

DOUTORAMENTO
CIÊNCIAS BIOMÉDICAS

Functionalities and interplay of actin-filament crosslinkers *in vivo*

Ana Filipa Sobral

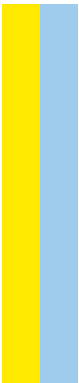
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CROSSLINKERS *IN VIVO***

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CIÊNCIA, TECNOLOGIA
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DECLARAÇÃO DE HONRA

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Porto, Dezembro de 2021

Ana Filipa Fernandes Ferreira Sobral

“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less“

- Marie Curie

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LISTA DE PUBLICAÇÕES/LIST OF PUBLICATIONS

Esta tese de Doutorado é uma compilação de resultados originais, publicados ou em fase de preparação para publicação em revistas internacionais indexadas. A autora declara que teve participação activa na concepção, execução, análise, interpretação, discussão e redacção dos trabalhos aqui apresentados. Alguns dados foram obtidos em colaboração com a Dr. Fung-Yi Chan.

Faz parte integrante desta tese o seguinte artigo publicado:

This Doctoral thesis is a compilation of original data, published or in the process of preparation to submit for publication in international peer-reviewed journals. The author declares that she had an active role in the conception, execution, analysis, interpretation, discussion and writing of the work here presented. Some of the data presented were obtained in collaboration with Dr. Fung-Yi Chan.

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Additionally, work developed during the production of this thesis was published in the following articles that are not an integrant part of this thesis:

- Leite J., Chan F. Y., Osório D. S., Saramago J., **Sobral A. F.**, Silva A. M., Gassmann R., Carvalho A. X. (2020). Equatorial non-muscle myosin II and plastin cooperate to align and compact F-actin bundles in the cytokinetic ring. *Frontiers in Cell and Developmental Biology*, 25;8:573393. doi: 10.3389/fcell.2020.573393.
- Osório D. S., Chan F. Y., Saramago J., Leite J., Silva A. M., **Sobral A. F.**, Gassmann R., Carvalho A. X. (2019). Crosslinking activity of non-muscle myosin II is not sufficient for embryonic cytokinesis in *C. elegans*. *Development*, 12;146(21):dev179150. doi: 10.1242/dev.179150.

SUMMARY

Cells rely on contractile actomyosin networks to move, divide, adhere, change their shape to adapt to the environment, perform intracellular processes like endocytosis, and to promote tissue-scale behavior, such as muscle contraction. The underlying machinery requires the fine-tuned crosstalk between actin filaments (F-actin), myosin motor protein, F-actin crosslinkers and other regulatory proteins. By bridging F-actin, crosslinkers are key players in the building of actomyosin networks with different conformations adapted to each cellular context. At the same time, crosslinkers maintain the overall interconnectivity of the formed networks to allow the efficient propagation of force and contractility. Despite the increasing number of studies on this subclass of cytoskeletal proteins, an integrative study of the individual and combined functionalities and interplay of co-existing crosslinkers in actomyosin-dependent cellular contexts *in vivo* was still missing.

In this thesis, using *C. elegans* as an *in vivo* model organism, advanced genetic manipulation techniques and confocal microscopy, the localization, functional diversity and contribution of multiple well-conserved F-actin crosslinkers were explored. The analysis was done in different cellular contexts, with a special focus on embryonic cytokinesis. Crosslinkers were shown to have different expression profiles and to impact the actomyosin networks' behavior in a tissue-dependent manner. While some affect cytokinesis and/or the animal's reproductive system, others seem to be selectively involved in muscle morphology or animal mobility. Moreover, a novel interplay between a small-sized crosslinker (plastin) and a large-sized crosslinker (β -Heavy-spectrin), which is essential for cytokinesis, was unraveled and thoroughly characterized. These two crosslinkers were found to cooperate not only to organize but also to stabilize the F-actin bundles that form the contractile ring that physically divides the cell. The collapse of the actomyosin network observed in the co-inhibition of plastin and β -Heavy-spectrin led to recurrent cytokinesis failure. A failure in this process culminates in multinucleated cells, which are a hallmark of diseases as cancer. Subsequently, it was found that most plastin's functions could be performed by another small-sized crosslinker, α -actinin, which indicates that different crosslinkers with similar size can perform identical functions during cytokinesis.

In summary, the work conducted during this thesis resulted in three main achievements. First, the localization profiles and loss-of-function phenotypes of the distinct crosslinkers in different actomyosin-dependent contexts disclose the necessity of their diversity and specificity *in vivo*. Second, the uncovering of a novel interplay between different-sized crosslinkers to interconnect and stabilize the actomyosin network during cytokinesis, as well as the revelation of spectrins as players in animal cell cytokinesis, represent valuable advances in the understanding of the mechanisms that govern actomyosin network

behavior during this cellular process. Third, this work reinforces the idea that the size of crosslinkers is a relevant biophysical feature that defines the performance of specific functions during cytokinesis. The gained insights open new doors for the future study of contractile actomyosin networks' biomechanics and can shed light into the molecular mechanisms that go awry in diseases where F-actin crosslinkers are altered.

Keywords: Crosslinkers; F-actin; actomyosin networks; cytokinesis; *C. elegans*

RESUMO

As células dependem de redes contrácteis de actina e miosina para se moverem, dividirem, aderirem, mudarem de forma para se adaptarem ao ambiente, realizarem processos intracelulares como a endocitose, e para promoverem o funcionamento de tecidos, como a contração muscular. Para tal, é necessária uma interação entre filamentos de actina (F-actina), a proteína motora miosina, os conectores de F-actina e outras proteínas regulatórias. Os conectores de F-actina, ao fazerem pontes entre os filamentos de actina, são intervenientes chave na construção das redes de actomiosina com diferentes conformações, adaptadas a cada contexto celular. Simultaneamente, os conectores mantêm a interconectividade global das redes formadas de forma a permitir a propagação eficiente de forças e contractilidade. Apesar do número crescente de estudos sobre esta subclasse de proteínas do citosqueleto celular, ainda estava em falta um estudo integrativo sobre as suas funções individuais e combinadas, bem como sobre a existência de interações entre os conectores co-existentes em contextos celulares dependentes de actina e miosina.

Nesta tese, usando *C. elegans* como organismo modelo *in vivo*, técnicas avançadas de manipulação genética e microscopia confocal, explorou-se a localização, diversidade funcional e a contribuição de múltiplos conectores de F-actina em diferentes contextos celulares, com especial foco na citocinese embrionária. Os conectores mostraram ter diferentes perfis de expressão e impactaram o comportamento das redes de actomiosina de uma forma tecido-dependente. Enquanto uns afectam a citocinese e/ou o sistema reprodutor, outros parecem estar selectivamente envolvidos na morfologia do tecido muscular ou na mobilidade do animal. Além disso, uma nova interação entre conectores de tamanho pequeno (plastina) e de tamanho grande (β -espectrina-pesada), que é essencial para a realização da citocinese, foi descoberta e caracterizada. Estes dois conectores mostraram cooperar na organização e na estabilização dos filamentos de actina que formam o anel contráctil que divide a célula. O colapso da rede de actomiosina observado na co-inibição de plastina e β -espectrina-pesada levou à falha recorrente da citocinese. Uma falha neste processo culmina em células multinucleadas, um traço presente, por exemplo, em muitas células de cancro. Subsequentemente, descobriu-se que a maioria das funções da plastina podem ser realizadas por outro conector de tamanho pequeno, a α -actinina. Isto mostra que conectores diferentes mas de tamanho similar podem desempenhar funções idênticas durante a citocinese.

Em conjunto, o trabalho realizado nesta tese resultou em três conquistas principais. Primeiro, os perfis de localização e os fenótipos de perda-de-função dos diferentes conectores em diferentes contextos dependentes de actomiosina revelam

que a sua diversidade e especificidade são necessárias *in vivo*. Segundo, a descoberta de uma nova interação entre conectores de diferentes tamanhos para interconectar e estabilizar as redes de actomiosina durante a citocinese, bem como a descoberta do envolvimento da espectrina na citocinese de células animais, representam valiosos avanços na compreensão dos mecanismos que governam o comportamento das redes de actomiosina durante este processo celular. Terceiro, este trabalho reforça a ideia de que o tamanho dos conectores é um aspecto biofísico relevante que define a realização de funções específicas durante a citocinese. Os conhecimentos adquiridos abrem novas portas para estudos futuros sobre a biomecânica da contractilidade das redes de actomiosina e podem ajudar a esclarecer os mecanismos moleculares que correm mal em doenças onde os conectores de F-actina estão alterados.

Palavras-chave: Conectores, F-actina; redes de actomiosina; citocinese; *C. elegans*

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LIST OF SYMBOLS AND ABBREVIATIONS

°C – Degree Celsius	ESCRT – Endosomal sorting complexes required for transport
% – Percentage	F-actin – Filamentous actin
α – Alpha	FRAP – Fluorescence recovery after photobleaching
β – Beta	G-actin – Globular actin
Δ – Delta	gDNA – Genomic deoxyribonucleic acid
ABD – Actin binding domain	GEF – Guanine nucleotide exchange factor
ADP – Adenosine Diphosphate	GFP – Green fluorescent protein
AHR – Anillin-homology region	GST – Glutathione S-transferase
ATP – Adenosine Triphosphate	GTP – Guanosine-5'-triphosphate
BSA – Bovine serum albumin	GTPase – Guanosine triphosphatase
<i>C. elegans</i> – <i>Caenorhabditis elegans</i>	HDR – Homology Directed Repair
cDNA – Complementary deoxyribonucleic acid	HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
CGC – <i>Caenorhabditis</i> Genetics Center	HRP – Horseradish peroxidase
CH – Calponin homology	Hz – Hertz
CI – Confidence interval	Ig repeat – Immunoglobulin-like repeat
CRISPR/Cas9 – Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9	IPTG – Isopropyl β-D-1-thiogalactopyranoside
crRNA – Crispr RNA	kD – Kilodalton
<i>D. discoideum</i> – <i>Dictyostelium discoideum</i>	L, mL, μL – Liter, milliliter, microliter
<i>D. melanogaster</i> – <i>Drosophila melanogaster</i>	LB – Luria/Lysogenic broth
DAPI – 4',6-diamidino-2-phenylindole	LED – Light emitting diode
DNA – Deoxyribonucleic acid	mg, μg, ng – Milligram, microgram, nanogram
DsRNA – Double-stranded RNAs	mW – Milliwatt
DTT – Dithiothreitol	MHC – Myosin heavy chain
<i>E. coli</i> – <i>Escherichia coli</i>	MosSCI – Mos1-mediated single copy insertion
ECL – Enhanced chemiluminescence	mRNA – Messenger ribonucleic acid
EDTA – Ethylenediamine tetraacetic acid	NGM – Nematode growth media
EGTA – Egtazic acid	NHEJ – Non-Homologous End-Joining
ELC – Myosin essential light chain	
EM – Electron microscopy	

NLS – Nuclear localization sequence	SEM – Standard error of the mean
nm, μ m, mm – Nanometer; micrometer, millimeter	SgRNA – Single guide RNA
NRK – normal rat kidney	SH3 – Src-homology domain
OD600 – Optical density at 600 nm	siRNA – small interference RNA
ORF – Open reading frame	SOC – Super Optimal broth with Catabolite repression
PBS-TG – Phosphate-buffered saline with Triton X-100 and glycine	SP-UT – Spermatheca-uterine
PCR – Polymerase chain reaction	spGFP – split GFP
PH – Pleckstrin homology domain	SR – Spectrin repeat
Pi – Inorganic phosphate	ssRNA – Single-stranded RNA
PIV – Particle Image Velocimetry	TAE – Mixture of Tris, acetic acid and EDTA
RISC – RNA-induced silencing complex	TBST – Tris-buffered saline with Tween
RLC – Myosin regulatory light chain	TIRFM – Total internal reflection microscopy
RNA – Ribonucleic acid	TMHC – Tetramisole hydrochloride
RNAi – RNA interference	tracrRNA – Trans-activating crRNA
RNP – Ribonucleoprotein	Tris – Tris(hydroxymethyl)aminomethane
ROI – Regions of interest	μ M, mM, M – Micromolar, millimolar, molar
rpm – Revolutions per minute	pN - Piconewton
(RT)-PCR – Reverse-Transcription-PCR	UTR – Untranslated region
s – seconds	v/v – Volume per volume
<i>S. cerevisiae</i> – <i>Saccharomyces cerevisiae</i>	VIL – Villin
<i>S. pombe</i> – <i>Schizosaccharomyces pombe</i>	w/v – Volume per weight
SD – Standard deviation	WD repeat – Tryptophan (W), aspartic acid (D) repeat
SDS – Sodium dodecyl sulfate	WT – Wild-type
SDS-PAGE – Sodium dodecyl sulfate - polyacrylamide gel electrophoresis	<i>X. laevis</i> – <i>Xenopus laevis</i>

CHAPTER I

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. Cytoskeleton

In 1903, the biologist Nikolai Koltsov pioneered the idea of a cytoskeleton, the term proposed for the network of tubules that determined the shape of the cells. Present in every cell type, the cytoskeleton is nowadays known to be a dynamic filamentous network, not only responsible for maintaining the overall integrity of the cell but also to define the cell spatial internal organization, control its shape, help the cell to mechanically sense and interact with the environment, and provide mechanical support that allows the cell to perform all its functions. It extends from the plasma membrane to the nucleus throughout the cytoplasm, and its behavior varies according to the biological needs of each cell type and its environment (Fletcher & Mullins, 2010). The eukaryotic cytoskeleton consists of three major classes of filamentous proteins that, together, form a cohesive and complex network: microtubules, a group of filaments collectively known as intermediate filaments, and actin filaments. Microtubules, composed of tubulin subunits, are a thick (~25 nm of diameter) and stiff type of cytoskeletal filaments, essential to form and arrange the mitotic spindle that segregates the chromosomes during cell division, to position organelles and the nucleus and to direct intracellular transport (Brouhard & Rice, 2018; Pollard & Goldman, 2018). Intermediate filaments are a family of mid-sized filaments (~10 nm of diameter), whose main function is to provide mechanical support and strength to the membrane and to the other cytoskeletal proteins. Intermediate filaments types vary according to the cell type - examples of this type of filaments are keratins, vimentin and lamins (Lowery et al., 2015). Actin filaments, composed of the globular protein actin, are the thinnest (~7 nm of diameter) filaments of the cytoskeleton. They form a dynamic but robust specialized network at the peripheral cell cortex, which supports a variety of cell processes that require force generation, contractility and cell shape changes, namely cytokinesis, cell motility and morphogenesis (Fischer & Fowler, 2015; Svitkina, 2018).

In the present thesis, I will focus on the actin filament network.

1.1.1. Actin

Actin is the monomeric subunit of actin filaments and it is a highly conserved and abundant protein in all organisms. The assembly of actin filaments starts with the polymerization of actin in its 42 kD free globular monomeric form (G-actin) into a double-stranded helical filamentous actin (F-actin) through hydrolysis of adenosine triphosphate (ATP) (Straub & Feuer, 1950; Fujii et al., 2010; Holmes et al., 1990; Oda et al., 2009). Actin monomers are always incorporated in the same orientation, generating polar actin filaments

with two different ends designated barbed (+) and pointed (-) ends. When filament polymerization reaches an equilibrium, monomers are preferentially added to the barbed end, while actin disassembly mostly happens at the pointed end (Begg et al., 1978; Fujiwara et al., 2018; Wegner, 1976). This process is highly dynamic, as actin filaments are constantly being polymerized and depolymerized, allowing for the rapid assembly and disassembly of F-actin networks. F-actin dynamics are regulated by its motor protein, myosin, and by multiple accessory actin-binding proteins, which have the function to nucleate, elongate, branch, cap, sever, stabilize or crosslink the F-actin (Blanchoin et al., 2014; Pollard et al., 2000; Shekhar et al., 2016; Figure 1.1). Thus, the actin cytoskeleton is an interconnected network in constant remodeling. The dynamic nature and interaction of F-actin with its regulatory proteins allows the F-actin network to readily adapt to intracellular and extracellular cues (Harris et al., 2018; Stossel et al., 2006).

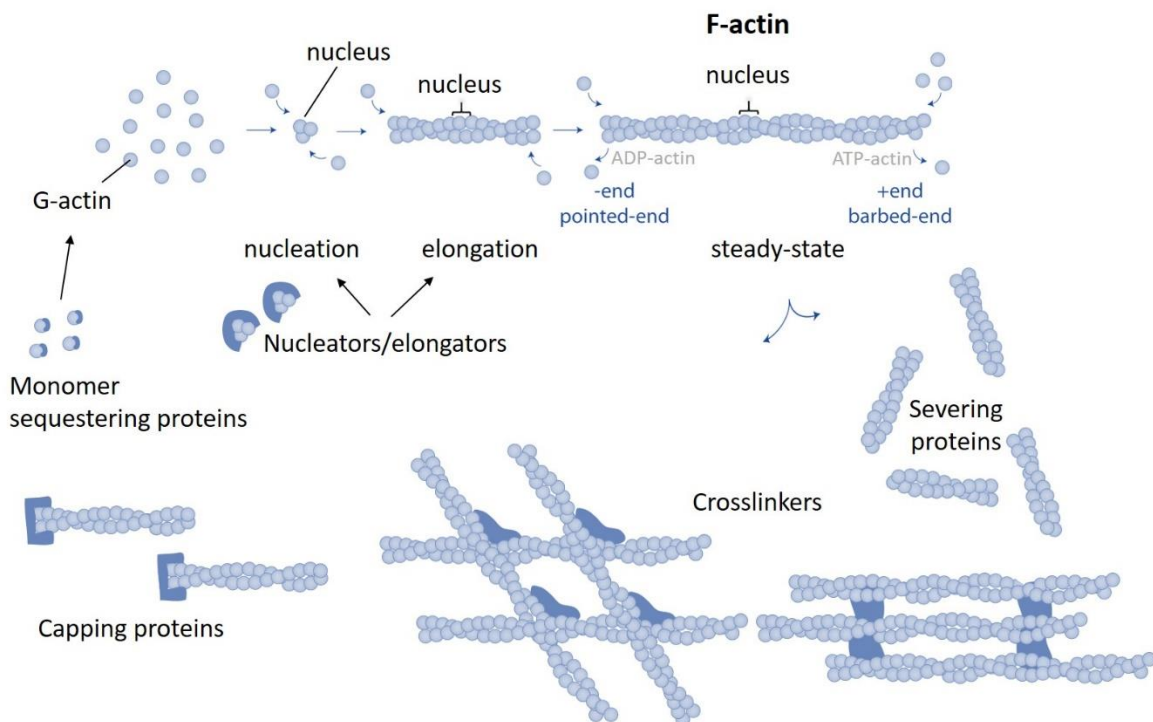


Figure 1.1. Schematic illustration of the regulation of F-actin dynamics by actin binding proteins. Globular actin (G-actin) monomers are polymerized and elongated into filaments (F-actin) with the help of actin nucleators and elongators, until they reach an equilibrium between the polymerization at its barbed-end and depolymerization at its pointed-end (steady-state). Actin filaments can further be bridged and organized into a network by crosslinkers, severed to reduce the filament size or disassemble networks by severing proteins, and capped to prevent F-actin elongation by capping proteins. Actin monomer sequestering proteins can also bind to actin monomers to prevent/regulate actin polymerization. Image from the Cytoskeletal Dynamics Group, i3S.

1.1.2. Myosin

Myosins constitute a superfamily of actin motor proteins that bind and translocate F-actin by generating mechanical force, which results in F-actin network contraction. Thus, myosins are essential proteins for all contractile-dependent cellular and tissue processes, such as muscle contraction, cytokinesis and cell motility (Hartman & Spudich, 2012). In the work presented in this thesis, only non-muscle myosin II (NMM-II) will be mentioned, as a part of the actomyosin networks studied. NMM-II (myosin, hereafter) is a conventional and ubiquitous myosin that functions as an hexameric complex composed of: two myosin heavy chains (MHC), each with a globular head and a coiled-coil tail/rod domain; two regulatory light chains (RLCs), involved in the regulation of myosin activity; and two essential light chains (ELCs), which stabilize the complex conformation (Vicente-Manzanares et al., 2009). This complex has three functional parts: the globular heads that contain the motor domain where actin and ATP bind, the neck region where the myosin light chains or calmodulin bind, and the tail which mediates the self-assembly of myosin into bipolar filaments in order to interact with F-actin at each end and exert tension on the network (Leite et al., 2019; Vicente-Manzanares et al., 2009).

Myosin activity is mainly regulated through phosphorylation of its RLCs by serine/threonine kinases, in particular by RhoA effectors, such as Rho-associated protein kinase (ROCK) and Citron Rho-interacting kinase (CIT-K). These phosphorylations result in myosin activation by opening its hexameric conformation (Kimura et al., 1996; Yamashiro et al., 2003). Upon activation, myosin is free to form bipolar filaments that are able to bind F-actin. After binding F-actin via their head domains, myosin's heads progressively move towards the F-actin barbed-ends. Thereby, bipolar myosin filaments are able to slide antiparallel actin filaments past one another, generating network tension and contraction. This movement is generated at the expense of ATP hydrolysis by myosin that converts chemical energy into mechanical force (Niederman & Pollard, 1975; Vicente-Manzanares et al., 2009). The same myosin bipolar filament is able to bind different actin filaments simultaneously, and, therefore, myosin is also able to crosslink and maintain the network interconnected (Leite et al., 2019).

1.1.3. F-actin crosslinkers

F-actin crosslinkers (hereafter crosslinkers) are a group of actin binding proteins that bridge F-actin and maintain actin networks interconnected. Depending on their size, flexibility and abundance, crosslinkers are able to form F-actin networks with different organizations (Lieleg et al., 2010). Smaller and intermediate-sized crosslinkers such as

fascin, plastin/fimbrin (hereafter plastin) and α -actinin tend to form tightly packed F-actin bundles, with filaments oriented in a parallel, antiparallel or mixed polarity fashion, depending on the crosslinker (Hampton et al., 2007; Skau et al., 2011; Winkelman et al., 2016). On the other hand, long crosslinkers, such as spectrins and filamin, are able to build larger and flexible meshworks (Kasza et al., 2010; Machnicka et al., 2014; Figure 1.2). These different organizations are necessary to ensure that the cell effectively performs essential actomyosin-based functions, such as the assembly of a linear and compact actomyosin contractile ring during cell division or the formation of a lattice-like F-actin network in erythrocytes to support mechanical deformation in circulation (Leite et al., 2020; Machnicka et al., 2014).

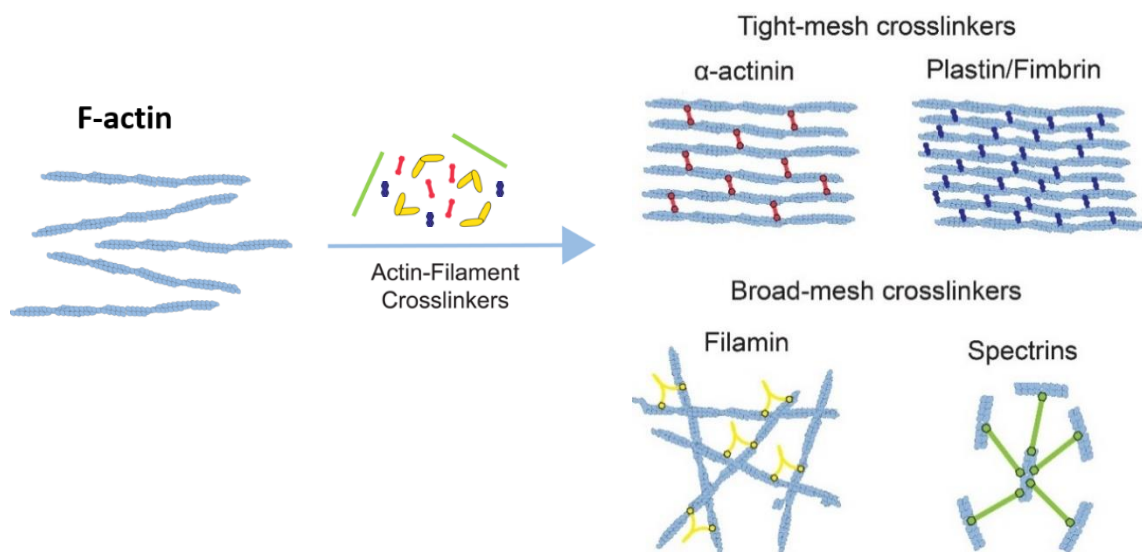


Figure 1.2. Schematic of different F-actin networks originated by distinct types of F-actin crosslinkers. F-actin crosslinkers have distinct sizes and conformations. They bind and bridge F-actin, creating networks of different architectures. Smaller crosslinkers tend to build tight F-actin bundles while larger crosslinkers are able to form more loose and flexible meshes. Image from the Cytoskeletal Dynamics Group, i3S.

Studies have reported that multiple crosslinkers populate a given cell type simultaneously and can operate in the same subcellular localization (for instance, α -actinin, filamin and fascin are all present in stress fibers), giving rise to the idea that crosslinkers can be redundant (Elkhatib et al., 2014; Foley & Young, 2014; Lamsoul et al., 2020). Combinations of crosslinkers could be advantageous to tune F-actin network dynamics or mechanical properties in order to optimize the overall biological response to different external stimuli. Although redundancy has already been documented in a limited number of studies when two crosslinkers localize to the same network (Rivero et al., 1999; Wu et al., 2001), not all crosslinkers are found in the same type of cells or tissues, imposing some degree of specificity and sorting of crosslinkers to different networks. Structural and functional differences that may be determinant for crosslinkers' sorting to different networks

are: a) their size, since the distance imposed by a crosslinker's length was shown to be a major segregating factor, with crosslinkers with similar sizes being able to populate the same actin filament and setting a filament spacing that limits the binding of other crosslinkers of different sizes (Winkelman et al., 2016); b) the type of actin-binding domain (ABD) - while many crosslinkers have one or two ABDs composed of two well conserved calponin-homology (CH) regions each (such as spectrins, α -actinin, filamin, plastrin, dynacortin), others (like anillin or septins) have singular types of ABDs that may have different affinity to F-actin or be regulated differently (Harris et al., 2019; Jananji et al., 2017; Smith et al., 2015); c) their functional form, some crosslinkers function as monomers, others dimerize and spectrins tetramerize; d) their structural domains - some only crosslink F-actin whereas others, like anillin and β -spectrins, have additional domains that confer them other functionalities, like membrane-binding through the PH domains that target them to the cell membrane (Liu et al., 2012; Machnicka et al., 2014); e) different kinds of cell deformations require distinct responses to stimuli: for instance, myosin and α -actinin respond to dilation, while filamin mainly reacts to shear and β -heavy-spectrin senses tension (Duan et al., 2018; Luo et al., 2013).

Crosslinkers are known to participate in multiple cellular processes that rely on actomyosin contractility, such as cytokinesis (see section 1.2); morphogenesis (Adams et al., 1991; Krueger et al., 2019; Norman & Moerman, 2002); cell migration (Honda et al., 1998; Feng & Walsh, 2004; Garbett et al., 2020; Lamb et al., 2021, Biorxiv); endocytosis (Ghisleni et al., 2020; Skau et al., 2011); ciliogenesis (Jia et al., 2019); polarity establishment (Ding et al., 2017; Thomas & Williams, 1999); and cell signaling (Feng & Walsh, 2004). Also, crosslinkers are required to crosslink F-actin in cellular dynamic structures like stress fibers, focal adhesions (Edlund et al., 2001; Choi et al., 2008; Pavalko et al., 1989), lamellipodia, filopodia, microvilli (Loomis et al., 2003; Vignjevic et al., 2006; Svitkina et al., 2003; Johnson et al., 2015) and at cell-cell contacts (Wang et al., 2015). They are key structural components of tissues like muscle, neurons, brain and the reproductive system of animals (Priti et al., 2018; Ribeiro et al., 2014; Wirshing & Cram, 2018; Xu et al., 2013).

Despite the increasing knowledge on individual crosslinkers, it is still not clear whether the large number of crosslinking proteins that coexist in the same cell and tissues reflects similar/redundant roles or if there is a necessary diversification *in vivo*.

The biological relevance of crosslinkers *in vivo* is highlighted by their association with disease, as different crosslinkers were found to be mutated or expressed in abnormal levels in tumors and in neurodegenerative, kidney, cardiac or muscular disorders (Hall et al., 2005; Machnicka et al., 2014; Miyakawa et al., 2012; Kaplan et al., 2000). This issue will be detailed in section 1.4.

1.1.4. Actomyosin networks

Actomyosin networks are contractile three-dimensional meshes of interconnected F-actin. Interconnectivity is ensured by F-actin crosslinkers and contractility by the motor myosin. Other accessory proteins ensure the capability of remodelling of the network, to generate a diversity of dynamic conformations that include parallel or antiparallel bundles, crisscrossed or polygonal networks, branched networks and more (Blanchoin et al., 2014).

The F-actin cytoskeleton forms its networks mainly in the cell periphery, on the cell cortex just beneath the plasma membrane – this allows for strict communication with the plasma membrane through transmembrane proteins, facilitates mechanosensing and signaling transduction of stimuli due to the proximity to external environmental stimuli and provides mechanical support to membrane deformations (Doherty & McMahon, 2008).

In terms of mechanical properties, the actomyosin networks are found to be viscoelastic materials. They can extend and go back to the original shape (for example in the epidermis), but they can also change conformation if the pressure applied to the tissue is long enough (for example during morphogenesis; Lardennois et al., 2019; Blanchoin et al., 2014; Gardel et al., 2004; Tharmann et al., 2007). Studies so far state that the two main regulators of the mechanical properties of actin networks in cells are myosin and F-actin crosslinkers (Ahmed & Betz, 2015; Lieleg et al., 2010; Mizuno et al., 2007). On one hand, myosin motors govern the actomyosin cortical tension, which stiffens the cell surface (Kelkar et al., 2020). On the other hand, crosslinkers were shown (*in silico* and *in vitro*) to alter the network mechanical properties in terms of stiffness and elasticity, depending on their size, number (concentration), F-actin binding kinetics and consequently, on how tight or loose they crosslink a network (Ahmed & Betz, 2015; Lieleg et al., 2010; Wachsstock et al., 1994; Wagner et al., 2006). In practical terms, up to a certain level, the more crosslinking exists, the more elastic the network becomes (Figure 1.3). However, above an optimal crosslinking level or under certain pressure/tension, the network stiffness increases. Essentially, the microscopic biophysical interactions between crosslinking proteins and F-actin affect the viscoelastic behavior of the F-actin network at a large-scale (Lieleg et al., 2010).

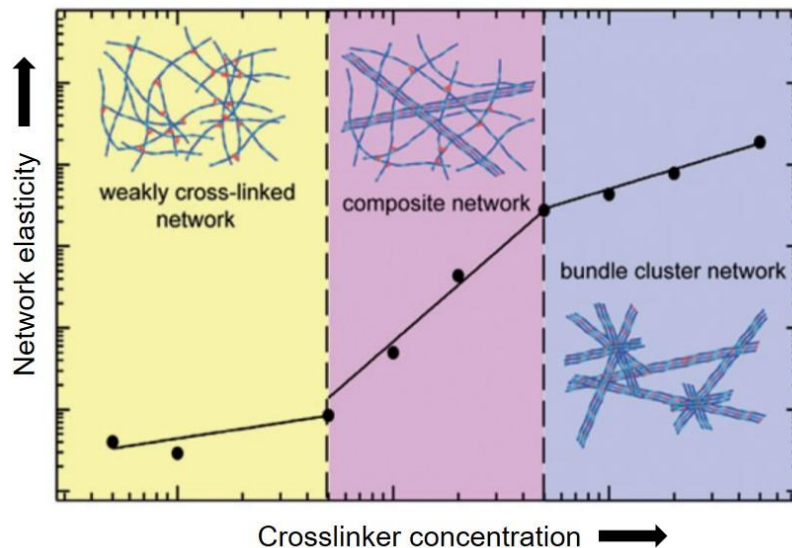


Figure 1.3. Illustrative graph depicting the correlation between crosslinkers concentration and network elasticity. At very low crosslinker concentrations, weakly crosslinked networks behave similarly to F-actin in solution without crosslinkers. Above a certain crosslinker concentration, there is an increase in the networks elasticity, which coincides with the transition of networks to composites or bundles. Adapted from Lieleg et al., 2010; used under permission.

Overall, the actomyosin networks are essential to conduct a broad range of vital cellular and tissue processes that require force generation and cell shape remodeling or stabilization (Munjal & Lecuit et al., 2014; Leite et al., 2019; Salbreux et al., 2012; Huber et al., 2013). Therefore, it is not unexpected that defects in the actin cytoskeleton are linked to several pathological conditions, such as neurodegenerative disorders, cancer and infections (reviewed in Blanchoin et al., 2014).

1.1.5. Contractility in interconnected actomyosin networks

In terms of contractility, most of the current knowledge on how actin and myosin together generate contraction comes from muscle sarcomeres, perhaps the most well-characterized contractile actomyosin structure. In sarcomeres, actin filaments are organized in an antiparallel fashion with the barbed-ends anchored to the Z-discs that limit the sarcomere, and the pointed-ends pointing towards the center of the sarcomere. Myosin bipolar filaments are anchored to the M-line in the center of the sarcomere and intercalate the actin filaments. As myosin binds actin filaments and moves toward their barbed-ends at both sides of the sarcomere, the actin filaments are pulled to the center of the sarcomere leading to sarcomere (and muscle) contraction (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954).

Other actomyosin contractile structures in non-muscle cells, such as the cytokinetic contractile ring (see section 1.2.1) that forms at the end of cell division, may constrict differently. In the contractile ring, linear and packed filaments of actin are interdigitated by myosin bipolar filaments or stacks of filaments, where myosin slides F-actin to drive its constriction in a way compatible with a purse-string contractile mechanism (Henson et al., 2017; Sanger & Sanger, 1980; Schroeder, 1972, 1973). Nevertheless, contrasting to sarcomeric structures, the contractile ring seems to maintain its thickness and width during constriction, implying disassembly of the actin bundles as the ring decreases in size (Carvalho et al., 2009; Schroeder, 1972). In this scenario, contractility is proposed to occur in the context of an actomyosin network organized in sequential equal-sized contractile units or in a continuous array of randomly oriented F-actin, both of which shortening over time due to F-actin disassembly/depolymerization (Carvalho et al., 2009; Lenz et al., 2012; Mendes Pinto et al., 2012; Ennomani et al., 2016). Different hypotheses are debated to explain F-actin disassembly during ring constriction. Myosin has been implicated in F-actin networks disassembly and constriction, likely due to the generation of compressive forces that lead to F-actin buckling (Lenz et al., 2012; Murrel et al., 2015). F-actin buckles are likely more prone to severing by cofilin due to its higher curvature, which promotes filament disassembly (Schramm et al., 2017). Actin filament continuous turnover (polymerization/depolymerization) can also in principle generate network stress and contraction in a myosin-independent manner if the actin filaments are bound by end-tracking passive crosslinkers (crosslinkers that remain bound to depolymerizing filament ends; Zumdick et al., 2007; Oelz et al., 2015).

