic pump that was adjusted to place a constant amount of NGM in each plate. The plates were left at room temperature for 2-3 days to dry and to detect possible contaminants. To complete the preparation of the plates, the plates were seeded with *E.coli* OP50 strain using sterile procedures. For the preparation of medium plates, we used 0,05 ml of *E. coli* OP50 liquid culture and for the preparation of large plates we used 0,1 ml of *E.coli* OP50 liquid culture. The plates were left at room temperature to dry during one day before storage.

C. elegans general stocks were maintained at a temperature range between 16 ºC and 25 ºC, more frequently at 20 ºC. The temperature was adjusted according to the planned experiments to control the speed of growth of animals, as it is known that *C.*

elegans grow 2.1 times faster at 25 ºC than at 16 ºC and 1.3 times faster at 20 ºC than 16 ºC (Wood 1983). All plates were identified with the strain and date. Eventually, *C. elegans* plates can become contaminated with other bacteria, yeast or in more hostile cases, mites, that make it difficult to visualize phenotypes and transfer worms to other plates. In this case, we used an alkaline bleach protocol to remove the contamination. This protocol consisted in putting several adult hermaphrodites from the contaminated strain into a drop of a hypochlorite solution on a new plate, for 24 hours to clean the worms. During this procedure, the hypochlorite solution kills the contaminants and the hermaphrodites, however the embryos remain intact because they are protected by the eggshell. In the next day, the eggs that hatched have moved onto the OP50 lawn and are transferred to a new clean NGM plate seeded with OP50.

In table 1 are listed all strains used in this study and their respective genotype.

Table 1 - List of *C. elegans* strains used in this study.

Strain	**Genotype**	**Source/ reference**
N2	Ancestral	
GCP819	cyk-1[prt132(V279D)]III; hT2 [bli-4(e937) let-?(q782) qIs48](I;III)	This study
GCP896	cyk-1[prt153(Asp1250-end deletion)]III; hT2 [bli-4(e937) let-?(q782) qIs48](I;III)	This study
GCP741	prtSi14[pAC70; Pcyk-1:cyk1reencoded::GFP::StrepTagII::3'UTRcyk-1; cb-unc-119(+)]II; ltIs37 [pAA64; pie-1/mCHERRY::his-58; unc-119 (+)] IV; nmy-2(cp52[nmy-2::mkate2+ LoxP unc-119(+) LoxP]I; unc-119(ed3) III	This study
GCP820	cyk-1[prt132(V279D)]III; prtSi14[pAC70; Pcyk-1:cyk-1reencoded::GFP::StrepTagII::3'UTRcyk-1; cb-unc-119(+)]II; ltIs37 [pAA64; pie-1/mCHERRY::his-58; unc-119 (+)] IV; nmy-2(cp52[nmy-2::mkate2+ LoxP unc-119(+) LoxP]I; unc-	This study

	119(ed3) III	
GCP880	unc-119(ed3)III; prtSi14[pAC70; Pcyk-1:cyk-1reencoded::GFP::StrepTagII::3'UTRcyk-1; cb-unc-119(+)]II; zbIs2(pie-1::lifeACT::RFP)	This study
GCP883	cyk-1[prt132(V279D)]III; prtSi14[pAC70; Pcyk-1:cyk-1reencoded::GFP::StrepTagII::3'UTRcyk-1; cb-unc-119(+)]II; ltIs37 [pAA64; pie-1/mCHERRY::his-58; unc-119 (+)] IV; zbIs2(pie-1::lifeACT::RFP)	This study
GCP928	cyk-1[prt153(Asp1250-end deletion)]III; prtSi14[pAC70; Pcyk-1:cyk-1reencoded::GFP::StrepTagII::3'UTRcyk-1; cb-unc-119(+)]II; ltIs37 [pAA64; pie-1/mCHERRY::his-58; unc-119 (+)] IV; nmy-2(cp52[nmy-2::mkate2+ LoxP unc-119(+) LoxP]I; unc-119(ed3) III	This study
GCP929	cyk-1[prt153(Asp1250-end deletion)]III; unc-119(ed3)III; prtSi14[pAC70; Pcyk-1:cyk-1reencoded::GFP::StrepTagII::3'UTRcyk-1; cb-unc-119(+)]II; zbIs2(pie-1::lifeACT::RFP)	This study
JK2739	mcm-4(e1466) dpy-5(e61) I/hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)	CGC

Freezing *C. elegans* Stocks

To perform the freezing of *C. elegans* strains we picked 15 young adults and placed them into two large plates seeded with OP50. The plates were kept at 20 ºC to allow the development of the worms until the food is completely depleted and the progeny in a starved stage. At this point, the plates had a lot of L1s and L2s that are the worms that are suitable to be frozen. Besides L1 and L2, the plates should also have some unhatched eggs, which ensures that the plates were not without food for a long time. Using sterile procedures, the plates were rinsed twice with 10 mL S-Basal

[100 mM NaCl, 50 mM potassium phosphate (pH 6.0), 5 mg/L cholesterol] and transferred to 50 mL conical tubes. Animals were left until they migrated to the bottom of the tubes. After that, the supernatant was removed until the mark of 2.5 mL and mixed with an equal amount of freezing medium [100 mM NaCl, 50 mM potassium phosphate (pH 6.0), 30 % (v/v) glycerol]. Worms were aliquoted in cryovials (1 mL of worm suspension per cryovial) and then stored at - 80 ºC in a Coolcell container to allow a gradual decrease of temperature required for survival.

Generation of new *C. elegans* strains expressing CYK-1(V279D) and CYK-1(Δ1250-1437) by CRISPR-CAS9-mediated direct editing of the *C. elegans* genome

Regarding this method, the candidate only contributed to the screening of the strains and therefore that part is described in more detail.

To study the mechanism of regulation of CYK-1 in *C. elegans*, two different strains were constructed: a strain carrying a point mutation in a conserved residue of valine 279 of the N-terminal GBD of CYK-1(V279D) and a strain expressing a truncated CYK-1 mutant with a deletion of the C-terminal region (Δ1250-1437). To generate these strains, we used CRISPR-CAS9 endogenous genome editing co-conversion strategy (Dickinson et al. 2013; Arribere et al. 2014; Ran et al. 2013). Single guide RNAs (sgRNA), complementary to the site desired to be cleaved and repair templates consisting of mutation to be inserted, restriction enzyme site for screening and flanking regions were designed and were already available in the lab (Table 2). A mix of Cas9 and sgRNAs and repair template for the desired mutations was injected in N2 animals. The mix also contained sgRNAs and repair template for a mutation that confers a phenotype of “rolling” to the worms if integration is successful.

After the injection of the mix, three animals (F0) per plate were left to grow for 3-4 days at 25 ºC. The progeny (F1) from injected mothers that reached the adult stage were observed and the plates that contained worms with the roller phenotype were selected and kept overnight at 20 ºC to allow worms to lay embryos. In the following day, we screened the F1 adult rollers to assess their mutation status. The screening was done using Polymerase Chain Reaction (PCR). First, each worm was lysed in a PCR tube with a 10 µL reaction that contained 0.5 µL of 20 mg/ml Proteinase K and 9.5 µL of a lysis buffer (10 mM Tris pH 8.5 mM KCl, 1.5 mM $MgCl_2$). The reactions were spin down in a minifuge to assure that the worms settled at the bottom of the tube. The tubes were then placed in a thermocycler with the following conditions: 65 ºC for 90

minutes and 95 ºC for 15 minutes (to inactivate Proteinase K). After the worms were lysed, they were subject to PCR using the oligonucleotides and conditions listed on tables 3 and 4. The PCR product from *cyk-1(V279D)* mutant screen was digested with SpeI restriction enzyme (digestion reaction 20 µL, 37 ºC for 2 hours: 16.9 µL of distilled H_2O, 2 µL 10x Fast Digest Green Buffer (Thermo Scientific), 1 µL of PCR product and 0.1 µL of restriction enzyme SpeI (Thermo Scientific).

If the repair template carrying the mutation was rightly integrated in the genome, a SpeI site must have been integrated also and digestion of the PCR product should detect the generation of fragments of 619 and 340 bp when ran on a 1% agarose gel. In cases where the repair template was not successfully integrated, the band should be of 959 bp when ran on a 1% agarose gel. In the case of proper integration of the repair template for the 1250-1437 truncation the PCR product was just ran on a 1% agarose gel. The mutated version should be 627 bp and the wild-type version should be of 911 bps.

After screening of the F1 progeny, we obtained six positive samples for CYK-1 (V279D) and one positive sample for CYK-1(Δ1250-1437).

Table 2 - List of CRISPR/Cas9 single guide RNAs (sgRNAs) and repair templates used in this study.

Gene/Mutation	**Repair template***	**Diagnosis PCR and restriction enzyme**	**sgRNA sequence**
cyk-1(V279D)	GGGAGAAATATTGAAAACGAAAA ACATTCCGGAATGCAAGCAGGAT ATTGTTACTGTtCGaGaTCAaCTAG TaGGTCAgGGTGTTTCATTTCTTA ATAAGGTTCGTTTTTTAAGATAGA TATTGTTACTATTAAAATAC	Forward primer: CCGTTCCT ATGGTGC TAAGATG Reverse primer: GAGAGTT CCGTCGC AATACAA Restriction enzyme: SpeI	sgRNA#1 CTTGGGGT TCAGCTAG TTGGTCAA AACTGACC AACTAGCT GAACCC sgRNA#2 CTTGCTGT ACGGGTTC AGCTAGTA AACACTAG CTGAACCC GTACAG sgRNA#3 CTTGCAGG ATATTGTTA CTGTAC

			AAACGTAC AGTAACAA TATCCTG
*cyk(*Δ1250-1437)	GTTTCGACTGGTGAGTTGATATTT TTGAAATTGTTTTTTAATATAATTT TTTCAGCTGCCTGAAAGCTCACAT CGGTTGTCTTATCTATAGATCTAG TCATTCTCCGTCTCTATTTTC	Forward primer: TTGAGACA CAGAAGC GGAAG Reverse primer: GAAGAGG GACCCTG TGAATG	sgRNA#1 CTTGTCGA GCACACCA GCCTTAT AAACATAA GGCTGGTG TGCTCGA sgRNA#2 CTTGGCTG GTGTGCTC GATGAGTA AACACTCA TCGAGCAC ACCAGC sgRNA#3 CTTGCGAG AAGATCGT CTGTTGAA AACTCAAC AGACGATC TTCTCG

*Bases in lowercase indicate silent mutations introduced to avoid repair template recognition by Cas9 and/or to introduce a restriction site (underlined bases) for diagnostic PCR of genomic edits.

Table 3 - List of CRISPR/Cas9 single guide RNAs (sgRNAs) and repair templates used in this study.

PCR reaction for CYK-1(V279D) mutant	
Component	Volume (µL)
Lysed DNA	2,5
2x Master Mix (NZYTech)	7.5
10 µM oligonucleotide (oAC 1609)	0.75
10 µM oligonucleotide (oAC 1610)	0.75
distilled H_2O	3.5

Cycles	Thermocycler conditions	Time
	Step 1: 95 °C	2 minutes
10	Step 2: 92 °C	30 seconds
	Step 3: 63 °C	30 seconds
	Step 4: 72 ºC	1 minute
25	Step 5: 92 °C	30 seconds
	Step 6: 53 °C	30 seconds
	Step 7: 72 ºC	1 minute
	Step 8: 72ºC	5 minutes
	Step 9: 8ºC	Pause

Table 4 - Components and conditions used in the PCR reaction to identify CYK-1(Δ1250-1437) mutation.

PCR reaction for CYK-1(Δ1250-1437) mutant	
Component	Volume (µL)
Lysed DNA	2.5
2x Master Mix (NZYTech)	7.5
10 µM oligonucleotide (oAC 1034)	0.75
10 µM oligonucleotide (oAC1035)	0.75
distilled H_2O	3.5

Cycles	Thermocycler conditions	Time
	Step 1: 95 °C	2 minutes
10	Step 2: 92 °C	30 seconds
	Step 3: 63 °C	30 seconds
	Step 4: 72 ºC	1 minute and 30 seconds
25	Step 5: 92 °C	30 seconds
	Step 6: 53 °C	30 seconds
	Step 7: 72 ºC	1 minute and 30 seconds
	Step 8: 72ºC	5 minutes
	Step 9: 8ºC	Pause

Crossing *C. elegans* strains

Outcrossing positive hits for CYK-1(V279D) and CYK-1(Δ1250-1437). We crossed the F2 progeny from positive animals containing the desired mutations with the wild type N2 strain. This outcross was repeated six times with the aim of cleaning the background genome from potential off-target mutations. To confirm the presence of the desired modifications, the PCR products performed as described above were sent to sequence (GATC). As *cyk-1(V279D)* homozygous animals were sterile and *cyk-1(Δ1250-1437)* homozygous animals generated unviable eggs, we had to depart the outcrossing from heterozygous animals. The outcross procedure was done in the same way for both CYK-1 mutants.