Indeed, besides myosin, generation and propagation of contractile forces also requires passive crosslinkers to interconnect filaments into fine-tuned contracting networks, so that when myosin pulls on F-actin, tension is created and efficiently propagated into the crosslinked network, allowing its deformation as a whole. Several theoretical models show that in the absence or excess of interconnectivity, cells are mechanically unstable and consequently, contractility and force propagation in space are less effective (Belmonte et al., 2017; Descovich et al., 2018; Ennomani et al., 2016). Moreover, it was observed that connectivity is directly correlated with contractility and that intermediately interconnected networks present optimal contraction (Descovich et al., 2018; Ding et al., 2017; Ennomani et al., 2016; Li et al., 2016; Figure 1.4). In support of these views, *in vitro* studies using micropatterned actin rings composed of parallel actin filaments of mixed polarities and myosin, which resembled contractile rings, only contracted in the presence of the crosslinker α -actinin (Ennomani et al., 2016). *In vivo* studies in cytokinesis also support these models (see section 1.2.2). Overall, crosslinkers determine the global architecture and connectivity of an actomyosin network and, consequently, modulate its contractility.

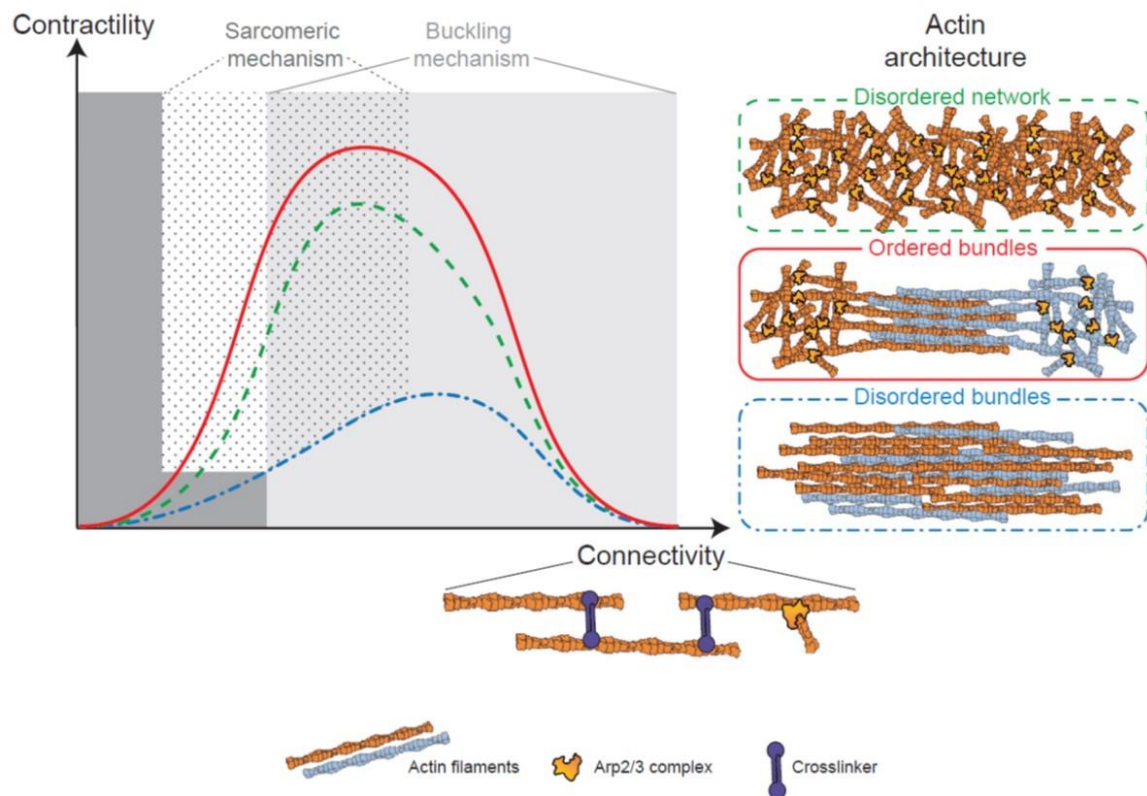


Figure 1.4. Schematic depicting a theoretical model for the correlation between network connectivity and network contractility in three different network architectures. Theoretical model showing that the contractile response of a network is a function of the networks' connectivity by crosslinkers: too much or too low interconnected networks are less contractile than intermediately interconnected networks. The amplitude of the contractile response depends on the F-actin organization, as it is maximal for ordered bundles, intermediate for disordered networks and lowest for disordered bundles. The contractile mechanism is sarcomeric-like for low or intermediate connectivity or buckling-like at intermediate to high connectivity. Adapted from Ennomani et al., 2016; used under permission.

1.2. Cytokinesis

Cytokinesis is the name of the last step of cell division in which a mother cell physically separates into two daughter cells. It is an absolutely essential process that has been studied for more than 100 years, first designated by Whitman in 1887. Cytokinesis starts at anaphase onset, when the sister chromatids aligned at the cell center in the metaphase plate separate from each other and move towards opposite cell poles (Green et al., 2012). In animal cells, cytokinesis relies on an actomyosin contractile ring, a contractile circular structure that assembles beneath the plasma membrane and that, as it constricts, brings the plasma membrane behind it. In brief, as the chromatids segregate, the cytokinesis machinery (actin, myosin and their regulatory components) is recruited to the cell equator

to assemble the contractile ring (Zhou & Wang, 2008). Cytokinesis machinery recruitment to the equator is promoted by an equatorial zone of active RhoA. RhoA activation, by the local GTPase exchange factor (GEF) Ect2, initiates the recruitment of effector proteins, such as Rho kinase and formin, which promote the activation of myosin and the elongation of non-branched (linear) F-actin, respectively (Basant & Glotzer, 2018). Once the actomyosin ring is assembled, it starts to constrict, creating a cleavage furrow that ingresses toward the center of the cell and physically separates the content of the mother cell between the two daughter cells. The positioning of the cleavage furrow is set by mitotic spindle cues. These cues include positive and negative signals: central spindle signaling promotes the local activation of RhoA at the equator while the astral microtubules suppress RhoA activity at the cell poles (Basant & Glotzer, 2018; Bringmann & Hyman, 2005; Chen et al., 2008; Mangal et al., 2018; Werner et al., 2007; Bement et al., 2005; Foe & von Dassow, 2008; von Dassow et al., 2009). After complete furrow ingression, abscission process takes place to seal the remaining narrow cytoplasmic connection between the two daughter cells, separating them at last (Figure 1.5; Green et al., 2012; Mierzwa & Gerlich, 2014). Failure of cytokinesis leads to the formation of multinucleated and polyploid cells, widely associated with cancer (Fujiwara et al., 2005; Steigemann et al., 2009). As cancer is one of the major and most complex pathologies in the current century worldwide, understanding the mechanisms of cytokinesis is of great importance.

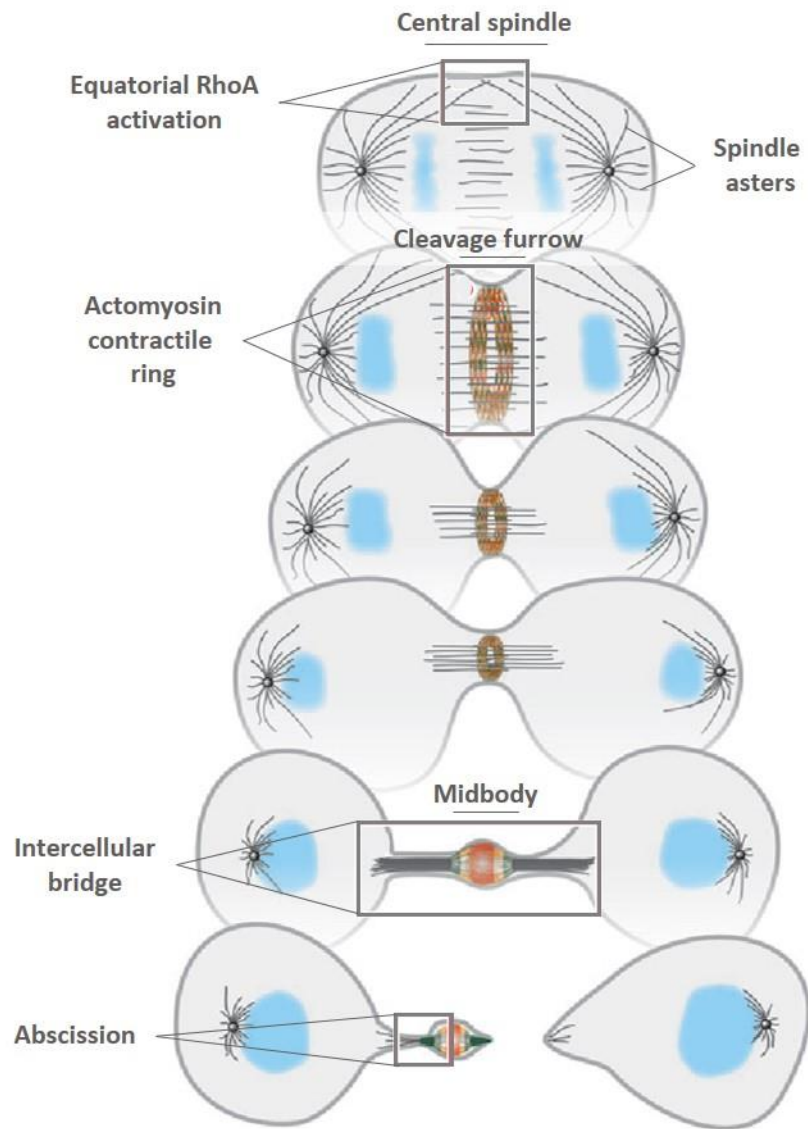


Figure 1.5. Illustration of cytokinesis in animal cells. Signaling from the central spindle and cortex generates an equatorial zone of active RhoA between the separating chromosomes, activated by its Rho-GEF, Ect2. Active RhoA leads to the assembly of the actomyosin contractile ring beneath the plasma membrane, which constricts and changes the cell shape, forming a cleavage furrow. The contractile ring constriction and the cleavage furrow narrowing compact the spindle midzone into a dense structure called midbody and an intercellular bridge is formed. The abscission occurs when the ESCRT family of proteins cleave the membrane in each side of the midbody leading to the complete separation of the two daughter cells. Adapted from Green et al., 2012, used under permission.

1.2.1. Actomyosin Contractile Ring

The contractile ring is a thin layer (0.1-0.2 μm) of differentiated cell cortex that assembles beneath the plasma membrane (Schroeder, 1968, 1972, 1973). It assembles in a circumferential fashion, parallel to the cleavage plane, around the equatorial region of the cell, after anaphase onset (Figure 1.6). The basic toolbox of proteins that composes the

contractile ring is common to many organisms throughout evolution (Eggert et al., 2006). The ring is a filamentous structure essentially composed of formin-nucleated non-branched actin filaments associated to the motor myosin and bundled by crosslinkers (Fujiwara et al., 1978; Garo et al., 2021, Biorxiv; Green et al., 2012; Henson et al., 2017). The use of actin-depolymerizing drugs inhibits cytokinesis, demonstrating that F-actin is essential for the formation of a competent contractile ring (Carter, 1967; Carvalho et al., 2011; Leite et al., 2019). Myosin inhibition also precludes cytokinesis in several systems (De Lozanne & Spudich, 1987; Mabuchi & Okuno, 1977; Shelton et al., 1999; Straight et al., 2003).

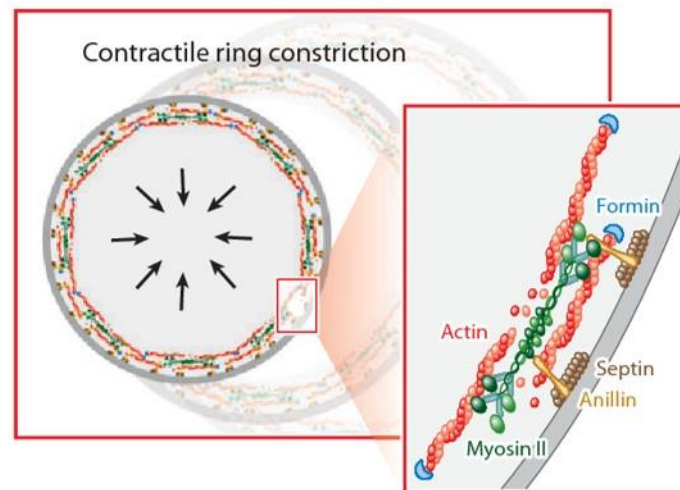


Figure 1.6. Illustration of the contractile ring network. The contractile ring is a filamentous network composed of formin-nucleated F-actin, bipolar filaments of motor myosin II and interconnected by crosslinkers, such as septin and anillin, which are also thought to anchor the contractile ring to the plasma membrane. During constriction, the contractile ring becomes smaller in size, pulling the plasma membrane behind it. Reprinted from Green et al., 2012 used under permission.

Although the contractile ring has been more extensively studied in the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), how its components organize and function in animal cells is less well understood (Leite et al., 2019; Pollard, 2017). Combined super-resolution and Electron Microscopy (EM) data of human cultured U2OS cells and demembranized sea urchin embryos showed that F-actin in the contractile ring is organized in mostly well aligned parallel and non-branched tightly packed filaments, while myosin progressively transitions from an organization in clusters into bipolar filaments or stacks of filaments that interdigitate the linear F-actin (Fenix et al., 2016; Henson et al., 2017).

The crosslinkers more usually related to the functioning of the contractile ring are anillin and septins, which are thought to act as F-actin crosslinkers, scaffolding proteins and anchors of the contractile ring to the membrane. Both localize in the cleavage furrow and bind F-actin, myosin, the plasma membrane and one another (Figure 1.6; Field & Alberts, 1995; Makoto Kinoshita et al., 2002; Mostowy & Cossart, 2012; Piekny & Maddox, 2010).

Recent super-resolution work in demembranized sea urchin embryos showed that anillin and septin mostly co-localize with myosin in the contractile ring (Garno et al., 2021, Biorxiv). Lack of anillin or septins lead to different outcomes in different organisms, ranging from cytokinesis failure to milder phenotypes. These two crosslinkers will be further described in section 1.4.

1.2.2. The role of crosslinkers during cytokinesis

The understanding on how crosslinkers regulate the dynamic properties of the contractile ring during cytokinesis has been increasing. Different crosslinkers were reported in the actomyosin contractile rings of animal cells, such as α -actinin (Fujiwara et al., 1978), plastin (Ding et al., 2017) and anillin (Field & Alberts, 1995). Consistently with theoretical and *in vitro* data that reveal that maximum network contractility was attained with intermediate amounts of connectivity/crosslinkers (Descovich et al., 2018; Ding et al., 2017; Ennomani et al., 2016), the same seems to apply *in vivo*. For instance, in *C. elegans* zygotes, decreasing anillin levels up to a certain point results in faster speeds of constriction, and the lack of plastin slowed cytokinesis total time (Descovich et al., 2018; Ding et al., 2017). In tissue cultured normal rat kidney (NRK) epithelial cells, α -actinin overexpression slowed down or precluded ring constriction while its depletion speeded up ring furrowing (Mukhina et al., 2007). In fission yeast, α -actinin mutants with lower bundling capacity also presented slower ring constriction (Li et al., 2016). Altogether, these studies suggest that crosslinking proteins can both drive or attenuate ring closure speed, depending on their levels. The reason why rings can constrict faster with intermediate amounts instead of physiological amounts of myosin or crosslinkers is still unclear.

Apart from ring kinetics, crosslinkers were shown to affect ring assembly and the connectivity of the surrounding cortex during cytokinesis. In *C. elegans*, plastin was shown to be a major contributor to cortical connectivity during zygote polarization and cytokinesis: a null mutant of this crosslinker presented a less contractile cortex and weaker actomyosin cortical flows. Inefficient recruitment of myosin to the equatorial region for timely and robust actomyosin ring assembly was also observed. Collectively, these alterations culminated in delayed or sometimes failed cytokinesis (Ding et al., 2017). Recently, studies from our laboratory in *C. elegans* additionally showed that plastin and myosin cooperate during cytokinesis to align and compact the equatorial F-actin bundles that form the contractile ring (Leite et al., 2020). In the absence of plastin, embryos partially depleted of myosin are unable to align F-actin bundles along the division plane preventing cytokinesis progression. After contractile ring assembly, plastin seems to be dispensable for contractile ring constriction (Ding et al., 2017). The crosslinker α -actinin seems to be dispensable for

cytokinesis in this animal model (Ding et al., 2017), but in tissue cultured NRK cells α -actinin is important for the integrity of the cortical actin network during cell division: its depletion led to decreased density of F-actin throughout the cortex. Conversely, its overexpression led to increased density of F-actin throughout the cortex, causing cytokinesis failure (Mukhina et al., 2007). In *Dictyostelium discoideum* (*D. discoideum*), the crosslinkers plastin and dynacortin were shown to modulate the cell mechanical properties (as viscoelasticity and cortical tension) in a myosin-dependent manner during cytokinesis (Reichl et al., 2008). Also in *D. discoideum*, myosin and the crosslinker cortexillin were shown to depend on each other to accumulate at regions of increased mechanical load through their mechanosensing abilities and to cooperate for cytokinesis completion: cortexillin stably connects the F-actin network so that myosin can exert more tension (Ren et al., 2009). Additionally, crosslinkers were shown to present differential kinetics in the equator versus surrounding cortex: crosslinkers in the equatorial region (such as cortexillin) have longer lifetimes and maintain more stable associations with F-actin while global crosslinkers that also populate the cortical cell poles (plastin and dynacortin) have shorter lifetimes and are quickly released from F-actin (Reichl et al., 2008).

Altogether, these data support the idea that network connectivity, mechanical properties, and cooperation between different players, such as crosslinkers and myosin, are critical to modulate cytokinesis. In spite of the advances in the comprehension of the mechanisms that drive cytokinesis, contracting actomyosin networks are most likely populated by more than one crosslinker, thus a systematic analysis of which crosslinkers are involved and how they contribute to cytokinesis, individually or in cooperation, is mandatory.

1.3. *Caenorhabditis elegans*: an ideal model system

1.3.1. *C. elegans* as an animal model

In 1963, Sydney Brenner and colleagues proposed the roundworm *Caenorhabditis elegans* (*C. elegans*) as a model organism to perform primarily neuronal development studies and soon dived into molecular and developmental biology (Brenner, 1974). Nowadays, *C. elegans* is extensively used as a laboratory animal model and it is one of the most popular model organisms in the research fields of genetics, evolution, neuroscience, cell and developmental biology and ageing.

C. elegans is a small soil nematode - 0.25 mm long as newly hatched larvae and ~1 mm long as an adult - found worldwide. It is a multicellular eukaryotic organism with numerous evolutionary conserved cellular and molecular processes.

In the laboratory, worms can be easily grown and maintained between 15 °C and 25 °C on inexpensive NGM (nematode growth medium) plates seeded with the bacterium *Escherichia coli* (*E. coli*) as their food supply (Stiernagle, 2006). In a favorable environment, these worms have a fast life-cycle that involves: embryonic development in uterus (~14-16 hours) until the egg is laid; four larval stages (L1 to L4 stages; ~2-3 days); and adulthood with a short fertile period of 2-3 days with brood size of up to 300 progeny per worm (Figure 1.7). Their growth rate is slower at lower temperatures and faster at higher temperatures, allowing the researchers to easily control their development. In the lack of food, adult worms take a few weeks to completely starve as they use their own fat storage to survive. The younger larvae can get into an alternative state called dauer, where they arrest their development, which enables them to survive for up to six more months (Cassada & Russell, 1975). If needed, starved worms can be placed on a new plate with bacteria and resume their development as soon as they start eating. Additionally, L1 stage larvae can be easily frozen at -80 °C for long-term storage (for several years), facilitating the accommodation of strains without keeping them in culture at all times. Defrosted larvae will also resume their growth within a few hours in the presence of food (Stiernagle, 2006).

In addition to inexpensive and easy maintenance, *C. elegans* is also a great model for live-imaging: it is a transparent organism that allows visual access to virtually every cell of its body under a microscope (Corsi et al., 2015). Also, the introduction of fluorescent tags allows visualization of cellular and subcellular details.

Another key advantage of *C. elegans* is the fact that its whole genome is completely sequenced (by *C. elegans* Sequencing Consortium, 1998), enabling straightforward genetic manipulation, generation of mutants or the utilization of RNA interference (RNAi) to deplete any desired gene (Fire et al., 1998). Moreover, the *C. elegans* research community benefits from a comprehensive database, the Wormbase, which includes all genes, phenotypes, genetic interactions, mutants and strains characterized so far. Any generated strains can be deposited at the Caenorhabditis Genetics Center (CGC), becoming available for purchase by any researcher.

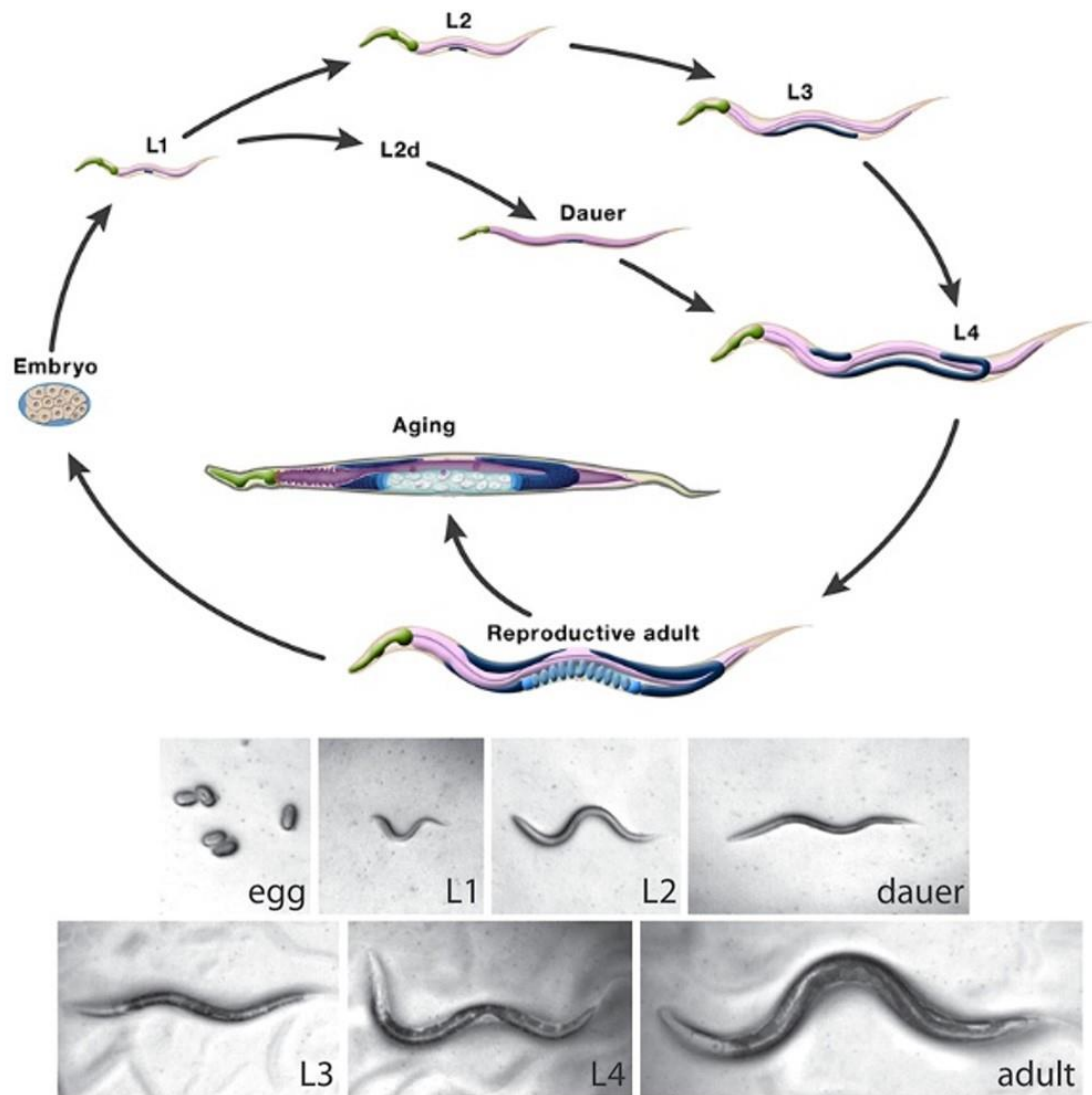


Figure 1.7. Illustration of *C. elegans* life cycle. After embryonic development, *C. elegans* eggs hatch into a larva. Larval development proceeds through four larval stages (L1 to L4). L4 larvae molt into adults that reproduce for a limited period of time. L1 larvae may enter in the alternate dauer pathway under difficult environmental conditions, which allows long-term survival until they reach a more favorable environment and resume reproductive development. Adult worms survive after the reproductive stage for more 3-4 weeks as aged animals. Top: reprinted from WormAtlas (Herndon et al., 2018, Introduction to Aging in *C. elegans*); bottom: adapted from Fielenbach & Antebi, 2008; used under permission.

C. elegans are mostly self-fertilizing hermaphrodites, although males occur naturally in <0.2 % of the progeny or in higher percentages if L4 stage hermaphrodites are subjected to heat-shock conditions (temperatures above their optimal – 30 to 34 °C) for a few hours (Corsi et al., 2015). Moreover, mating of males with hermaphrodites will render ~50 % of males in the progeny. Thus, building new strains with different genetic backgrounds by mating hermaphrodites with males is possible, since genetic crossing follows the rules of Mendelian segregation. This nematode only has five pairs of autosomes and one pair of

sex chromosomes (XX if hermaphrodites or XO if males; Corsi et al., 2015). Many genes and essential cellular and molecular processes are conserved in *C. elegans*, with 60-80 % of human genes (including genes linked to human diseases) having an ortholog in *C. elegans* (Culetto & Sattelle, 2000; Kaletta & Hengartner, 2006). The number of somatic cells and their developmental fate in *C. elegans* has been mapped and is invariable (Giurumescu & Chisholm, 2011; Sulston et al., 1983; Sulston & Horvitz, 1977). This stereotypical cell lineage facilitates the identification and study of genes whose mutation or depletion cause observable phenotypes. These studies are possible due to the efficiency of RNAi and genome editing techniques, such as CRISPR/Cas9 (Waaijers & Boxem, 2014).

In its general anatomy, *C. elegans* is surprisingly complex and retains most of the main tissues expected of higher multicellular organisms, as a mouth, pharynx, gut, anus, gonad, uterus, muscles, neurons, epidermis (called hypodermis), a collagenous cuticle exoskeleton and a fluid filled body cavity. Still, they miss circulatory and respiratory systems, like all nematodes (Corsi et al., 2015).

In sum, *C. elegans* is an inexpensive, low maintenance, transparent nematode that provides powerful genetics and visual accessibility to multiple conserved and stereotypical cellular processes, making it a perfectly suitable model organism to study F-actin network dynamics *in vivo*.

1.3.2. Early embryonic cytokinesis in *C. elegans*

1.3.2.1. Advantages of studying cytokinesis in *C. elegans* early embryos

Several appealing aspects compelled cell biologists to start studying cell division and cytokinesis in the *C. elegans* early embryos in the past decades. In contrast to cell cultures, in which cellular divisions occur within an artificial two-dimensional context of cells adhered to a substrate, cell division in *C. elegans* embryos occurs in a physiological context. Moreover, early embryonic cell divisions are stereotypical, facilitating the detection of even mildly abnormal phenotypes. Imaging early embryos is advantageous to study cytokinesis, since the embryo is still not crowded with cells that complicate the visualization of the dividing ones. Embryos are large (~50 µm length and ~30 µm wide) and expression of fluorescently tagged cytokinesis proteins for fluorescence microscopy is straightforward. Moreover, the possibility to perform gradual depletion of proteins by RNAi or inactivate them at a desired stage of cytokinesis using temperature-sensitive mutants is a valuable asset to study protein functions during this process (Davies et al., 2014; Fire et al., 1998). Embryos can also be permeabilized and treated with drugs of interest before or during cytokinesis (Carvalho et al., 2011). Besides all advantageous technical aspects, *C. elegans* cytoskeletal

proteins and cytokinesis mechanisms are generally well conserved, thus studies in this model are translatable to other organisms.

1.3.2.2. Actomyosin contractile ring in *C. elegans* early embryos

C. elegans embryonic cytokinesis relies on the formation of an actomyosin contractile ring at the cell equator, followed by the ingression of a cleavage furrow as the contractile ring constricts, and culminates with abscission. The process from anaphase onset until the end of the contractile ring constriction takes less than 5 minutes in one-cell embryos (Figure 1.8).

This nematode encodes five closely related and ubiquitous actin proteins (ACT-1-5), some with redundant roles in the embryos (Velarde et al., 2007; Willis et al., 2006). During cytokinesis, there are two pools of F-actin: branched and non-branched/linear. Branched F-actin is nucleated by the ARP2/3 complex and populates the cell cortex in the form of bright puncta, except in the equatorial region. Linear F-actin is nucleated by formin (CYK-1) and is arranged in bundles that enrich in the contractile ring at the equatorial region of the embryos (Chan et al., 2019). F-actin is indispensable for cytokinesis in *C. elegans* embryos, as depolymerization of all F-actin pools with the use of the drug Latrunculin A prevents cytokinesis (Carvalho et al., 2011; Leite et al., 2019).

The major non-muscle myosin II involved in cytokinesis in *C. elegans* is NMY-2. NMY-2 represents the myosin heavy chain, which forms a complex with the regulatory myosin light chain (MLC-4) and the essential myosin light chain (MLC-5). The phosphorylation of MLC-4 on the highly conserved residues threonine 17 and serine 18 (18/19 in the case of mammals) controls the activity and localization of NMY-2 (Gally et al., 2009). NMY-2 localizes at the overall cell cortex at anaphase onset, with a more prominent accumulation at the anterior side of the cell. Then it concentrates in the form of patches at the cleavage furrow (Ding et al., 2017; Osorio et al., 2019). Similarly to F-actin, NMY-2 is essential for cytokinesis as its depletion/inactivation or depletion/inactivation of MLC-4 lead to cytokinesis failure (Davies et al., 2014; Osorio et al., 2019; Shelton et al., 1999). Recent studies from our laboratory suggest that NMY-2 motor activity, rather than its crosslinking activity, is a requirement for cytokinesis completion, since embryos expressing motor-dead NMY-2 that is still able to bind F-actin do not complete cytokinesis (Osorio et al., 2019).

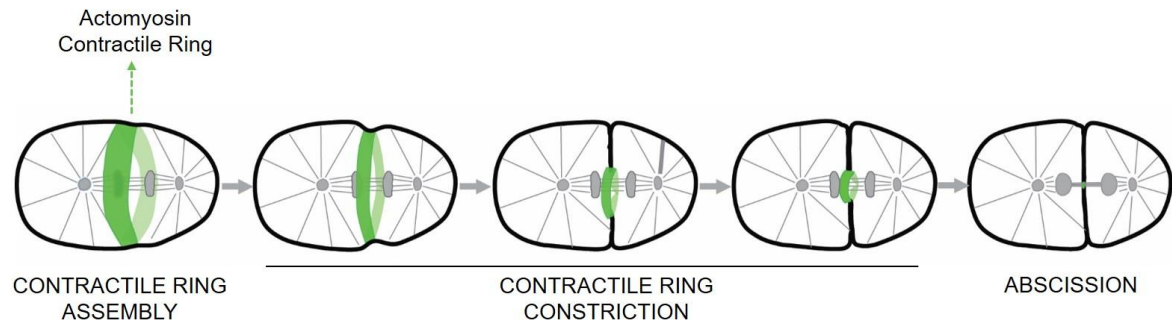


Figure 1.8. Schematic of *C. elegans* one-cell embryos undergoing cytokinesis. Cytokinesis in *C. elegans* embryos relies on the assembly and constriction of an actomyosin contractile ring at the equatorial region of the cell. Unlike tissue culture cells, furrow ingression is asymmetric and there is no formation of an intercellular bridge before abscission (Thieleke-Matos et al., 2017). Cytokinesis completes with the formation of a midbody and the physical separation of the two daughter cells. Image from the Cytoskeletal Dynamics Group, i3S.

In *C. elegans*, contractile ring assembly also initiates with the activation of the small GTPase RhoA (RHO-1), via its GEF, ECT-2 (Pintard & Bowerman, 2019). Active RhoA at the cell cortex activates three effectors: a) formin CYK-1, which promotes nucleation/elongation of linear F-actin in the equatorial region of the cell (Chan et al., 2019); b) Rho-associated protein kinase ROCK (LET-502), which activates NMY-2 via phosphorylation of the MLC-4, leading to the open (active) conformation of the myosin complex (Piekny & Mains, 2002); c) and anillin (ANI-1), which acts as a scaffold protein, crosslinks F-actin, recruits septins, binds to the membrane and to other regulators (Nguyen et al., 2000; Piekny & Maddox, 2010).

As referred in section 1.2.2, another crosslinker, plastin (PLST-1), was shown to be a major participant in the maintenance of cortical connectivity and in the compaction of equatorial F-actin bundles to ensure timely and efficient contractile ring assembly (Ding et al., 2017; Leite et al., 2020). Whether additional crosslinkers contribute to cytokinesis in this animal was unknown and was an object of the present study.

1.3.2.3. The role of the cortex and cortical flows in cytokinesis

The cellular cortex is a thin (100-200 nm on average) F-actin meshwork that underlies and is connected to the plasma membrane of the cell (Svitkina, 2020). Actomyosin cortical tension allows the cells to sense or resist external stress. Also, gradients of cortical tension lead to the generation of cortical flows that are thought to be important drivers of cell shape changes, such as mitotic cell rounding and cytokinetic ring formation. Cortical tension gradients are also associated with increased contractility and contractile pulses of the

cortex, important for example during *C. elegans* polarization or epithelial morphogenesis (Chugh & Paluch, 2018; Kelkar et al., 2020).

In cytokinesis, studies are mostly focused on the contractile ring and less attention is paid to the surrounding actomyosin cortex. Nevertheless, the contractile ring specializes from and remains integrated in the surrounding cortex. Unlike the contractile ring, which is a linear array of F-actin bundles, the cortex is mainly composed of a crisscross F-actin meshwork (Henson et al., 2017). How the surrounding cortex impacts ring constriction is still poorly investigated.

A well-documented cortical behavior during cytokinesis in one-cell embryos of *C. elegans* is the existence of long-range actin and myosin cortical flows. Cortical flows in cytokinesis move from regions of relaxation towards regions of tension. These persistent, initially unidirectional (anterior-directed) and then bidirectional (equator-directed) flows of F-actin during contractile ring assembly were suggested to be the main drivers of F-actin alignment at the equatorial region (Reymann et al., 2016). This hypothesis was challenged by recent work from our lab, where F-actin alignment and compaction at the equatorial region was proposed to be primarily driven locally by myosin in cooperation with plastrin. Although the contribution of F-actin flows was not excluded, they were also not essential for contractile ring formation, furrowing, myosin accumulation at the contractile ring nor cytokinesis completion (Leite et al., 2020).

1.3.3. Other actomyosin-dependent contexts in *C. elegans*

1.3.3.1. Gonadogenesis, oogenesis and embryogenesis

The reproductive system of the adult hermaphrodite *C. elegans* requires coordinated contractility and tissue organization in order to produce and fertilize oocytes, store the developing embryos and expel them at the right stage. The tissue organization and contractility are actomyosin-dependent and, therefore, this is an excellent model to study actomyosin network connectivity by crosslinkers (Kelley et al., 2020; Kovacevic & Cram, 2010; Priti et al., 2018). This system is composed of two U-shaped tubular gonad arms, each one containing a spermatheca at the most proximal region. Between the two spermathecae there is a common uterus and vulva (Figure 1.9; Strome, 1986). The gonad arms supply the oocytes, the spermatheca stores the sperm and is the place where the oocytes are fertilized, and the uterus stores the developing embryos until they are laid. The oocytes are produced and gradually mature in the proximal region of the gonads' arms, in a process called oogenesis. The most mature and proximal oocyte is ovulated and fertilized inside the spermatheca. Then, the spermatheca contracts to push the embryo into the

uterus through the spermatheca-uterine (SP-UT) valve (McCarter et al., 1999). In the uterus, several different-staged embryos are developed in a process called embryogenesis. The formation of the gonad, which starts at the L1 stage and completes in adult animals, is called gonadogenesis.

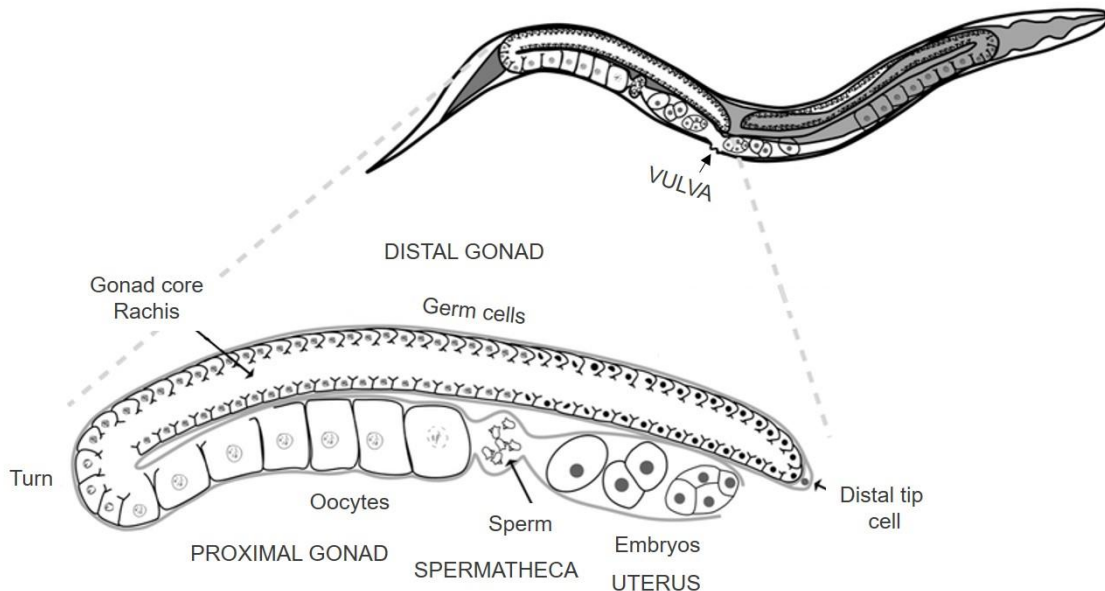


Figure 1.9. Illustration of the adult *C. elegans* hermaphrodite reproductive system. (Upper) Adult *C. elegans* hermaphrodite with the gonad and intestine (dark grey) depicted. (Bottom) Zoom in on the U-shaped structure of *C. elegans* gonad depicting the distal and the proximal gonad, the spermatheca and part of the uterus. The distal gonad contains a distal tip somatic cell, a syncytium with peripherally arranged germ cells and the gonad core, the rachis. The germ cells are partially enclosed in the plasma membrane with a ring canal that allows cytoplasmic exchange with the rachis and expand in size around the gonad's turn to become individualized and mature oocytes in the proximal gonad. Mature oocytes enter the spermatheca to be fertilized and are stored in the uterus during initial embryonic development. Adapted from Huelgas-Morales et al., 2016; used under permission.

The distal arm of the gonad consists of a cytoplasmic core with a tubular shape, called rachis, surrounded by a syncytium of germ cells that are partially cellularized. Each germ cell has a nucleus and shares its cytoplasm with the rachis through a rachis bridge, which is formed upon an incomplete cytokinesis-like process. The cytoplasm in the rachis delivers all the material required for the germ cells to expand and individualize along the gonad into mature oocytes (Wolke et al., 2007). Several somatic sheath cells outline the gonad arms. Actomyosin network contractility in these cells is essential to guarantee the entry of embryos into the spermatheca. If the actomyosin cytoskeleton is defective and affects gonadogenesis, it leads to obvious phenotypes in the gonad's architecture and its actomyosin-dependent processes, such as cellularization, cell size expansion and oocyte individualization. Impairment of these processes may also affect the amount of oocytes and

embryos produced and their proper development, thereby affecting the brood size of the animal and the embryonic viability of its eggs.

1.3.3.2. Body wall muscles

C. elegans locomotion is ensured by its body wall muscles. These muscles are equivalent to vertebrate skeletal muscles. The other *C. elegans* muscles: the vulval muscles, the pharyngeal muscle cells, the somatointestinal muscles, the anal sphincter muscle, the anal depressor muscle, the uterine muscles and contratile gonadal sheath cells, will not be addressed in this thesis (Gieseler et al., 2017).

As in all other nematodes, the body wall muscles of *C. elegans* are obliquely striated. They are formed by a longitudinal layer of muscle cells that run from head to tail, arranged in four quadrants, two dorsal and two ventral (Figure 1.10; Gieseler et al., 2017). Each quadrant has a double row of muscle cells. These are beneath the epidermal cells, separated by a basal lamina.

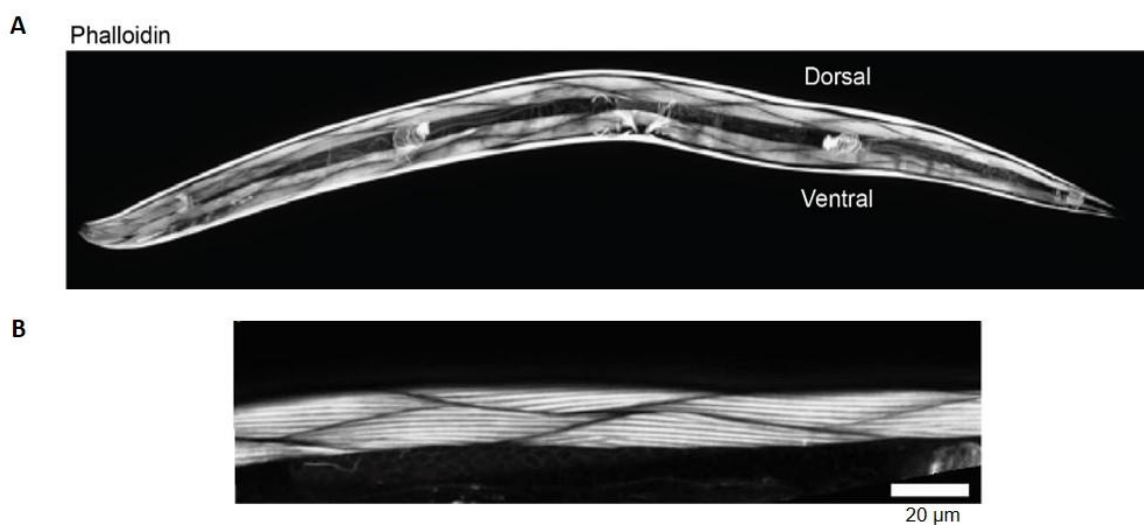


Figure 1.10. Image of striated body wall muscles of adult *C. elegans* hermaphrodite. (A) Actin stained with Phalloidin demarks the dorsal and ventral longitudinal body wall muscles of an adult *C. elegans* hermaphrodite along the entire animal body. (B) Zoom in on part of the body wall muscles depicting muscle cells with striated morphology.

The striated muscle cells are composed of multiple sarcomeres, the muscle contractile units. These contain regularly distributed attachment points called dense bodies (analogous to vertebrate Z-discs) and M-lines, which anchor the actin and myosin filaments, respectively, to the muscle cell basal membrane and to the underlying epidermis and cuticle, in order to transmit the contractile forces to the exoskeleton. For coordinated

movement, the force generated by contraction must also be transmitted to the neighbouring muscle cells by the lateral attachment plaques. The attachment complexes contain actin-binding proteins as integrins, vinculin, talin (which interact with each other to anchor actin to the cell plasma membrane) and the F-actin crosslinker α -actinin, a major component of the dense bodies (Altun & Hall, 2009; Gieseler et al., 2017). The overall structure, regulation and functioning of the sarcomere apparatus is conserved from *C. elegans* to vertebrates. The pulling of F-actin by myosin heads generates contraction of the muscles (Gieseler et al., 2017). Body wall muscle contractions in *C. elegans* are modulated by neuronal transmission from motor neurons, which are present in the nerve cords that innervate the body wall muscles along the animal body or in the nerve ring in the head (Altun & Hall, 2009).

Most body wall muscle cells develop during embryogenesis and only a few develop post-embryonically. Then they grow in size during larval development, adding new sarcomeres to and enlarging the muscle cells. Body wall muscles are important for full elongation of the animal's body (Gieseler et al., 2017).

Overall, the *C. elegans* body wall muscles are a contractile, interconnected actomyosin-dependent tissue required for proper animal elongation and movement, including locomotion on solid or semi-solid surfaces and body bending during thrashing on liquids. Defects in the actomyosin network of body wall muscles, such as loss of connectivity by loss of crosslinkers, should render morphological, mobility and body bending phenotypes that can be observed and quantified by standardized assays.

1.3.4. RNAi pathway in *C. elegans*

The RNAi pathway was first discovered and characterized in plants and in *C. elegans* (Baulcombe, 1996; Fire et al., 1998). Present in most eukaryotic cells, RNAi is a regulatory mechanism in which cells use small double-stranded RNA (dsRNA) molecules to silence a gene's activity via an homology-dependent pathway. In brief, naturally occurring long dsRNA molecules are recognized and cut by Dicer, an RNase endonuclease, into small fragments (small interference RNAi or siRNA). These then bind to a complex, called RNA-induced silencing complex (RISC) that includes the protein Argonaute. The siRNA-RISC are directed to messenger RNAs (mRNAs) that share complementary homology with the siRNA fragment and they pair. Argonaute then cleaves the mRNA, which is degraded, blocking its transcription (Kim & Rossi, 2008; Neumeier & Meister, 2020). This mechanism was further shown to be important during viral infections, for instance, as long dsRNAs in the cytosol are hallmarks for virus replication inside the cells (Schlee & Hartmann, 2016).

In *C. elegans*, RNAi-mediated depletion of a gene is triggered by the administration of synthetic dsRNAs via injection, feeding or soaking of the worms. Taking advantage of the anatomy of the animals' reproducing system, the reduction in the activity of a gene occurs in a controlled manner. The corresponding mRNA degradation is gradual in the progeny: during the treatment, oocytes and embryos will continue to load the target protein already existing in the gonads until all tissues are depleted and there is no more protein left to be loaded (Oegema & Hyman, 2006). This gradual decrease in protein levels in newly fertilized embryos allows the characterization of partial to more severe phenotypes. Additionally, double and triple RNAi depletions can be performed simultaneously in *C. elegans*. The depletion of each target can be checked by immunoblotting, Reverse-Transcription (RT)-PCR or phenotypical analysis.

1.3.5. CRISPR/Cas9 in *C. elegans*

Genome editing by CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) is also considerably straightforward in *C. elegans*.

The CRISPR/Cas9 system is primarily an antiviral defense mechanism in some bacteria and archaea. These prokaryotes use the DNA of invading viruses to create DNA sequences arranged in regularly interspaced arrays to detect and destroy similar viruses that may subsequently infect them. To do so, they use the CRISPR-associated protein 9 (Cas9), which is an enzyme (endonuclease) that recognizes CRISPR segments in the viral DNA and cleaves them to stop viral replication.

In the past decade, this system was adapted to a genome-editing tool on several organisms, including mammals and invertebrates (like *C. elegans*), revolutionizing biomedical research (Jinek et al., 2012). Its application as a gene-editing technique was awarded with a Nobel Prize in Chemistry in 2020. Currently, it is used to generate insertions, deletions or modifications in the endogenous loci of target genes - thus modified proteins are expressed from their original genomic context.

CRISPR/Cas9 genome-editing system relies on the recognition and cleavage of genomic DNA sequences by the enzyme Cas9. The Cas9 is guided to the sequence of interest by a single guide RNA (sgRNA). Custom-made sgRNAs are generated to direct the Cas9 to a specific genomic location. Cas9 induces double strand breaks in the target DNA, which is then repaired via non-homologous end-joining (NHEJ) DNA repair pathway or homology directed repair (HDR). The NHEJ repair is error-prone and insertions and/or deletions that may be introduced can disrupt gene function, resulting in truncated or knocked-out genes. The HDR is used when specific insertions (of fluorescent tags for instance) or deletions are desired (Sander & Joung, 2014). To do so, a custom-made repair

template with homology arms to each side of the cut site is given to the system. This repair template can be delivered in the form of a single-stranded DNA sequence or as a PCR product with single stranded flanking regions (Figure 1.11; Dickinson et al., 2013; Dokshin et al., 2018).

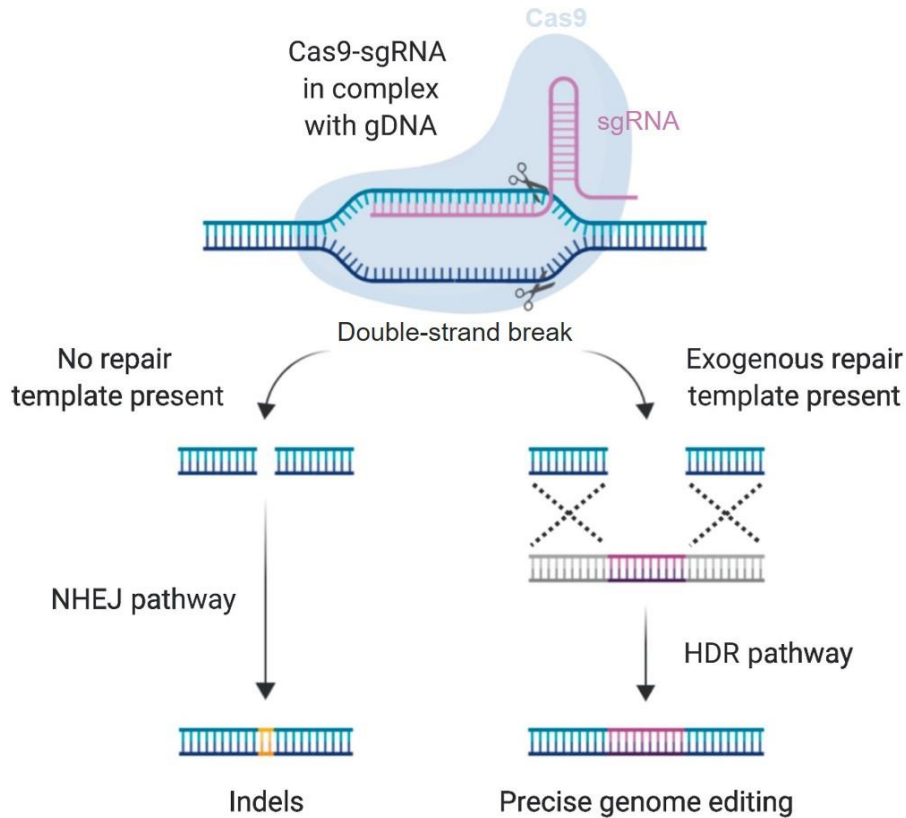


Figure 1.11. CRISPR/Cas9 system. The sgRNA binds to Cas9 nuclease and directs it to specific genomic regions of homology. The Cas9 generates a double-strand break in the DNA, which is repaired via the NHEJ or HDR pathways. NHEJ-mediated repair is error prone and frequently results in small insertions or deletions (indels) at the cut site, affecting the gene function. The HDR is a high-fidelity repair mechanism that uses a DNA template to accurately repair the double-strand break. This mechanism is very useful to knock-in exogenous DNA elements, such as tags, or to delete portions of the genome without affecting the remaining gene function. Adapted from van Kampen & van Rooij, 2019; used under permission.

The CRISPR/Cas9 system delivery in *C. elegans* requires the injection of animals with a mix containing purified Cas9 (or a plasmid carrying the Cas9 sequence), one or more sgRNAs and, usually, a repair template. Variants of the CRISPR/Cas9 method include co-conversion of the *dpy-10* gene (a sgRNA and a repair template to modify the *dpy-10* gene is introduced in the injection mix together with the sgRNA and the repair template of the desired gene to be modified). The *dpy-10* modified gene will give a roller phenotype to the progeny of well-injected mothers, facilitating the screening of well-injected animals that may carry the desired modification as well (Arribere et al., 2014). The *dpy-10* modification can

be lost either after the initial screening or by outcrossing the final strain with a wild-type strain, by selecting non-roller progeny carrying the modification of interest in both cases. Also, a co-CRISPR variant technique can be used to facilitate the detection of genome-editing events, and consists of adding plasmids carrying fluorescent markers to the injection mix. Inside the animal these sequences will tandemize into extrachromosomal arrays and help to identify the well injected animals by fluorescence screening. These extrachromosomal arrays only persist for a few generations (Dokshin et al., 2018; Kim et al., 2014). In all cases, hermaphrodites are injected as young adults in both gonads to increase the chances of getting the modified gene in their progeny.

In *C. elegans*, CRISPR/Cas9 is routinely used as an alternative or complementary technique to transgenesis methods, such as microparticle gene bombardment (Praitis et al., 2001) or Mos1 single-copy insertion of transgenes (MosSCI; Frøkjær-Jensen et al., 2008).

1.4. Overview of conserved crosslinker orthologues

There are multiple F-actin crosslinkers reported in vertebrates, each one with several genes encoding for different isoforms. This diversity complicates functional studies of these proteins in vertebrate models. In *C. elegans*, the number of conserved crosslinkers is reduced and they are represented by one, two or three isoforms at most.

The crosslinkers studied in this project were selected based on homology studies, literature data of all proteins that contain an ABD and have been proved to be able to crosslink/bundle F-actin *in vitro*. Other proteins containing an ABD with presumed crosslinking activity but without conclusive crosslinking/bundling *in vitro* data were not considered. Neither were considered proteins that merely branched F-actin or linked F-actin to the membrane, transmembrane proteins, microtubules or other structures. From a total of 16 proteins found to match our criteria, 12 crosslinkers exist in mammalian cells. Of those, 7 have well-conserved orthologues in *C. elegans* and were selected to be the focus of this study. They are: α -actinin ATN-1, plastin PLST-1, β -spectrins SMA-1 and UNC-70, filamin FLN-1, anillin ANI-1, septins UNC-59/UNC-61, and coronins COR-1 and POD-1 (Table 1.1). *C. elegans* expresses two more anillins, ANI-2 and ANI-3, but these have no ABD and are poorly conserved (Maddox et al., 2005). There is also a second filamin, FLN-2, which is nematode specific (DeMaso et al., 2011). Additionally, POD-1 coronin-like protein ended up being excluded from this study, since its depletion caused severe osmotic sensitive embryos, which precluded a satisfactory investigation of cytokinesis.

Table 1.1. List of proteins qualified as F-actin crosslinkers in all organisms with known orthologues in mammalian and conserved orthologues in *C. elegans*.

All organisms	Mammalian	<i>C. elegans</i>	Refs supporting crosslinking
α -actinin	ACTN	ATN-1	(Meyer & Aebi, 1990)
Fimbrin/Plastin	PLS/LCP	PLST-1	(Adams et al., 1991; Bretscher, 1981; Goode et al., 1999; Janji et al., 2006; Nakano et al., 2001)
Spectrins	SPT	UNC-70, SMA-1, (SPC-1)*	(Brown et al., 2015)
Filamin	FLN	FLN-1, FLN-2 [†]	(Nakamura et al., 2007; Wang & Singer, 1977)
Anillin	ANLN	ANI-1, ANI-2 [†] , ANI-3 [†]	(Dorn et al., 2016; Jananji et al., 2017)
Septins	SEPT	UNC-59, UNC-61	(Mavrakakis et al., 2014)
Coronins	CORO	COR-1, POD-1 [†]	(Cai, et al., 2007b; Goode et al., 1999)
Fascin	FSCN		
Espin	ESPN		
Eplin	LIMA		
Villin	VIL		
Dematin	DMTN		
Palladin	PALLD		
Cortexillin			
Dynacortin			
Scruin			

* Does not contain an ABD but it functions in a complex with other crosslinkers (details in section 1.4.3).

[†] Proteins that did not integrate this study.

1.4.1. α -actinin

α -actinin is a ubiquitous and well conserved F-actin crosslinker of approximately 100 kD, present in diverse organisms from protists to mammals. Vertebrates have at least four α -actinin genes (ACTN1-4) which are selectively expressed in muscle cells (ACTN2 and ACTN3) or non-muscle cells (ACTN1 and ACTN4), while *C. elegans* has only one, *atn-1* (Barstead et al., 1991; Ribeiro et al., 2014; Sjöblom et al., 2008). Due to their similar ABDs

and spectrin repeats (SRs), α -actinin belongs to the spectrin superfamily of proteins along with spectrins, plastin, filamin and dynactin (Broderick & Winder, 2005; Dubreuil, 1991). α -actinin contains one ABD composed of two CH regions in the N-terminus, followed by four SRs and two pairs of EF-hand motifs in the C-terminus (Blanchard et al., 1989; Meyer & Aebi, 1990; Sjöblom et al., 2008). While the ABD is responsible for its F-actin binding and crosslinking ability, the series of SRs give the molecule its rod shape, and interact with transmembrane proteins, such as vinculin or integrin (Foley & Young, 2014). The EF-hands form a calmodulin-binding sequence which is regulated by calcium binding, important to trigger conformational changes in the protein that either allow or inhibit its interactions with F-actin or other targets (Sjöblom et al., 2008). Yet, throughout evolution, many EF-hand motifs have diverged in a way that they partially or completely lost calcium binding capacity and are therefore calcium insensitive (Nakayama & Kretsinger, 1994). In humans, for instance, only the non-muscle ACTN1 and ACTN4 retained calcium-binding, which negatively regulates its binding to F-actin (Burrige & Feramisco, 1981; Tang et al., 2001). In order to function, α -actinin forms antiparallel dimers (around 35 nm long), with the ABDs at each end of the dimer to bridge F-actin. Due to its size and characteristics, it preferentially forms F-actin parallel or antiparallel bundles (Hampton et al., 2007; Meyer & Aebi, 1990; Wachsstock et al., 1993).

The first reports of α -actinin were in skeletal muscle cells, where it plays a structural role in crosslinking F-actin in the sarcomere Z-discs and in stabilizing the muscle apparatus (Masaki et al., 1968; Squire, 1997). It is now known that α -actinin is a major crosslinker in non-muscle cells as well, involved in lamellipodia, stress fibers, cytokinesis, cell-cell and cell-matrix contacts, and interacting with multiple proteins for cytoskeletal remodeling (Foley & Young, 2014). In the fission yeast *S. pombe*, α -actinin is calcium insensitive and it is involved in cell division, cooperating with plastin for normal cytokinetic ring formation. Double mutants of α -actinin and plastin fail cytokinesis and are therefore unviable (Laporte et al., 2012; Wu et al., 2001). *D. discoideum* without α -actinin are viable with mild developmental defects, but when combined with null mutants of the crosslinker filamin, much more severe defects occur, including slow growth, abnormal morphogenetic processes and motility defects (Ponte et al., 2000; Rivero et al., 1999). In *Drosophila melanogaster* (*D. melanogaster*), null α -actinin alleles that knock out both muscle and non-muscle isoforms complete embryogenesis but are lethal within two days after hatching due to severe defects in muscle tissues and paralysis (Roulier et al., 1992). Nevertheless, they do not cause significant defects in non-muscle cells (Wahlström et al., 2004, 2006).

Mutations in human α -actinins cause serious pathologies in several tissues. Some of these include the inherited autosomal-dominant form of the kidney disease Focal Segmental Glomerulosclerosis, myopathies such as hypertrophic or dilated

cardiomyopathy, and blood platelets disease Congenital Macrothrombocytopenia (Chiu et al., 2010; Kaplan et al., 2000; Kunishima et al., 2013; Mohapatra et al., 2003).

1.4.1.1. ATN-1 in *C. elegans*

In *C. elegans*, α -actinin ATN-1 is a 107 kD protein with a well conserved structure with vertebrates (information retrieved from Uniprot.org, gene code Q23158 and Barstead et al., 1991; Francis & Waterston, 1985; Figure 1.12). Also consistent with its vertebrate homologues, ATN-1 is localized to the striated muscle dense bodies (analogous to Z-discs) of these animals (Francis & Waterston, 1985; Moulder et al., 2010). A null *atn-1* mutant was shown to present abnormally shorty and broad dense bodies and abnormal F-actin accumulations in the muscle cell boundaries. The rest of the muscle cells displayed normal striated morphology (Moulder et al., 2010). Although they presented normal locomotion on agar or liquid surfaces, a reduction in the locomotion of *atn-1* null animals was observed in more sensitive bending assays, most likely associated to the muscle abnormalities or defects in the transmission of muscle contraction (Moulder et al., 2010). Its mild phenotype indicates that other crosslinkers might play a similar or redundant role in the same context. ATN-1 was also implied in the maintenance of vulva muscle morphology (Wang et al., 2020). This crosslinker was shown to be dispensable for early embryo polarity establishment, cortical contractility and cytokinesis, suggesting that it does not alter the mechanics or dynamics of F-actin networks in the *C. elegans* zygote (Ding et al., 2017).

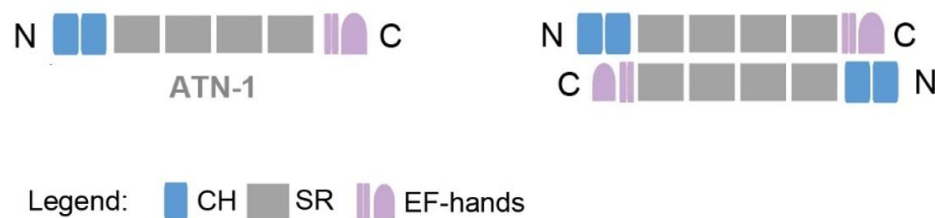


Figure 1.12. Schematic structure of ATN-1. (Left) Structure of ATN-1 monomer. (Right) Scheme of ATN-1 antiparallel dimer. CH: calponin homology domain; SR: spectrin repeat; N: N-terminus; C: C-terminus.

1.4.2. Plastin/Fimbrin

Plastin, also known as fimbrin, is a small globular protein of approximately 70 kD and 9-12 nm in length, which crosslinks F-actin into tightly packed parallel or antiparallel bundles (Bretscher, 1981; de Arruda et al., 1990; Glenney et al., 1981; Matsudaira et al., 1983; Skau et al., 2011). Plastin canonical structure is one the most basic for an actin-binding protein: it contains two N-terminal EF-hands followed by two conserved ABDs (two pairs of CH

sequences), which comprise the remainder of the protein. As the ABDs compose most of the protein, plastin is presumed to have no other functions beyond crosslinking F-actin. Yet, plastin (as other crosslinkers) has also been implicated in F-actin networks' dynamics. Plastin has been reported to stabilize F-actin *in vitro* and *in vivo*, either by protecting against depolymerization or by decreasing F-actin disassembly rate and reducing the access of the F-actin-severing protein cofilin to the network (Goode et al., 1999; Lebart et al., 2004; Giganti et al., 2005; Young et al., 2004). In contrast, work in fission yeast shows that plastin modulates F-actin turnover by competing with tropomyosin, a protein that stabilizes F-actin polymers, facilitating cofilin-mediated F-actin disassembly (Christensen et al., 2017; Skau & Kovar, 2010). Whether the F-actin crosslinking and F-actin stabilization/destabilization are independent functions of the protein is unclear.

Mammals have three plastins expressed in a tissue-specific manner: I-plastin (PLS1), restricted to intestine and kidney, L-plastin (PLS2), expressed in leukocytes and T-plastin (PLS3), expressed in solid tissues as the brain (de Arruda et al., 1990; Shinomiya, 2012). Plastins are known to localize in the intestine microvilli, lamellipodia, auditory hair cell stereocilia, fibroblast filopodia and podosomes, among others (Coluccio & Bretscher, 1989; Evans et al., 2003; Matsudaira et al., 1983; Matsudaira, 1994; Shinomiya, 2012). Unlike mammals, invertebrates like *D. melanogaster* and *C. elegans* contain only one plastin gene.

Plastins of different organisms were reported to play a role in distinct actomyosin-dependent processes. In *D. melanogaster*, plastin is important for proper actomyosin network remodeling that occurs during the embryonic cellularization process. This process requires the transition of an hexagonal-shaped actomyosin network array into contractile rings that individualize each cell by closing. Loss of plastin results in a permanent hexagonal-shaped network that constricts slower (Krueger et al., 2019). In *D. discoideum*, plastin localizes to cortical actin patches and also modulates the furrow speed during cytokinesis (Reichl et al., 2008). In the unicellular *Tetrahymena thermophile*, plastin localizes in the cleavage furrow of the dividing cell, suggesting a role in cell division (Shirayama & Numata, 2003). As previously referred, plastin is also important in fission yeast cytokinesis in cooperation with α -actinin, but also by itself in controlling the dynamics of F-actin via tropomyosin antagonization and promotion of cofilin-mediated F-actin severing (Christensen et al., 2017; Laporte et al., 2012; Skau & Kovar, 2010; Wu et al., 2001). In the same model, plastin was shown to localize to endocytic actin patches and to mildly affect cell polarity, suggesting a role in endocytosis and polarization (Skau et al., 2011). Plastin has also been implicated in host defense mechanisms, as L-plastin-deficient mice were reported to be unable to control bacterial infection (Chen et al., 2003).

In terms of human disease, plastins were mostly linked to cancer, as alterations in its expression levels were found in different tumor cells. For instance, overexpression of L-

plastin was found in leukemias, while downregulation of T-plastin was related to colorectal cancer development. For this reason, plastin is considered a potential biomarker for cancer (de Arruda et al., 1990; Goldstein et al., 1985; Sasaki et al., 2002; Shinomiya, 2012).

1.4.2.1. PLST-1 in *C. elegans*

C. elegans encodes a single plastin gene, *plst-1*. It shares >60 % similarity to the human homologues and possesses the same canonical structure, although the EF-hand sequences are less well conserved (Ding et al., 2017; Figure 1.13). PLST-1 is expressed ubiquitously from one-cell embryos, enriching at cell-cell contacts. Throughout embryogenesis it is enriched at the buccal region of the epidermis, pharynx and intestine. In adulthood it is expressed in various tissues like the pharynx, intestine, gonad, vulva and neurons (Ding et al., 2017).

Plastin was shown to bind to branched and non-branched cortical F-actin of one-cell embryos and to influence embryonic polarization, cortical contractility, cortical flows and cytokinesis, as previously referred (Ding et al., 2017; Leite et al., 2020). Apart from embryonic cytokinesis, PLST-1 was also shown to act in the *C. elegans*' gonad. In this context, PLST-1 was necessary for maintaining proper actomyosin architecture and an even distribution of actin and myosin along the tissue, smoothening the distribution of tension and, consequently, of contractility (Priti et al., 2018).



Figure 1.13. Schematic structure of PLST-1. Structure of PLST-1 monomer; CH: calponin homology domain; N: N-terminus; C: C-terminus.

1.4.3. Spectrins

Spectrins' name comes from their initial isolation from membrane ghosts of red blood cells, where they were first described as one of the structural components of erythrocytes' membranes (Byers & Branton, 1985; Marchesi & Steers, 1968). Nevertheless, spectrins are expressed ubiquitously. Spectrins are large and flexible crosslinkers that consist of two antiparallel heterodimers of α and β subunits that assemble into a tetrameric structure (Ipsaro et al., 2010; Morrow et al., 1980; Speicher et al., 1980). The spectrin tetramers are elongated rods with an ABD (composed of two CH domains) at either side, on the β -spectrin N-terminus, to allow F-actin binding and crosslinking. β -spectrins also contain a pleckstrin homology (PH) domain in the C-terminus, which binds to phosphatidylinositol lipids and

localizes spectrin to the plasma membrane (Wang et al., 1996). Contrasting to β -spectrins, α -spectrin has C-terminal EF-hand domains that are juxtaposed to the β -spectrins ABD in their tetrameric assembly. Additionally, α -spectrin and, in some invertebrates, the heavier isoform of β -spectrin (β H-spectrin) contain a Src homology 3 domain (SH3), which is thought to mediate signaling (Cortese et al., 2017; Machnicka et al., 2014). Multiple SRs, each composed of ~106 amino acids that fold into three α -helices linked by flexible loops, are present in both spectrin subunits, conferring them their flexible and elastic structure (Speicher & Marchesi, 1984). Besides its functional domains, spectrins directly interact with spectrin-associated proteins that assist spectrins in binding F-actin and the membrane, such as protein band 4.1, adducin and ankyrin (Machnicka et al., 2014; Speicher & Marchesi, 1984). The ABD region of β -spectrin was found to form a stable ternary complex with actin and adducin, a membrane-skeletal protein that caps F-actin barbed ends and recruits spectrins to the ends of the filaments (Li & Bennett, 1996). Protein 4.1 was also found to bind to this complex and mediate its connection to the membrane through Glycophorin C, a transmembrane protein (Baines, 2009; Hemming et al., 1995). The PH domain was shown to bind to phosphatidylinositol lipids and to localize spectrin to the plasma membrane (Wang et al., 1996). Additionally, β -spectrin is able to bind to the membrane through an interaction it establishes with ankyrin, which in turn binds to the transmembrane protein Band 3 (Bennett & Lorenzo, 2016; Bennett & Stenbuck, 1979b; Davis et al., 2009). Functionally, the interactions of spectrin with actin, adducin, ankyrin and protein 4.1 are thought to be implicated in aspects of membrane trafficking, cell signaling, cell adhesion and cortical stability (Baines, 2009).