On the first outcross, we performed a mating with 8 hermaphrodites on L4-stage from the progeny of a heterozygous mother (mut/wt) with 16 N2 males on L4-stage, overnight at 25 ºC. On the following day, the 8 hermaphrodites were singled out

and left to lay embryos at 25 ºC for 20 hours. After laying some embryos, we screened the hermaphrodite mothers to assess their CYK-1 mutation status. Plates that contained progeny derived from a mother that scored positive for the mutation by PCR were kept and the others discarded. Of these, those with 50% of males (where mating was successful) were selected. On the following outcrosses, 16 males on L4-stage (+/+ ; mut/+) from positive plates of the first outcross were chosen to mate with 16 N2 hermaphrodites on L4-stage (1 male L4-stage (+/+; mut/+) and 1 hermaphrodite animal L4-stage N2 per plate). The plates were kept overnight at 25 ºC. On the following day, the males were screened for CYK-1 mutation and the hermaphrodite N2 animals were left on the plates, in order to lay embryos for 20 hours. Male progeny from the mating plates whose father scored positive on the PCR were chosen to perform the next outcross. The outcrosses were repeated four more times. At the end of the sixth outcross, *cyk-1(V279D)* homozygous animals continued to be sterile and *cyk-1(∆1250-1437)* homozygous animals continued to generate unviable eggs and they could not be propagated. To maintain these mutants two strategies were used. In the first approach, mut/+ were kept in heterozygosity after balancing. The balancing consisted in crossing heterozygotic mutants (mut/+) with GFP-marked balancer strain JK2739 to generate the strains GCP819 and GCP896. The balancer strain facilitates the identification of homozygous mutant worms, and promotes the propagation of only the balanced allele that labels the animal's pharynx with GFP. Thus, after the crossing of heterozygous mutants for the desired modifications with the balancer strain, the worms generated contained one allele that comes from the heterozygous mutant and one allele that comes from the balancer strain (bal). The progeny of these worms includes the following genotypes: bal/bal, which are lethal, bal/mut, which are wild-type looking and have GFP-labeled pharynx, and mut/mut that are GFP-negative in the pharynx and are sterile in the case of *cyk-1(V279D)* or lay dead eggs in the case of *cyk-1(∆1250-1437)*. Using this strategy we were able to propagate the balanced strain (as heterozygous mutant worms were fertile and easily identifiable) and to isolate sterile homozygous *cyk-1(V279D)* animals for analysis of the gonad as shown in figures 8B and homozygous *cyk-1(∆1250-1437)* embryos to calculate percentage of embryonic lethality (Figures 13B and 13E). In the second approach, we were able to keep the *cyk-1(V279D)* and *cyk-1(∆1250-1437)* alleles in homozygosity after crossing the strains GCP819 and GCP896 with GCP741 to generate the strains GCP820 and GCP928, respectively. The resulting animals expressed the point mutation V279D or deletion 1250-1437 from the endogenous cyk-1 gene in chromosome III and a wild-type copy of GFP-labelled cyk-1 in chromosome II. The presence of the wild-type version of cyk-1 allows the *cyk-1(V279D)* worms to be fertile and *cyk-1(∆1250-1437)* to develop viable

embryos. Specific depletion of the transgenic wild-type CYK-1 by RNAi (see below) decreased the levels of wild-type protein in the embryos, which led to embryonic lethality in *cyk1(V279D)*.

Embryonic Viability test

The embryonic viability test was performed on *cyk-1(V279D),* cyk-*1(∆1250-1437)* and wild type animals. To check embryonic viability of *cyk-1(V279D)* embryos compared to controls, twenty L4-stage animals of the strain GCP741 (that constitute the control situation) or GCP820 were singled out on two different kinds of plates: plates seeded with OP50 and plates seeded with bacteria expressing cyk-1_RNA#2 (Table 5). All plates were kept at 20 ºC for 40 hours. To check embryonic viability of *cyk-1(∆1250-1437)* embryos, fourteen wild type L4-stage animals or nine L4-stage mut/mut homozygous of the strain GCP896 (negative for GFP signal on the pharynx) were singled out on plates seeded with OP50 and kept at 20 ºC for 40 hours. After the period of 40 hours, the adults of all conditions were transferred to new plates seeded with OP50 at 20 ºC for 8 hours to allow the worms to lay embryos (1 adult per plate). The adults were then removed and embryos on the plate were kept at 20 ºC for 48 hours to allow for their development. At the end of the 48 hours, the number of hatched progeny and dead embryos were counted.

RNA interference

RNAi was performed by feeding worms with bacteria that expressed the dsRNA of interest. DNA fragments of interest were cloned into the L4440 vector and these were transformed into HT115 *E. coli*. In this project we used, a cyk-1_RNA#1 to target F11H8.4 (cyk-1) gene and a cyk-1_RNA#2 to target cyk-1:GFP reencoded (cyk-1::GFP^{re}) (Table 5). For cyk-1_RNA#1, the L4440 vector was obtained from the Ahringer library (Source Bioscience) and sequenced to confirm gene target. For cyk-1_RNA#2, the cyk-1 DNA fragment was amplified from a synthesized re-encoded region and was cloned into the EcoRV site in the L4440 vector. RNAi treatment conditions were different depending on the use of cyk-1_RNA#1 or cyk-1_RNA#2. In experiments where specific depletion of transgenic CYK-1::GFP^{re} was desired, cyk-1_RNA#2 was used in GCP741, GCP820, GCP880, GCP883, GCP928 L4 stage animals at 20 °C during 52 hours (Figures 10,11,12 and 14).

In cyk-1 (RNAi) experiments where the purpose was to deplete endogenous CYK-1, cyk-1_RNA#1 was used in GCP741 L4 stage animals at 20 °C for 48 hours (Figure 9).

Table 5 - List of dsRNAs used in this study.

Name	**Gene target**	**Forward primer**	**Reverse primer**	**RNAi**
cyk-1_RNA#1	F11H8.4 (cyk-1)	----------	----------	feeding (Ahringer library)
cyk-1_RNA#2	cyk-1:GFP reencoded (cyk-1::GFP^{re})	CCCCCGATATCGGAATTCTCCAAGGCTC	CCCCCGATATCCATTCGCATGTCAAATGC	feeding

Results

The experimental work I describe here was conducted by myself and Fung Yi Chan. The immunoblots, live imaging, characterization of the CYK-1::GFP^{re} transgenic version and protein alignments were done by Fung Yi Chan alone.

1 - Animals expressing CYK-1(V279D) animals are sterile

Mammalian diaphanous formins are known to be regulated by Rho (Kühn and Geyer 2014). We started this work by checking that CYK-1 is also regulated by Rho. If that is the case, it is expected that embryos expressing mutant CYK-1 that lacks the ability to bind active Rho1 should behave as a constitutively inactive mutant and consequently generate similar effects to those observed in embryos where CYK-1 is depleted, which are unable to complete cytokinesis (Chan et al. 2018; Severson, Baillie, and Bowerman 2002; Davies et al. 2014; Swan et al. 1998). To test the veracity of this hypothesis, we generated a Rho-binding defective mutant of CYK-1, through mutation of residue V279D in the DID domain using CRISPR/Cas9 technology. According to *in vitro* studies, the modification of the hydrophobic residue of valine to the hydrophilic residue of aspartate in mDia1(V161D) and in mDia2(V180D) causes strong deficient Rho-binding without affecting the DID-DAD interaction (Otomo, Otomo, et al. 2005; Seth, Otomo, and Rosen 2006; S. Watanabe et al. 2010). This residue of valine in the DID domain is conserved in CYK-1 (Figure 8A). We were able to generate heterozygous animals expressing CYK-1(V279D) (Figure 8B). Homozygous animals developed into sterile adult worms: by differential interference contrast microscopy, we observed that the gonad and vulva of N2 (wild type) animals developed properly and produced embryos; in contrast, the homozygous mutant animals had non-functional gonads, presented a protruded vulva, did not produce embryos and did not move normally (Figure 8C).

To assess the protein expression levels of the wild-type and *cyk-1(V279D)* animals, we performed an immunoblotting using an antibody against CYK-1. The results revealed that the levels of CYK-1 were similar in wild type and homozygous cyk-1(V279D) animals, suggesting that the introduction of the mutation V279D in CYK- 1 did not affect protein stability (Figure 8D).

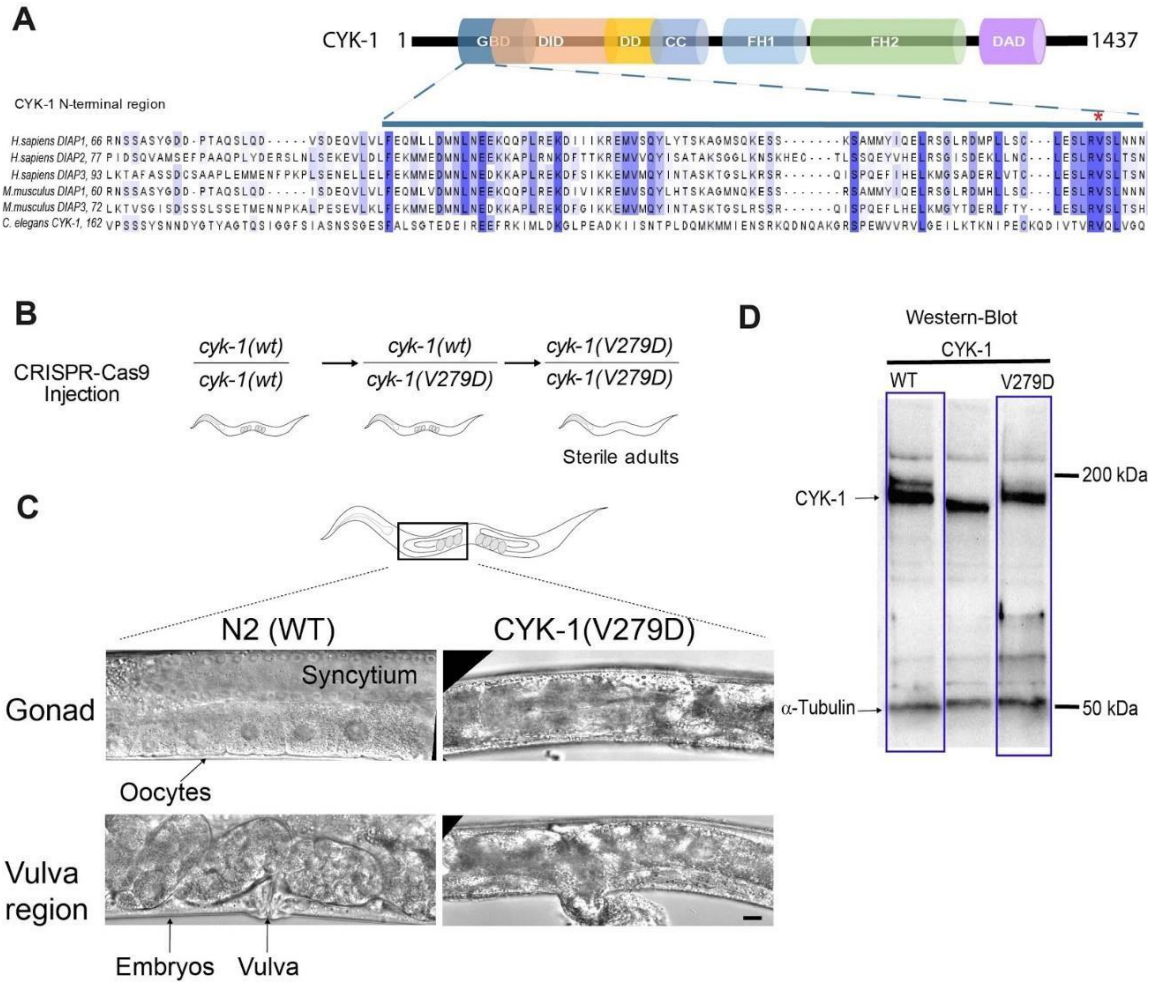

Figure 8 – Expression of CYK-1(V279D) leads to animal sterility.A. Protein sequence alignment of GBD domains of Homo sapiens formin DIAP1-3 paralogs, Mus musculus formins DIAP1 (also known as mDIA1), DIAP3 (also known as mDIA2) and *C. elegans* formin CYK-1. Residue mutated to alanine is marked by a red asterisk. **B.** Schematic illustrating the isolation of Cyk-1(V279D) animals. Homozygous progeny were able to develop into adults but were sterile. **C.** Differential interference contrast (DIC) images of gonad and vulva of wild type and cyk-1(V279D) animals**. D.** Immunoblot showing the levels of CYK-1 in wild type and homozygous cyk-1(V279D) animals. α-Tubulin is used as a loading control. Scale bar, 10 µm.