Consistent with these characteristics, spectrins were found to form two-dimensional networks under the plasma membrane, held together by short F-actin and associating simultaneously to the membrane and membrane-bound proteins. Due to the formation and nature of its flexible yet robust polygonal lattice-like meshwork in erythrocytes, the spectrins network is often referred to as the spectrin cytoskeleton (Fowler, 2013). In axons, spectrins build longitudinal networks periodically interspaced by approximately 190 nm, along the axon, enabling the formation of periodic circular actin structures called axonal actin rings (Han et al., 2017; He et al., 2016; Xu et al., 2013). Recently, β -spectrin and actin were shown to display complementary distribution patterns at the cortex of a variety of mammalian cell types in interphase, by total internal reflection microscopy (TIRFM). This study revealed the existence of plasma membrane mesoscale territories occupied either by actin or by the spectrin cytoskeleton, which was likely associated with very short actin filaments (Ghisleni et al., 2020). In *D. melanogaster* muscle cells, β -spectrin cytoskeleton was shown to fence F-actin protrusions during the muscle cells' fusion process (Duan et al., 2018). These studies are consistent with the idea that spectrin and actomyosin

cytoskeletons might be spatially distinct but cooperate to form a cohesive cortex beneath the plasma membrane, particularly in response to mechanical challenges.

The spectrin tetramers are about 80 nm at resting but can reach 200 nm when stretched (Pan et al., 2018; Shen et al., 1986). This expansibility usually occurs in response to tension/forces and it was modelled to work as a woven finger trap (Brown et al., 2015). This is supported by experimental data reporting SR unfolding, under shear stress in erythrocytes (Johnson et al., 2007; Law et al., 2003). Therefore, these cells have remarkable mechanical properties that allow them to be robust while in high speed flow in the blood circulation and also to be flexible when crossing the smallest blood vessels, the capillaries.

Besides the well known mechanoprotective function in erythrocytes and axons, spectrins contribute to a variety of cell processes in humans, such as cell signaling and cell adhesion (Machnicka et al., 2012). In *D. melanogaster*, spectrins were shown to be involved in tissue morphogenesis (Lee et al., 2010; Ng et al., 2016; Thomas & Williams, 1999; Zarnescu & Thomas, 1999), in the stabilization of apical membranes (Médina et al., 2002) and in signaling pathways (Chen et al., 2009; Fletcher et al., 2015; Forest et al., 2018; Lee & Thomas, 2011). It was also shown to have mechanosensitive properties and to accumulate in regions of higher tension in *D. melanogaster* muscle cells (Duan et al., 2018). In *C. elegans*, spectrins are necessary for ciliogenesis (Jia et al., 2019), animal body embryonic elongation (McKeown et al., 1998; Moorthy et al., 2000; Norman & Moerman, 2002; Praitis et al., 2005), and for the integrity of neurons, body wall muscles and spermatheca (Hammarlund et al., 2000; Wirshing & Cram, 2018). They are also involved in touch sensation, suggesting a role in the response to external mechanical stimuli (Krieg et al., 2014). In sum, spectrins are multifunctional proteins with a role in different processes/contexts.

In mammals, there are seven kinds of spectrin genes: two α -spectrins (αI and αII), four conventional β -spectrins (βI , βII , βIII and βIV) and one βH -spectrin (βV). Several combinations of α/β -spectrins can exist. The αI is erythrocyte-specific, while the others are found in non-erythrocyte cells and are expressed in a complex temporal- and tissue-specific manner (Machnicka et al., 2012; Winkelmann & Forget, 1993). Besides localizing at the cell surface, spectrin isoforms can be found in the Golgi apparatus, vesicles, the endoplasmic reticulum and the nucleus (Bennett & Baines, 2001; Hayes et al., 2000; Machnicka et al., 2012; Stankewich et al., 1998). In contrast, invertebrates like *D. melanogaster* and *C. elegans* only have a single gene of each spectrin type. In *C. elegans*, α -spectrin, conventional β -spectrin and βH -spectrin are represented by the genes *spc-1* (similar to mammalian αII), *unc-70* (resembling mammalian βII) and *sma-1* (similar to mammalian βV), respectively (Dubreuil & Grushko, 1998; see sections 1.4.3.1 to 1.4.3.3 for detailed

descriptions). Therefore, only two kinds of tetramers are formed in this animal model: SPC-1/UNC-70 or SPC-1/SMA-1.

In all organisms, the main difference between the conventional β -spectrins and the β H-spectrins isoform relies on their size, related to the number of SRs that these proteins contain. While conventional β -spectrins contain 17 SRs, β H-spectrins can contain up to 30 SRs (Dubreuil et al., 1990; McKeown et al., 1998). The α -spectrins, conventional β -spectrins and β H-spectrins have approximately 280 kD, 260 kD and 480 kD, respectively.

Several pathological conditions in humans were associated with spectrin perturbations. Mutations in β III lead to abnormalities in glutamate signaling and consequent development of Spinocerebellar ataxia type 5 (Dick et al., 2012). β V spectrin was reported to be involved in Usher syndrome type IB, which is characterized by deafness and blindness (Papal et al., 2013). Mutations in erythroid spectrins cause hemolytic anemia such as hereditary elliptocytosis, pyropoikilocytosis and spherocytosis (Winkelmann & Forget, 1993). Also, spectrins may serve as a biomarker for detecting brain related injuries, since α -spectrin's breakdown products were found in the cerebrospinal fluid of patients with aneurisma (Papa et al., 2018).

1.4.3.1. UNC-70 in *C. elegans*

UNC-70 is the *C. elegans* orthologue of the conventional non-erythrocyte β -spectrins in vertebrates and *D. melanogaster*. It is a ~267 kD protein (information retrieved from Uniprot.org, gene code Q9U9J8), with an N-terminal ABD composed of two CH domains, a middle region with 17 SRs and a C-terminal PH domain (Hammarlund et al., 2000; Moorthy et al., 2000; Figure 1.14). UNC-70 was firstly reported by immunofluorescence to be expressed from early stage embryos (starting at the first cell division), at cell-cell contacts (Hammarlund et al., 2000; Moorthy et al., 2000). More recently, a strain expressing endogenously tagged GFP::UNC-70 shows minimal levels and a dispersed localization pattern of this protein during the first embryonic divisions until mid-embryogenesis (Jia et al., 2019). Then, UNC-70 expression increases during embryonic development in all cells, enriching at the cell-cell contacts. In adults, UNC-70 is also detected in most tissues (Hammarlund et al., 2000; Moorthy et al., 2000). Animals depleted of UNC-70 or expressing mutated UNC-70 are not viable, as they are not able to survive past the L1 stage under normal conditions. Feeding these animals with a more liquid food (*E. Coli* HB101 freshly seeded bacteria) seems to enable their slow growth, allowing the study of loss of *unc-70* phenotypes in adult animals, which are still severely uncoordinated. The defects caused by loss of UNC-70 include: muscle dystrophy with impaired myofilament organization; weakening and misorientation of F-actin bundles in the myoepithelial cells of the

spermatheca (a contractile organ of the reproductive tissue); defective ciliogenesis; defects in sensory neurons morphology and their response to mechanical stimuli; and spontaneous breakage of axons caused by animal locomotion (Hammarlund et al., 2000; Moorthy et al., 2000; Coakley et al., 2020; Jia et al., 2019; Krieg et al., 2014, 2017; Wirshing & Cram, 2018). UNC-70 was also shown to stabilize thick peripheral F-actin bundles formed in the spermatheca of animals lacking another crosslinker, filamin, suggesting a potential compensatory mechanism between these crosslinkers (Kelley et al., 2020). Altogether, these results show that UNC-70 plays important roles to support the functioning of diverse tissues after embryogenesis.

1.4.3.2. SMA-1 in *C. elegans*

The β H-spectrin orthologue in *C. elegans* is SMA-1, a 482 kD protein (information retrieved from Uniprot.org, gene code G5EFW3). Similarly to UNC-70, it contains an ABD at the N-terminus composed of two CH domains and a C-terminal PH domain. The middle region is composed of 29 SRs, 12 more than in UNC-70 (McKeown et al., 1998; Figure 1.14). Additionally, SMA-1 has an SH3 domain within the 4th and 5th SRs. This domain, thought to be involved in signaling processes, is found in a folding transition state that can be unfolded when mechanical forces are applied (Jagannathan et al., 2012; Martínez & Serrano, 1999). The ankyrin-binding region, which mediates an interaction with the plasma membrane in β -spectrins, is poorly conserved in SMA-1 (McKeown et al., 1998). SMA-1 is primarily expressed during embryogenesis, starting at the first cell stage (this thesis, Chapter III) and it is restricted to epithelial tissues during elongation, such as epidermis, intestine and pharynx (McKeown et al., 1998; Praitis et al., 2005). In epithelia, it is apically located, contrary to UNC-70, which is more basolateral. *Sma-1* null mutants are small in size (reason why the gene is called “sma”), have a round head and a decreased elongation rate during embryogenesis (McKeown et al., 1998). That is because the actin cytoskeleton association to the apical membranes is disrupted during embryonic elongation in the absence of SMA-1 (Praitis et al., 2005), revealing a role in the maintenance of the F-actin cytoskeleton. In post-development tissues, SMA-1 was shown to localize in the spermatheca and spermatheca-uterine (SP-UT) valve where, together with UNC-70 and SPC-1, helps to maintain the F-actin organization during contractility (Wirshing & Cram, 2018). In the intestine, SMA-1 acts together with Ezrin-Radixin-Moesin *C. elegans* homologue ERM-1 to anchor the F-actin at the lumen and ensure its proper formation (Göbel et al., 2004). Nevertheless, SMA-1 is not essential for life, since the null mutant animals live to adulthood and propagate with mild fertility problems (McKeown et al., 1998).

1.4.3.3. SPC-1 in *C. elegans*

In *C. elegans*, the gene *spc-1* encodes for α -spectrin, SPC-1, a protein of 282 kD (information retrieved from Uniprot.org, gene reference Q21408). It is more than 70 % similar to the human non-erythroid form of α -spectrin and to *D. melanogaster* single α -spectrin (Norman & Moerman, 2002). It is composed of 20 SRs, an SH3 domain (inside the SR9) and two C-terminal EF-hands (Figure 1.14). Its expression is detected at the earliest stage of embryogenesis and persists throughout embryogenesis at the cells' surface. Its localization pattern overlaps those from SMA-1 and UNC-70. In epithelial tissues, it is found both basolaterally, like the conventional β -spectrin, UNC-70, and apically, like the β H-spectrin, SMA-1 (McKeown et al., 1998; Moorthy et al., 2000; Norman & Moerman, 2002). Animals lacking this protein die after hatching, presenting a combination of defects caused by the simultaneous loss of UNC-70 and SMA-1 (Norman & Moerman, 2002). Recently, SPC-1 was shown to play an important role in embryogenesis, namely in the stabilization of the cell shape changes that occur in epidermis cells during embryonic elongation, allowing for proper tissue remodeling (Lardennois et al., 2019). This may be a feature of the SPC-1/SMA-1 tetramers, as SMA-1 is also necessary for embryonic elongation (McKeown et al., 1998; Norman & Moerman, 2002).

Altogether, these reports indicate that: 1) the spectrin cytoskeleton depends on the formation of tetramers (Figure 1.14); 2) α/β H- and α/β -spectrin tetramers are required in different tissues and for different purposes. α/β -spectrin tetramers localize to all tissues, except the apical membrane in epithelial tissues, and are necessary for the organization and development of body wall muscles and neurons. α/β H-spectrin tetramers localize to the apical membrane of epithelial cells and are required for embryonic elongation and epidermal organization (Hammarlund et al., 2000; Moorthy et al., 2000; Norman & Moerman, 2002; Praitis et al., 2005).

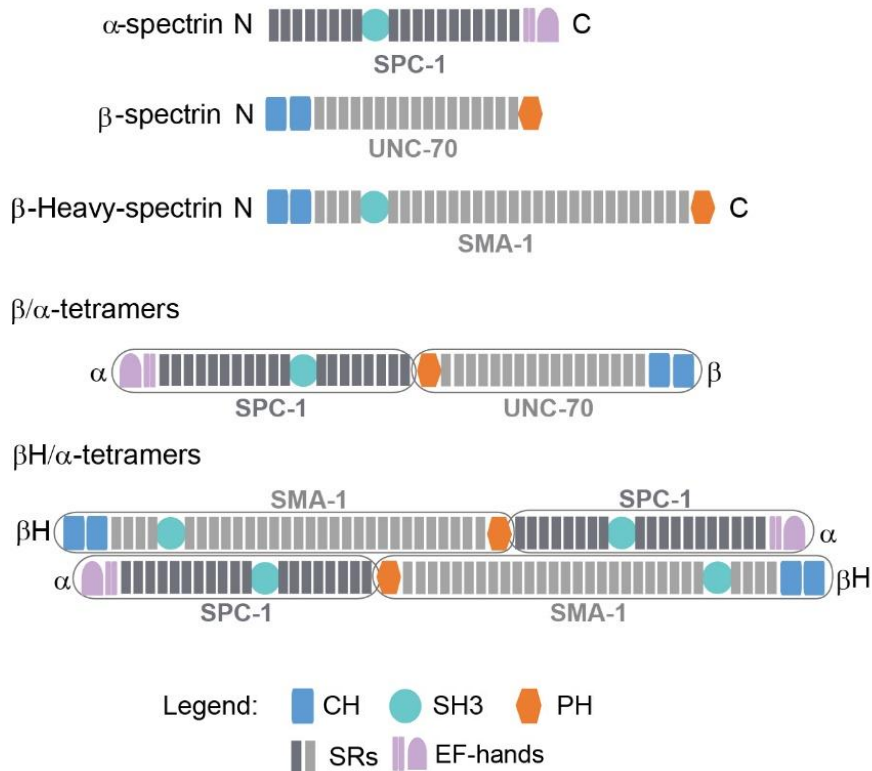


Figure 1.14. Schematic structure of *C. elegans* spectrins. (Top) Structure of SPC-1 (α -spectrin), UNC-70 (β -spectrin) and SMA-1 (β -Heavy-spectrin) monomers. (Bottom) Scheme of SPC-1/UNC-70 (α/β -spectrin) tetramer and SPC-1/SMA-1 (α/β H-spectrin) tetramer. CH: calponin homology domain; SH3: Src homology 3 domain; PH: pleckstrin homology domain; SRs: spectrin repeats; N: N-terminus; C: C-terminus.

1.4.4. Filamins

Filamin is a large protein that assembles into dimers and crosslinks F-actin in dynamic three-dimensional structures (Hartwig et al., 1980; Stossel et al., 2001; Tyler et al., 1980; Wang et al., 1975). It is also reported to be a versatile signaling scaffold protein, able to directly interact with more than thirty proteins of great functional diversity, including transmembrane proteins as integrins, transmembrane receptors and signaling proteins as Rho GTPases (Stossel et al., 2001; van der Flier & Sonnenberg, 2001). In mammals, filamin's molecular weight ranges from 240 to 280 kD and the dimer is up to 160 nm long when extended (Stossel et al., 2001; van der Flier & Sonnenberg, 2001). Structurally, filamin consists of a well conserved N-terminal ABD (composed of two CH domains) followed by a rod-like domain of 4-24 immunoglobulin-like (Ig) repeats, depending on the organism. These repeats are formed by antiparallel β -sheets organized into β -sandwiches, and function as interface for protein-protein interactions (Feng & Walsh, 2004; Stossel et al., 2001; van der Flier & Sonnenberg, 2001). In vertebrates and in *D. melanogaster*, two flexible

loops (~90-100 amino acids) form hinge structures in two different regions of the rod-like domain that provide filamin crosslinked networks with remarkable elasticity (Gardel et al., 2006; Schmoller et al., 2009). The last repeat is responsible for protein dimerization which allows the formation of a V-shaped flexible structure that crosslinks F-actin into high-angle orthogonal or perpendicular networks (Hartwig et al., 1980; Nakamura et al., 2007; Stossel et al., 2001).

Mammals have three filamins (FLNA, B and C), widely expressed and with overlapping patterns of localization, although the isoform C is more restricted to skeletal and cardiac muscles, more specifically to Z-discs and in association with the cortical actin cytoskeleton near the membrane (Thompson et al., 2000; van der Flier & Sonnenberg, 2001). In non-muscle cells, filamins are also expressed in actin-based structures as stress fibers, lamellipodium and cortical actin, and they are important for cortical stability, formation of cell-cell junctions, cell adhesion, mechanical stretch sensing and cell migration (Baldassarre et al., 2009; Cunningham et al., 1992; Lamsoul et al., 2020; Matsudaira, 1994; Tu & You, 2014; Ehrlicher et al., 2011; Xu et al., 2010). In *D. melanogaster*, filamin is required for proper organization of actin and the formation of ring canals in ovaries, which are important for normal oogenesis and fertility (Li et al., 1999; Sokol & Cooley, 1999). More recently, filamin was also shown to be required for proper actomyosin network patterning and cell configuration during embryonic cellularization (Krueger et al., 2019). In *D. discoideum*, loss of filamin results in several anomalies, such as impaired F-actin protrusions' formation, altered motility and defective phagocytosis (Cox et al., 1992, 1996; Khaire et al., 2007; Ponte et al., 2000). In mice, loss of filamin A expression causes embryonic lethality due to cardiovascular and bone malformations (Hart et al., 2006). Human cultured cells lacking filamin display impaired cell migration and adhesion, reduced actin cortical stability and less mechanical resistance (cells bleb and deform more easily; Cunningham et al., 1992; Flanagan et al., 2001; Kainulainen et al., 2002; Lamsoul et al., 2020). Furthermore, mutations or altered levels of human filamins are associated with a wide range of congenital malformations in several organs. These include periventricular heterotopia, in which neurons fail to migrate to the cerebral cortex, defective bone morphogenesis, familiar cardiac vulvar dystrophy and other cardiac diseases (Kyndt et al., 2007; Robertson et al., 2003; Yamak et al., 2020). Mutants or aberrant expression of filamin were also associated with metastatic cancer progression (Jiang et al., 2013; Lamsoul et al., 2020; Yue et al., 2013).

1.4.4.1. FLN-1 in *C. elegans*

Filamin is encoded by two genes in *C. elegans*, *fln-1* and *fln-2*. While FLN-1 is well conserved when compared to vertebrate filamins, FLN-2 is nematode specific (DeMaso et al., 2011) and thus will not be addressed in this thesis. FLN-1 larger isoform (FLN-1A) is a 246 kD protein (information retrieved from Uniprot.org, gene code D0IMZ5), with a conserved ABD and 20 Ig-like repeats, similarly to vertebrate filamins (Figure 1.15). To note, smaller isoforms B and C have 8 and 9 Ig-like repeats, respectively, and isoform C lacks the ABD. There is insufficient homology to specify which human filamin the FLN-1 is most similar to (DeMaso et al., 2011).

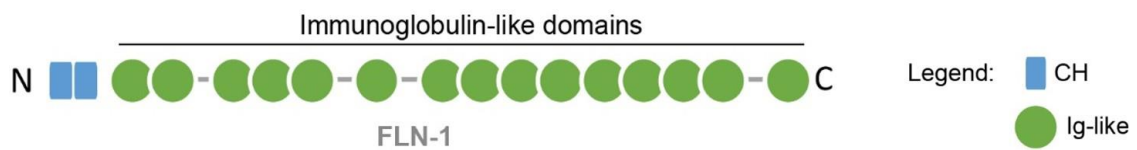


Figure 1.15. Schematic structure of FLN-1. Structure of FLN-1 monomer. CH: calponin homology domain; Ig-like: immunoglobulin-like domains; N: N-terminus; C: C-terminus.

GFP-tagged FLN-1A being expressed under its endogenous promoter and 3'UTR from an extrachromosomal array showed that FLN-1A expression starts at the L4 stage and that it is mostly expressed in the somatic reproductive tissues, namely the uterus, spermatheca and proximal gonad sheath (Kovacevic & Cram, 2010). No expression was observed in the germline, but that can be attributed to germline silencing (Kelly & Fire, 1998; Seydoux & Schedl, 2001). The subcellular localization of FLN-1 in the reproductive system showed that it has a punctate pattern that partially co-localizes with F-actin bundles in the spermathecal and uterine cells. Although FLN-1 is not necessary for the development of these organs, it is required for the formation and maintenance of the actin networks in the spermatheca and uterus (Kelley et al., 2020; Kovacevic & Cram, 2010). FLN-1 deficient animals have severe fertility problems attributed to a defect in the passage of the embryos from the spermatheca to the uterus. The long permanence of embryos inside the spermatheca leads to their physical damage and to the accumulation of unfertilized or damaged eggs inside the gonad (Kovacevic & Cram, 2010). Additionally, FLN-1 was shown to act together with phospholipase PLC-1, a protein that triggers calcium release and promotes contractility within cells, suggesting that FLN-1 may sense the mechanical stretch caused by the oocyte inside the spermatheca and trigger a biochemical signal that allows contraction so that the fertilized oocyte exits the spermatheca (Kovacevic & Cram, 2010, 2013). Moreover, FLN-1

loss also results in disrupted spatial organization of several organelles in the spermatheca, such as the nucleus, endoplasmic reticulum and mitochondria (Kelley et al., 2020).

1.4.5. Anillins

Anillin got its name from the Spanish word “anillo” which means ring, in reference to its enrichment in the contractile ring during cytokinesis. It is a multidomain protein that was firstly shown to bind F-actin *in vitro* (D’Avino, 2009; Miller et al., 1989). Beyond crosslinking, anillin also performs scaffolding functions within the cells (D’Avino, 2009; Piekny & Maddox, 2010). This is possible due to the presence of an N-terminal myosin binding domain, followed by an ABD, and a C-terminal Anillin-homology region (AHR) that comprises RhoA- and septin-binding sequences followed by a well conserved PH domain (Field & Alberts, 1995; Oegema et al., 2000; Piekny & Glotzer, 2008). Anillin is therefore able to bind F-actin, myosin, RhoA, septins and phospholipids in the plasma membrane. Moreover, consensus nuclear localization sequences (NLSs) and an SH3 motif were found in human and *D. melanogaster* anillin (Piekny & Maddox, 2010). There is also evidence that anillin binds to microtubules *in vitro* and interacts with formins, with the mitotic spindle component MgcRacGAP and with the RhoA activator Ect2, in human or *D. melanogaster* cultured cells, evidencing its complex scaffolding functions (Chen et al., 2017; D’Avino et al., 2008; Frenette et al., 2012; Gregory et al., 2008; Triplet et al., 2014; Watanabe et al., 2010).

In contrast to the spectrin superfamily of proteins, the ABD of anillin does not contain conserved CH domains, yet it binds F-actin through multiple binding sites (Jananji et al., 2017; Matsuda et al., 2020; Oegema et al., 2000). Anillin can also directly bind myosin and regulate its localization to the equatorial cortex during cytokinesis (Garno et al., 2021, Biorxiv; Piekny & Glotzer, 2008; Straight et al., 2005). The AHR mediates anillin interaction with RhoA, as well as with microtubules, MgcRacGAP and Ect2 (D’Avino et al., 2008; Frenette et al., 2012; Gregory et al., 2008; Piekny & Glotzer, 2008; Triplet et al., 2014). The PH domain of anillin is thought to regulate anillin’s localization and mediate its anchorage to the plasma membrane (Liu et al., 2012; Oegema et al., 2000; Sun et al., 2015). The PH domain also contains a septin-binding sequence, which allows septin filaments to be recruited to F-actin bundles and to the contractile ring (Field et al., 2005; Goldbach et al., 2010; Hickson & O’Farrell, 2008; Kinoshita et al., 2002; Maddox et al., 2005; Oegema et al., 2000).

Humans’ single anillin is 124 kD and, as in other systems, it localizes at the contractile ring and is an important regulator of cytokinesis (Oegema et al., 2000). Its absence does not impede contractile ring assembly but affects furrowing speed and leads to cytokinesis failure (Liu et al., 2012; Oegema et al., 2000; Piekny & Glotzer, 2008; Piekny & Maddox,

2010). In *D. melanogaster* embryos and S2 cells, anillin's depletions or mutations also result in cytokinesis defects and failure (D'Avino, 2009; Echard et al., 2004; Field et al., 2005).

Both in human HeLa cells, *D. melanogaster* S2 cells and in fission yeast, anillin intracellular localization is dynamic between the nucleus in interphase cells, and the cell cortex in dividing cells (Beaudet et al., 2020; Chen et al., 2015; Field & Alberts, 1995; Hickson & O'Farrell, 2008; Wu et al., 2003). When at the cortex, anillin is cleared from the cell poles and restricted to the equator during cytokinesis due to astral microtubules' signaling (Mangal et al., 2018; Triplet et al., 2014). Anillin is also implicated in abscission in HeLa cells and *D. melanogaster* S2 cells (Amine et al., 2013; Kechad et al., 2012; Zhao & Fang, 2005; Echard et al., 2004; Gai et al., 2011). After mitotic exit, anillin levels drop drastically in human HeLa cells (Zhao & Fang, 2005).

Beyond cytokinesis, anillin was also shown to localize to cell-cell junctions in human epithelial cells and *Xenopus laevis* (*X. laevis*) embryonic cells, where it is necessary for the organization and integrity of the actomyosin cytoskeleton, and proper distribution of other cytoskeletal proteins, such as spectrin and adducin (Arnold et al., 2019; Reyes et al., 2014; Wang et al., 2015). In *D. melanogaster*, anillin is required for normal cellularization during embryogenesis (Field et al., 2005).

Although anillin is commonly referred to as a RhoA effector, it might act as a RhoA regulator or as a RhoA stabilizer as well, since anillin prevents RhoA membrane dissociation and depletion of anillin dysregulates cortical RhoA distribution in adherens junctions and in the furrow of cultured dividing cells and *X. laevis* embryos (Arnold et al., 2017; Budnar et al., 2019; Piekny & Glotzer, 2008; Reyes et al., 2014). Conversely, anillin accumulation at the contractile ring during cytokinesis depends on RhoA activity, suggesting the existence of a feedback loop between these two elements (Piekny & Glotzer, 2008). Anillin and RhoA also cooperate during cytokinesis to enhance formin-mediated F-actin nucleation: anillin binds formin after RhoA and this binding increases formin's F-actin nucleation activity at the contractile ring (Chen et al., 2017; Watanabe et al., 2010).

Anillin is overexpressed in several types of human solid tumors, such as breast, pancreatic, colorectal, lung, gastric, urothelial and liver cancer (Hall et al., 2005; Olakowski et al., 2009; Suzuki et al., 2005). Besides, its overexpression is associated with worse prognosis, increased tumor progression, increased migration/metastatic potential and increased stemness potential. The mechanisms are yet to be clarified but often imply impaired division of cancer cells, altered cell signaling cross-talks and actomyosin cytoskeletal remodeling that favours migrating metastatic cancers (Naydenov et al., 2021).

1.4.5.1. ANI-1 in *C. elegans*

Three different genes encode anillins in *C. elegans* (*ani-1*, *ani-2* and *ani-3*). All have conserved AHR and PH domains at the C-terminus. However, only ANI-1 bears homology of the N-terminal ABD, which is similar to the ones from vertebrate and *D. melanogaster* anillins (Maddox et al., 2005; Figure 1.16). In *C. elegans*, ANI-1 is expressed in the germline and neuronal tissues and it is enriched in the cortex and cytokinetic furrows of the dividing embryos and neuroblasts (Fotopoulos et al., 2013; Maddox et al., 2005). In early embryos, anillin is necessary for polar body extrusion during meiosis, for cortical contractility during polarization and for the asymmetric ingression of the contractile ring during cytokinesis, a stereotypical feature in *C. elegans* embryonic cell divisions (Dorn et al., 2010, 2016; Maddox et al., 2005, 2007). Moreover, during cytokinesis in the one-cell embryo, ANI-1 localizes to the equatorial region and to the contractile ring independently of septins or myosin. ANI-1 recruits septins, but not myosin, to the contractile ring (Maddox et al., 2005). Although cytokinesis takes longer in the absence of ANI-1, this protein is not essential for cytokinesis to complete in early embryos (Maddox et al., 2005). Nevertheless, increased embryonic lethality was associated with the lack of ANI-1, which might be largely attributed to reported meiotic defects, namely polar body extrusion defects, in addition to the accumulation of other defects or subsequent failures in cell division during embryogenesis (Maddox et al., 2005). Consistent with a role in later stages of development, ANI-1-deficient adult worms display defective morphological phenotypes, such as malformed cuticles, uncoordinated movement, protruding vulvae and extruded gonads (Maddox et al., 2005). Additionally, ANI-1 was shown to regulate neuroblast cytokinesis and epidermal morphogenesis during mid-embryonic development (Fotopoulos et al., 2013). Although the mechanisms remain unclear, this reinforces ANI-1 as an important player during mid to late-stages of embryogenesis.

In post-embryonic tissues, during neuronal development, ANI-1 was shown to accumulate in the cell cortex and to stabilize the F-actin cytoskeleton by antagonizing the F-actin severing activity of cofilin in the leading edge of neurons, promoting neuroblast migration and neurite outgrowth (Tian et al., 2015). Migration of the neuroblasts is a dynamic stereotypical process, where two progenitor cells migrate and successively divide upon larva's hatching to form the sensory neurons, mechanosensory neurons and interneurons.

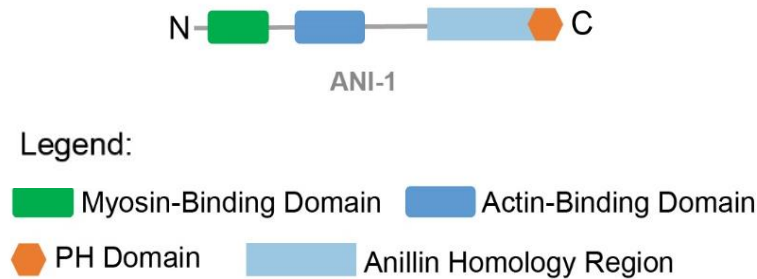


Figure 1.16. Schematic structure of ANI-1. Structure of ANI-1 monomer. PH: pleckstrin homology domain; N: N-terminus; C: C-terminus.

1.4.6. Septins

Septins were discovered by Hartwell in 1971, in *Saccharomyces cerevisiae* (*S. cerevisiae*), in a screen for genes involved in cell division (Hartwell, 1971). Its name came from its localization, in ~10 nm thick filaments, around the septating bud neck of the dividing yeast cell (Byers & Goetsch, 1976; Haarer & Pringle, 1987; Kim et al., 1991). Since then, multiple septins were identified in most eukaryotes, and they are highly conserved between species, with a size range of 30-65 kD (Cao et al., 2007; Pan et al., 2007). Septins encode a GTPase family of proteins, as they have a GTP-binding and hydrolysis function (Weirich et al., 2008). They have multiple domains: an N-terminal proline rich domain, a basic region known to bind phosphoinositides in the plasma membrane, followed by a GTP-binding core domain, a highly conserved septin unique region and a C-terminal coiled-coil domain of varying length, known to participate in protein-protein interactions (Casamayor & Snyder, 2003; Gönczi et al., 2021; Versele & Thorner, 2004). The F-actin binding and crosslinking region of septins is still not well characterized. Indeed, this region was only determined in one of the human septins, and that region corresponds to a non-conserved N-terminal basic domain that is not present in other paralogs (Smith et al., 2015).

Vertebrates encode as much as 17 septins which are either ubiquitous or tissue-specific (Cao et al., 2007). In humans, 13 septin genes are described, divided in four groups according to homology: SEPT2 (SEPT1, 2, 4 and 5), SEPT3 (SEPT3, 9 and 12); SEPT6 (SEPT6, 8, 10, 11 and 14) and SEPT7 (Hall et al., 2005). In invertebrates, as *D. melanogaster* or *C. elegans*, only five or two septins are encoded, respectively.

Septins are considered the fourth component of the cytoskeleton. Yet, unlike F-actin and microtubules, septins are non-polar and can polymerize at both ends, similarly to intermediate filaments (Mostowy & Cossart, 2012). They are also more stable in terms of turnover dynamics (Hagiwara et al., 2011). Septin monomers can assemble into non-polar oligomeric complexes through their GTP-binding domain, N-terminal and C-terminal

regions. These complexes then join laterally to form heterofilaments, which are considered to be the predominant and functional form of septins (Bridges et al., 2014; Garo et al., 2021, Biorxiv; Mostowy & Cossart, 2012; Sellin et al., 2011). Moreover, complexes of septin filaments can further self-assemble into bundles and rings (Kinoshita et al., 2002; Mostowy & Cossart, 2012). The formation of septin filaments is facilitated by their interaction with the plasma membrane (Bertin et al., 2010; Bridges et al., 2014; Byers & Goetsch, 1976; Tanaka-Takiguchi et al., 2009; Zhang et al., 1999), F-actin (Kinoshita et al., 2002; Mavrakakis et al., 2014; Smith et al., 2015), microtubules (Bai et al., 2013; Bowen et al., 2011; Nagata et al., 2003; Spiliotis et al., 2008; Surka et al., 2002), anillin (Field et al., 2005; Kinoshita et al., 2002; Oegema et al., 2000) and myosin (Joo et al., 2007). *In vitro*, septins were shown to assemble F-actin into curved bundles similar to those existing in cytokinetic rings, thus they are predicted to contribute to the assembly of the contractile ring during cytokinesis (Mavrakakis et al., 2014). Interestingly, septins were also found to recognize and preferentially bind membranes with a curvature like the ones existing at the base of protrusive structures, such as filopodia and dendritic spines (Bridges et al., 2016; Cannon et al., 2019). The regulation of septin filament assembly and disassembly is still poorly understood.

Septins have been reported to participate in numerous cellular processes both in mitotic and non-mitotic cells, such as cytokinesis, exocytosis, ciliogenesis, neurotransmission and morphogenesis (Gönczi et al., 2021; Mostowy & Cossart, 2012). Although septins form F-actin bundles (Mavrakakis et al., 2014), they are mostly known to act as scaffolds at the membrane and cytoplasm to recruit other proteins and/or as barriers limiting the diffusion of membrane-associated proteins. For example, in budding yeast, septins form a ring-like barrier at the bud neck site to limit the diffusion of components from the mother cell to the daughter cells to ensure proper mitosis (Barral et al., 2000; Takizawa et al., 2000). In mice, a septin diffusion barrier exists in cilia and in sperm, and septin ring structures are also found during phagocytosis and in the base of dendritic spines in neurons, where they might also act as diffusion barriers (Beise & Trimble, 2011).

In cytokinesis, septins are localized at the division site and are involved in the organization of the actomyosin contractile ring in multiple systems. In mammalian HeLa cells, inhibition of septins leads to delayed or failed cytokinesis, defects in chromosome segregation, in spindle elongation and in abscission (Estey et al., 2010; Kinoshita et al., 1997; Spiliotis et al., 2005; Surka et al., 2002). Septins are also essential for mitotic spindle positioning and cytokinesis in budding yeast but not in fission yeast, although they are required for cell separation in the latter (Wu et al., 2010). In *C. elegans* embryos, septins localize at the contractile ring and in the midbody, and have a role in asymmetric furrow ingression (Maddox et al., 2007; Nguyen et al., 2000). In *D. melanogaster*, septins are localized in the cleavage furrow of dividing ovarian follicle

cells and lymph gland cells, and are required for post-embryonic cytokinesis (Neufeld & Rubin, 1994). The role of septins in cell division may also account for the several morphological defects observed upon septin perturbations either in the bud morphology of budding yeast, in the cell shape and morphogenesis of embryos of *D. melanogaster*, in adult tissues of *C. elegans*, and in mammalian embryogenesis, where mutations of some septins are lethal (Adam et al., 2000; Dolat et al., 2014; Hartwell, 1971; Nguyen et al., 2000).

In interphase cells, septins are also suggested to spatially organize actin and microtubules, to be involved in the formation and maintenance of stress fibers, in the formation of dendritic spines and of phagocytic cups (Kinoshita et al., 2002; Spiliotis, 2018; Surka et al., 2002).

Other than F-actin, myosin, membranes and microtubules, septins directly interact with anillin, which has an important function in the recruitment of septins. In fact, anillin is required to target septins to the plasma membrane in the cleavage furrow of dividing mammalian HeLa cells (Liu et al., 2012). In *D. melanogaster*, mutants of anillin fail to localize septin to the contractile ring of dividing embryonic cells and to the cellularization front during embryogenesis (Field et al., 2005; Oegema et al., 2000). In *C. elegans* embryos, the anillin ANI-1 is also necessary to target septins to the contractile ring (Maddox et al., 2005). In *S. pombe*, anillin stabilizes septins in the contractile ring (Berlin et al., 2003; Tasto et al., 2003).

Septins have been linked to several pathologies. Altered expression or truncations of septins are linked to different cancer types. Expression changes in septins are also associated with neurological disorders, such as Parkinson's disease. Mutations in septins have been identified in hereditary neuralgic amyotrophy, male infertility and neuromuscular disorders (Dolat et al., 2014; Peterson & Petty, 2010). Moreover, septins also play a role in the development and physiological function of many cells/tissues/organs in the human body, which is understandable given their tissue-specific expression patterns (Dolat et al., 2014). Finally, septins play a role in infection and host-defense mechanisms, since they can prevent pathogens from causing harm in healthy cells by forming a cage-like structure around the invading microbes (Mostowy & Cossart, 2012).

1.4.6.1. UNC-59 and UNC-61 in *C. elegans*

The *C. elegans* genes *unc-59* and *unc-61* encode the two septins expressed in this animal model. Electron microscopy data of these proteins show that they form a non-polar tetrameric complex (UNC-59/UNC-61/UNC-61/UNC-59; (John et al., 2007). They have an approximate molecular weight of 53 kD (information retrieved from Uniprot.org, codes Q9U334 and Q25AR8, respectively) and they share a common structure with other septin

orthologues, comprising an N-terminal basic domain, a GTP-binding domain, a septin unique region and a C-terminal coiled-coil domain (Figure 1.17). UNC-59 and UNC-61 additionally have a low complexity region in the C-terminus and N-terminus, respectively (John et al., 2007). Studies report that the UNC-59 and UNC-61 require each other for proper localization (John et al., 2007; Nguyen et al., 2000).

In vivo both septins localize in the contractile ring and in the midbody and play a role in cytokinesis. In early embryos, together with anillin, septins regulate the stereotypical asymmetric ingression of the contractile ring but no further cytokinesis defects are displayed in mutants of both septins (Dorn et al., 2016; Maddox et al., 2007; Nguyen et al., 2000). Other than embryonic cytokinesis, pleiotropic abnormalities in post-embryonic tissues are found in *unc-59* and/or *unc-61* mutants or depletions of UNC-59 or UNC-61, including vulva protrusion, extruded gonad, egg-laying defects and abnormal male tail (Nguyen et al., 2000). Most of the related abnormalities are at least partially associated with defective or failed post-embryonic cytokinesis during tissues' development. Additionally, uncoordinated movement is reported in septin mutants, which is attributable to reported impaired axonal migration and guidance during the development of motor neurons, affecting muscle coordination (Finger et al., 2003). The septins are also expressed in the migrating distal tip cells on the animal's gonad, and mutations on these proteins affect the morphology of the distal tip cells, their migration and guidance during gonadogenesis, in a similar manner as in the axons (Finger et al., 2003). Recently, septins, along with CYK-1 (formin), were shown to play a role in the chiral cortical rotation of the actomyosin cytoskeleton during anaphase in one-cell embryos, which is an intrinsic feature of cells and tissues that emerges from cortical flows or contractions (Zaatri et al., 2021).



Figure 1.17. Schematic structure of UNC-59 and UNC-61. Putative structure of UNC-59 or UNC-61 monomer. N: N-terminus; C: C-terminus.

1.4.7. Coronins

Coronin is an actin-binding protein with F-actin crosslinking activity which also participates in actin cytoskeleton remodeling. It was first identified in actomyosin rich crown-like structures isolated from *D. discoideum* cell surface and it was then shown to bind F-

actin *in vitro* and *in vivo* (Cai et al., 2007b; de Hostos et al., 1991; Goode et al., 1999). Coronins have a β -propeller tertiary structure formed by N-terminal WD-repeats of approximately 40 amino acids ending in a tryptophan (W) and an aspartic acid (D). WD-repeats serve as platforms for interactions between proteins and no catalytic activity was found in them. Yet, the only binding partner found for coronin's WD-repeats was F-actin. Both vertebrate and invertebrate coronin homologues have a WD-repeat structural core that differs in the number of WD-repeats (Eckert et al., 2011; Uetrecht & Bear, 2006). In the C-terminus, most coronins have a coiled-coil region, which allows coronins to oligomerize in dimers or trimers. Oligomerization is thought to be the way by which coronins crosslink F-actin, despite some models suggesting that F-actin binding sites are found in the coiled-coil region as well, and that coronins do not necessarily oligomerize to bundle F-actin (Gandhi et al., 2009; Liu et al., 2006). The coiled-coil region of coronin is also known to mediate its direct interaction with the Arp2/3 complex *in vitro* and *in vivo*. This interaction regulates Arp2/3 complex activity and thus regulates F-actin dynamics (Cai et al., 2005; Cai et al., 2007a; Humphries et al., 2002). Between the N-terminal WD-repeats and the C-terminal coiled-coil region, each coronin has a unique and divergent region, whose number of amino acids varies according to the species (ranging from 22 amino acids in *D. discoideum* to 194 in budding yeast, for example). Mammalian coronins' unique region has as many as 50 amino acids. The function of this region remains poorly understood (de Hostos et al., 1991).

While seven different coronin genes are reported in mammals (Coronin 1A-C, Coronin 2A-B, Coronin 6 and Coronin 7), other organisms, such as *D. discoideum*, *D. melanogaster* and *C. elegans*, have 2 or 3 coronin genes, and *S. cerevisiae* and *S. pombe* only have a single coronin gene (Uetrecht & Bear, 2006). Due to their structural and phylogenetic differences, coronins were grouped in 3 types (I, II and III), whereas type I and type II have the classic structure (β -propeller WD-repeats region, unique region and coiled-coil domain), being the type II more divergent in the unique region. Type III coronins (*Coro7* in mammals, *CorB* in *D. discoideum*, *Dpod1* in *D. melanogaster* and *pod-1* in *C. elegans*) lack a coiled-coil domain but instead have two β -propeller WD-repeat regions and are much longer (Chan et al., 2011).

Disruption of coronins in several models revealed its importance in a myriad of actin-related cell processes and cellular functions. In *D. discoideum*, coronin deletion impairs cell migration, endocytosis, phagocytosis and leads to cytokinesis failure (de Hostos et al., 1993; Maniak et al., 1995). In *D. melanogaster*, coronin (*coro*) mutations are lethal in 90 % of the animals, with the ones that survive displaying severe morphological defects. The other *D. melanogaster* coronin gene, *Dpod1*, is required for axonal guidance. In mammals, coronins have distinct expression patterns indicating different functions for each isoform: for instance, Coronin 1 in humans is mostly expressed in hematopoietic cells (Rybakin &

Clemen, 2005). Therefore, different coronin mutations or depletions lead to variable defects depending on the cell. These include defective lymphocyte motility, impaired phagocytosis and chemotaxis in neutrophils, reduced actin flow, cell migration and membrane ruffles in fibroblasts, and altered actomyosin organization of the cell-cell junctions in endothelial cells (Gandhi & Goode, 2008; Michael et al., 2016; Priya et al., 2016; Werner et al., 2020; Yan et al., 2007). Of note, *S. cerevisiae* and *D. melanogaster* coronins were shown to bind to microtubules as well (Goode et al., 1999; Rothenberg et al., 2003).

One of the roles proposed for type I coronins is the coronin-mediated spatial regulation of Arp2/3 activity and protection against F-actin disassembly through cofilin. It is proposed that, at the ATP-rich front of F-actin networks (such as in lamellipodium), coronin protects F-actin from being severed by cofilin while recruiting Arp2/3 complex to those filaments in order to nucleate and branch them. In contrast, at the rear ADP-rich F-actin, coronin binds to Arp2/3 stabilizing it in an open and inactive conformation, inhibiting F-actin nucleation. At the same time, at the ADP-rich F-actin, coronin seems to prime the filaments for cofilin by accelerating Pi release and enhancing cofilin-mediated F-actin disassembly (Cai et al., 2007b; Gandhi et al., 2009; Gandhi & Goode, 2008; Liu et al., 2011; Mikati et al., 2015; Rodal et al., 2005; Tang et al., 2020). Regulation of cofilin and coronin activities is also achieved through phosphorylation/dephosphorylation of both proteins, yet these mechanisms will not be addressed for simplicity. Overall, coronins contribute to the regulation of the dynamics of F-actin networks.

In terms of clinical implications, coronins were suggested to have a role in the mammalian and *D. discoideum* immune system, yet the mechanisms remain unclear. Some studies indicate that intracellular pathogens recruit coronins to facilitate the trafficking and survival inside the macrophages. Other data show coronin as a host protecting protein in infections (Jayachandran et al., 2007; Mori et al., 2018; Solomon et al., 2003). In mammals, inhibition of coronins was also associated with severe immunodeficiencies, such as loss of T-cells (Mueller et al., 2011; Pieters et al., 2013). Increased levels of coronin 1A are found in patients with cystic fibrosis and overexpression of this same protein increases neutrophil resistance to apoptosis (Moriceau et al., 2009). In mice, a coronin mutation at the β -propeller WD-repeat region (that might affect actin crosslinking) suppressed the phenotype of autoimmune diseases, such as lupus erythematosus (Haraldsson et al., 2008).

1.4.7.1. COR-1 in *C. elegans*

C. elegans encodes two coronin genes: *cor-1* (type I-like) and *pod-1* (type III-like). *Pod-1* is the most well characterized. It fits the type III category of coronins, since it does not have a coiled-coil domain. In embryos, it co-localizes with cortical actin and ARP2/3

complex, exhibiting a punctate cytoplasmic pattern (Eckert et al., 2011; Rappleye et al., 1999). In neuroblasts it is enriched at the leading edges, where it also co-localizes with ARP2/3. Deletion of *pod-1* causes severe defects during early embryonic development, such as osmotic defects, impaired embryonic cell polarity, blocked ARP2/3 motility, impaired myosin cortical flows, abnormal cytokinesis, defective eggshell formation, abnormal protrusions in the cellular membrane, impaired vesicular trafficking of polar granules, accumulation of abnormal endocytic vesicles in the cytoplasm, and embryonic lethality (Chan et al., 2011; Rappleye et al., 1999; Xie et al., 2021). During larval development, loss of POD-1 also impairs neuroblasts morphology and migration (Xie et al., 2021). As the loss of POD-1 affects the embryo's permeability and leads to severe osmotic defects at the earlier stages of development, this precluded adequate studies of this protein and it ended up being excluded from this study.

COR-1 is a 67 kD protein (information retrieved from Uniprot.org, code Q21624) with a canonical coronin type I structure (Figure 1.18). Less explored than POD-1, COR-1 is also known to localize at the leading edge and to play a role in the migration of *C. elegans* neuroblasts, by promoting cofilin F-actin severing activity (Shen et al., 2014; Wang et al., 2013). Knock-out of *cor-1* results in abnormal morphology of the migrating neuroblasts, with ectopic formation of actin bundles and the formation of misoriented neurites (Cheng et al., 2013; Shen et al., 2014; Wang et al., 2013). Although COR-1 is present in early embryos, it differs from POD-1, as it does not co-localize with ARP2/3 and does not seem to play a role in polarity (Xie et al., 2021).

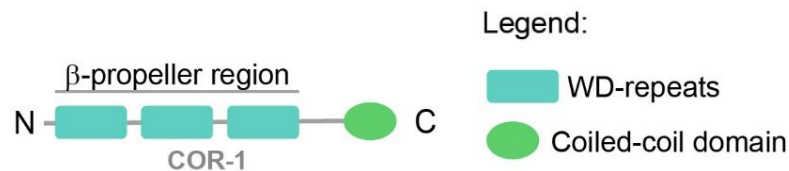


Figure 1.18. Schematic structure of COR-1. Putative structure of COR-1 monomer. N: N-terminus; C: C-terminus.

1.5. References

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CHAPTER II

AIMS AND OVERVIEW

2. AIMS AND OVERVIEW

The mechanistic understanding of actomyosin networks' functioning remains a central challenge in cell biology and it is mandatory to understand the basis of the problems that lead to a variety of actin cytoskeleton-related disease conditions.

In my doctoral studies, I focused on a subclass of actin-binding proteins: the F-actin crosslinkers. F-actin crosslinkers mediate the essential interconnection between the filaments of actin that compose an actomyosin network. Different types of crosslinkers with variable characteristics co-exist in the same cell or tissues. How the actomyosin networks organize and behave in the presence of multiple crosslinkers *in vivo* is underexplored.

I was driven by this major question: *what is the individual and combined contribution of different crosslinkers to a range of actomyosin-dependent cellular processes in vivo?* To answer this question, I aimed to perform the most comprehensive functional characterization of F-actin crosslinkers in an animal model to date. Using the powerful *C. elegans* system, I intended to perform these studies in different actomyosin-dependent contexts, with a main focus on the last step of cell division, cytokinesis.

The first aim of my project was to dissect the individual contribution of multiple well-conserved crosslinkers in *C. elegans*: α -actinin ATN-1, plastin PLST-1, β -spectrins SMA-1 and UNC-70, filamin FLN-1, anillin ANI-1, septins UNC-59/UNC-61, and coronin COR-1. These studies were performed across a range of cellular contexts: cytokinesis in the one-cell embryo, organization of the oocyte-producing gonad and the body wall muscles, using RNAi-depletion and genome-editing approaches to inhibit crosslinkers' function.

The second aim of my project was to perform a more in-depth study of the role of crosslinkers during the first embryonic division. Individual depletions led to the conclusion that PLST-1 was the crosslinker that on its own most contributed to cytokinesis. Combined depletions of other crosslinkers in a *p/st-1* mutant background led to the discovery of a novel synergy between PLST-1 and SMA-1. In chapter IV, it is described how this discovery came about and the phenotypic characterization and mechanistic dissection performed to understand it.

Given the different structural and biophysical properties of each crosslinker, the third aim of my project was to test whether crosslinkers of approximately the same size can replace for one another during early embryonic cytokinesis. In chapter V, I will describe our approach to test whether ATN-1 (normally not expressed at this stage) can substitute PLST-1.

CHAPTER III

UNRAVELLING THE DIVERSITY OF ACTIN-FILAMENT CROSSLINKERS *IN VIVO*

3. UNRAVELLING THE DIVERSITY OF ACTIN-FILAMENT CROSSLINKERS *IN VIVO*

3.1. Abstract

Actin filaments are interconnected by crosslinkers, which determine the different architectures adopted by actomyosin networks required for cell and tissue remodeling. How different crosslinkers contribute to a range of cellular processes *in vivo* is poorly explored. It is also unknown whether the expression of several crosslinkers reflects functional redundancy or a necessary diversification. We dissected the contribution of 9 crosslinkers in actomyosin-dependent cellular contexts in *C. elegans*, such as the reproductive system and the body wall muscles. Loss-of-function phenotypes are studied after RNAi-mediated depletion of each crosslinker or in animals expressing crosslinker versions that lack the actin-binding domain, generated by CRISPR/Cas9. Our results show that distinct tissues use different crosslinker sets to function: while plastin, filamin and anillin are important for the organization of the actomyosin network of the gonad and animal fertility, α -actinin is required for proper muscle organization and animal motility. We conclude that crosslinkers' diversity is necessary for the dynamic organization of different actin architectures *in vivo*.

3.2. Introduction

The actin cytoskeleton is responsible for the structural integrity of the cells. The actin filaments (F-actin) that compose the actin cytoskeleton can be arranged into complex networks in the presence of F-actin crosslinkers, and these networks become contractile in the presence of myosin motors. The differently organized actomyosin networks have very dynamic properties and are responsible for all processes that require force generation and cell shape remodeling, including cell division, motility, invasion and coordinated cellular deformations during tissue morphogenesis (Blanchoin et al., 2014; Svitkina, 2018). Stemming from this range of functionalities, dysregulation of the actomyosin cytoskeleton has been linked to a variety of pathological conditions such as metastatic cancer, immunodeficiencies, birth defects, deafness, microbial infections and neurodegenerative disorders (Blanchoin et al., 2014).

Several types of crosslinkers exist and promote the formation of different F-actin architectures with distinct mechanical and dynamic properties, depending on the crosslinkers' characteristics. For instance, smaller crosslinkers tend to pack F-actin into bundles whereas larger crosslinkers can form more complex networks by bridging them at different angles. More or less loose networks allow different degrees of filaments sliding and

contractility, affecting the behavior of the network. Adding to their ability to organize actin networks with different architectures, crosslinkers with different structural and functional characteristics may also present specific subcellular localizations (Fishkind et al., 1990; Kovacevic & Cram, 2010; Reichl et al., 2008; Xu et al., 2013), different actin-binding kinetics, and selective recruitment upon different external stimuli (Duan et al., 2018; Luo et al., 2013; Ren et al., 2009). All of these favor the view of differential roles in the dynamic organization of actin architectures and functionalities.

How each crosslinker individually contributes to the organization and functioning of actomyosin networks of different tissues and cellular contexts *in vivo* is still poorly explored. It is not known whether the number of crosslinking proteins within a same organism reflects functional redundancy to guarantee cell survival, or diversify the cell capabilities to respond to a variety of different stimuli. In this Chapter, we aimed to dissect the functional roles of 9 well-conserved crosslinker homologues in distinct actomyosin-dependent contexts in *C. elegans*: α -actinin (ATN-1), plastin (PLST-1), filamin (FLN-1), β -spectrin (UNC-70), β -heavy(β H)-spectrin (SMA-1), anillin (ANI-1), septins (UNC-59 and UNC-61) and coronin (COR-1). To do so, loss-of-function phenotypes were analyzed upon RNAi-mediated depletion or in mutants expressing crosslinker versions lacking their actin-binding capacity, generated by endogenous genome editing via CRISPR/Cas9 technique.

We took advantage of the *C. elegans* cellular and tissue simplicity and well conserved actomyosin-dependent processes, to expand the knowledge on these proteins in an *in vivo* context. We chose the *C. elegans* reproductive system, which is rich in processes that require actomyosin dynamics and contractility. This system is composed of two U-shaped tubular gonad arms, each containing a spermatheca at the most proximal region to store sperm. Between the two spermathecae there is a common uterus to store the developing embryos and a vulva to expel them (Strome, 1986). The oocytes are produced and gradually mature at the proximal region of the gonad arms in a process called oogenesis. The most mature and proximal oocyte is ovulated and fertilized inside the spermatheca and then pushed into the uterus through the spermatheca-uterine (SP-UT) valve (McCarter et al., 1999). In the uterus, several different-staged embryos are developed in a process called embryogenesis. The maintenance of the gonad actomyosin organization is crucial to ensure that the oogenesis process occurs normally. The impairment in this process affects the amount of oocytes and embryos produced, thereby affecting the brood size of the animal. The second *in vivo* tissue we looked at was the body wall muscles. The striated body wall muscle cells are composed of multiple sarcomeres, the contractile units, which contain regularly distributed dense bodies (analogous to vertebrate Z-discs) and M-lines, which anchor the actin and myosin filaments, respectively, to the muscle cell basement membrane and to the connected epidermis and cuticle, in order to transmit the contractile forces to the

exoskeleton (Altun & Hall, 2009; McCarter et al., 1999). Thus, the body wall muscles are a contractile actomyosin-dependent tissue required for proper animal elongation and movement. An impairment in the actomyosin network of body wall muscles caused by loss of crosslinkers is expected to render morphological defects in the structural organization of sarcomeres, as well as defects in animal movement and body bending.

3.3. Methods

3.3.1. *C. elegans* strains

The *C. elegans* strains used in this Chapter are listed in Table 3.1. Strains were maintained at 16 °C or 20 °C on nematode growth medium (NGM) plates seeded with OP50 *E. coli* bacteria as food source (Stiernagle, 2006). Bacteria was obtained by streaking out *E. coli* OP50 from a glycerol stock onto an agar plate (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast, 5 g/L NaCl and 15 g/L agar, pH 7.5). Bacteria colonies were left to grow overnight at 37 °C (Byerly et al., 1976). A single colony of OP50 was inoculated on Luria Broth liquid medium (LB) and grown overnight at 37 °C. The bacterial suspension was used to seed NGM plates. Medium size plates (60 mm diameter) were used for general strains maintenance and large plates (100 mm diameter) were used to grow larger quantities of worms for freezing purposes. NGM (3 g/L NaCl, 17 g/L agar, and 2.5 g/L peptone) was sterilized by autoclaving at 110 °C for 30 minutes. After cooling, 1 M CaCl₂, 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL of 1 M MgSO₄ and 25 mL of 1 M KH₂PO₄ pH 6 were added. Using sterile procedures, NGM solution was dispensed in each plate at a constant amount into plates using a peristaltic pump (Wheaton Science Products). Plates were left to dry at room temperature for 2-3 days, seeded with 250 µL of *E. coli* OP50 liquid culture in the case of medium NGM plates or 750 µL in the case of large NGM plates, and left at room temperature for one day before storage. When *C. elegans* strains in culture became contaminated, a standard alkaline bleach protocol was used to clean up the animals (Stiernagle, 2006). The bleach kills the contaminants and hermaphrodites but soaks onto the plate before their embryos hatch. The next day the larvae crawl onto the *E. coli* OP50 lawn and are transferred to a clean seeded NGM plate.

3.3.2. RNA interference

RNAi was performed either by feeding hermaphrodites with HT115 *E. coli* bacteria expressing double-stranded RNA (dsRNA) or by injecting animals with dsRNA.

To produce dsRNA for injection, oligonucleotides with tails containing T3 and T7 RNA polymerase promoters were used to amplify regions from N2 genomic DNA (gDNA) or

complementary DNA (cDNA). The oligonucleotides used are listed in Table 3.2. PCR products were separated on agarose 1 % gels containing 2 μ L of Midori Green (DNA staining solution) per 100 mL of 1x TAE buffer (40 mM Tris, 20 mM Glacial acetic acid, 1mM EDTA, pH 8). The gel was then visualized on a Gel doc system (Biorad). The bands of interest were excised with a scalpel under UV light and the DNA was purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Manufacturer's instructions were followed. Purified PCR products were used as templates for independent T3 and T7 *in vitro* transcription reactions (MEGAscript, Invitrogen). The transcription reaction was left incubating for 5 hours at 37 °C and the resulting single-stranded (ssRNA) was then purified using the NucleoSpin RNA Clean-up (Macherey-Nagel) according to manufacturer's instructions. Purified ssRNA products were annealed in soaking buffer (3 $\times$ soaking buffer: 10.9 mM Na₂HPO₄, 5.5 mM KH₂PO₄, 2.1 mM NaCl, 4.7 mM NH₄Cl) by incubating at 68 °C for 10 minutes and then at 37 °C for 30 minutes. Double-stranded RNA (dsRNA) was aliquoted in working volumes and kept at - 80 °C until used. Injection of the dsRNA of interest was performed in the neck of L4 stage *C. elegans* and worms were left in treatment for 48 hours at 20 °C prior to dissection for imaging. Injection of dsRNA was used to deplete the crosslinkers PLST-1 or UNC-70.

RNAi by feeding was conducted by feeding worms with bacteria that expressed the dsRNA of interest. L4440 vectors carrying part of the sequence of *atn-1*, *fln-1*, *unc-70*, *ani-1*, *unc-59*, *unc-61* or *cor-1* were obtained from the Ahringer library (Kamath & Ahringer, 2003); distributed by Source BioScience, United Kingdom) and sequenced to confirm the gene target. L4440 carrying the *sma-1* sequence was built by cloning a *sma-1* cDNA fragment amplified with primers specified in Table 3.2 from N2 cDNA into the EcoRV restriction enzyme site: L4440 plasmid and amplified DNA fragments were digested with EcoRV (Fermentas Fast Digest®) for 1 hour at 37 °C and the digestion products were separated in agarose gels and the bands purified as described previously. Digested L4440 was incubated with calf intestinal phosphatase (Roche®) for 30 minutes at 37 °C. The purified DNA fragment was incubated with T4 Polynucleotide Kinase (Thermo Scientific) for 20 minutes at 37 °C and for 10 minutes at 75 °C. Ligation reactions were carried out with T4 DNA ligase (New England Biolabs) with the supplied buffer, using a 7:1 insert to plasmid ratio and overnight incubation at 16 °C (total volume 10 μ L). For transformations, 100 μ L aliquots of TOP10 *E. coli* competent cells were thawed on ice and mixed with 5 μ L of the ligation reactions. The mixture was incubated for 20 minutes on ice, heat-shocked at 42 °C for 1 minute and cooled down on ice for 2 minutes. Then, 500 μ L of Super Optimal broth with Catabolite repression (SOC) was added, and the cells were incubated on a shaking incubator at 37 °C for 45 minutes. After incubation, the suspension was spread on agar plates containing 100 mg/mL ampicillin and 5 mg/mL tetracycline. The plates were

incubated overnight at 37 °C for 16 hours. Single colonies were tested for correct insertion and final plasmids were transformed into HT115 *E. coli*, which were used to prepare RNAi feeding plates (as described below) and feed the worms after Isopropyl β -D-thiogalactopyranoside (IPTG) induction. L4 stage animals were fed in plates with the dsRNA of interest and incubated at 20 °C for 48-54 hours prior to dissection for imaging.

To prepare feeding plates, bacteria expressing the dsRNA of interest were grown overnight at 37 °C in LB containing 12.5 μ g/mL tetracycline and 100 μ g/mL ampicillin. The overnight culture was grown until an OD₆₀₀ of 1.6, then pelleted at 2500 xg for 10 minutes and resuspended in LB with 12.5 μ g/ml tetracycline, 100 μ g/mL ampicillin, and 1 mM IPTG. Resuspended bacteria was spread onto 6 cm NGM agar plates (~75 μ L/plate), previously spread with 100 μ L of a 1:1:1 mixture of 100 mg/mL ampicillin, 5 mg/mL tetracycline and 1 M IPTG. Plates were dried at room temperature and in the dark for 1-3 days to induce RNA expression before usage at 20 °C or storage at 4 °C.

3.3.3. Generation of endogenously tagged SMA-1 using CRISPR/Cas9

To generate an endogenous fluorescent version of SMA-1, we used the self-complementing split GFP system (Kamiyama et al., 2016). We inserted 6 copies of GFP11 in tandem downstream of the *sma-1* open reading frame using plasmid-based CRISPR/Cas9-mediated genome editing, as described above. The plasmid template used for homology recombination was pAC571, which was built using Gibson assembly (Gibson et al., 2009). The fragments used for pAC571 assembly were as follows: pBluescript backbone, amplified with primers 5'-CATGTGAGCAAAAGGCCAGC-3' and 5'-ATCGCCCTTCCCAACAGTTG-3'; 1 kb left homology region in the *sma-1* open reading frame, amplified from N2 genomic DNA (gDNA) with primers 5'-TCAGGCTGCGCAACTGTTGGGAAGGGCGATAGCTTTCTTCCCTCCGTC-3' and 5'-CTTTGAATGTTTGGATCCACG**TTTAAACAAGGATCCGAATCCTTTTCGTTCTG**-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9); 6xGFP11, amplified from pDC592 with primers 5'-**CTTGTTTAAACGTGGATCCAAACATTCAAAGGGAGGGAGGGCCGGCTCTG**-3' and 5'-GGTGATTCCAGCAGCGTTAACGTACTCAT-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9; bases in italics indicate part of the linker region); and 1 kb right homology region in the *sma-1* 3'UTR, amplified from N2 gDNA with primers 5'-ATGAGTACGTTAACGCTGCTGGAATCACCTAAATAC**GTCACCACACGCTGATCTTC**-3' and 5'-CTGGCCTTTTGCTGGCCTTTTGCTCACATGCCATTTTGTGGATTGCTC-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by

Cas9). Animals expressing SMA-1::6xGFP11 were crossed with animals expressing GFP1-10 from the *mex-5* promoter (GCP794 strain). GCP794 was obtained by subjecting the strain OD4016 (kindly provided by Arshad Desai) to six rounds of outcrossing with N2 animals.

3.3.4. Generation of mutants using CRISPR/Cas9

For generation of *atn-1(prt87)*, *unc-70(prt88)*, *plst-1(prt89)*, *fln-1(prt102)*, *ani-1(prt129)* and *unc-59(prt131)*, we used a plasmid-based approach, in which a single guide RNA (sgRNA) and the Cas9 protein are co-expressed from pDD162 (Dickinson et al., 2013). Guide sequences were chosen using the online tools <http://crispr.mit.edu> and <http://crispor.tefor.net/>. Single-stranded repair templates (Integrated DNA Technologies) included 50-60 bp homology regions flanking the Cas9 cleavage site and, when necessary, silent mutations to avoid repair template recognition by Cas9. Repair template and guide sequences targeting are listed in Table 3.3.

Repair templates and pDD162 were injected into gonads of young adult N2 hermaphrodites. To facilitate the identification of successfully injected animals, injection mixes also contained a repair template and pJA58 (a gift from Andrew Fire, Addgene plasmid #59933; <http://n2t.net/addgene:59933>; RRID:Addgene_59933) to generate the R92C mutation in *dpy-10* gene, which causes a dominant roller phenotype in mutated F1 progeny (Arribere et al., 2014; Levy et al., 1993).

All mutants were verified by DNA sequencing (sequences of the mutated regions are listed in Table 3.3). To remove potential off-target mutations, mutants were subjected to six rounds of outcrossing with wild-type N2 animals. Combined mutations and fluorescent markers were subsequently introduced by mating.

3.3.5. Brood size and embryonic viability

Brood size and embryonic viability tests were performed in L4-stage animals at 20 °C.

In Figure 3.2B-C, L4-stage animals were injected or fed with dsRNAi for 48 hours before being singled out onto fresh plates (with bacteria expressing the corresponding dsRNA in the cases of RNAi by feeding). Adult animals were left to lay eggs for 24 hours before being removed.

In Figure 3.5A-B, L4-stage mutant animals were grown for 44 hours in OP50 *E. coli* plates or in feeding plates expressing dsRNA in the cases where this treatment was performed, before being singled out onto fresh plates. Adult animals were left to lay eggs for 8 hours before being removed.

For both, the laid embryos were left to hatch at 20 °C for another 24 hours, after which the total number of progeny (brood size) and the number of embryos that hatched (embryonic viability) on each plate were counted in a Nikon SMZ800 dissection scope. Percent embryonic viability was calculated by dividing the number of hatched embryos by the total number of embryos in that plate and averaged for the total number of plates counted.

3.3.6. Fixed and live-imaging

For live-imaging of knock-in animals (GCP960, COP1506, RZB213, GOU3103, ZAN348 and NK2225) and the hermaphrodite gonads (GCP113, GCP567, GCP570, GCP602, GCP633, GCP701, GCP717 and GCP1210) in Figures 3.1A-F, 3.6A, 3.6B and 3.6C, synchronized and intact adult gravid hermaphrodites were placed in 5 μ L of 1 mg/mL tetramisole hydrochloride (TMHC; Sigma L9756) for 5-10 minutes and then mounted on 2 % agarose pads overlaid with a 18 mm $\times$ 18 mm coverslip (No. 1.5H, Marienfeld). For fixed animal imaging in Figures 3.6D-E and 3.7A, fluorescence staining and fixation of the whole animal was performed as described below in Section 3.3.8, prior to imaging. Images were acquired at 20 °C on a spinning disk confocal system (Andor Revolution XD Confocal System; Andor Technology) with a confocal scanner unit (CSU-X1; Yokogawa Electric Corporation) mounted on an inverted microscope (Ti-E, Nikon) equipped with a 60x (for knock-in animals and gonads) or 40x (for fixed animals) oil-immersion Plan-Apochromat objective (N.A. 1.4) and solid-state lasers of 488 nm (50 mW) and 561 nm (50 mW). For image acquisition, an electron multiplication back-thinned charge coupled device camera (iXon Ultra 897; Andor Technology) with 1x1 binning was used. Acquisition parameters, shutters and focus were controlled by Andor iQ3 software. Multiple 1 μ m z-stacks were collected to cover the entire animal weight.

For Figure 3.7B, time-lapse imaging of N2 and mutant animals (GCP466, GCP506, GCP564, GCP577, GCP626, GCP700, GCP716, GCP788 and AZ30) in liquid culture was used to assess changes in thrashing behavior. For each condition, L4 animals in standard or dsRNA feeding plates, depending on the condition, were grown for 48 hours prior to imaging. A single adult was placed in a 5 μ L droplet of M9 on a slide and left to acclimatize for 1 minute. Thrashing was recorded for 60 s at ~40 fps, with exposure time of 1 ms and binning of 4x4, using a SMZ 745T stereoscope (Nikon) equipped with a QIClic CCD camera (QImaging), controlled by Micro-Manager open source microscopy software.

3.3.7. Image processing and quantitative analysis

Image processing and analysis was performed using Fiji (ImageJ; National Institutes of Health; Schindelin et al., 2012).

Consecutive images of the animals in Figures 3.1A-F, 3.6A-E and 3.7A were stitched in order to reconstruct larger images, using the Pairwise Stitching plugin with Linear Blending (Preibisch et al., 2009).

Z-stacks taken in Figures 3.1A-F, 3.6D-E and 3.7A were projected using the maximum intensity projection tool.

Distal arm gonad images in Figures 3.6B and 3.6C were scaled equally to the respective controls.

Body bending frequency in Figure 3.7B was automatically quantified using the open source wrMTrck plugin with standard parameters (Nussbaum-Krammer et al., 2015). The background in each acquired image was removed by subtracting the maximum intensity projection of the stack using the Image Calculator tool. Animals were segmented using the Otsu intensity thresholding. A single thrash was defined as a complete change in the direction (to the left or right) of the body down the longitudinal body midline.

Graph plotting was performed with Prism 9.0 (GraphPad Software).

3.3.8. F-actin and DNA staining

F-actin staining with Phalloidin shown in Figures 3.6D-E and 3.7A was done in adult hermaphrodites of N2 or mutant strains GCP466, GCP506, GCP564, GCP577, GCP626, GCP700, GCP716, GCP788 and AZ30. Depending on the condition, 48 hours of dsRNAi treatment by feeding was performed prior to staining.