2 - Embryos expressing CYK-1(V279D) are not viable

To assess the effects of expressing CYK-1(V279D) during embryogenesis and cytokinesis in the early embryos, we crossed the heterozygous *cyk-1(V279D) -/+* animals with animals expressing a transgenic version of CYK-1 that is fused to GFP and has part of its ORF re-encoded. The latter had been previously generated in our lab using the Mos-1 mediated single-copy insertion (MosSCI) technique that allows for insertion of a transgene in single copy and in a defined chromosome locus (Frøkjær-Jensen et al. 2008). Briefly, the transgene constructed by our group was integrated into chromosome II and it was constituted by the entire cyk-1 endogenous locus containing

the open-reading frame (ORF), promoter and 3'-untranslated region (3'UTR), as well as a GFP-tag to follow the localization of CYK-1 during cytokinesis. The transgene also included a region of approximately 400 base pairs that was re-encoded in order to confer RNAi specificity. This feature was crucial to enable the specific depletion of endogenous CYK-1 or transgenic CYK-1 as desired (Figure 9A). To confirm the functionality of the transgenic protein CYK-1::GFP^{re} (re standing for re-encoded), embryos expressing CYK-1::GFP^{re} were tested for viability after depletion of endogenous CYK-1 using RNAi#1 (see methods). Embryos were viable, which indicates that CYK-1::GFP^{re} is functional (Figure 9).

Crossing *cyk-1(V279D)-/+* animals with animals expressing CYK-1::GFP^{re} allowed us to homozygoze the mutant. We obtained a stable strain called GCP821 carrying both copies of *cyk-1(V279D)* and two copies of cyk-1::gfp^{re}. In the presence of CYK-1::GFP^{re}, *cyk-1(V279D)* embryos were viable (Figure 10 A). However, when we specifically depleted CYK-1::GFP^{re}, using cyk-1_RNA#2 (see methods), without affecting the expression of CYK-1(V279D), all embryos failed to hatch (Figure 11B). We conclude that expression of CYK-1(V279D) alone cannot support embryo development.

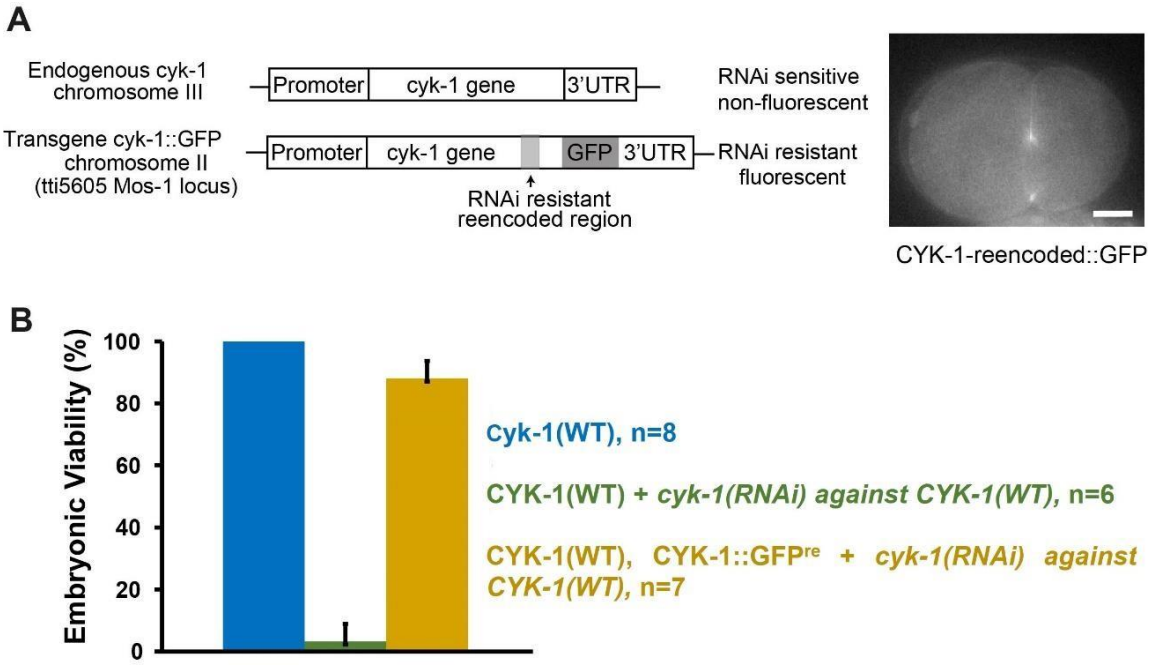

Figure 9 – CYK-1::GFP^{re} probe localizes in the contractile ring and rescues embryonic viability upon depletion of endogenous CYK-1 by RNAi. A. Schematic of the endogenous and transgenic cyk-1 loci. The transgenic cyk-1 containing a re-encoded region for RNAi resistance and fused to GFP (CYK-1::GFP^{re}) was introduced in single copy in a defined position of chromosome II using MosSCI (left). Transgenic CYK-1::GFP^{re} probe localizes in the contractile ring at the tip of the cleavage furrow of the 1-cell *C. elegans* embryo (right). **B.** Embryonic viability test. Cyk- 1(RNAi) was performed in the wild-type strain (N2 strain) and in the strain expressing CYK-1::GFP^{re}. CYK-1::GFP^{re} is

functional because it rescues embryonic viability after RNAi of endogenous CYK-1.Scale bar, 10 µm.

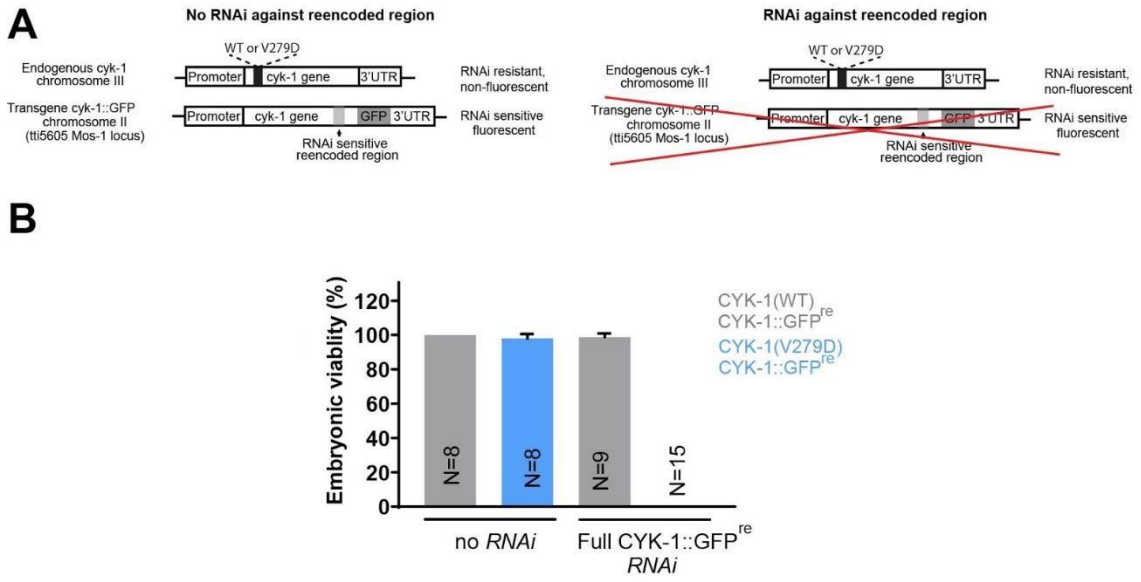

Figure 10 - CYK-1 RHO-binding deficient mutant causes embryo lethality.A. Schematic diagram of the endogenous and transgenic cyk-1 loci in wild-type and mutants (left). Specific deletion of CYK-1::GFP^{re} by RNAi#1 leads to the expression of the endogenous version (right). B. Embryonic viability of progeny of animals expressing wild- type (grey) or cyk-1(V279D) (blue) from endogenous locus and CYK-1::GFP^{re} from the transgene upon depletion or not of CYK-1::GFP^{re}.

3 - CYK-1(V279D) does not support cytokinesis in 1-cell embryos

After observing that CYK-1(V279D) had a negative impact on the viability of the embryos, we next intended to understand the repercussions of this mutation on cytokinesis.

To assess cytokinesis, we performed live imaging in 1-cell embryos expressing wild-type or mutant CYK-1 from the endogenous locus and CYK1::GFP^{re} from the transgene (Figure 11A). These embryos were also expressing mKate2-labeled non-muscle myosin II (NMY-2::mKate2) to monitor the contractile ring and mCherry- labeled histone H2B to monitor the chromosomes and cell cycle stage (H2B::mCherry). Before filming, embryos were specifically depleted of CYK1::GFP^{re}. In embryos expressing wild-type CYK-1 and CYK1::GFP^{re}, our control situation, penetrant depletion of CYK1::GFP^{re} did not affect the success of cytokinesis. In these embryos, the contractile ring formed normally, as NMY-2::mKate2 accumulated in the equatorial region of the cell, and the timing of cytokinesis occurred like in wild-type embryos.

In embryos expressing CYK-1(V279D) and CYK1::GFP^{re}, penetrant depletion of CYK1::GFP^{re} caused cytokinesis failure and no furrow ingression was observed. This phenotype is similar to that observed when embryos completely lack CYK-1 (Figure 11B). We conclude that in the absence of wild-type CYK-1, CYK-1(V279D) does not support cytokinesis in 1-cell embryos.