Animals were collected and washed twice in M9 buffer, fixed with 4 % paraformaldehyde (20 % aqueous solution, Electron Microscopy Sciences) diluted in 1x cytoskeleton buffer (10 mM MES-KOH, pH 6.1, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA) containing 0.32 M sucrose (Cramer & Mitchison, 1993) for 15 minutes, permeabilized with acetone at -20 °C for 5 minutes, washed with PBS containing 0.5 % Triton X-100 and 30 mM glycine (PBS-TG) for 10 minutes, and stained with 1:40 Oregon Green 488 Phalloidin (Molecular Probes, Thermo Fisher Scientific) in PBS-TG for 30 minutes. After three 10 minute washes with PBS-TG, animals were mounted with ProLong Antifade containing DAPI to label the DNA (Molecular Probes, Thermo Fisher Scientific).

3.3.9. Preparation of worm protein extracts and immunoblotting

For Figure 3.4, fifty L4 worms of each of the strains N2, GCP700 and GCP716 were grown on NGM plates for 48 hours at 20 °C. Animals were collected in M9 medium with 0.1 % Triton X-100 and pelleted at 750 xg for 3 minutes. Then, animal pellets were washed 3 more times in M9 medium and twice in M9 with 0.1 % Triton X-100. To 25 µL of worm suspension, 25 µL 2x SDS-PAGE sample buffer [125 mM Tris-HCl, pH 6.8, 15 % (v/v) glycerol, 4 % (w/v) SDS, 100 mM DTT and 0.02 % (w/v) bromophenol blue] were added. To the 50 µL of worm suspension with SDS-PAGE buffer, approximately 1/3 of the volume of glass beads (Sigma-Aldrich, 425-600 µm) was added to mechanically lyse the animals. Samples were subjected to three 5-minute cycles of alternating boiling at 95 °C and vortexing. After centrifugation at 20000 x g for 1 minute at room temperature, supernatants were collected. Protein samples were resolved by SDS-PAGE in a 10 % acrylamide gel and transferred to 0.2 µm nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5 % non-fat dry milk in TBST (20 mM Tris, 140 mM NaCl, and 0.1 % Tween, pH 7.6) and probed at 4 °C overnight with the following primary antibodies: 1:5000 anti-UNC-59 antibody (rabbit polyclonal) or 1:5000 anti-ANI-1 antibody (rabbit polyclonal) and 1:5000 anti-tubulin antibody (mouse monoclonal DM1- α , Sigma). Membranes were washed three times with TBST, incubated with HRP-conjugated secondary antibodies mouse anti-rabbit 1:5000 or goat anti-mouse 1:5000 (Jackson ImmunoResearch) for 1 hour at room temperature and washed again three times with TBST. Proteins were visualized by chemiluminescence using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and imaged in a ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad).

3.3.10. Statistical analysis

Statistical analysis was performed with Prism 9.0 software (GraphPad). Error bars represent 95 % confidence interval of the mean (95 % CI). Statistical significance tests were performed using one-way ANOVA followed by Dunnett's multiple comparison test, as indicated in the figure legends.

Table 3.1. List of *C. elegans* strains used in this study.

Name	Genotype	Source/ reference
N2	Ancestral	<i>Caenorhabditis</i> Genetics Center (CGC)
GCP113	nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	Our lab (Silva et al., 2016)
GCP466	unc-70(prt88) V; unc-5(e53) IV/nT1 [qls51] (IV;V); dpy-11(e224) V/nT1 [qls51] (IV;V)	This thesis
GCP506	atn-1(prt87) V	This thesis
GCP508	atn-1(prt87) V; nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
GCP564	plst-1(prt89) IV	This thesis (published in Leite et al., 2020)
GCP567	unc-70(prt88) V; unc-5(e53) IV/nT1 [qls51] (IV;V); dpy-11(e224) V/nT1 [qls51] (IV;V); nmy-2(cp13[nmy-2::gfp + LoxP]) I; unc-119(ed3) III (?)	This thesis
GCP570	plst-1(prt89) IV; nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
GCP577	atn-1(prt87) V; plst-1(prt89) IV	This thesis
GCP602	atn-1(prt87) V; plst-1(prt89) IV; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
GCP626	fln-1(prt102) IV	This thesis
GCP633	fln-1(prt102) IV; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
GCP700	ani-1(prt129) III	This thesis
GCP701	ani-1(prt129) III; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
GCP716	unc-59(prt131) I	This thesis
GCP717	unc-59(prt131) I; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); ltIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis

GCP788	atn-1(prt87) V; plst-1(prt89) IV; unc-59(prt131) I; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); lts37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
GCP794	ltSi1188[pDC600; Pmex-5::GFP1-10::3'UTR-tbb-2::gpd-2/3 operon linker::mCherry-his-15::3'UTR-unc-54; cb-unc-119(+)] II; unc-119(ed3) III (this is OD4016 after 6 outcrosses)	This thesis
GCP960	sma-1::GFP11-6x [pAC571(prt159)]V; ltSi1188[pDC600; Pmex-5::sGFPS1-10::tbb-2_3'UTR::gpd-2/3 operon linker::mCherry-his-15::unc-54-3'UTR;; cb-unc-119(+)] II; unc-119(ed3) III	This thesis
GCP1210	sma-1(ru18) V; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); lts37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis
AZ30	sma-1(ru18) V	CGC (McKeown et al., 1998)
NK2225	unc-59(qy50[unc-59::GFP::AID]) I	CGC (Chen et al., 2019)
MDX29	ani-1(mon7[mNeonGreen^3xFlag::ani-1]) III	Maddox Lab (Rehain-Bell et al., 2017)
COP1506	atn-1(knu478[degron::mKate2]unc-119); unc-119(ed3) III	Zaidel-Bar Lab
OD4016	ltSi1188 [pDC600; Pmex-5::GFP1-10::3'UTR-tbb-2::gpd-2/3 operon linker::mCherry::his-15::3'UTR-unc-54; cb-unc-119(+)] II; unc-119(ed3) III	OD Lab
RZB213	plst-1(msn190[plst-1::GFP]) IV	Zaidel-Bar Lab (Ding et al., 2017)
GUO3103	unc-70(cas962[GFP::unc-70]) V	Ou Lab (Jia et al., 2019)

* unc-119(ed3) III (?) - ed3 allele was present in one of the paternal strains, but its presence was not checked after the genetic cross.

Table 3.2. List of dsRNAs used in this study.

Gene	Oligonucleotide 1 (5'-3')	Oligonucleotide 2 (5'-3')	RNAi
W04D2.1 (<i>atn-1</i>)	AGATGCTGATCGCGAGTTTT	GGAGTAGGCGTGAGTTGCTC	Feeding

Y49E10.19 (<i>ani-1</i>)	CAAACCTCAATGGAGAGGACAATC	CATGTTCACTGACAACTGGGATA	Feeding
Y66H1B.2 (<i>fln-1</i>)	TCTCCAGTTCATAAGCATCCAAT	ACGTGAAAGACTCACCATTGACT	Feeding
R01H10.3 (<i>cor-1</i>)	ACCAGCCGATCTATGTTTGG	TTCAAAAACTCACGCAGTCG	Feeding
Y104H12BR.1 (<i>p/st-1</i>)	aattaaccctactaaaggACTCGGAGT CCATGGAAATG	taatacgactcactataggCACCATTTTT GGCTTCACCT	Injection
R31.1 (<i>sma-1</i>)	CCCCCGATATCGACACCCTTTCA AATG	CCCCCGATATCCCTTCTGTTCCA TCTCGTTTC	Feeding
W09C5.2 (<i>unc-59</i>)	AGCATCTTCATGGACGTGTTAAT	CATTACAACGGGGAAAATTGTA	Feeding
Y50E8A.4 (<i>unc-61</i>)	AATAACGATGGGATGGGGAT	TCAGACTCCAAGCCCTCGA	Feeding
K11C4.3 (<i>unc-70</i>)	CAACGTCTTGCTGGACTTCA	CGTGGTTGTCGATTTTCATTG	Feeding
K11C4.3 (<i>unc-70</i>)	aattaaccctactaaaggCACTGTCTTG AGAACGTTGAG	taatacgactcactataggAATGGATTTC TCATCAGGTTGG	Injection

* T3 and T7 promoter sequences are in lowercase.

Table 3.3. List of CRISPR/Cas9 sgRNAs, crRNAs, repair templates and diagnostic primers used in this study.

<i>sma-1::6xGFP11</i>	
Strategy design	6 tandem insertions of GFP11 downstream of the <i>sma-1</i> open reading frame
Repair template sequence (5'-3')	pAC571 (see text for details)
sgRNAs (5'-3')	TCGGATCGTTATTCAAGCG; GGTATCTACTTTGAATGTT; TGAAGATCAGCGTGTGGTG
Diagnostic primers (5'-3')	Fwd GCGAATTCATTACTTGGGTAGA; Rev GTCTTCCAGCTTTGGTGATAG
Obtained sequence (5'-3')	1kb left homology arm (last 50 bps: AACGAAAAGGATTTCGGATCCTTGTAAACGTGGATCCAAACATTCAAAG) + GGAGGGAGGGCCGGCTCTGGAGGCTCTGGA + 6xGFP11 separated by linkers (AGAGATCATATGGTCCTCCACGAATACGTCAACGCTGCCGGGATCACTGGAGGAT CTGGAGGACGTGACCATATGGTCCTCCACGAATACGTCAATGCCGCCGGAATCAC CGGAGGTTCCGGAGGACGTGATCACATGGTCCTCCACGAATACGTCAACGCTGCC GGGATCACTGGAGGAAGCGGAGGACGCGATCATATGGTCCTCCACGAGTACGTTA ACGCCGCTGGAATCACCGGAGGATCCGGAGGTAGAGACCATATGGTCCTTCACGA

	ATACGTCAACGCCGCTGGAATCACCGGTGGATCCGGTGGACGTGATCACATGGTT CTTCATGAGTACGTAAACGCTGCTGGAATCACCTAA +1kb right homology arm (first 50 bp: <u>atacgtcaccacacgctgacttcatatcttctgtaggcgaagattgaaa</u>)
<i>atn-1(prt87)</i>	
Strategy design	Removal of ABD
Repair template sequence (5'-3')	CATACCATCAGCCGGGCTACGACTACACTCAACAAGAGGAAGAATGGGACCGTGA AGGATTACTGGATCCTGCCTGGGAGGCATTTTCGTAACATGCGTGATCCTCCACCA CCAGTTATTGCGCAACCACCACCACAGCGTGTGTTGTTGCTCCACCTCCAGAG
sgRNAs (5'-3')	AGGACTGTTAGATCCAGCA;GGAAGTGTAGATCCAGCAT; ATGCTGGATCTAACAGTCC
Diagnostic primers (5'-3')	Fwd CCTTCTTCACACCGTTCATC; Rev GAGACAAGGACAATGGACATTTA
Obtained sequence (5'-3')	CATACCATCAGCCGGGCTACGACTACACTCAACAAGAGGAAGAATGGGACCGTGA AGGATTACTGGATCCTGCCTGGGAGGCATTTTCGTAACATGCGTGATCCTCCACCA CCAGTTATTGCGCAACCACCACCACAGCGTGTGTTGTTGCTCCACCTCCAGAG
<i>unc-70(prt88)</i>	
Strategy design	Removal of ABD
Repair template sequence (5'-3')	cattttaatgcgaatttttgggttaattcttctaaaacatgtattttcagATGAACGTGAATACTTCAACAACT GAAACAAGATAACATCCAAGGAAAACGTATCGGAAAAGTGATTAATG
sgRNAs (5'-3')	AAATGGGTCAACTCACACT;AATGGATTTCTCATCAGGT; GTAACATACATAAGTGATAA
Diagnostic primers (5'-3')	Fwd GTCGCTAGTCCTGGTATCTATC Rev ACTCGGTGAGCTGTCTTT
Obtained sequence (5'-3')	cattttaatgcgaatttttgggttaattcttctaaaacatgtattttcagATGAACGTGAATACTCGTGCAAAAGAA AACATTTACAAAATGGGTCAACTCACACTTGGTTTCGAGTCAGCTGCAAAGTACAAGt gagtaaatattttcgaaacccggtttattaaaattaataatttcagGACCTCTACATGGATATGCGAGACGG AAAAATGCTCCTCCGACTCCTCGCAGTTCTTTCCGGTGAACGTCTGgtgagttgtattttttt caaaaaattattcaaaagcgtcatttcaaaaaaaaactcctcctcaaaaaaatcaatgagacgagggcagcgtcttttg caagcagcttgcaagcttttccggaagaaaagagatagactggcccgagaaggaaaactaattgaattcaaaaatgt gcaaactttGAGAAATCCATTATCACTTATGTAGTTACATATTATCATTACTTCAACAAAC TGAAACAAGATAACATCCAAGGAAAACGTATCGGAAAAGTGATTAATG
<i>plst-1(prt89)</i>	
Strategy design	Removal of ABDs: insertion of an extra nucleotide to introduce a frameshift and premature stop at the beginning of the Wormbase's <i>plst-1</i> coding sequence (version WS276), correspondent to the amino acid 262 in the CH2 domain of PLST-1 protein according to Ding et al., 2017. Note: <i>plst-1</i> sequence in WormBase lacks the first part of the gene corresponding to the EF-hands and CH1 domain of the PLST-1 protein (Ding et al., 2017).
Repair template sequence (5'-3')	ctctgaaaaatccacaaatttcagactgctccgcgacggtgaaactctgaagatctcaggcgtctgtcccagaagaat tctcATGAAGAGCTAGCTGGGTTAATTATCATTTAGAACGTGCTAGAACTCAACGTCG CCTACACAATTTACATCAGATATTGTCGATTCAGAGATTATAC
sgRNAs (5'-3')	TTCTTCTGGGGACAGACGC;ATTATCATTTGGAACGTGC; AGATGGGTAAATTATCATT
Diagnostic primers (5'-3')	Fwd CATGGCCTAGAAACCACTTT Rev GCTTCTCGGCTTCATCTAAC Followed by Digestion with NheI restriction enzyme

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Obtained sequence (5'-3')	ctctgaaatccacaaatttcagactgctccgagcgggtgaaactctgaagatctccggcgtctgtcccagaagaat tctcATG A AGA GCTAGC TGGGTTAATTATCATTT A GAACGTGCT A GAACCTCAACGTCTG CCTACACAATTTACATCAGATATTGTCGATT C AGAGATT T ATAC
<i>fln-1(prt102)</i>	
Strategy design	Removal of ABD
Repair template sequence (5'-3')	gtatttatttcagaagactatacaacttttaacataaatatttcagGTTGTTCC AGCCATTAGACACGACG ATGCGGAATGGAAGACAAAACCAATTATAATGAA ACCGAAAGTCCAGGCG ACTATA TCAAATTTGGACAAAATTCTCCAAGTCAATGATCCTAGAGAGTTTGATTG
sgRNAs (5'-3')	CGTCATCGTGACGGATCGC;CCATTTCGGCGTCATCGTGA; AAATAGTCGCTTGAACCTT; CCTTTGGCTTCATTATTAT
Diagnostic primers (5'-3')	Fwd ctctcattctcagcttcttctatc Rev TCATCTCGGATCGAGACTTTC
Obtained sequence (5'-3')	gtatttatttcagaagactatacaacttttaacataaatatttcagGTTGTTCC AGCCATTAGACACGACG ATGCGGAATGGAAGACAAAACCAATTATAATGAA ACCGAAAGTCCAGGCG ACTATT TCAAATTTGGACAAAATTCTCCAAGTCAATGATCCTAGAGAGTTTGATTG
<i>ani-1(prt129)</i>	
Strategy design	Knock-down: insertion of extra nucleotides to introduce a frameshift and premature stop at the beginning of <i>ani-1</i> coding sequence
Repair template sequence (5'-3')	gttttgatgagattttctggaacattgtttcttcgagaactacactgtaatacaATG ACGTGCTAGCG TATCA ATTCGATTCTCTAATGCAACGCATACGAGTTTCGGCAAGCCGAAATGTCAGGCGAA GGAGAGGTGGCAAAGGAGAATGG
sgRNAs (5'-3')	CTACACTGTAAATACAATGG;GAACTACACTGTAAATACAA; GGATCAATTCGATTCTCTAA
Diagnostic primers (5'-3')	Fwd GCGTCGAGACCTGGTTG Rev GAGTTGTCTCCTTTGGCTCTT Followed by Digestion with NheI restriction enzyme
Obtained sequence (5'-3')	gttttgatgagattttctggaacattgtttcttcgagaactacactgtaatacaATG ACGTGCTAGCG TATCA ATTCGATTCTCTAATGGAACGCATACGAGTTTCGGCAAGCCGAAATGTCAGGCGAA GGAGAGGTGGCAAAGGAGAATGG
<i>unc-59(prt131)</i>	
Strategy design	Knock-down: insertion of an extra nucleotide to introduce a frameshift and premature stop at the beginning of <i>unc-59</i> coding sequence
Repair template sequence (5'-3')	gtatttatatacacatccttttctcaaatattgttcattcaatgtacttctcagcttcttcaactcttttctctgagtcacatcaaa atttccaatttcagcaaaaaATG TAGCGCTAGC AGTCTGACTGCAAAATAGCTCGTCTAGAAA CGATGAATCTTTGAGAACCGGGCAGCACAAAGAAAATC
sgRNAs (5'-3')	TGACTCGCAGAGAAAAGAAG; CTTCTTTTCTCTGCGAGTCA
Diagnostic primers (5'-3')	Fwd CAGCTGTCATTTGCTCAG Rev GGAACAGAGTGTGATGAATGT Followed by Digestion with NheI restriction enzyme
Obtained sequence (5'-3')	gtatttatatacacatccttttctcaaatattgttcattcaatgtacttctcagcttcttcaactcttttctctgagtcacatcaaa atttccaatttcagcaaaaaATG TAGCGCTAGC AGTCTGGACTGCAAAATAGCTCGTCTAGAAA CGATGAATCTTTGAGAACCGGGCAGCACAAAGAAAATC

*Bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9 or to maintain codon balance.

** Bases in lowercase in repair templates or obtained sequences indicate intron regions; underlined bases in lowercase indicate 3'UTR.

*** Bases in bold and underlined correspond to restriction enzyme sequences introduced for screening purposes.

**** Bases in bold, underlined and italic correspond to insertions of extra nucleotides to introduce a frameshift and premature stop in the sequence.

+ Bases in italic correspond to linkers between *sma-1* gene and 6xGFP11 or between the 6 copies of GFP11.

3.4. Results

3.4.1. Crosslinkers have different expression patterns in the *C. elegans* tissues

To explore the contribution of the crosslinkers to different actomyosin-dependent contexts, we first assessed the expression pattern of six fluorescent-labeled crosslinkers, in the entire *C. elegans* body, using a confocal spinning-disk microscope. The investigated crosslinkers were α -actinin (ATN-1::mKate2), plastin (PLST-1::GFP), β -spectrin (GFP::UNC-70), β H-spectrin (SMA-1::spGFP), anillin (mNeonGreen::ANI-1) and septin (UNC-59::GFP; Figure 3.1). In all these strains, the fluorescent tag is on the genomic locus of each crosslinker gene and all isoforms are labelled, except isoform C of UNC-70.

Each crosslinker presented its unique expression pattern.

ATN-1::mKate2 is enriched in the *C. elegans* body wall muscles that run from head to tail, presenting a discontinuous signal that is consistent with its known localization to the dense bodies (Figure 3.4; Moulder et al., 2010). It is also present in the pharynx, intestine, vulva and, curiously, in structures that appear to be part of the nervous system, namely the amphid or labial sensilla in the mouth and the phasmid sensilla in the tail (Figure 3.1A).

PLST-1::GFP localization pattern was previously reported in Ding et al., 2017. PLST-1::GFP is ubiquitously expressed within the organism, enriching in the cell-cell contacts during embryogenesis, in the gut (pharyngeal-intestinal valve, intestine and intestinal-rectal valve), in the syncytial gonad and oocytes, in the vulva, and in structures of the nervous system, previously referred in Ding et al., 2017 as representing the socket cells of amphid and phasmid neurons, in the mouth and tail, respectively (Figure 3.1B).

The expression pattern of GFP::UNC-70 in the strain GOU3103 was previously reported in Jia et al., 2019. It starts accumulating in the cell membranes near cell-cell contacts of embryos around the gastrulation time during embryogenesis and enriches in the nervous system in larvae and adult animals along the entire body, both in sensory and motor neurons, axons, dendrites and cilia. It is also localized to the spermatheca and spermatheca-uterine valve (sp-ut valve), as reported in Wirshing & Cram, 2018, and in the vulval region, which is also a target of motor neurons (Figure 3.1C). In this strain, we did

not observe evident expression of UNC-70 in body wall muscles. This is in contrast with what was reported by Hammarlund et al., 2000, where a transgenic *gfp::unc-70* under the *unc-70* promoter localized to all muscles including the pharyngeal, vulval, uterine and the body wall muscles.

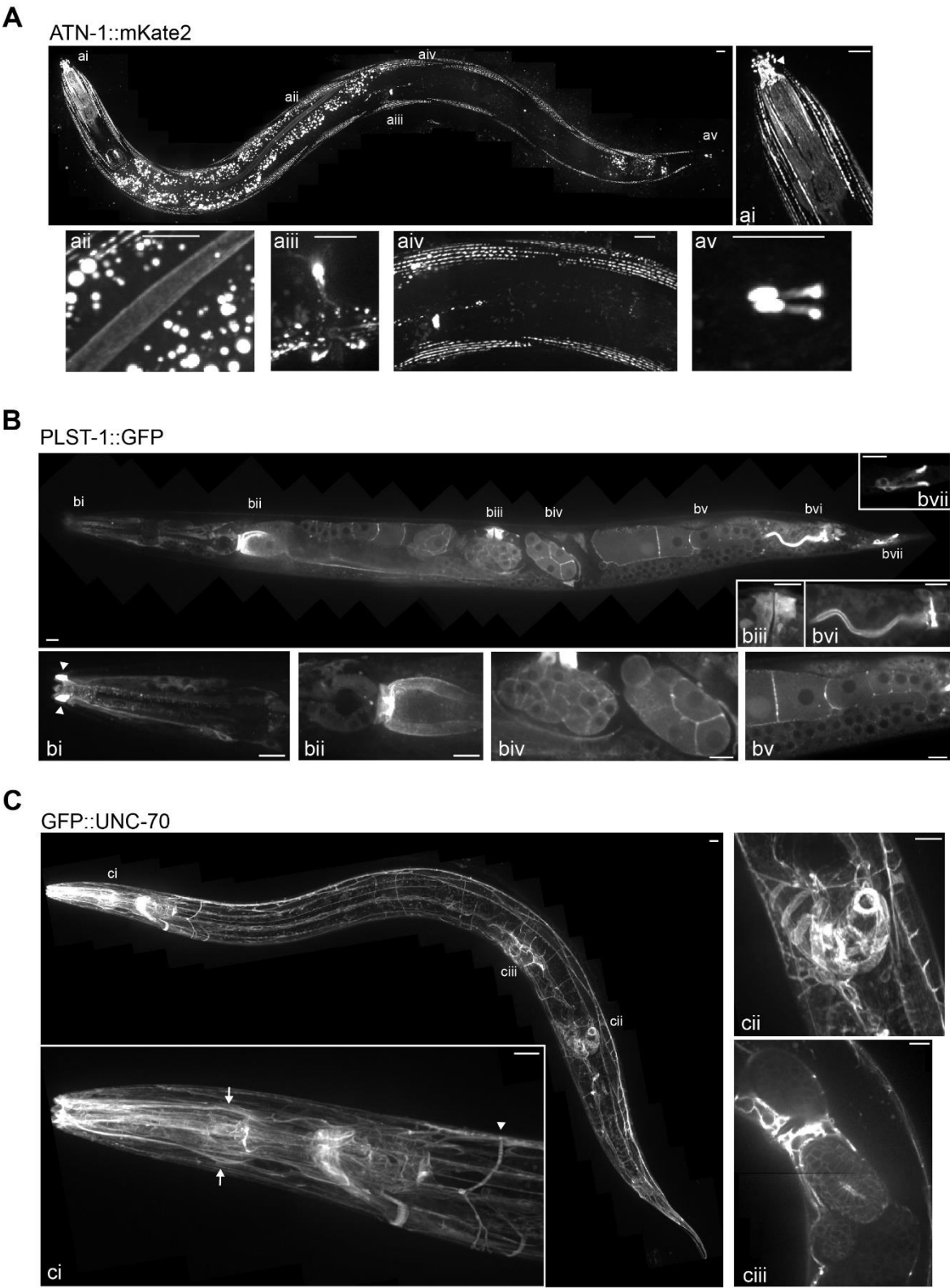
To assess SMA-1 localization pattern, we attempted to generate a fluorescent version of SMA-1 expressing the full length GFP. We were not successful so far. Therefore, we tagged SMA-1 using a split GFP (spGFP) approach. In this approach, the 11-stranded β -barrel structure of GFP is reconstituted from two separately expressed and self-complementing fragments of GFP: GFP1-10 (β -strands 1-10) and GFP11 (β -strand 11; (Kamiyama et al., 2016)). In this case, 6 tandem copies of GFP11 were inserted downstream of *sma-1* open reading frame by CRISPR/Cas9 and the animals expressing SMA-1::6xGFP11 were crossed with a strain expressing transgenic GFP1-10 under the *mex-5* (zinc finger protein involved in the regulation of protein localization) promoter and *tbb-2* (β -tubulin) 3'UTR, in order to promote SMA-1 expression in the germline. We observed that SMA-1::spGFP is faintly expressed in the germline starting at one-cell embryos, where it is localized to the contractile ring. It is also localized to the syncytial gonad, more specifically to the rachis bridges, and at the oocytes cell-cell contacts. Moreover, SMA-1::spGFP enriches in the pharyngeal-intestinal valve, intestine lumen and vulva (Figure 3.1D). Previous studies report that SMA-1 is also localized to the epidermis, pharynx and intestine already during embryogenesis (McKeown et al., 1998). Although there is fluorescent signal in the pharynx lumen of SMA-1::spGFP animals (as well as in mNeonGreen::ANI-1 and UNC-59::GFP animals), this signal pattern is also observed in wild-type N2 animals, thus it likely represents autofluorescence of this tissue.

The localization pattern of mNeonGreen::ANI-1 shows a clear enrichment in the syncytial and cellularized gonad with strong accumulation at the distal tip region of the gonad. mNeonGreen::ANI-1 is also expressed in oocytes and throughout embryogenesis from early embryos, localizing mostly at cell-cell contacts and in the midbodies (Figure 3.1E). Furthermore, ANI-1 localizes to the pharyngeal-intestinal valve, intestine lumen and in the vulva.

Similarly to mNeonGreen::ANI-1, we observed that UNC-59::GFP is also largely expressed in the *C. elegans* gonad, especially in the rachis bridges and distal tip region. It is also expressed in oocytes and embryos throughout embryogenesis. In embryos, it localizes to contractile rings and midbodies (Figure 3.1E). However, ANI-1 and UNC-59 localization patterns are not completely overlapping: UNC-59::GFP does not localize to the cell-cell contacts of the embryonic cells or oocytes. It also localizes to the pharynx lumen, pharyngeal-intestinal valve, intestine and vulva (Figure 3.1F). UNC-59::GFP was also shown to be expressed in the seam cells and sperm (Chen et al., 2019).

We have not characterized tagged versions of FLN-1 and COR-1 by CRISPR/Cas9. Nevertheless, recent work from Ou's laboratory, revealed that GFP::COR-1 is expressed in the cortex of early embryos (starting at the one-cell embryo) and in migrating neuroblasts during larval development (Xie et al., 2021). The localization pattern of FLN-1 was also previously described with strains either expressing a transgenic full-length GFP::FLN-1A under the endogenous promoter and 3'UTR or carrying transgenic constructs of the *fln-1A-B* or *fln-1C* promoters and GFP. This work revealed that full length GFP::FLN-1A and GFP under the expression of the *fln-1A-B* promoter are strongly expressed in the somatic gonad, namely in the spermatheca, uterus and sheath cells, starting at the L4 stage. GFP under the expression of the *fln-1C* promoter is expressed in the body wall muscles, vulval muscle and epidermis (DeMaso et al., 2011; Kovacevic & Cram, 2010).

These results show differentiated expression and localization patterns of distinct crosslinkers in *C. elegans*, consistent with the idea that crosslinkers have distinct functionalities.



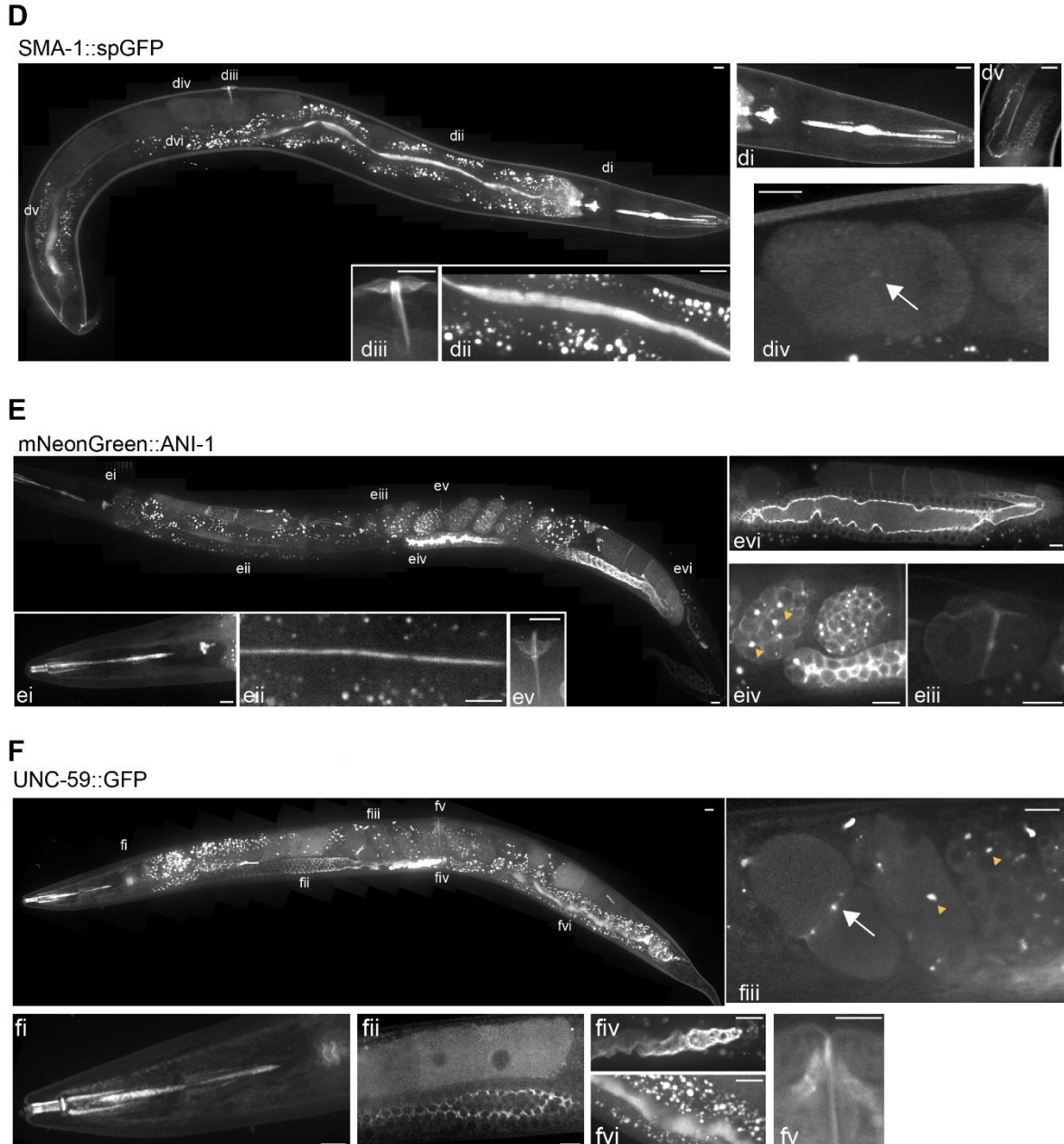


Figure 3.1. Expression patterns of crosslinkers in *C. elegans*. (A) ATN-1::mKate2 expression in the adult hermaphrodite showing enrichment in the mouth, arrowhead pointing to terminal doty structures that may belong to the sensilla of the head neuronal system (ai), pharynx (ai), intestine (aai), vulva (aiii), body wall muscles (aiv), and terminal structure that may belong to the sensilla of the tail neuronal system (av). (B) PLST-1::GFP is expressed in amphid and phasmid socket cells of mouth and tail neurons (bi and bvii, respectively; white arrowheads point to amphid socket cells), in cell-cell contacts during embryogenesis (biv), in the gut (pharyngeal-intestinal valve, intestine and intestinal-rectal valve; bii and bvi), in the syncytial gonad and oocytes (bv), and in the vulva (biii). (C) GFP::UNC-70 accumulates in the nervous system (white arrows point to sensory neuronal dendrites in the head and white arrowhead points to a motor neuron in the neck, magnified in ci), in the spermatheca and sp-ut valve (cii), in cell membranes during gastrulation (cii) and in the vulva (cii). (D) SMA-1::spGFP (see text for details) is expressed in the pharyngeal-intestinal valve (di), in the intestine lumen (dii), in the vulva (diii), in embryos (div; white arrow points to the contractile ring) and in the rachis bridges of the syncytial gonad (dv). (E) mNeonGreen::ANI-1 enriches in the pharyngeal-intestinal valve and intestine lumen

(ei and eii, respectively), in early embryos and throughout embryogenesis, localizing mostly at membranes (eiii) and midbodies (eiv, orange arrowheads), in the vulva (ev) and in the syncytial gonad and oocytes (evi), with stronger accumulation at the distal tip region of the gonad. (F) UNC-59::GFP is expressed in the pharyngeal-intestinal valve (fi), in the rachis bridges and in the distal tip region of the gonad (fii and fiv, respectively), in oocytes (fii), in embryos throughout embryogenesis with stronger accumulation at the contractile rings and midbodies (fiii; white arrow points to contractile ring; orange arrowheads point to midbodies), in the vulva (fv) and in the intestine (fvi). Scale bars, 10 μ m.

3.4.2. Depletion of some crosslinkers leads to a reduction in brood size and embryonic viability

The evaluation of brood size and embryonic viability provide information on how crosslinkers individually contribute to the animal's development and serve as read-outs for defects in oogenesis and embryogenesis (Figure 3.2A). We performed these assays after RNAi-mediated depletion of the nine proteins under evaluation in this study: ATN-1; PLST-1; FLN-1; UNC-70; SMA-1; ANI-1; UNC-59; UNC-61 and COR-1. Of note, the efficacy of each RNAi was confirmed as in Chapter IV - Figure S4.1.

The brood size was not affected after depletion of ATN-1, UNC-61 or COR-1. Evident reductions in brood size were observed after *plst-1(RNAi)* (69 ± 11 embryos), *sma-1(RNAi)* (83 ± 37 embryos), *ani-1(RNAi)* (67 ± 28 embryos) and *unc-59(RNAi)* (94 ± 39 embryos), when compared to control (151 ± 34 embryos; Figure 3.2B). Depletions of PLST-1, FLN-1 and ANI-1 also caused a significant reduction in the embryonic viability, consistent with previous literature data [(83 ± 5 % in *plst-1(RNAi)*, 10 ± 7 % in *fln-1(RNAi)*, 13 ± 8 % in *ani-1(RNAi)* and 96 ± 5 % in controls; Figure 3.2C; Ding et al., 2017; Kovacevic & Cram, 2010; Maddox et al., 2005)]. These results indicate that SMA-1 and UNC-59 are important to ensure normal oogenesis, FLN-1 is required for proper embryogenesis, and PLST-1 and ANI-1 are required for both.

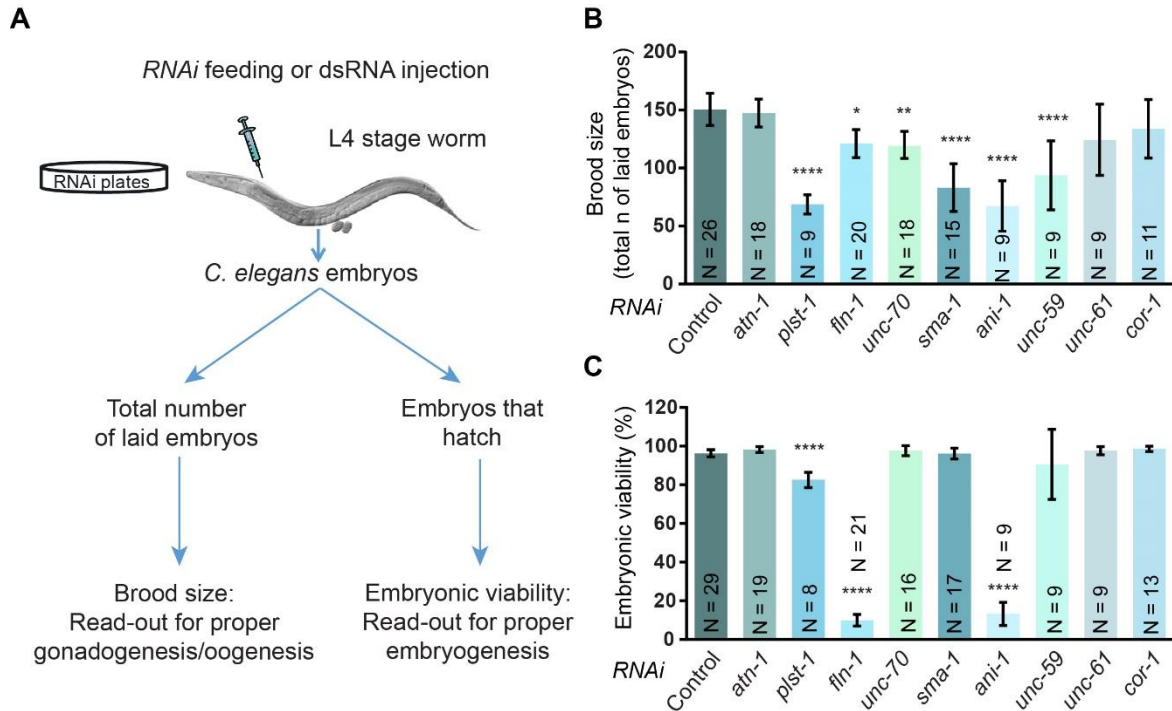


Figure 3.2. Impact of depleting crosslinkers in animal brood size and embryonic viability. (A) Diagram depicting the strategy used to perform brood size and embryonic viability assays by RNAi-mediated depletion of crosslinkers in *C. elegans* hermaphrodites. Brood size corresponds to the total number of laid embryos over 24 hours. Embryonic viability corresponds to the percentage of laid embryos over 24 hours that is viable (number of hatched embryos over total number of progeny*100). (B) Brood size quantification (mean ± 95 % CI) in control or crosslinker-depleted animals. (C) Percentage of embryonic viability (mean ± 95 % CI) in control or crosslinker-depleted animals. N is the number of embryos analyzed. Statistical significance between control and each RNAi condition was determined using one-way ANOVA followed by Dunnett's multiple comparison test: P values *P < 0.05, ** P < 0.01, **** P<0.0001.

3.4.3. Generation of crosslinker mutants

To specifically study the crosslinking capability of the crosslinkers without affecting their other potential functionalities within the cell, we next aimed to generate mutants lacking the actin-binding domains (ABDs) by CRISPR/Cas9. We successfully generated mutants of ATN-1, PLST-1, FLN-1, UNC-70, ANI-1 and UNC-59 (Figure 3.4).

The proteins encoded by *atn-1*(prt87) and *fln-1*(prt102) lack their entire ABDs, composed of two tandem calponin-homology (CH) domains, with the remainder of the gene left intact (Figure 3.3A and 3.3C). While *atn-1*(prt87) animals propagate normally, *fln-1*(prt102) animals propagate much slower, probably due to decreased brood size and embryonic viability (Figure 3.5A-B).

The protein encoded by *plst-1*(prt89) has a stop codon after the first CH domain of its first ABD (Figure 3.3B). A single CH domain does not compose a functional ABD, thus this protein presumably lacks its actin-binding capacity. Homozygous *plst-1*(prt89) animals have

a slower growth rate and propagate slower than wild-type animals, likely due to increased embryonic lethality (Figure 3.5B).

The editing of the *unc-70* locus did not occur as initially intended. In the *unc-70(prt88)* allele, only half of the CH1 and most of CH2 domain sequences were deleted (Figure 3.3D). Nevertheless, the strain was unable to be propagated in homozygosity, which is in agreement with what previously described for *unc-70* null mutants that arrest at the L1 larvae stage (Hammarlund et al., 2000). Although some *unc70(prt88)* homozygous L1 larvae exist in the progeny of an heterozygous *unc-70(prt88)* mother, we were unable to propagate them, even under optimized conditions [it was reported that *unc-70* null mutants could propagate in HB101 freshly seeded bacteria (Hammarlund et al., 2000), yet we were unable to reproduce it in *unc-70(prt88)*]. Therefore, the mutation was kept in heterozygosity, using a balancer chromosome. In order to study *unc-70(prt88)* balanced mutant without the interference of the wild-type copy of the gene present in the balancer chromosome, we designed a dsRNA that specifically targets the wild-type copy only, producing the dsRNA against the region that is absent in the mutant allele but is still present in the wild-type allele of the gene. By using this strategy, *unc-70(prt88);unc-70(RNAi)* should only express the mutated UNC-70.

In the cases of *ani-1* and *unc-59* we deleted the whole genes. Our attempts to generate an *ani-1* mutant lacking its ABD were not successful and the ABD of *unc-59* is not well defined in *C. elegans* (Figure 3.3E and 3.3F). Therefore, a frameshift/stop codon was inserted at the beginning of their open reading frames to block protein expression and function. The obtained *ani-1(prt129)* animals propagate normally without evident phenotypic alterations, which is different from previous literature reports of ANI-1 perturbations that describe protruded vulva and slow growth (Maddox et al., 2005). This strongly indicated that our mutant did not correspond to a null. The *unc-59(prt131)* animals grow and propagate slowly and the worms present pleiotropic phenotypes, ranging from wild-type-looking to sick-looking, longer, sterile with a protruded vulva or extruded gonad. These phenotypes are in agreement with previously described UNC-59 perturbations (Finger et al., 2003; Nguyen et al., 2000).

defects, propagate slowly and sometimes display a roller phenotype (McKeown et al., 1998; Praitis et al., 2005).

Additionally, by crossing *atn-1(prt87)* with *plst-1(prt89)* mutant strains, we obtained *atn-1(prt87);plst-1(prt89)* animals co-expressing the two crosslinker mutants, which allowed us to study potential redundancy between these proteins. These animals propagate similar to single *plst-1(prt89)* animals. By crossing these with *unc-59(prt131)* animals, we obtained triple mutants [*atn-1(prt87);plst-1(prt89);unc-59(prt131)*], which are sick-looking and slow growing worms, presenting a worst phenotype than the single and double mutants.

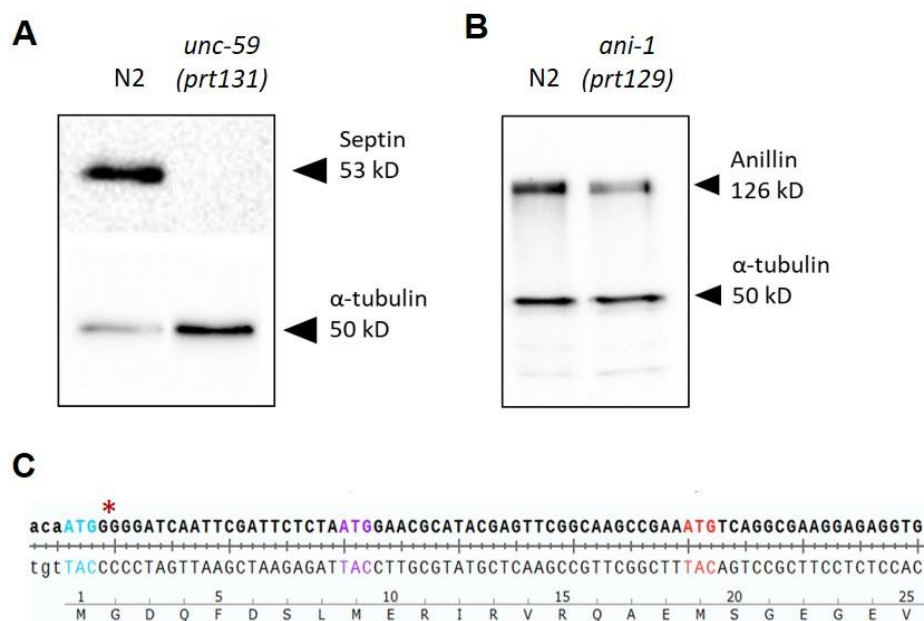


Figure 3.4. Protein expression levels in *unc-59(prt131)* and *ani-1(prt129)* animals assessed by immunoblot. (A) Immunoblot with an antibody against the septin UNC-59 showing protein levels in wild-type (N2) versus *unc-59(prt131)* animals. α-tubulin was used as a loading control. (B) Immunoblot with an antibody against anillin ANI-1 showing protein levels in wild-type (N2) versus *ani-1(prt129)* animals. α-Tubulin was used as a loading control. (C) DNA sequence that encodes for the first 25 amino acids of the *ani-1* gene with the 3 start codons (ATG) depicted in colors. The red asterisk marks the site that we edited to insert a stop codon and cause a frameshift.

3.4.4. Crosslinker mutant animals present reduced brood size and embryonic viability

Next, we performed brood size and embryonic viability assays in these mutants. Similarly to the RNAi depletions, most mutants presented a significant reduction in their brood size, with the exception of *atn-1(prt87)* and *ani-1(prt129)* [35 ± 10 embryos in *atn-1(prt87)*, 39 ± 7 embryos *ani-1(prt129)* vs 38 ± 13 embryos in controls; Figure 3.5A].

Compared to the RNAi-depletion results, this reduction was more pronounced in the cases of *fln-1(prt102)* and the *unc-70(prt88);unc-70(RNAi)* [23 ± 10 embryos in *fln-1(prt102)*, 13 ± 6 embryos in *unc-70(prt88);unc-70(RNAi)*]. Double and triple mutants [*atn-1(prt87);plst-1(prt89)* and *atn-1(prt88);plst-1(prt89);unc-59(prt131)*, respectively] did not have a lower brood size than the single mutants [35 ± 10 embryos in *atn-1(prt87)*, 19 ± 8 embryos in *plst-1(prt89)*, 11 ± 9 embryos in *unc-59(prt131)*; 26 ± 11 embryos in *atn-1(prt87);plst-1(prt89)*, 11 ± 10 embryos in *atn-1(prt88);plst-1(prt89);unc-59(prt131)*].

A decrease in the embryonic viability of *plst-1(prt89)*, *unc-70(prt88);unc-70(RNAi)* and *sma-1(ru18)* animals was observed when compared to controls and RNAi-treated animals, indicating that RNAi depletions were not fully penetrant [52 ± 17 % in *plst-1(prt89)*, 38 ± 15 % in *unc-70(prt88);unc-70(RNAi)*, 58 ± 21 % in *sma-1(ru18)* vs 99 ± 3 % in controls; Figure 3.5B]. Similarly to the brood size, the embryonic viability of *atn-1(prt87)* and *ani-1(prt129)* animals was not affected [95 ± 7 in *atn-1(prt87)*, 98 ± 2 % in *ani-1(prt129)*]. The fact that the results obtained in *ani-1(prt129)* are not consistent with those obtained after *ani-1(RNAi)* is explained by the expression levels of ANI-1 in the mutant, which might be sufficient to allow proper oogenesis and embryogenesis. Although the brood size of the *unc-59(prt131)* mutants was reduced, the majority of the embryos produced were viable (98 ± 4 %; Figure 3.5B). Similar to what has been described in other *unc-59* alleles (Nguyen et al., 2000), some *unc-59(prt131)* animals displayed extruded gonads, protruded vulvas and were sick-looking and/or sterile, which explains the observed variability (Figures 3.2B-C, 3.5A). The embryonic viability of the *fln-1(prt102)* mutant was severely reduced, consistent with the results obtained after *fln-1(RNAi)* (9 ± 8 %). The double mutant *atn-1(prt87);plst-1(prt89)* animals did not present a significant difference in embryonic viability when compared to single *plst-1(prt89)* animals (58 ± 19 %). The triple mutant *atn-1(prt88);plst-1(prt89);unc-59(prt131)* animals presented slightly reduced embryonic viability when compared to the single *plst-1(prt89)* mutant (38 ± 32 %). These results reveal that most crosslinkers are involved in embryo production and development, suggesting a role in gonadogenesis, oogenesis and/or embryogenesis processes.

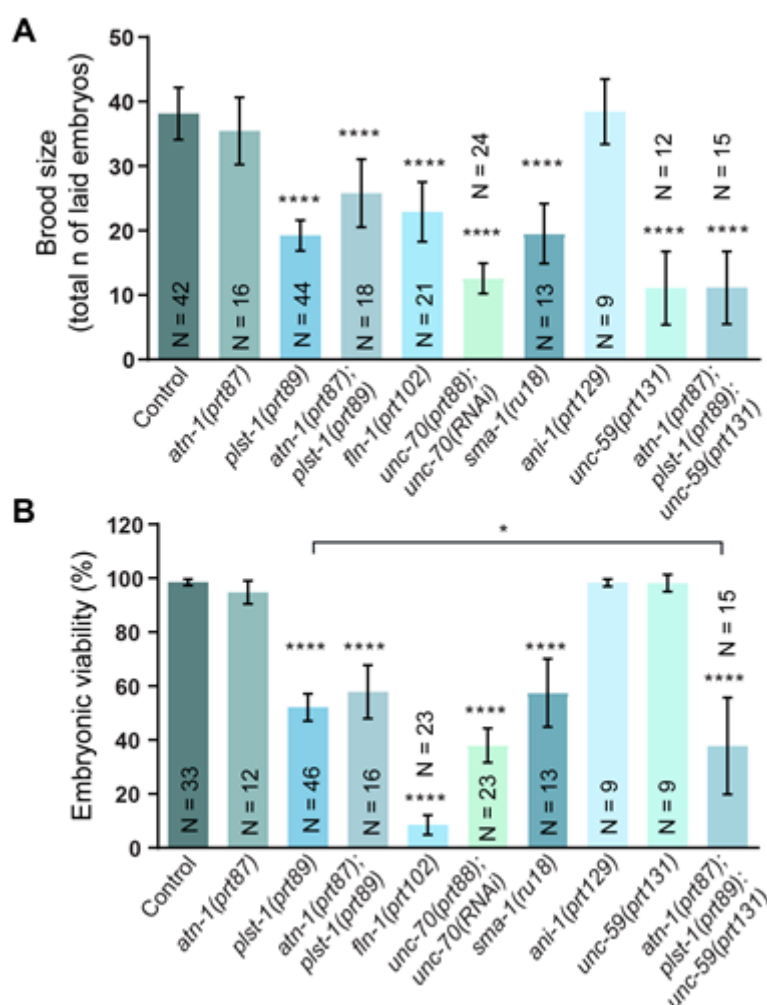


Figure 3.5. Impact of crosslinker mutations in animals' brood size and embryonic viability. (A) Brood size quantification (mean $\pm$ 95 % CI) in control or crosslinker mutant animals. Brood size corresponds to the total number of laid embryos over 24 hours. (B) Percentage of embryonic viability (mean $\pm$ 95 % CI) in control or crosslinker mutant animals. Embryonic viability corresponds to the percentage of laid embryos over 24 hours that is viable (number of hatched embryos over total number of progeny*100). N is the number of embryos analyzed. Statistical significance between control and each mutant was determined using one-way ANOVA followed by Dunnett's multiple comparison test: P values *P < 0.05, **** P<0.0001.

3.4.5. Inhibition of PLST-1, FLN-1 and ANI-1 impairs *C. elegans* gonad morphology

The *C. elegans* gonads have a tubular morphology composed of a distal arm, a turn and a proximal arm (Figure 3.6A). The distal gonad is a syncytium with peripherally arranged germ cells and a core called rachis. The peripheral germ cells are compartmentalized but not fully enclosed, with a rachis bridge that allows cytoplasmic exchange with the rachis. These compartments expand in size in the gonad's turn to become individualized into mature oocytes in the proximal gonad. Mature oocytes enter the spermatheca to be

fertilized and are stored in the uterus during initial embryonic development before they are laid through the vulva. The gonads' morphology and functioning relies on a robust actomyosin network, presumably interconnected by crosslinkers. In order to assess the role of each crosslinker in the gonad morphology, we imaged this tissue in the crosslinker mutants expressing fluorescent non-muscle myosin II (NMY-2::GFP) and histone H2B (mCherry::H2B), to allow the visualization of the actomyosin network and DNA, respectively.

The *plst-1(prt89)* adult animals showed a disorganized rachis in the distal arm of the gonad, with germ cell compartments distributing in more than one layer (compartment multilayer) [59 % of *plst-1(prt89)* vs 10 % of control animals], compartment loss [35 % of *plst-1(prt89)* vs 5 % of control animals] and compartments forming a bridge across the rachis cytoplasm [29 % of *plst-1(prt89)* vs 0 % of control animals]. The morphology of the rachis bridges was also disturbed, presenting irregular size and a more angular shape [76 % of *plst-1(prt89)* vs 0 % of control animals, Figure 3.6B]. The proximal gonad and oocytes were well organized, although an increased number of oocytes would be expected to be formed at this adult stage (~4 to 6 oocytes). A decreased number of oocytes might be explained by the slower growth rate of this mutant.

Consistent with the fact that ATN-1 was not detected in the gonad, the gonad morphology of the double mutant *atn-1(prt87);plst-1(prt89)* was similar to that in *plst-1(prt89)*. In 17 % of the analyzed gonads we noticed oocytes with variable size, which we have not noticed in *plst-1(prt89)* animals (Figure 3.6B).

The *fln-1(prt102)* animals presented a disorganized rachis, with germ cell compartment multilayering and loss of compartments in 33 % of the analyzed gonads. Moreover, the myosin levels seemed to be increased in the entire rachis surface when compared to controls. Premature compartment expansion was observed, with gonads presenting significant compartment enlargement before the turn instead of displaying a gradual increase in compartment's size in the turn (67 % of *fln-1(prt102)* vs 25 % of control animals). In the proximal gonad, damaged oocytes or embryos were observed in all animals (Figure 3.6B). Previous reports show that FLN-1 is necessary to allow the exit of fertilized oocytes from the spermatheca to the uterus and that *fln-1* mutant animals often lay dead or damaged embryos, or unfertilized oocytes, due to impaired functioning of the spermatheca (Kovacevic & Cram, 2010). Labeling with phalloidin (binds F-actin) in fixed animals confirmed that our *fln-1* mutant presented disturbed F-actin morphology in the spermatheca when compared to controls, and that the spermatheca often had one embryo trapped inside already undergoing the first cell division, confirming the defects in timely expelling the embryos into the uterus (Figure 3.6D).

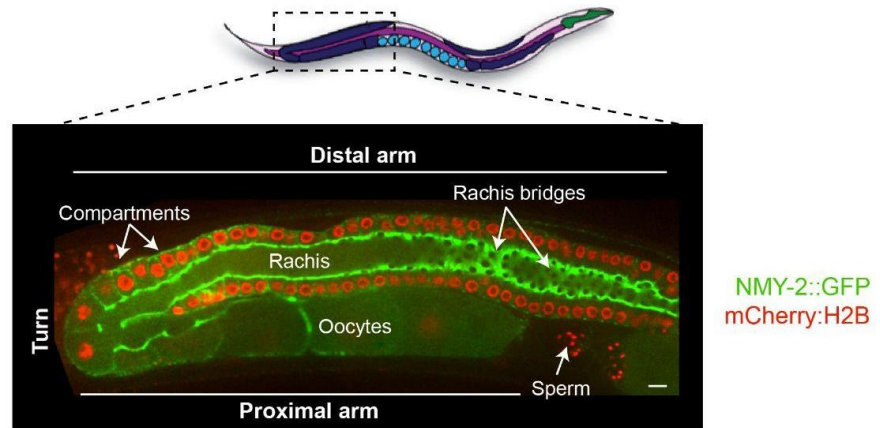
To test the impact of depleting ANI-1 in the gonad, we performed *ani-1(RNAi)* in *ani-1(prt129)* animals. *ani-1(prt129);ani-1(RNAi)* animals did not present significant defects in

the distal gonad but defects in the turn and proximal gonad were observed, such as premature compartment expansion and, more frequently, multinucleated oocytes with irregular sizes (Figure 3.6C). These animals had an abnormal accumulation of embryos inside the uterus (bagging phenotype), indicating an egg laying defect, possibly due to problems in the vulva, which was frequently protruded (Figure 3.6E).

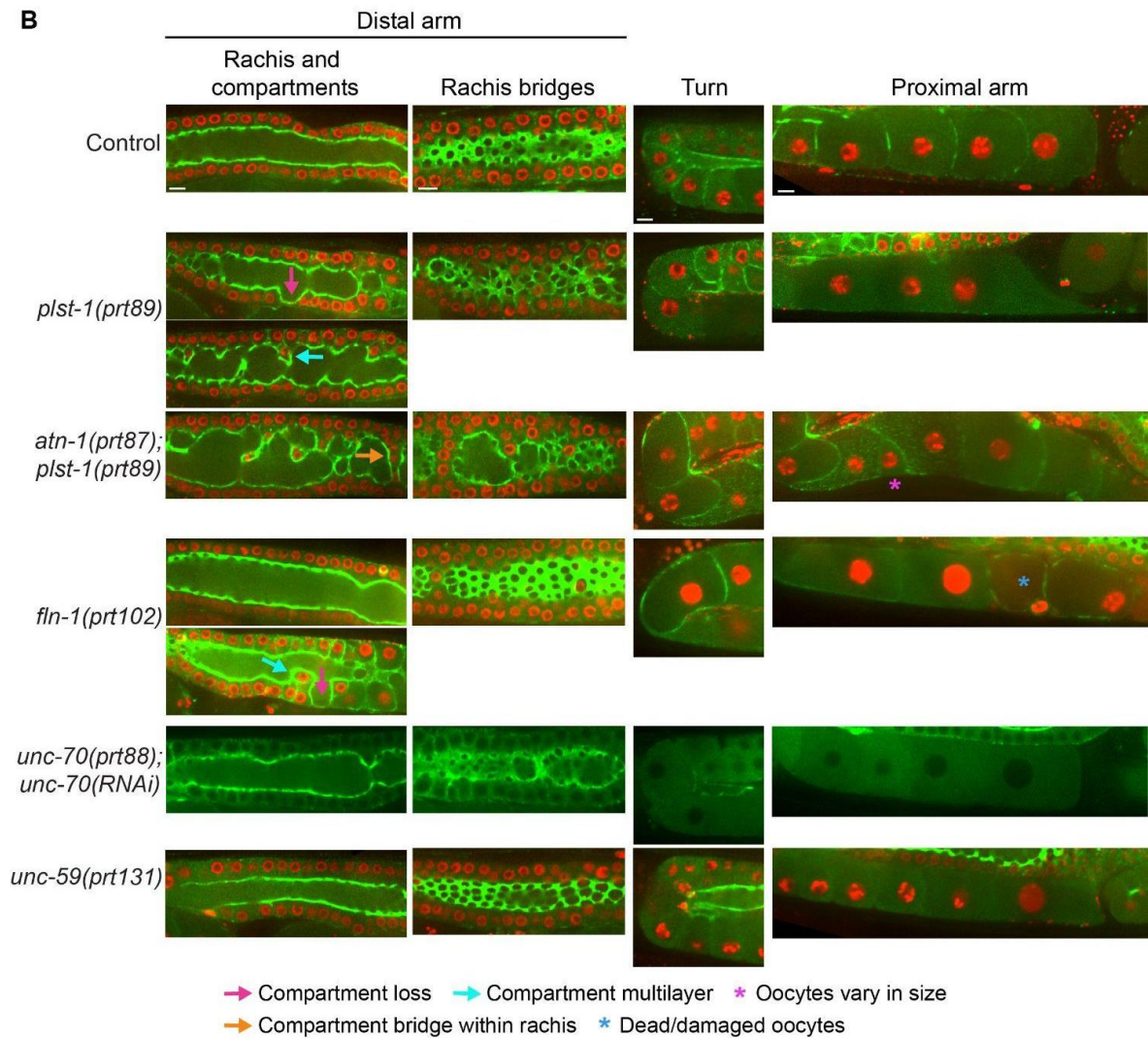
The *unc-70(prt88);unc-70(RNAi)* presented well-formed gonads (Figure 3.6B), but the F-actin bundles in the spermathecal cells of this mutant animals seemed to be slightly disoriented, less visible in the center and more prominent at the periphery, as reported in Whirshing et al., 2018 (Figure 3.6D). This mutant strain was not crossed with mCherry::H2B and the DNA cannot be observed. Despite the reduced embryonic lethality, the *unc-59(prt131)* and *sma-1(ru18)* mutants analysed did not present significant alterations in the gonads' morphology (Figure 3.6B-C). Nevertheless, as referred, several *unc-59(prt131)* mutant animals presented extruded gonads (not imaged), protruded vulvas, bagging phenotype (Figure 3.6E) or were sick-looking and/sterile animals, which is indicative of developmental defects. Due to the lack of mutants, we assessed *unc-61* and *cor-1* roles in gonads' morphology by RNAi-mediated depletion but no significant alterations were observed (Figure 3.6C).

These results indicate that PLST-1, FLN-1 and ANI-1 are the crosslinkers that most impact the morphology and actomyosin organization of the *C. elegans* gonad.

A



B



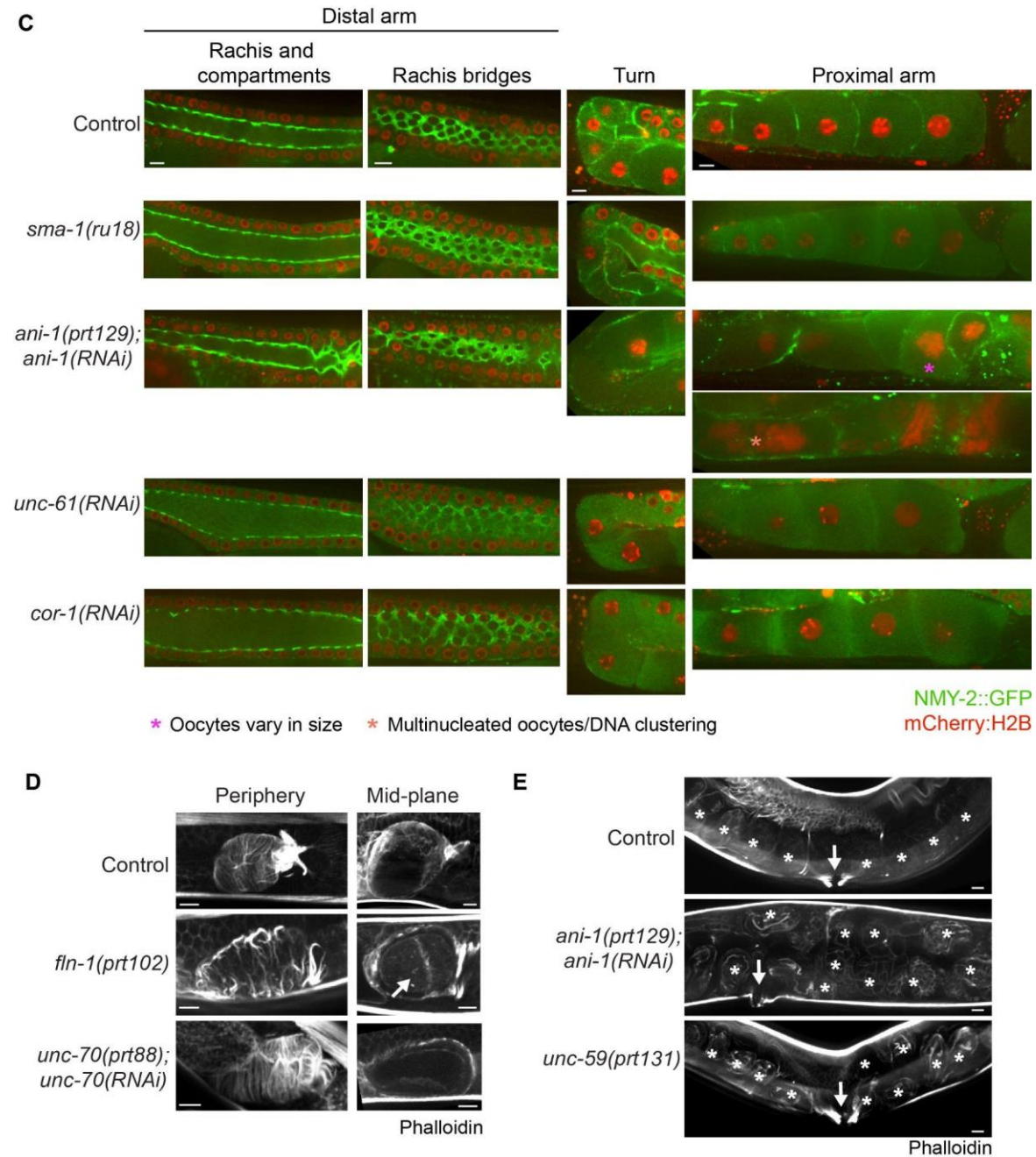


Figure 3.6. *C. elegans* gonad morphology in crosslinker mutant and/or depleted animals. (A) Central plane of an adult *C. elegans* hermaphrodite gonad. Non-muscle myosin II (NMY-2) is shown in green and DNA (H2B) in red. Different regions and processes throughout the gonad are depicted. (B,C) Selected stills of the distal arm, turn and proximal arm of control or mutant animals. Defective phenotypes are indicated by arrows or asterisks and labeled below the stills. Images of the distal arm are scaled equally. (D) Stills of the spermatheca in control, *fln-1(prt102)* and *unc-70(prt88);unc-70(RNAi)* animals stained with Phalloidin. Images of the cortical plane are shown on the left and of the central plane on the right. White arrow points to an embryo undergoing the first cell division inside the *fln-1(prt102)* spermatheca. (E) Stills of control, *ani-1(prt129);ani-1(RNAi)* or *unc-59(prt88)* animals stained with Phalloidin. White arrows point to the vulva [protruded in *ani-1(prt129);ani-1(RNAi)*] and asterisks represent the embryos inside the uterus. *ani-1(prt129);ani-1(RNAi)* and *unc-59(prt88)* animals have abnormal accumulation of late-stage embryos inside the uterus (bagging phenotype). Scale bars, 10 μ m.

3.4.6. ATN-1 is important for proper body wall muscle morphology in *C. elegans*

The *C. elegans* body wall muscles are responsible for the animal's movement, which is achieved through actomyosin contractility forces. The *C. elegans* body wall muscles are arranged in four quadrants, two dorsal and two ventral. Each quadrant is composed of a double layer of obliquely striated muscle cells, longitudinally arranged from head to tail (Figure 3.7A). To study the role of crosslinkers in the support and organization of the body wall muscle actomyosin network, we performed muscle F-actin staining with phalloidin in the crosslinker mutants and/or after crosslinker depletion by RNAi. Additionally, we quantified the animal body bending frequency in a liquid thrashing assay, as a read-out for proper animal movement.

Results demonstrated an impairment in the body wall muscle morphology of *atn-1(prt87)* animals. Although the overall muscle cells displayed a striated morphology, both ventral (not shown) and dorsal muscle filaments were curly and presented abnormal F-actin accumulation at the cell boundaries (Figure 3.7A). In the liquid thrashing assay, the *atn-1(prt87)* animals presented a slightly decreased body bending frequency [1.12 ± 0.33 Hz in *atn-1(prt87)* vs 1.48 ± 0.2 Hz in controls; Figure 3.7B]. These results are in agreement with previous published data, where similar phenotypes were reported for an *atn-1* null mutant (Moulder et al., 2010). None of the other single mutant or RNAi-depleted crosslinkers displayed significant morphological defects in the body wall muscles. Nevertheless, *unc-70(prt88);unc-70(RNAi)*, *sma-1(ru18)* and *unc-59(prt131)*, had a significantly decreased body bending frequency when compared to controls (0.73 ± 0.3 Hz, 0.9 ± 0.26 Hz and 0.86 ± 0.68 Hz, respectively; Figure 3.7B). Defects in the structural organization of body wall muscles, which presented reduced number and increased width of sarcomeres, impaired arrangement of the myofilament lattice (with clustered or absent thick filaments) and narrower dense bodies, were previously documented for an *unc-70* mutant, however we do not observe the same (Hammarlund et al., 2000). It could be that the mobility defects that we observe in *unc-70*, *sma-1* and *unc-59* mutants are due to minor defects in myofilament organization that we could not detect by phalloidin staining. Alternatively, those defects can be due to problems in the nervous system. Indeed, UNC-70 and UNC-59 are expressed in neurons (the former during embryogenesis and post-embryonic development, and the latter during larval development; Figure 3.1; Finger et al., 2003). In the case of *sma-1(ru18)*, the mobility defects may be due to the defective elongation of the embryo during embryogenesis, which can have implications on post-embryonic movement of the animal. Similar to the *atn-1(prt87)* animals, double and triple mutants *atn-1(prt87);plst-1(prt89)* and

atn-1(prt87);plst-1(prt89);unc-59(prt131) presented curly F-actin structures in the body wall muscles, as well as a reduction in their body bending frequency.

Altogether, these results suggest that ATN-1 is the main crosslinker involved in body wall muscles striated F-actin morphology and that proper animal movement also requires the septin UNC-59 and the spectrins UNC-70 and SMA-1.