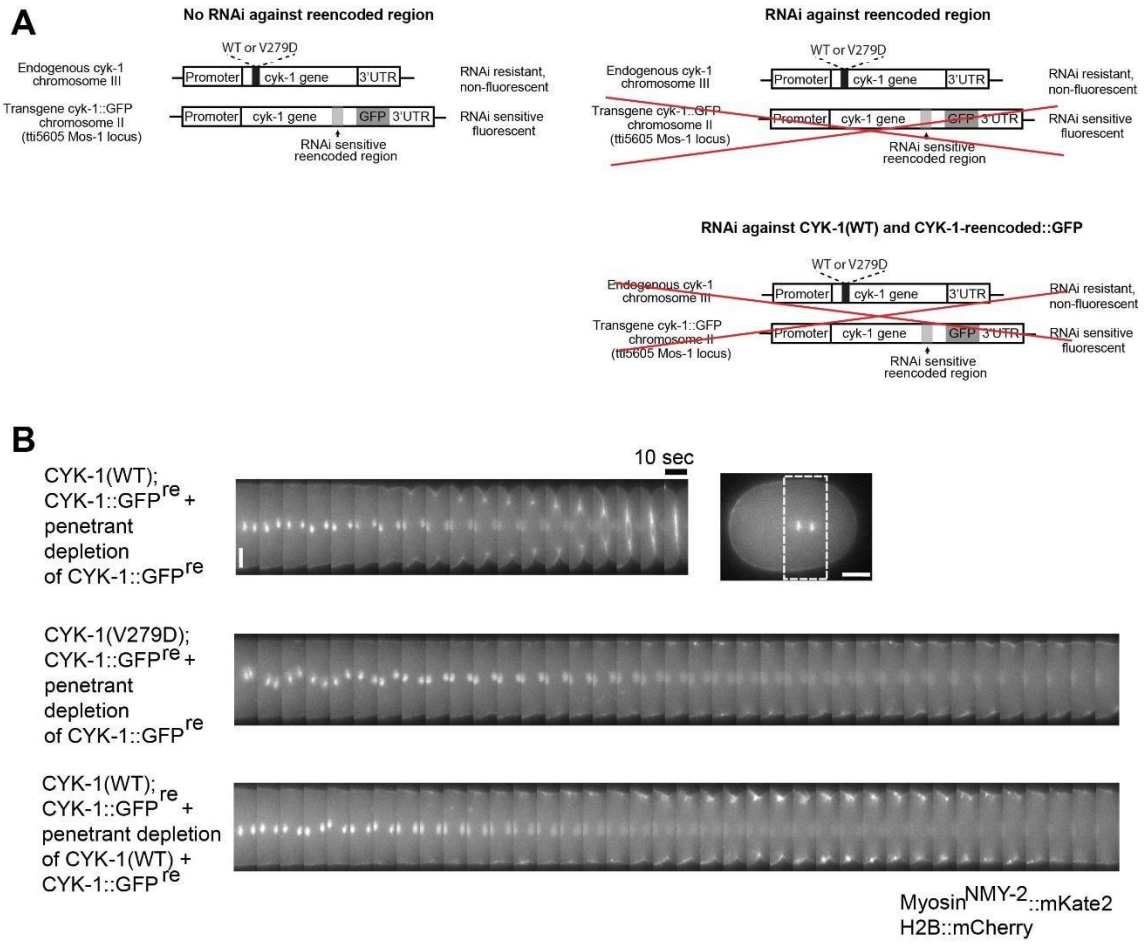

Figure 11 - CYK-1(V279D) leads to failure of cytokinesis in 1-cell embryos. A. Schematic of the endogenous and transgenic cyk-1 loci (left)**.** Specific depletion of CYK1::GFP^{re} allows for the embryos to only express CYK-1(WT) or CYK-1(V279D) (right top). Depletion of CYK1::GFP^{re} and CYK-1 allows for the embryos to express no CYK-1 at all (right bottom). **B.** Kymographs of the equatorial region of 1-cell control embryos (top) or cyk-1(V279D) embryos (middle) after penetrant depletion of CYK1::GFP^{re}. In the third row an example of an embryo expressing wildtype CYK-1 and CYK1::GFP^{re} after depletion of both versions of CYK-1 is shown. All embryos express myosin NMY-2::mKate2 and H2B::mCherry. First frame corresponds to anaphase onset. Scale bars, 10 µm.

4- CYK-1(V279D) does not elongate actin filaments bundles at the cell equator

To evaluate the effect of Rho-binding CYK-1(V279D) on actin filaments, we generated embryos co-expressing wild-type or mutant CYK-1, CYK1::GFP^{re} and a fluorescent probe to follow the localization of actin (LifeAct::mRFP). The cortex of these embryos was filmed while embryos underwent division after penetrant depletion of CYK1::GFP^{re}. In embryos expressing wild-type CYK-1 and CYK1::GFP^{re} after penetrant depletion of CYK1::GFP^{re}, we observed that actin filament bundles were scattered throughout the cortex and 50 seconds after this event, the actin filament bundles started to accumulate in the equatorial region. This accumulation of actin in the equatorial region and reduction of its levels in the surrounding regions corresponds to the formation of the contractile ring. In embryos expressing wild-type CYK-1 and CYK1::GFP^{re} after penetrant depletion of CYK1::GFP^{re}, we observed that the accumulation of actin filaments in the equatorial region never happened (Figure 12).

These results suggest that the mutation V279D produced a constitutively inactive version of CYK-1, in the presence of which cytokinesis fails because the contractile ring fails to form. Thus, these findings strengthen the evidence that RHO-1 is an upstream regulator of CYK-1 and needs to bind to CYK-1 to promote its activation, ensure the nucleation and elongation of F-actin linear filaments and form the contractile ring in the equatorial region of the cell.

All these results together demonstrate that the binding of RHO-1 to the formin CYK-1 is fundamental and indispensable for proper formation of the gonad and vulva in *C. elegans* as well as for embryonic cytokinesis. RHO-1 is therefore the major upstream regulator of CYK-1, like in mammalian cells.

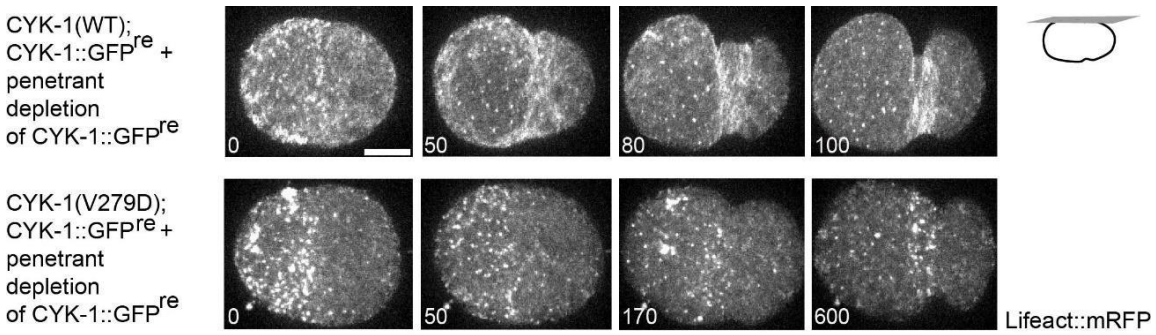

Figure 12 - CYK-1(V279D) causes cytokinetic failure due to incapacity to recruit and nucleate F-actin linear filaments at the cell equator. Time lapse imaging series showing the cortex of 1-cell control embryos (top) and cyk-1(V279D) (bottom) embryos after penetrant depletion of CYK1::GFP^{re}. All embryos express Lifeact::mRFP. The

interval shown is from anaphase onset (time point 0 s). Scale bar, 10 µm.

5 - Animals expressing CYK-1(∆1250-1437) generate unviable progeny

Given our results described above and the accepted model for mammalian formin activation, we expect that removal of the DID or the DAD domains of CYK-1 should result in a constitutively activated CYK-1. Above we described what happened in the presence of a constitutively inactive version of CYK-1, next, we attempted at generating a constitutively active CYK-1 where DAD cannot interact with DID. To do this we generated a truncated CYK-1 lacking residues 1250-1437. The truncated region corresponds to the DAD domain and the C-terminus region of the protein (Figure 13A). This truncation was chosen because previous studies indicate that basic residues downstream of DAD also contribute to mDia1 auto-inhibition as they bind to a conserved acidic region within the DID (Nezami, Poy, and Eck 2006). This truncation should not affect CYK-1 to mediate actin filament elongation because FH1 and FH2 domains, responsible for recruitment and nucleation of F-actin filaments are not modified.

To check protein levels of CYK-1 and CYK-1(∆1250-1437) in wild-type and *cyk-1(∆1250-1437)* animals, respectively, we performed an immunoblot using an antibody against CYK-1. The results of this assay revealed that the levels of CYK-1 were similar in both types of animals (Figure 13D).

Observations by differential interference contrast revealed that in contrast to embryos laid by wild-type animals, embryos laid by *cyk-1(∆1250-1437)* animals seem to lack compartments (Figure 13C). Indeed, assessing embryonic viability revealed that all these embryos were not viable, which is a strong indication that this mutation promotes defects during cytokinesis (Figure 13E).

These results demonstrate that CYK-1(∆1250-1437) does not support embryo development.

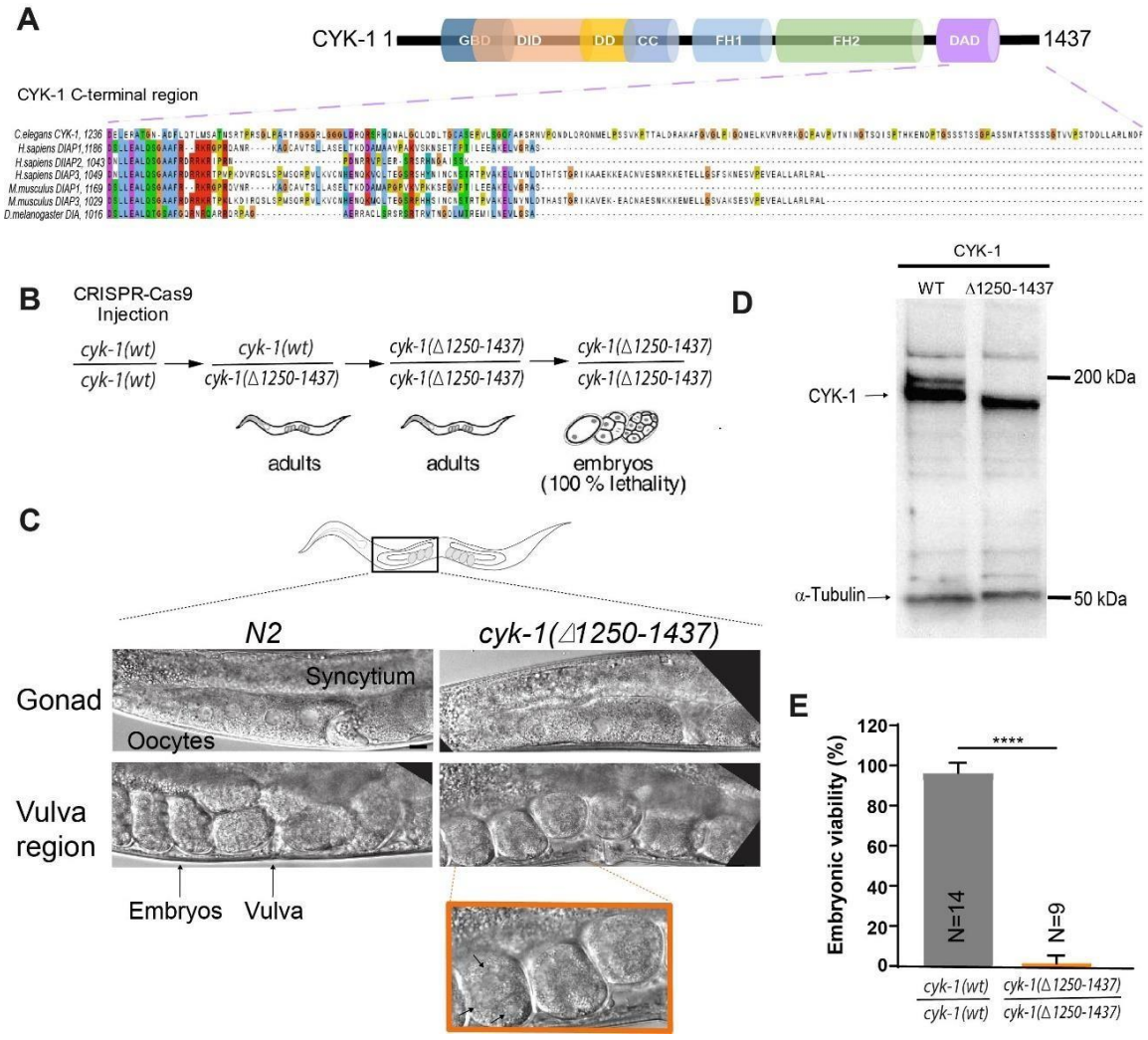

Figura 13 – Cyk-1(Δ1250-1437) causes embryo lethality due to inability to proceed cytokinesis after first division. A. Protein sequence alignment of DAD domain and C-terminus of Homo sapiens formin DIAP1-3 paralogs, Mus musculus formins DIAP1 (also known as mDIA1), DIAP3 (also known as mDIA2) and *C. elegans* formin CYK-1. **B.** Schematic illustrating the isolation of cyk-1(Δ1250-1437) animals. Homozygous progeny was able to develop into adults but developed unviable embryos **C.** Differential interference contrast (DIC) images of gonad and vulva of the wild type and cyk-1(Δ1250-1437) animals**. D.** Immunoblot showing the levels of CYK-1 in wild type and homozygous cyk-1(Δ1250-1437). α-Tubulin is used as loading control. **E.** Embryonic viability test of wild type (grey) and cyk-1(Δ1250-1437)/cyk-1(Δ1250-1437) mutants (orange) (right). Scale bar, 10 µm.

6 - Embryos expressing CYK-1(Δ1250-1437) fail to complete cytokinesis after first division

To study the influence of cyk-1(Δ1250-1437) mutation on cytokinesis, we successfully obtained heterozygous animals expressing CYK-1(Δ1250-1437) (Figure 13B). From this animals progeny, homozygotic worms expressing CYK-1(Δ1250-1437) were generated and as described above, embryos laid by homozygotic adults were not able to hatch. Thus, to be able to assess the impact of the CYK-1(Δ1250-1437) mutation on embryogenesis and cytokinesis, we crossed the homozygous *cyk-*

1(Δ1250-1437) animals with animals expressing a transgenic version of CYK-1 that is fused to GFP and has part of its ORF re-encoded. This region is sensitive to a specific RNAi and allows us to promote the specific depletion of endogenous CYK-1 or transgenic CYK-1 as desired.

To confirm that cyk-1(Δ1250-1437) mutation caused perturbations during cytokinesis, we did a live imaging assay with 1-cell embryos expressing CYK-1 wild-type from the endogenous locus and CYK1::GFP^{re} from the transgene, our control situation, and 1-cell embryos that expressed CYK-1(Δ1250-1437) from endogenous locus and CYK1::GFP^{re} from the transgene to follow cytokinesis *in vivo* (Figure 14A).

All these embryos also expressed the fluorescent probes mKate2-labeled non-muscle myosin II (NMY-2::mKate2) and (H2B::mCherry) to follow the formation of the contractile ring and to monitor the cell cycle stage, respectively. Before proceeding to the filming, all embryos were submitted to a penetrant depletion of CYK1::GFP^{re}. In control embryos, penetrant depletion of CYK1::GFP^{re} did not impact cytokinesis, allowing for normal contractile ring formation, as NMY-2::mKate2 accumulated in the equatorial region of the cell, and the timing of furrow ingression was similar compared to that in wild type embryos in absence of RNAi. Oppositely, the footage of 1-cell embryos expressing CYK-1(Δ1250-1437), after penetrant depletion of CYK1::GFP^{re}, caused a slight delay compared to the control situation to complete furrow ingression (Figure 14B).

After completing the furrow ingression, embryos of control situation were able to proceed to the later stages of cytokinesis normally, while the 1-cell embryos of the homozygous mutant exhibit a regression in the furrow 820 seconds after anaphase onset that causes cytokinesis failure.

These results indicate that CYK-1 needs to be activated during the early stages of cytokinesis to nucleate and polymerize linear actin bundles for the assembly of the contractile ring and subsequent constriction. When CYK-1 is constitutively active, cytokinesis fails in the later stages, presumably during abscission, which suggests that CYK-1 needs to be inactive during this stage for cytokinesis to occur properly (Figure 14C).

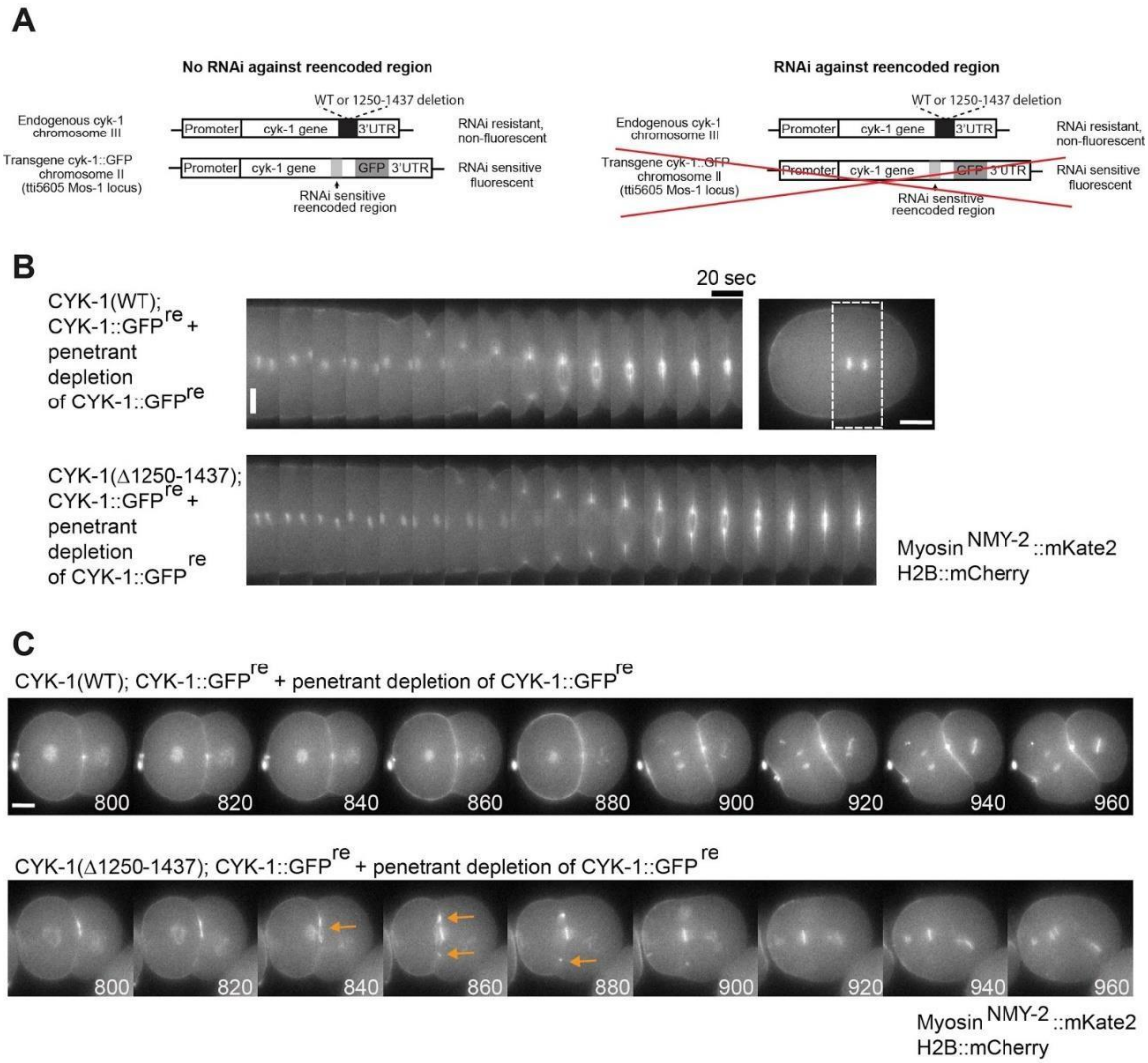

Figure 14 - Expression of CYK-1(Δ1250-1437) causes furrow regression after complete furrow ingression. A. Schematic of the endogenous and transgenic cyk-1 loci (left)**.** Specific depletion of CYK1::GFP^{re} allows for the embryos to only express CYK-1(WT) or CYK-1(Δ1250-1437) (right). **B.** Kymographs of the equatorial region of 1-cell embryos expressing wild type or CYK-1(Δ1250-1437) after penetrant depletion of CYK1::GFP^{re}. All embryos co-express NMY-2::mKate2 and H2B::mCherry. First frame corresponds to anaphase onset. **C.** Live imaging of 1-cell embryos expressing wild type (top) or cyk-1(Δ1250-1437) after penetrant depletion of CYK1::GFP^{re} (bottom). First still corresponds to time point after complete furrow ingression. Orange arrows point at furrow regression. Time is in seconds after anaphase onset. Scale bars, 10 µm.

Discussion and Future perspectives

Cytokinesis is a fundamental process in all eukaryotic cells to allow their development in size and complexity, ensuring the viability and proper growth of eukaryotic organisms. Defects in this process cause crucial perturbations, as it provides the occurrence of successive defective mitosis and chromosomal instability that are responsible for the emergence of multiple diseases, including cancer (Lacroix and Maddox 2012)

These features reflect why the study of cytokinesis is so fundamental. Although in the last decades many components have been identified and fundamental steps characterized, the field of cytokinesis research is still full of interrogations that need to be addressed (Pollard 2017).

Model organisms that have been used in the cell division field include the fission yeast *Schizosaccharomyces pombe*, the amoeba *Dictyostelium discoideum* and the metazoan *Caenorhabditis elegans*. Cytokinesis is the last step of cell division, which depends on the assembly of an actomyosin ring to generate the contractile force to divide one into two daughter cells (Cheffings, Burroughs, and Balasubramanian 2016). The assembly of the contractile ring requires the activity of formins, a highly conserved family of proteins that are known for their crucial role in promoting rapid assembly of non-branched actin filaments (S. Watanabe et al. 2008; Pruyne et al. 2002; Severson, Baillie, and Bowerman 2002). To perform its precise function during cytokinesis, formins need to be highly regulated, in order to promote assembly of actin structures at the right place and time.

Within formins, a predominant subclass of Diaphanous related formins (DRFs), in mammalians cells is auto-regulated. DRFs auto-regulation consists in an intramolecular interaction between the C-terminal Diaphanous autoregulatory domain (DAD) and the N-terminal Diaphanous inhibitory domain (DID). The interaction between DID and DAD keeps the DRF molecule in an inactive state, which prevents it from elongating actin filaments (Alberts 2001; Schönichen et al. 2006; Vaillant et al. 2008; W. Liu et al. 2008). The regulatory mechanism for mammalian DRF's activity is relatively well characterized: binding of Rho-GTP to the N-terminal GTPase binding domain in the DRF, prevents the DID-DAD interaction and consequently enables the formins to assemble linear actin filaments (N. Watanabe et al. 1997, 1999; S. Watanabe et al. 2010; Rose et al. 2005; Otomo, Otomo, et al. 2005; Lammers et al. 2005)**.** Although this may be an important regulatory mechanism, other mechanisms have been shown to exist.

Due to indications of the existence of various mechanisms that regulate formin activity, in this project we investigated the regulatory mechanism of formin CYK-1 specifically during cytokinesis, using dividing 1- cell *C. elegans* embryos. This system has been reported as a powerful system that offers many advantages to the study of cell division processes (Hattersley et al. 2018). It is important to study how CYK-1 formin is regulated in *C. elegans* because it is known that in this model organism, CYK-1 formin localizes in the contractile ring and it was shown that a temperature sensitive mutant of CYK-1 and penetrant depletions of CYK-1 by RNAi lead to lack of F-actin in the equatorial cell cortex and hence cytokinesis failure (Swan et al. 1998; Severson, Baillie, and Bowerman 2002; Davies et al. 2014; Chan et al. 2018). This suggests that in *C. elegans*, CYK-1 is the only formin required for nucleation of the actin filaments that form the contractile ring during cytokinesis (Swan et al. 1998; Severson, Baillie, and Bowerman 2002; Davies et al. 2014; Chan et al. 2018). To proceed with our investigation, we prioritized the verification of a mechanism dependent on RHO-1, the analogous of RhoA in *C. elegans*. The reasons behind this decision are related to the facts that formin activation by a Rho-dependent mechanism seems to be the major mechanism for activation of several mammalian formins. Moreover, an analogous protein to Sid2 does not seem to be conserved in *C. elegans*; this is expected because Sid2 is a kinase involved in the assembly of the septum in yeast, which does not occur in *C. elegans*. To proceed with our investigation, we verified that the GBD domain of CYK-1 is only 25 % similar to that of human mDia3 (DIAP2). However, the secondary structure of GBD is similar in both proteins, consisting of five alpha-helices. Moreover, the residue described above as V161 in mDia1 and V180 in mDia2 that when mutated to aspartate (D) revealed a Rho-binding deficient behavior (Otomo, Otomo, et al. 2005; Seth, Otomo, and Rosen 2006; S. Watanabe et al. 2010) was conserved in CYK-1 and corresponds to V279. To see if RhoA had an impact in regulating CYK-1, we generated embryos expressing CYK-1(V279D) and our results show that CYK-1 is directly regulated by RHO-1 during cytokinesis. In the future, we should prove that CYK-1 GBD binds RHO-1 but CYK-1(V279D) does not bind *in vitro* assays to confirm that CYK-1(V279D) is a Rho-binding deficient mutation.

Our results strongly indicate that binding to RHO-1-GTP is the major mechanism for CYK-1 activation during early embryonic cytokinesis. In the future, we should look at additional factors that may assist RHO-1 in formin activation. Of the factors that are known to assist RHOA in activation of mammalian formins (see above), only the study of anillin and ROCK are relevant in *C. elegans*, because the Fli-I binding sequence that is conserved in diverse formins from several organisms is only present in the *C. elegans* formin Daam that is not required for cytokinesis (Higashi et al. 2010).

A kinase analogous to the ROCK, designed LET-502, exists in *C. elegans* and it will be interesting to investigate if LET-502 and CYK-1 could interact by co-immunoprecipitation assay. Besides, other kinases in mammalians such PKA or PKG contain domains that can interact with the sequence of DAD domain in a similar way to ROCK (Pearce, Komander, and Alessi 2010) and these possible interactions could be also tested in *C. elegans,* as both proteins have homologues in *C. elegans:* KIN-1 and PKG-2, respectively. In this case, an *in vitro* kinase assay can be used to see if similar phosphorylation occurs in worms. As for the anillin pathway, *C. elegans* contains three proteins with homology to anillin (ANI-1, ANI-2 and ANI-3) but only ANI-1 seems to be essential for viability of the early embryo (Maddox et al. 2005). Thus, we should also do a co-immunoprecipitation assay to check if ANI-1 and CYK-1 interact.

We also observed that *cyk-1(V279D)* homozygous animals presented abnormal gonads and were therefore sterile. Moreover, these animals also had locomotion problems. These evidence support the idea that CYK-1 activation by RHO is also important in other cellular contexts.

In fact, these are in agreement with previous studies that reported that CYK-1 was required for maintenance of adult body wall muscle (BWM) actin organization and for syncytial germline architecture (Mi-Mi et al. 2012; Priti et al. 2018). Animals expressing mutants of CYK-1 exhibited movement defects and long-term depletion of CYK-1 by RNAi led to worm paralysis or death (Mi-Mi et al. 2012). The syncytial germline architecture is regulated by contraction of a corset-like actomyosin structure surrounding the rachis. It was found that CYK-1 localizes at the rachis envelope and temperature sensitive mutants of CYK-1 show significant reductions of F-actin levels and leads to defects in the early meiotic region of the germline, while depletion of CYK-1 by RNAi results in failure of germ cell cellularization and leads to sterility (Priti et al. 2018)).

Other studies revealed that CYK-1 is also required in other tissues, like the intestine. It was shown that CYK-1 is essential for actin organization in intestinal epithelia, where PTRN-1, a microtubule minus end binding protein, is a positive regulator of actin polymerization through a specific interaction with CYK-1. PTRN-1 overlaps with CYK-1 in punctate structures and its depletion significantly reduces actin structures. It was shown *in vitro* that in the absence of PTRN-1, CYK-1 only displays a moderate capacity to promote actin polymerization. Further, it was demonstrated that PTRN-1 binds to CYK-1 through an interaction between the PTRN-1 N-terminal CH domain and the N-terminal GBD of CYK-1, which suggests that PTRN-1, like RHOA could disrupt CYK-1 autoinhibition (Gong et al. 2018).

In the future, we should check the localization of CYK-1(V279D) in muscles and whether F-actin levels are affected just like in muscles where CYK-1 was depleted. We should also look at PTRN-1 localization in early embryos to see if it co-localizes with CYK-1 and verify whether PTRN-1 could also regulate CYK-1 activity during cytokinesis.

After showing that regulation by RHO-1 binding is most likely the main mechanism for CYK-1 activation during early embryonic cytokinesis, we wanted to investigate what would be the consequences of having CYK-1 always active during cytokinesis. In mammalians DRFs, the DAD contains a conserved sequence "MDXLLXXL" that constitutes the core region of DAD and downstream to this sequence a basic region "RRKR" was also identified. Both regions were found to be essential for the binding to DID and thus regulate DRFs activity *in vivo* (Alberts 2001; Wallar et al. 2006) (Bohnert et al. 2013; Yonetani et al. 2008). If the DAD-like region of CYK-1 acts like DAD of mDia1, we expected to be able to make a constitutively active CYK-1 by generating the mutant CYK-1(Δ1250-1437). This mutant lacks DAD and the downstream region and therefore should not be able to be in the closed conformation. As the FH2 domain is not affected, CYK-1(Δ1250-1437) should still be able to nucleate and elongate F-actin. We expected that the constitutively active mutant of CYK-1 had a negative effect during cytokinesis as increased levels of actin filaments may be expected and this may have disadvantageous for cytokinesis (Bohnert et al. 2013; Yonetani et al. 2008). Embryos expressing CYK-1(Δ1250-1437) failed cytokinesis by furrow regression after completion of furrow ingression. This indicates that CYK-1(Δ1250-1437) leads to defects in abscission. These results are consistent with multinucleation being observed in mammalian cells after expression of supposedly active mDia2 (DeWard and Alberts 2009). This study also showed that mDia2 is degraded at the end of mitosis and that its ubiquitination targets it for degradation. The similar results exhibited by CYK-1(Δ1250-1437) indicate that we succeeded in generating a constitutively form of CYK-1 that may behave similarly to its homologue in mammalian cells. How active CYK-1 could prevent abscission from happening is unclear. It is possible that non-branched actin has to stop being polymerized for the contractile ring remnant to disappear. This may be important to seal the plasma membranes from both sides of the cleavage furrow.

Another possibility to explain cytokinesis failure in *cyk-1(Δ1250-1437)* embryos, comes from studies *in vitro* with fragments of the *Drosophila* DRF Daam1 that suggests that DAD and C-terminal region together play a role in actin assembly regulation due to binding to actin and contribute to assist the FH2 domain in nucleating actin filaments (Vig et al. 2017). This study showed that Daam1 FH1-FH2 fragment is able to nucleate

actin filaments, but the DAD-C-terminus region considerably increases the process of F-actin nucleation. This finding raises the possibility that our CYK-1(Δ1250-1437) mutant may not be able to nucleate sufficient actin filaments during cytokinesis. However, since contractile ring assembly and constriction seem to occur normally in embryos expressing this mutant, we find this possibility less likely.

In the future, we need to prove that CYK-1(Δ1250-1437) is indeed a constitutively active version of CYK-1. To do this, we need to generate embryos co-expressing CYK-1(Δ1250-1437), CYK1::GFP^{re} and a fluorescent probe to follow the localization of actin (LifeAct::mRFP) and film cytokinesis. If CYK-1(Δ1250-1437) is constitutively active, we could do a penetrant depletion of RHO-1 by RNAi in these embryos and check again the levels of actin. Rho1 is essential for cytokinesis and therefore cytokinesis should fail. However, contrary to RHO-1 depletion in wild type embryos, in *cyk-1(Δ1250-1437)* embryos, the supposedly constitutively active CYK-1 should be insensitive to RHO-1 and therefore should be able to elongate F-actin at the cell equator. Additionally, we can perform *in vitro* pyrene assays to measure actin polymerization of CYK-1(Δ1250-1437) versus CYK-1. If CYK-1(Δ1250-1437) is constitutively active, a significant increase in actin polymerization should be observed in the absence of RHO-1.

Conclusion

After the completion of this project, we can conclude that the objectives that were initially proposed were successfully achieved.

Thus, we have strong evidence that CYK-1, the formin of *C. elegans* that is required during the cytokinesis process, is regulated through a RHO-1 dependent-mechanism of activation, in a similar way, to the mechanism of activation that occurs in mammalian formins which is better described in formin mDIA1.

That way, we have indications that active RHO-1 is necessary to disrupt an intramolecular interaction between the N-terminal DID and C-terminal DAD domains and posteriorly activate CYK-1. The process of activation of CYK-1 needs to be regulated in place and time. After the reorganization of the mitotic spindle during anaphase onset, CYK-1 needs to be recruited for the equatorial region of the cell, where it will assist in the formation of the actomyosin ring that will be necessary for the following stages of cytokinesis. Our investigation also suggests that CYK-1 needs to be inactivated during the later stages of cytokinesis, in order to complete cytokinesis.

In the future, these results need to be clarified, once it is necessary to completely prove that the CYK-1 (V279D) and CYK-1(Δ1250-1437) constitutes a Rho-binding deficient mutant and a constitutively active mutant of CYK-1, respectively. Only in this way, it will be possible to validate our hypotheses, which would become of crucial relevance, once the mechanism by which CYK-1 is regulated has never been solved.

These results would be an asset for the laboratory community that worked with *C. elegans*, once this model organism is widely used in laboratories worldwide. If in the future, we could completely prove that RHO-1 triggers the activation of CYK-1, our research would comprise new goals that would pass for the identification of possible additional factors that could assist RHO-1 in activation of CYK-1.

References

Alberts, A. S. 2001. “Identification of a Carboxyl-Terminal Diaphanous-Related Formin Homology Protein Autoregulatory Domain.” *The Journal of Biological Chemistry* 276 (4): 2824–30.

Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi. 1996. “Phosphorylation and Activation of Myosin by Rho-Associated Kinase (Rho-Kinase).” *The Journal of Biological Chemistry* 271 (34): 20246–49.

Ankeny, Rachel A., and Sabina Leonelli. 2011. “What’s so Special about Model Organisms?” *Studies in History and Philosophy of Science. Part B. Studies in History and Philosophy of Modern Physics* 42 (2): 313–23.

Arribere, Joshua A., Ryan T. Bell, Becky X. H. Fu, Karen L. Artiles, Phil S. Hartman, and Andrew Z. Fire. 2014. “Efficient Marker-Free Recovery of Custom Genetic Modifications with CRISPR/Cas9 in Caenorhabditis Elegans.” *Genetics* 198 (3): 837–46.

Bhaya, Devaki, Michelle Davison, and Rodolphe Barrangou. 2011. “CRISPR-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation.” *Annual Review of Genetics* 45 (1): 273–97.

Bogdan, Sven, Jörg Schultz, and Jörg Grosshans. 2013. “Formin’ Cellular Structures: Physiological Roles of Diaphanous (Dia) in Actin Dynamics.” *Communicative & Integrative Biology* 6 (6): e27634.

Bohnert, K. Adam, Agnieszka P. Grzegorzewska, Alaina H. Willet, Craig W. Vander Kooi, David R. Kovar, and Kathleen L. Gould. 2013. “SIN-Dependent Phosphoinhibition of Formin Multimerization Controls Fission Yeast Cytokinesis.” *Genes & Development* 27 (19): 2164–77.

Breitsprecher, Dennis, and Bruce L. Goode. 2013. “Formins at a Glance” 126 (1): 1–7.

Brenner, S. 1974. “The Genetics of Caenorhabditis Elegans.” *Genetics* 77 (1): 71–94.

Bridges, Andrew A., and Amy S. Gladfelter. 2015. “Septin Form and Function at the Cell Cortex.” *The Journal of Biological Chemistry* 290 (28): 17173–80.

Byerly, L., R. C. Cassada, and R. L. Russell. 1976. “The Life Cycle of the Nematode Caenorhabditis Elegans. I. Wild-Type Growth and Reproduction.” *Developmental Biology* 51 (1): 23–33.

C. elegans Sequencing Consortium. 1998. “Genome Sequence of the Nematode C. Elegans: A Platform for Investigating Biology.” *Science* 282 (5396): 2012–18.

Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. “Green Fluorescent Protein as a Marker for Gene Expression.” *Science* 263 (5148): 802–5.

Chan, Fung-Yi, Ana Silva, Joana Saramago, Joana Sousa, Hailey E. Brighton, Marisa Pereira, Karen Oegema, Reto Gassmann, and Ana Xavier Carvalho. 2018. *The ARP2/3 Complex Prevents Excessive Formin Activity during Cytokinesis*. Vol. 30.

Chang, F., D. Drubin, and P. Nurse. 1997. “cdc12p, a Protein Required for Cytokinesis in Fission Yeast, Is a Component of the Cell Division Ring and Interacts with Profilin.” *The Journal of Cell Biology* 137 (1): 169–82.

Cheffings, Thomas H., Nigel J. Burroughs, and Mohan K. Balasubramanian. 2016. “Actomyosin Ring Formation and Tension Generation in Eukaryotic Cytokinesis.” *Current Biology: CB* 26 (15): R719–37.

Chesarone, Melissa A., Amy Grace DuPage, and Bruce L. Goode. 2009. “Unleashing Formins to Remodel the Actin and Microtubule Cytoskeletons.” *Nature Reviews. Molecular Cell Biology* 11: 62.

Chesarone, Melissa A., and Bruce L. Goode. 2009. “Actin Nucleation and Elongation Factors: Mechanisms and Interplay.” *Current Opinion in Cell Biology* 21 (1): 28–37.

Chircop, Megan. 2014. “Rho GTPases as Regulators of Mitosis and Cytokinesis in Mammalian Cells.” *Small GTPases* 5 (July). https://doi.org/10.4161/sgtp.29770.

Chiu, Hui, Hillel T. Schwartz, Igor Antoshechkin, and Paul W. Sternberg. 2013. “Transgene-Free Genome Editing in Caenorhabditis Elegans Using CRISPR-Cas.” *Genetics* 195 (3): 1167–71.

Corsi, Ann K., Bruce Wightman, and Martin Chalfie. 2015. “A Transparent Window into Biology: A Primer on Caenorhabditis Elegans” 200 (2): 387–407.

Courtemanche, Naomi. 2018. “Mechanisms of Formin-Mediated Actin Assembly and Dynamics.” *Biophysical Reviews* 10 (6): 1553–69.

Courtemanche, Naomi, and Thomas D. Pollard. 2012. “Determinants of Formin Homology 1 (FH1) Domain Function in Actin Filament Elongation by Formins.” *The Journal of Biological Chemistry* 287 (10): 7812–20.

Davies, Tim, Shawn N. Jordan, Vandana Chand, Jennifer A. Sees, Kimberley Laband, Ana X. Carvalho, Mimi Shirasu-Hiza, David R. Kovar, Julien Dumont, and Julie C. Canman. 2014. “High-Resolution Temporal Analysis Reveals a Functional Timeline for the Molecular Regulation of Cytokinesis.” *Developmental Cell* 30 (2): 209–23.

DeWard, Aaron D., and Arthur S. Alberts. 2009. “Ubiquitin-Mediated Degradation of the Formin mDia2 upon Completion of Cell Division.” *The Journal of Biological Chemistry* 284 (30): 20061–69.

Dickinson, Daniel J., and Bob Goldstein. 2016. “CRISPR-Based Methods for Caenorhabditis Elegans Genome Engineering.” *Genetics* 202 (3): 885–901.

Dickinson, Daniel J., Jordan D. Ward, David J. Reiner, and Bob Goldstein. 2013. “Engineering the Caenorhabditis Elegans Genome Using Cas9-Triggered Homologous Recombination.” *Nature Methods* 10 (10): 1028–34.

Douglas, Max E., and Masanori Mishima. 2010. “Still Entangled: Assembly of the Central Spindle by Multiple Microtubule Modulators.” *Seminars in Cell & Developmental Biology* 21 (9): 899–908.

Figel, Sheila, and Robert A. Fenstermaker. 2018. “Chapter 18 - Cell-Cycle Regulation.” In *Handbook of Brain Tumor Chemotherapy, Molecular Therapeutics, and*

Immunotherapy (Second Edition), edited by Herbert B. Newton, 257–69. Academic Press.

Frazier, Jennifer A., and Christine M. Field. 1997. “Actin Cytoskeleton: Are FH Proteins Local Organizers?” *Current Biology: CB* 7 (7): R414–17.

Frøkjær-Jensen, Christian, M. Wayne Davis, Christopher E. Hopkins, Blake J. Newman, Jason M. Thummel, Søren-Peter Olesen, Morten Grunnet, and Erik M. Jorgensen. 2008. “Single-Copy Insertion of Transgenes in Caenorhabditis Elegans.” *Nature Genetics* 40 (11): 1375–83.

Gong, Ting, Yanling Yan, Jing Zhang, Shuai Liu, Hang Liu, Jinghu Gao, Xin Zhou, Juan Chen, and Anbing Shi. 2018. “PTRN-1/CAMSAP Promotes CYK-1/formin-Dependent Actin Polymerization during Endocytic Recycling.” *The EMBO Journal* 37 (9). https://doi.org/10.15252/embj.201798556.

Goode, Bruce, and Michael Eck. 2007. *Mechanism and Function of Formins in Control of Actin Assembly*. Vol. 76.

Hattersley, Neil, Pablo Lara-Gonzalez, Dhanya Cheerambathur, J. Sebastian Gomez-Cavazos, Taekyung Kim, Bram Prevo, Renat Khaliullin, et al. 2018. “Employing the One-Cell C. Elegans Embryo to Study Cell Division Processes.” *Methods in Cell Biology* 144 (May): 185–231.

Hidalgo-Cantabrana, Claudio, Yong Jun Goh, and Rodolphe Barrangou. 2019. “Characterization and Repurposing of Type I and Type II CRISPR-Cas Systems in Bacteria.” *Journal of Molecular Biology* 431 (1): 21–33.

Higashi, Tomohito, Tomoyuki Ikeda, Takaaki Murakami, Ryutaro Shirakawa, Mitsunori Kawato, Katsuya Okawa, Mikio Furuse, Takeshi Kimura, Toru Kita, and Hisanori Horiuchi. 2010. “Flightless-I (Fli-I) Regulates the Actin Assembly Activity of Diaphanous-Related Formins (DRFs) Daam1 and mDia1 in Cooperation with Active Rho GTPase.” *The Journal of Biological Chemistry* 285 (21): 16231–38.

Higgs, Henry N. 2005. “Formin Proteins: A Domain-Based Approach.” *Trends in Biochemical Sciences* 30 (6): 342–53.

Kechad, Amel, Silvana Jananji, Yvonne Ruella, and Gilles R. X. Hickson. 2012. “Anillin Acts as a Bifunctional Linker Coordinating Midbody Ring Biogenesis during Cytokinesis.” *Current Biology: CB* 22 (3): 197–203.

Kitayama, Chikako, and Taro Q. P. Uyeda. 2003. “ForC, a Novel Type of Formin Family Protein Lacking an FH1 Domain, Is Involved in Multicellular Development in Dictyostelium Discoideum.” *Journal of Cell Science* 116 (Pt 4): 711–23.

Kovar, David R., Jeffrey R. Kuhn, Andrea L. Tichy, and Thomas D. Pollard. 2003. “The Fission Yeast Cytokinesis Formin Cdc12p Is a Barbed End Actin Filament Capping Protein Gated by Profilin.” *The Journal of Cell Biology* 161 (5): 875–87.

Kühn, Sonja, and Matthias Geyer. 2014. “Formins as Effector Proteins of Rho GTPases.” *Small GTPases* 5: e29513–e29513.

Lacroix, Benjamin, and Amy Shaub Maddox. 2012. “Cytokinesis, Ploidy and Aneuploidy.” *The Journal of Pathology* 226 (2): 338–51.

Lammers, Michael, Rolf Rose, Andrea Scrima, and Alfred Wittinghofer. 2005. “The Regulation of mDia1 by Autoinhibition and Its Release by Rho*GTP.” *The EMBO Journal* 24 (23): 4176–87.

Lee, I-Ju, Valerie C. Coffman, and Jian-Qiu Wu. 2012. “Contractile-Ring Assembly in Fission Yeast Cytokinesis: Recent Advances and New Perspectives.” *Cytoskeleton* 69 (10): 751–63.

Lemmens, Bennie B. L. G., and Marcel Tijsterman. 2011. “DNA Double-Strand Break Repair in Caenorhabditis Elegans.” *Chromosoma* 120 (1): 1–21.

Li, Fang, and Henry N. Higgs. 2003. “The Mouse Formin mDia1 Is a Potent Actin Nucleation Factor Regulated by Autoinhibition.” *Current Biology: CB* 13 (15): 1335–40.

Liu, Raymond, Elena V. Linardopoulou, Gregory E. Osborn, and Susan M. Parkhurst. 2010. “Formins in Development: Orchestrating Body Plan Origami.” *Biochimica et Biophysica Acta* 1803 (2): 207–25.

Liu, Wei, Akira Sato, Deepak Khadka, Ritu Bharti, Hector Diaz, Loren W. Runnels, and Raymond Habas. 2008. “Mechanism of Activation of the Formin Protein Daam1.” *Proceedings of the National Academy of Sciences of the United States of America* 105 (1): 210–15.

Lo, Te-Wen, Catherine S. Pickle, Steven Lin, Edward J. Ralston, Mark Gurling, Caitlin M. Schartner, Qian Bian, Jennifer A. Doudna, and Barbara J. Meyer. 2013. “Precise and Heritable Genome Editing in Evolutionarily Diverse Nematodes Using TALENs and CRISPR/Cas9 to Engineer Insertions and Deletions.” *Genetics* 195 (2): 331–48.

Lu, Jun, Wuyi Meng, Florence Poy, Sankar Maiti, Bruce L. Goode, and Michael J. Eck. 2007. “Structure of the FH2 Domain of Daam1: Implications for Formin Regulation of Actin Assembly.” *Journal of Molecular Biology* 369 (5): 1258–69.

Maddox, Amy Shaub, Bianca Habermann, Arshad Desai, and Karen Oegema. 2005. “Distinct Roles for Two C. Elegans Anillins in the Gonad and Early Embryo.” *Development* 132 (12): 2837–48.

Makarova, Kira S., Yuri I. Wolf, Omer S. Alkhnbashi, Fabrizio Costa, Shiraz A. Shah, Sita J. Saunders, Rodolphe Barrangou, et al. 2015. “An Updated Evolutionary Classification of CRISPR-Cas Systems.” *Nature Reviews. Microbiology* 13 (11): 722–36.

Makarova, Kira S., Yuri I. Wolf, and Eugene V. Koonin. 2018. “Classification and Nomenclature of CRISPR-Cas Systems: Where from Here?” *The CRISPR Journal* 1 (5): 325–36.

Martin, Sophie G., Sergio A. Rincón, Roshni Basu, Pilar Pérez, and Fred Chang. 2007. “Regulation of the Formin for3p by cdc42p and bud6p.” *Molecular Biology of the Cell* 18 (10): 4155–67.

Mierzwa, Beata, and Daniel W. Gerlich. 2014. “Cytokinetic Abscission: Molecular Mechanisms and Temporal Control.” *Developmental Cell* 31 (5): 525–38.

Miller, Ann L. 2011. “The Contractile Ring.” *Current Biology: CB* 21 (24): R976–78.

Mi-Mi, Lei, Sarahbeth Votra, Kenneth Kemphues, Anthony Bretscher, and David Pruyne. 2012. “Z-Line Formins Promote Contractile Lattice Growth and Maintenance in Striated Muscles of C. Elegans.” *The Journal of Cell Biology* 198 (1): 87–102.

Min, Kyoengwoo, and Junho Lee. 2007. “RNA Interference in C. Elegans: History, Application, and Perspectives.” *Integrative Biosciences* 11 (2): 99–104.

Nakano, Kentaro, Tadashi Mutoh, Ritsuko Arai, and Issei Mabuchi. 2003. “The Small GTPase Rho4 Is Involved in Controlling Cell Morphology and Septation in Fission Yeast.” *Genes to Cells: Devoted to Molecular & Cellular Mechanisms* 8 (4): 357–70.

Nezami, Azin G., Florence Poy, and Michael J. %J Structure Eck. 2006. “Structure of the Autoinhibitory Switch in Formin mDia1” 14 (2): 257–63.

Nurse, Paul. 2000. “A Long Twentieth Century of the Cell Cycle and Beyond.” *Cell* 100 (1): 71–78.

Oegema, Karen, and Anthony A. Hyman. 2006. “Cell Division.” *WormBook: The Online Review of C. Elegans Biology*, January, 1–40.

Otomo, Takanori, Chinatsu Otomo, Diana R. Tomchick, Mischa Machius, and Michael K. %J Molecular Cell Rosen. 2005. “Structural Basis of Rho GTPase-Mediated Activation of the Formin mDia1” 18 (3): 273–81.

Otomo, Takanori, Diana R. Tomchick, Chinatsu Otomo, Sanjay C. Panchal, Mischa Machius, and Michael K. Rosen. 2005. “Structural Basis of Actin Filament Nucleation and Processive Capping by a Formin Homology 2 Domain.” *Nature* 433 (7025): 488–94.

Pearce, Laura R., David Komander, and Dario R. Alessi. 2010. “The Nuts and Bolts of AGC Protein Kinases.” *Nature Reviews. Molecular Cell Biology* 11 (1): 9–22.

Pelham, Robert J., and Fred Chang. 2002. “Actin Dynamics in the Contractile Ring during Cytokinesis in Fission Yeast.” *Nature* 419 (6902): 82–86.

Petersen, J., O. Nielsen, R. Egel, and I. M. Hagan. 1998. “FH3, a Domain Found in Formins, Targets the Fission Yeast Formin Fus1 to the Projection Tip during Conjugation.” *The Journal of Cell Biology* 141 (5): 1217–28.

Piekny, Alisa, Michael Werner, and Michael Glotzer. 2005. “Cytokinesis: Welcome to the Rho Zone.” *Trends in Cell Biology* 15 (12): 651–58.

Pollard, Thomas D. 2017. “Nine Unanswered Questions about Cytokinesis” 216 (10): 3007–16.

Porta-de-la-Riva, Montserrat, Laura Fontrodona, Alberto Villanueva, and Julián Cerón. 2012. “Basic Caenorhabditis Elegans Methods: Synchronization and Observation.” *Journal of Visualized Experiments: JoVE*, no. 64 (June): e4019.

Pring, Martin, Marie Evangelista, Charles Boone, Changsong Yang, and Sally H. Zigmond. 2003. “Mechanism of Formin-Induced Nucleation of Actin Filaments.” *Biochemistry* 42 (2): 486–96.

Priti, Agarwal, Hui Ting Ong, Yusuke Toyama, Anup Padmanabhan, Sabyasachi Dasgupta, Matej Krajnc, and Ronen Zaidel-Bar. 2018. “Syncytial Germline Architecture Is Actively Maintained by Contraction of an Internal Actomyosin Corset.” *Nature Communications* 9 (1): 4694.

Pruyne, David. 2016. “Revisiting the Phylogeny of the Animal Formins: Two New Subtypes, Relationships with Multiple Wing Hairs Proteins, and a Lost Human Formin.” *PloS One* 11 (10): e0164067–e0164067.

Pruyne, David, Marie Evangelista, Changsong Yang, Erfei Bi, Sally Zigmond, Anthony Bretscher, and Charles Boone. 2002. “Role of Formins in Actin Assembly: Nucleation and Barbed-End Association.” *Science* 297 (5581): 612–15.

Ran, F. Ann, Patrick D. Hsu, Jason Wright, Vineeta Agarwala, David A. Scott, and Feng Zhang. 2013. “Genome Engineering Using the CRISPR-Cas9 System.” *Nature Protocols* 8 (11): 2281–2308.

Rose, R., M. Weyand, M. Lammers, T. Ishizaki, M. R. Ahmadian, and A. Wittinghofer. 2005. “Structural and Mechanistic Insights into the Interaction between Rho and Mammalian Dia.” *Nature* 435 (7041): 513–18.

Sagot, Isabelle, Saskia K. Klee, and David Pellman. 2002. “Yeast Formins Regulate Cell Polarity by Controlling the Assembly of Actin Cables.” *Nature Cell Biology* 4 (1): 42–50.

Salomoni, Paolo, and Federico Calegari. 2010. “Cell Cycle Control of Mammalian Neural Stem Cells: Putting a Speed Limit on G1.” *Trends in Cell Biology* 20 (5): 233–43.

Santos, Beatriz, Ana Belén Martín-Cuadrado, Carlos R. Vázquez de Aldana, Francisco del Rey, and Pilar Pérez. 2005. “Rho4 GTPase Is Involved in Secretion of Glucanases during Fission Yeast Cytokinesis.” *Eukaryotic Cell* 4 (10): 1639–45.

Schönichen, André, Michael Alexander, Judith E. Gasteier, Fanny E. Cuesta, Oliver T. Fackler, and Matthias Geyer. 2006. “Biochemical Characterization of the Diaphanous Autoregulatory Interaction in the Formin Homology Protein FHOD1.” *The Journal of Biological Chemistry* 281 (8): 5084–93.

Schönichen, André, and Matthias Geyer. 2010. “Fifteen Formins for an Actin Filament: A Molecular View on the Regulation of Human Formins.” *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1803 (2): 152–63.

Seth, Abhinav, Chinatsu Otomo, and Michael K. Rosen. 2006. “Autoinhibition Regulates Cellular Localization and Actin Assembly Activity of the Diaphanous-Related Formins FRLalpha and mDia1.” *The Journal of Cell Biology* 174 (5): 701–13.

Severson, Aaron F., David L. Baillie, and Bruce Bowerman. 2002. “A Formin Homology Protein and a Profilin Are Required for Cytokinesis and Arp2/3-Independent Assembly of Cortical Microfilaments in C. Elegans.” *Current Biology: CB* 12 (24): 2066–75.

Shimada, Atsushi, Miklós Nyitrai, Ingrid R. Vetter, Dorothee Kühlmann, Beáta Bugyi, Shuh Narumiya, Michael A. Geeves, and Alfred Wittinghofer. 2004. “The Core FH2 Domain of Diaphanous-Related Formins Is an Elongated Actin Binding Protein That Inhibits Polymerization.” *Molecular Cell* 13 (4): 511–22.

Staus, Dean P., Joan M. Taylor, and Christopher P. Mack. 2011. “Enhancement of mDia2 Activity by Rho-Kinase-Dependent Phosphorylation of the Diaphanous Autoregulatory Domain.” *Biochemical Journal* 439 (1): 57–65.

Swan, K. A., A. F. Severson, J. C. Carter, P. R. Martin, H. Schnabel, R. Schnabel, and B. Bowerman. 1998. “Cyk-1: A C. Elegans FH Gene Required for a Late Step in Embryonic Cytokinesis.” *Journal of Cell Science* 111 (Pt 14) (July): 2017–27.

Thompson, Morgan E., Ernest G. Heimsath, Timothy J. Gauvin, Henry N. Higgs, and F. Jon Kull. 2013. “FMNL3 FH2-Actin Structure Gives Insight into Formin-Mediated Actin Nucleation and Elongation.” *Nature Structural & Molecular Biology* 20 (1): 111–18.

Tucker, Morgan, and Min Han. 2010. “Caenorhabditis Elegans, A Simple Worm: Bridging the Gap Between Traditional and Systems-Level Biology.” In *Vogel and Motulsky’s Human Genetics*, edited by Michael R. Speicher, Arno G. Motulsky, and Stylianos E. Antonarakis, 787–94. Berlin, Heidelberg: Springer Berlin Heidelberg.

Vaillant, Dominique C., Sarah J. Copeland, Chris Davis, Susan F. Thurston, Nezar Abdennur, and John W. Copeland. 2008. “Interaction of the N- and C-Terminal Autoregulatory Domains of FRL2 Does Not Inhibit FRL2 Activity.” *The Journal of Biological Chemistry* 283 (48): 33750–62.

Vermeulen, Katrien, Dirk R. Van Bockstaele, and Zwi N. Berneman. 2003. “The Cell Cycle: A Review of Regulation, Deregulation and Therapeutic Targets in Cancer” 36 (3): 131–49.

Vig, Andrea Teréz, István Földi, Szilárd Szikora, Ede Migh, Rita Gombos, Mónika Ágnes Tóth, Tamás Huber, et al. 2017. “The Activities of the C-Terminal Regions of the Formin Protein Disheveled-Associated Activator of Morphogenesis (DAAM) in Actin Dynamics.” *The Journal of Biological Chemistry* 292 (33): 13566–83.

Wallar, Bradley J., and Arthur S. Alberts. 2003. “The Formins: Active Scaffolds That Remodel the Cytoskeleton.” *Trends in Cell Biology* 13 (8): 435–46.

Wallar, Bradley J., Brittany N. Stropich, Jessica A. Schoenherr, Holly A. Holman, Susan M. Kitchen, and Arthur S. Alberts. 2006. “The Basic Region of the Diaphanous-Autoregulatory Domain (DAD) Is Required for Autoregulatory Interactions with the

Diaphanous-Related Formin Inhibitory Domain." *The Journal of Biological Chemistry* 281 (7): 4300–4307.

Wang, Hongyan, Xie Tang, and Mohan K. Balasubramanian. 2003. "Rho3p Regulates Cell Separation by Modulating Exocyst Function in Schizosaccharomyces Pombe." *Genetics* 164 (4): 1323–31.

Wang, Ning, Mo Wang, Yi-Hua Zhu, Timothy W. Grosel, Daokun Sun, Dmitri S. Kudryashov, and Jian-Qiu Wu. 2015. "The Rho-GEF Gef3 Interacts with the Septin Complex and Activates the GTPase Rho4 during Fission Yeast Cytokinesis." *Molecular Biology of the Cell* 26 (2): 238–55.

Watanabe, N., T. Kato, A. Fujita, T. Ishizaki, and S. Narumiya. 1999. "Cooperation between mDia1 and ROCK in Rho-Induced Actin Reorganization." *Nature Cell Biology* 1 (3): 136–43.

Watanabe, N., P. Madaule, T. Reid, T. Ishizaki, G. Watanabe, A. Kakizuka, Y. Saito, K. Nakao, B. M. Jockusch, and S. Narumiya. 1997. "p140mDia, a Mammalian Homolog of Drosophila Diaphanous, Is a Target Protein for Rho Small GTPase and Is a Ligand for Profilin." *The EMBO Journal* 16 (11): 3044–56.

Watanabe, Sadanori, Yoshikazu Ando, Shingo Yasuda, Hiroshi Hosoya, Naoki Watanabe, Toshimasa Ishizaki, and Shuh Narumiya. 2008. "mDia2 Induces the Actin Scaffold for the Contractile Ring and Stabilizes Its Position during Cytokinesis in NIH 3T3 Cells." *Molecular Biology of the Cell* 19 (5): 2328–38.

Watanabe, Sadanori, Katsuya Okawa, Takashi Miki, Satoko Sakamoto, Tomoko Morinaga, Kohei Segawa, Takatoshi Arakawa, Makoto Kinoshita, Toshimasa Ishizaki, and Shuh Narumiya. 2010. "Rho and Anillin-Dependent Control of mDia2 Localization and Function in Cytokinesis." *Molecular Biology of the Cell* 21 (18): 3193–3204.

Wei, Bin, Brian S. Hercyk, Nicholas Mattson, Ahmad Mohammadi, Julie Rich, Erica DeBruyne, Mikayla M. Clark, and Maitreyi Das. 2016. "Unique Spatiotemporal Activation Pattern of Cdc42 by Gef1 and Scd1 Promotes Different Events during Cytokinesis." *Molecular Biology of the Cell* 27 (8): 1235–45.

Wiedenheft, Blake, Samuel H. Sternberg, and Jennifer A. Doudna. 2012. "RNA-Guided Genetic Silencing Systems in Bacteria and Archaea." *Nature* 482 (7385): 331–38.

Wiggan, O 'neil, Alisa E. Shaw, Jennifer G. DeLuca, and James R. Bamburg. 2012. "ADF/cofilin Regulates Actomyosin Assembly through Competitive Inhibition of Myosin II Binding to F-Actin." *Developmental Cell* 22 (3): 530–43.

Willet, Alaina H., Nathan A. McDonald, and Kathleen L. Gould. 2015. "Regulation of Contractile Ring Formation and Septation in Schizosaccharomyces Pombe." *Current Opinion in Microbiology* 28 (December): 46–52.

Wood, E. J. 1983. "Molecular Cloning. A Laboratory Manual by T Maniatis, E F Fritsch and J Sambrook. Pp 545. Cold Spring Harbor Laboratory, New York. 1982. $48 ISBN 0-87969-136-0." *Biochemical Education* 11 (2): 82–82.

Xu, Yingwu, James B. Moseley, Isabelle Sagot, Florence Poy, David Pellman, Bruce L. Goode, and Michael J. Eck. 2004. "Crystal Structures of a Formin Homology-2 Domain Reveal a Tethered Dimer Architecture." *Cell* 116 (5): 711–23.

Yonetani, Ann, Raymond J. Lustig, James B. Moseley, Tetsuya Takeda, Bruce L. Goode, and Fred Chang. 2008. "Regulation and Targeting of the Fission Yeast Formin cdc12p in Cytokinesis." *Molecular Biology of the Cell* 19 (5): 2208–19.