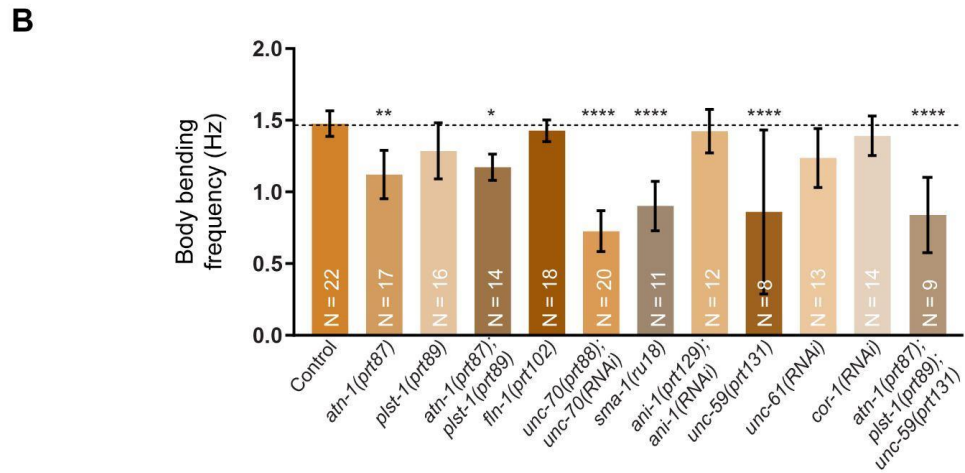
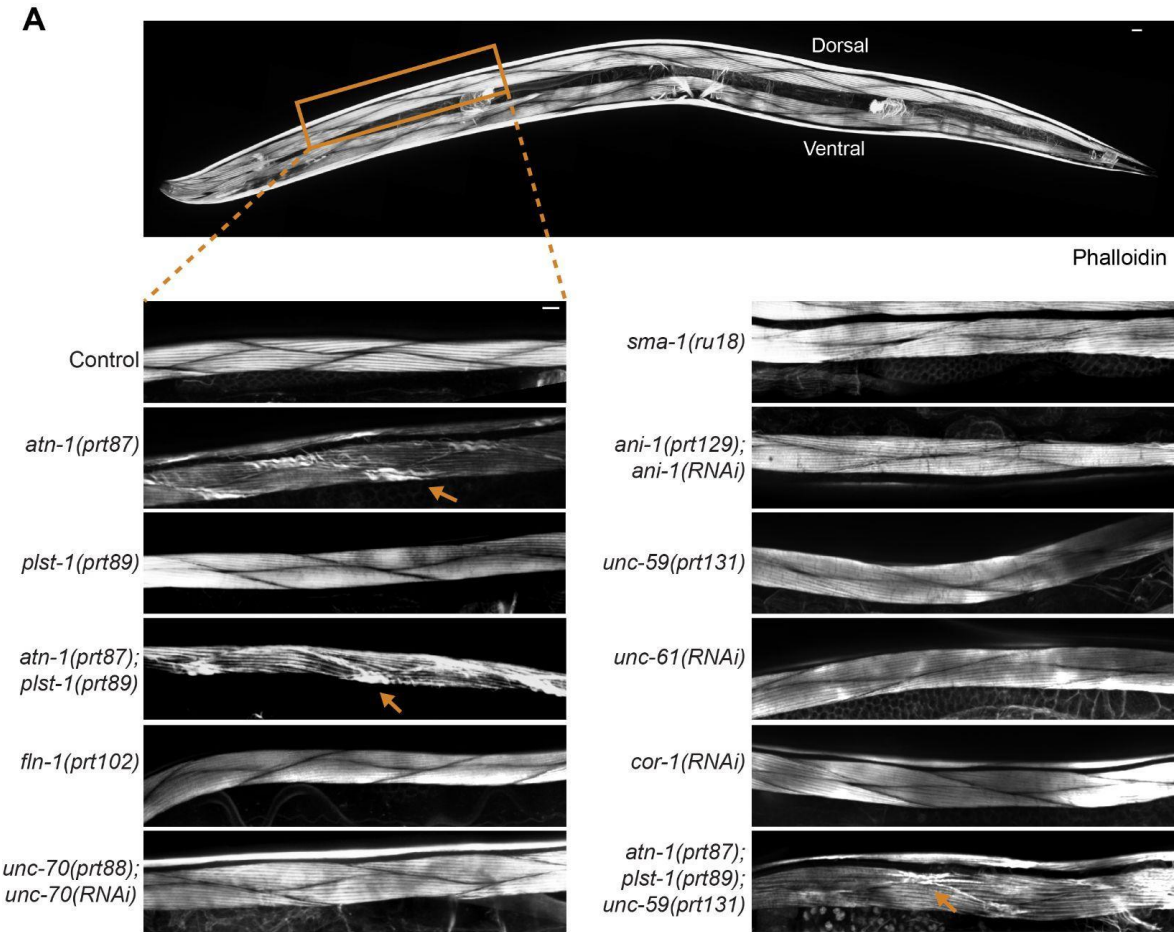


Figure 3.7. Body wall muscle morphology and animal mobility in crosslinkers' perturbations. (A) Phalloidin staining of the body wall muscles in a control animal (top). Orange box indicates the region from where magnifications of the dorsal muscle shown at the bottom were obtained. Orange arrows point at the cell boundaries where curly F-actin structures accumulate. (B) Analysis of body bending frequency in Hertz in controls or crosslinkers' perturbations. N is the number of embryos analyzed. Statistical significance between control and each mutant or RNAi condition was determined using one-way ANOVA followed by Dunnett's multiple comparison test: P values *P < 0.05, **P < 0.01, **** P<0.0001. Error bars represent 95 % CI. Scale bars, 10 μ m.

3.5. Discussion

The diversity of F-actin crosslinking proteins within an organism is presumed to be necessary for the formation of differently-organized F-actin networks. However, the contribution of each individual crosslinker to distinct actomyosin networks is not sufficiently investigated. Moreover, whether crosslinkers that co-localize and share structural similarities are able to play redundant roles or adjust their functionalities in the absence of other crosslinkers is underexplored. In this Chapter, we set out to explore the localization and contribution of a set of nine well-conserved crosslinkers in two actomyosin-dependent contexts *in vivo*, using *C. elegans* as a model organism.

In the past, the expression pattern of crosslinkers in living animals has been primarily assessed with the use of transgenic strains that did not necessarily express the crosslinkers at physiological levels. More recently, the CRISPR/Cas9 technique enabled the insertion of fluorescent tags in the endogenous loci of the genes of interest, which allow for more reliable studies of expression patterns in live-imaging. Here, we did a comprehensive examination and compilation of the localization patterns of several F-actin crosslinkers, using exclusively CRISPR/Cas9-generated strains. We observed that the examined crosslinkers have different expression patterns. Although several crosslinkers co-exist in the same tissue, some are exclusive of a certain context. For instance, ATN-1 was the only crosslinker detected in body wall muscles and UNC-70 was the only crosslinker strongly enriched throughout the nervous system, consistent with the idea that different crosslinkers play roles in distinct contexts. The specialization of crosslinkers may be due to their structural and biophysical characteristics: different crosslinkers have ABDs with different affinities for F-actin, which allied to their distinct sizes and ability to dimerize or tetramerize might favor binding under specific conditions only (Harris et al., 2019; Jananji et al., 2017; Winkelman et al., 2016); other functional domains, such as the PH domain that targets anillin and spectrins to membranes, may also affect crosslinker localization (Liu et al., 2012; Machnicka et al., 2014). Mechanosensing properties, expression of tissue-specific isoforms and distinct

binding partners are also sorting factors to different actomyosin processes (Duan et al., 2018; Stossel et al., 2001).

Due to their distinct localization patterns, indicative of a preferential involvement in specific contexts, different outcomes are expected when different crosslinkers are perturbed. Individual RNAi-mediated depletions of the crosslinkers under study revealed that the loss of PLST-1, FLN-1 and ANI-1 had a negative impact on the brood size and embryonic viability of the treated animals (as reported previously in Ding et al., 2017; Kovacevic & Cram, 2010; Maddox et al., 2005), which are indirect read-outs for impaired oogenesis and embryogenesis. Consistently, further analysis of the gonads' morphology and actomyosin organization revealed significant defects in the *plst-1*, *fln-1* and *ani-1* mutants. Along with the defects in the distal gonad of *plst-1(prt89)* animals, it is also described that *plst-1* mutant embryos have contractility and cytokinesis defects, and that these embryos frequently arrest during late-stages of embryogenesis (Chapter IV; Ding et al., 2017), thus PLST-1 seems to be important during both oogenesis and embryogenesis. The high embryonic lethality in *fln-1* mutant animals is likely due to the damage of the fertilized oocytes caused by the impaired functioning and actomyosin organization of the spermatheca (Kelley et al., 2020; Kovacevic & Cram, 2010). The *ani-1* mutant animals presented generally well-formed gonads but had a defective oogenesis, as abnormal oocytes (which presented large masses of diffuse DNA and multinucleation) were observed. Previous data with ANI-1 depletions showed well-formed gonads, but no defects in oocytes were observed and the embryonic lethality was attributed to defects in embryogenesis (Maddox et al., 2005). The fact that we depleted ANI-1 in an *ani-1* mutant may explain the aggravated loss-of-function phenotypes that we observed on this tissue. Although UNC-59::GFP is expressed in the gonads, we found this tissue to be well-formed in *unc-59(prt131)* animals. This might be explained by functional compensation by the other crosslinkers expressed in this tissue, and/or the other expressed crosslinkers perform more essential roles than UNC-59 in the gonads.

As previously reported, we also observed that UNC-70 is essential for life. Homozygous mutants of this crosslinker are unable to propagate past the first larval stage and balanced heterozygous mutants depleted of the wild-type copy lay fewer eggs, which are mostly dead. As we did not observe significant defects in the gonads' morphology of these RNAi-treated mutant animals, this suggests a role of this crosslinker during embryogenesis rather than gonadogenesis or oogenesis. The slightly disorganized F-actin network in the spermatheca of these animals may also indirectly account for further embryogenesis problems if the spermatheca's functioning gets impaired.

On the other hand, ATN-1 was shown to play a role in the maintenance of the body wall muscle morphology, similarly to its function in mammals and previously reported in *C.*

elegans (Moulder et al., 2010; Ribeiro et al., 2014), while other crosslinkers did not seem to be relevant in this tissue. Although previous data showed UNC-70 expression in muscles and myofilament organization defects in an *unc-70* mutant, we did not observe alterations in the striated morphology of the body wall muscles in *unc-70(prt88);unc-70(RNAi)* animals. Nevertheless, we did not examine myofilament organization, which requires electron microscopy analysis. We did observe a reduction in the ability of the animals to move in liquid (thrashing). This may be directly linked to myofilament defects in the body wall muscles' sarcomeres or might be indirectly caused by the loss of UNC-70 in the nervous system, which affects muscle functioning and animal mobility. Reports with *unc-70* mutants that were able to develop until adulthood showed defects in the morphology of sensory neurons and in their ability to respond to mechanical stimuli (Hammarlund et al., 2000; Krieg et al., 2014). Moreover, *unc-70* mutants were smaller and presented uncoordinated movement or paralysis (Hammarlund et al., 2000; Moorthy et al., 2000), spontaneous breakage of axons caused by the animal movement (Hammarlund et al., 2007; Krieg et al., 2014, 2017; Coakley et al., 2020) and defective ciliogenesis of ciliated neurons, which is required for the animal to "sense" the external environment (Jia et al., 2019). Therefore, the mobility reduction of *unc-70(prt88);unc-70(RNAi)* animals that we observed in the thrashing assay is likely to be a consequence of neuronal defects.

Loss of SMA-1 led to reduced brood size, embryonic viability and liquid thrashing. Nevertheless, we did not observe significant defects in the morphology of the gonad or body wall muscles in this crosslinker mutant. It is well documented that SMA-1 is necessary for proper embryonic elongation and animals lacking SMA-1 are smaller in size than wild-type animals, which may account for the reduced mobility of these animals (McKeown et al., 1998; Praitis et al., 2005). However, more detailed studies will be necessary to understand the defects observed in *sma-1(ru18)*.

The lack of UNC-59 also led to reduced brood size and thrashing. Since we did not observe defects in the morphology of the oocyte-producing gonad, reduced brood size could be because of problems during spermiogenesis, as this septin is known to be expressed in sperm (Chen et al., 2019). The reported impaired axonal migration and guidance during motor neurons' development may be responsible for the locomotion defects observed in *unc-59(prt131)*. Lack of UNC-59 has other detrimental effects on animal development, as previously referred. Mutant animals grow at a slower rate and display pleiotropic defects in different tissues, such as the vulva (several animals have a protruded vulva), male tail, and sensory neurons. Several *unc-59(131)* animals also present an extruded gonad, indicating a general reduced body integrity. The observed pleiotropic phenotypes may be attributed to the failure of post-embryonic cell divisions observed in other *unc-59* mutants and, in the case of the nervous system, to impaired cell migration and

pathfinding both in axons and distal tip cells (Finger et al., 2003; Nguyen et al., 2000). Similar pleiotropic phenotypes were observed in *unc-61* mutants, which is consistent with the fact that these two septins work as a complex (John et al., 2007; Nguyen et al., 2000). Yet, we did not observe the same phenotypes after *unc-61(RNAi)* possibly because the RNAi-mediated depletion was not penetrant.

The crosslinker COR-1 seemed to have non-essential roles in the studied contexts. Yet, it is known to be expressed in one-cell embryos and to localize and contribute to the migration of neuroblasts during larval development (Xie et al., 2021). Thus, it would be interesting to further explore the function of COR-1 in the context of embryonic development or in the nervous system of the adult animal.

Finally, in order to address potential redundant functions between crosslinkers, we crossed different mutant alleles. If two or more proteins perform the same function in a certain context, the removal of both is expected to cause worse phenotypes than each single inhibition. From the combinations obtained so far, the double *atn-1(prt87);plst-1(prt89)* mutant and the triple *atn-1(prt87);plst-1(prt89);unc-59(prt131)* did not display significantly aggravated phenotypes in the gonad or body wall muscle, when compared to the single mutants. The result with the double mutant can be explained by the fact that ATN-1 and PLST-1 do not co-express in these tissues. However, PLST-1 and UNC-59 are co-expressed in the gonad. In this case, this indicates that these two crosslinkers do not play redundant nor synergistic functions in the gonad. Yet further studies are required in other contexts. Taking into consideration their expression patterns, it would be of interest to examine these mutants in the context of the nervous system, as both ATN-1 and PLST-1 are apparently present in some nervous system structures and UNC-59 is known to play a role in the axonal migration (Finger et al., 2003). Moreover, the three seem to be expressed in the vulva, which is an attractive context to study organogenesis, tissue remodeling and cell-cell contacts. In general, it would be appealing to test combinations of crosslinker perturbations in contexts where crosslinkers co-localize.

We intended to take a step further from the previous studies by associating crosslinker function specifically to their ABDs in the case of ATN-1, PLST-1, FLN-1 and UNC-70, yet immunoblotting analysis will have to be done to assess protein expression levels in the corresponding mutants.

The contributions of crosslinkers in multiple actomyosin processes is transversal to other organisms: filamin and plastin in *D. melanogaster* both contribute, although differently, to the dynamic organization of the F-actin network during the cellularization process that occurs during embryogenesis. The actomyosin network during cellularization resembles the rachis bridges of *C. elegans*' gonad and it requires the transition of hexagonal-shaped canals into contractile rings that eventually individualize the cells after closing. Whereas

filamin knock-down in this system resulted in failure of forming the hexagonal-shaped canals and caused premature cell individualization, loss of plastin resulted in permanent hexagonal-shaped canals that constricted slowly (Krueger et al., 2019). Interestingly, we also observed that the rachis bridges of *plst-1(prt89)* were more angular-shaped rather than ring-shaped. Also in *D. melanogaster* muscle cells, β H-spectrin was shown to accumulate in regions of higher tension and to fence the F-actin network to promote the fusion between cells (Duan et al., 2018). Several other examples of human β -spectrin roles in cellular organization, F-actin network stability and signaling are well documented in erythrocytes, axons and epithelial cells (reviewed in Machnicka et al., 2012), indicating a role in specific cell types. Anillin and septins are reported as important protein scaffolds and regulators during cytokinesis from mammals to invertebrates, including *C. elegans* (Kinoshita et al., 1997; Nguyen et al., 2000; Oegema et al., 2000; Piekny & Maddox, 2010) and have also been implied in morphogenesis processes in *D. melanogaster*, for instance (Field et al., 2005). Thus, it seems that the diversity of crosslinkers is important to differently contribute to distinct processes/tissues but the same crosslinker can also act on various contexts.

In conclusion, our results and those of others show that PLST-1, FLN-1, ANI-1, SMA-1 and UNC-59 are important for proper oogenesis and/or embryogenesis, PLST-1, FLN-1 and ANI-1 are also necessary for the organization and maintenance of the actomyosin networks of the reproductive system, ATN-1 acts in the body wall muscles, UNC-70 is necessary for the maintenance of the F-actin network in the spermatheca, neuronal stability and ciliogenesis in ciliated neurons, and UNC-59 is needed for proper animal development (namely for cell division and proper motor neurons development) in *C. elegans*. Although we cannot rule out redundant functions, our results so far demonstrate that different crosslinkers contribute to the dynamic organization of F-actin architectures in distinct contexts *in vivo*. These results are in agreement with the crosslinkers' ability to build distinct actomyosin network conformations *in vitro* (Goode et al., 1999; Jananji et al., 2017; Mavrakis et al., 2014) and support the idea that the diversification of crosslinkers is necessary to optimize the cellular/tissue behavior to each situation. This study intended to expand the knowledge on these proteins, define their specific contributions and determine interplay between them in a more complex system with great translational potential to humans. The increasing knowledge of F-actin networks functioning is mandatory to understand disease conditions associated with defects in the actin cytoskeleton.

In the next chapters, we perform a thorough characterization of crosslinkers function during an essential and dynamic actomyosin-dependent process, cytokinesis. In the future, we will continue to explore the individual contribution of the different crosslinkers in other actomyosin networks, as the nervous system. Also, we will continue to test whether

crosslinkers can adjust their functionalities in situations where more than one crosslinker's function is affected.

3.6. References

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CHAPTER IV

PLASTIN AND SPECTRIN COOPERATE TO STABILIZE THE ACTOMYOSIN CORTEX DURING CYTOKINESIS

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4. PLASTIN AND SPECTRIN COOPERATE TO STABILIZE THE ACTOMYOSIN CORTEX DURING CYTOKINESIS

4.1. Abstract

Cytokinesis, the process that partitions the mother cell into two daughter cells, requires the assembly and constriction of an equatorial actomyosin network. Different types of non-motor F-actin crosslinkers localize to the network, but their functional contribution remains poorly understood. Here, we describe a synergy between the small rigid crosslinker plastin and the large flexible crosslinker spectrin in the *C. elegans* one-cell embryo. In contrast to single inhibitions, co-inhibition of plastin and the β H-spectrin (SMA-1) results in cytokinesis failure due to progressive disorganization and eventual collapse of the equatorial actomyosin network. Cortical localization dynamics of non-muscle myosin II in co-inhibited embryos mimic those observed after drug-induced F-actin depolymerization, suggesting that the combined action of plastin and spectrin stabilizes F-actin in the contractile ring. An *in silico* model predicts that spectrin is more efficient than plastin at stabilizing the ring and that ring formation is relatively insensitive to β H-spectrin length, which is confirmed *in vivo* with a *sma-1* mutant that lacks 11 of its 29 spectrin repeats. Our findings provide the first evidence that spectrin contributes to cytokinesis and highlight the importance of crosslinker interplay for actomyosin network integrity.

4.2. Introduction

The actin cytoskeleton is necessary for a wide range of cellular processes that require force generation and cell shape changes, including cell division, cell motility, and tissue morphogenesis (Blanchoin et al., 2014). Generation and propagation of contractile force requires myosin II motors and non-motor crosslinkers (crosslinkers hereafter). Bipolar myosin II filaments slide actin filaments past one another at the expense of ATP, while crosslinkers maintain F-actin network connectivity during filament sliding (Blanchoin et al., 2014).

Depending on their size, structural flexibility and concentration, crosslinkers bridge F-actin into networks of different complexities and mechanical properties (Lieleg et al., 2010). Smaller crosslinkers, such as α -actinin and plastin, tend to form tight parallel actin bundles, while larger crosslinkers, such as filamin and spectrins, organize F-actin into loosely crosslinked meshworks (Flanagan et al., 2001; Glenney et al., 1981; Pan et al., 2018;

Wachsstock et al., 1993). Crosslinkers may additionally differ in their subcellular localization and response to tension, and they may also have other functions apart from F-actin crosslinking. How F-actin networks organize and behave in the presence of multiple crosslinkers remains poorly explored.

During cytokinesis, animal cells assemble a contractile ring at the cell equator beneath the plasma membrane, and subsequent ring constriction ultimately leads to separation of the two daughter cells (Leite et al., 2019). The contractile ring consists of formin-nucleated non-branched F-actin that aligns circumferentially in a tight band. Besides non-muscle myosin II (hereafter myosin), other ring components include crosslinkers such as α -actinin, fimbrin/plastin (hereafter plastin), anillin, and septins (Leite et al., 2019). While myosin has long been known to be essential for cytokinesis (Leite et al., 2019), the role of crosslinkers remains unclear. When depleted individually, only cortexillins in *D. discoideum* and anillin in *D. melanogaster* and mammalian cultured cells have been shown to cause significant cytokinesis failure (Faix et al., 1996; Jananji et al., 2017; Piekny & Glotzer, 2008). However, cortexillins are not well conserved between species, and anillin is a multifunctional protein that interacts with several contractile ring components (Piekny & Glotzer, 2008), so its other functions also contribute to cytokinesis. The only known example of functional redundancy among crosslinkers during cytokinesis is between fission yeast α -actinin and fimbrin (Wu et al., 2001).

In the present study, we describe a synergy between plastin and spectrin, two crosslinkers that are highly conserved through evolution. Plastin and spectrin belong to the calponin homology (CH) domain superfamily of crosslinkers (like filamin, α -actinin, and dystrophin), whose actin-binding domains consist of a double calponin-like sequence.

Plastin is a small globular protein composed of two EF hands and two adjacent actin-binding domains (ABDs). Plastin binds branched and non-branched F-actin and assembles F-actin into tightly packed bundles in parallel or antiparallel orientation *in vitro* (Ding et al., 2017; Glenney et al., 1981; Matsudaira et al., 1983).

Plastin has been described to localize in cytokinetic rings, neurons, intestinal microvilli, hair cell stereocilia, and fibroblast filopodia, and to contribute to cytokinesis, invasion by pathogenic bacteria, endocytosis, and epidermal morphogenesis (Delanote et al., 2005; Ding et al., 2017; Leite et al., 2020; Shinomiya, 2012).

Spectrins are large and flexible crosslinkers that have not been previously implicated in cytokinesis. The functional form of spectrin consists of two antiparallel heterodimers of β -spectrin and α -spectrin (Machnicka et al., 2014). The resulting tetramers are elongated rods with ABDs at either end. Spectrins also contain a pleckstrin homology (PH) domain and

interact with plasma membrane-bound proteins, thus linking the actomyosin cytoskeleton to the plasma membrane (Machnicka et al., 2014). There are two types of β -spectrin, referred to as conventional β -spectrin and heavy β -spectrin (β H), which differ in the number of spectrin repeats and subcellular localization (Machnicka et al., 2012; Machnicka et al., 2014). Tetramers of conventional β -spectrin protect the cortex from mechanical stresses in erythrocytes and axons, where they form regular polygonal or longitudinal F-actin networks, respectively (Pan et al., 2018; Xu et al., 2013). They are also required for ciliogenesis and the ability to sense and respond to external mechanical stimuli in *C. elegans* (Jia et al., 2019; Krieg et al., 2014). β H-spectrin was shown to have mechanosensitive properties and to accumulate in regions of higher tension in *D. melanogaster* muscle cells (Duan et al., 2018). Furthermore, β H-spectrin is involved in embryonic development and tissue morphogenesis in *D. melanogaster* and *C. elegans* (McKeown et al., 1998; Zarnescu & Thomas, 1999).

C. elegans contains one ortholog each for plastin (*p/st-1*), α -spectrin (*spc-1*), conventional β -spectrin (*unc-70*), and β H-spectrin (*sma-1*) (Ding et al., 2017; McKeown et al., 1998; Moorthy et al., 2000; Norman & Moerman, 2002). This contrasts with vertebrates, which express three plastins, two α -spectrins, four β -spectrins, and one β H-spectrin in a tissue-specific manner (Machnicka et al., 2012; Machnicka et al., 2014; Shinomiya, 2012). The fact that these and other crosslinkers are represented by a single gene in *C. elegans* facilitates functional studies aimed at uncovering synergies among crosslinkers. Here, we find that, in contrast to single inhibitions, co-inhibition of plastin and β H-spectrin results in penetrant cytokinesis failure in the *C. elegans* one-cell embryo. Combining engineered β H-spectrin mutants with analysis of myosin and F-actin dynamics, we demonstrate that plastin and β H/ α -spectrin jointly organize and stabilize cortical F-actin at the cell equator, which is critical for contractile ring assembly. Our results emphasize the importance of understanding how different types of crosslinkers cooperate to organize actomyosin networks *in vivo*.

4.3. Methods (STAR Methods)

4.3.1. Key resources table

Table 4.1. Key Resources Table.

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti- α -tubulin	Sigma-Aldrich	Cat#T6199; RRID: AB_477583
Rabbit polyclonal anti-SMA-1 ABD	This paper	N/A

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Rabbit polyclonal anti-SMA-1 PH	This paper	N/A
Goat anti-rabbit IgG secondary HRP-linked	Jackson ImmunoResearch	Cat#111-035-003; RRID: AB_2313567
Goat anti-mouse IgG secondary HRP-linked	Jackson ImmunoResearch	Cat#115-035-003; RRID: AB_10015289
Donkey anti-rabbit IgG secondary labeled with Alexa Fluor 488	Jackson ImmunoResearch	Cat#711-545-152; RRID: AB_2313584
Donkey anti-mouse IgG secondary labeled with Alexa Fluor 594	Jackson ImmunoResearch	Cat#715-585-150; RRID: AB_2340854
Bacterial and virus strains		
<i>E. coli</i> OP50 <i>C. elegans</i> food source	Caenorhabditis Genetics Center	OP50
<i>E. coli</i> HT115 RNAi bacteria (targeting <i>sma-1</i>)	This study	R31.1 (ORF-ID)
<i>E. coli</i> HT115 RNAi bacteria (targeting <i>frm-1</i>)	Source Bioscience (Ahringer library)	ZK270.2 (ORF-ID)
<i>E. coli</i> HT115 RNAi bacteria (targeting <i>unc-44</i>)	Source Bioscience (Ahringer library)	B0350.2 (ORF-ID)
<i>E. coli</i> HT115 RNAi bacteria (targeting <i>perm-1</i>)	Source Bioscience (Ahringer library)	T01H3.4 (ORF-ID)
Chemicals, peptides, and recombinant proteins		
Recombinant Cas9 protein	In-house	N/A
Prescission protease	In-house	N/A
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	NZYTech	Cat#MB026
Latrunculin A	Sigma-Aldrich	Cat#L5163
FM4-64	Invitrogen	Cat#T13320
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific	Cat#32106
Nitrocellulose membrane	Amersham	Cat#GE10600001
Triton X-100	BDH Prolabo	Cat#437002A
Tween	BDH Prolabo	Cat#437082Q
Deposited data		
Code to run the simulation is available at https://gitlab.com/JulioMBeImonte/cytokinesis2021	N/A	N/A
Experimental models: Organisms/strains		
<i>C. elegans</i> N2 Bristol	Caenorhabditis Genetics Center (CGC)	N2
ItIs157 [pAC16;pie-1/LifeAct::GFP; unc-119 (+)]; ItIs37 [pAA64; pie-1/mCherry::	Our lab	GCP21

his-58; unc-119 (+)]; unc-119(ed3) III (?)		
ItIs157 [pAC16;pie-1/LifeAct::GFP; unc-119 (+)]; unc-119(ed3) III (?); prtSi2[pAC71;Pnmy-2:nmy-2reencoded::mCherry::StrepTagII::3'UTR-nmy-2; cb-unc-119(+)]II	Our lab	GCP22
nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	Our lab	GCP113
plst-1(prt89) IV	Our lab	GCP564
plst-1(prt89) IV; nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This study	GCP570
atn-1(prt87)V; plst-1(prt89) IV; nmy-2(cp13[nmy-2::GFP +LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This study	GCP602
plst-1(prt89) IV; ijmSi8[pJD362; Pmex-5::gfp::tbb-2; mCherry::his-11; cb-unc-119(+)] II; unc-119(ed3) III (?)	This study	GCP646
ItSi1188[pDC600; Pmex-5::GFP1-10::3'UTR-tbb-2::gpd-2/3 operon linker::mCherry-his-15::3'UTR-unc-54; cb-unc-119(+)] II; unc-119(ed3) III (this is OD4016 after 6 outcrosses)	This study	GCP794
sma-1ΔPH(prt133)V	This study	GCP808
sma-1ΔPH(prt133) V; nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; Ppie-1::mCherry::his-58; unc-119 (+)]	This study	GCP809
plst-1(msn190[plst-1::GFP]) IV; nmy-2(cp52[nmy-2::mKate2+ LoxP unc-119(+)] LoxP]; ItIs37 [pAA64; pie-1/mCherry::his-58 +unc-119(+)]; unc-119(ed3) III (?)	This study	GCP831
[plst-1(msn190[plst-1::GFP])] IV; ItIs37 [pAA64; pie-1/mCherry::his-58 +unc-119(+); unc-119(ed3) III (?)	This study	GCP832
plst-1(prt89) IV; sma-1ΔPH(prt133) V; nmy-2(cp13[nmy-2::GFP +LoxP]) I;	This study	GCP838

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unc-119(ed3) III (?); ItIs37 [pAA64; pie-1mCherry::his-58; unc-119 (+)] IV		
sma-1ΔABD#1(prt156) V	This study	GCP901
plst-1(prt89) IV; ItIs157 [pAC16; pie-1/LifeAct::GFP; unc-119 (+)]; prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'UTR-nmy-2; cb-unc-119(+)] II; unc-119(ed3) III (?)	Our lab	GCP927
sma-1ΔSH3(prt163) V	This study	GCP986
sma-1::6xGFP11(prt159) V; ItSi1188 [pDC600; Pmex-5::GFP1-10::3'UTR-tbb-2::gpd-2/3 operon linker::mCherry-his-15::3'UTR-unc-54; cb-unc-119(+)] II; unc-119(ed3) III (?); nmy-2(cp52[nmy-2::mKate2+LoxP unc-119(+)] LoxP) I	This study	GCP991
plst-1(prt89) IV; sma-1ΔSH3(prt163) V; nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)] IV	This study	GCP1014
sma-1ΔSH3(prt163) V; unc-119(ed3) III (?); nmy-2(cp13[nmy-2::GFP+LoxP]) I; ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)] IV	This study	GCP1036
sma-1Δ11SR(prt192) V	This study	GCP1102
sma-1ΔABD#2(prt185) V	This study	GCP1138
ItIs157 [pAC16;pie-1/LifeAct::GFP; unc-119 (+)]; unc-119(ed3) III (?); prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'UTR-nmy-2; cb-unc-119(+)] II; sma-1ΔSH3 (prt163) V	This study	GCP1163
plst-1(prt89) IV; ItIs157 [pAC16; pie-1/LifeAct::GFP; unc-119 (+)]; prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'UTR-nmy-2; cb-unc-119(+)] II; unc-119(ed3) III (?); sma-1ΔSH3 (prt163) V	This study	GCP1164
ItIs157 [pAC16;pie-1/LifeAct::GFP; unc-119 (+)]; unc-119(ed3) III (?); prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'UTR-nmy-2; cb-unc-119(+)] II; sma-1ΔSH3 (prt163) V	This study	GCP1169

TR-nmy-2; cb-unc-119(+) II; sma-1ΔPH(prt133) V		
plst-1(prt89) IV; ltIs157 [pAC16; pie-1/LifeAct::GFP; unc-119 (+)]; prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'U TR-nmy-2; cb-unc-119(+)] II; unc-119(ed3) III (?); sma-1ΔPH(prt133) V	This study	GCP1170
ltIs157 [pAC16;pie-1/LifeAct::GFP; unc-119 (+)]; unc-119(ed3) III (?); prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'U TR-nmy-2; cb-unc-119(+)] II; sma-1 ΔSR11(prt192)V	This study	GCP1176
plst-1(prt89) IV; ltIs157 [pAC16; pie-1/LifeAct::GFP; unc-119 (+)]; prtSi2[pAC71;Pnmy-2::nmy-2reencoded::mCherry::StrepTagII::3'U TR-nmy-2; cb-unc-119(+)] II; unc-119(ed3)III (?); sma-1 ΔSR11 (prt192) V	This study	GCP1187
sma-1::splitGFP (prt159) V; ltSi1188[pDC600; Pmex-5::sGFPS1-10::tbb-2_3'UTR::gpd-2/3 operon linker::mCherry-his-15::unc-54-3'UTR; cb-unc-119(+)] II; unc-119(ed3) III (?); plst-1(prt198(plst-1::linker::mCherry)) IV	This study	GCP1207
sma-1::splitGFP(prt159) V; ltSi1188[pDC600; Pmex-5::sGFPS1-10::tbb-2_3'UTR::gpd-2/3 operon linker::mCherry-his-15::unc-54-3'UTR; cb-unc-119(+)] II; unc-119(ed3) III (?); zbls2(pie-1::lifeAct::RFP)	This study	GCP1208
plst-1(prt198(plst-1::mCherry)) IV	This study	GCP1209
sma-1(ru18) V	CGC	AZ30
ijmSi8[pJD362; Pmex-5::GFP::tbb-2; mCherry::his-11; cb-unc-119(+)] II; unc-119(ed3) III	Dumont Lab	JDU21
unc-119(ed3) III; ltIs20 [pASM10; pie-1/GFP::unc-59; unc-119 (+)]	OD Lab	OD26
unc-119(ed3) III; ltIs28 [pASM14; pie-1/GFP-TEV-StrepTagII::ANI-1; unc-119	OD Lab	OD130

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(+)); ItIs37 [pAA64; pie-1::mCherry::his-58; unc-119 (+)]		
ItSi1188 [pDC600; Pmex-5::GFP1-10::3'UTR-tbb-2::gpd-2/3 operon linker::mCherry::his-15::3'UTR-unc-54; cb-unc-119(+)] II; unc-119(ed3) III	OD Lab	OD4016
spc-1(cas815[spc-1::GFP]) X	Ou Lab	GOU2936
unc-70(cas962[GFP::unc-70]) V	Ou Lab	GOU3103
plst-1(msn190[plst-1::GFP]) IV	Zaidel-Bar Lab	RZB213
plst-1(msn190[plst-1::gfp]) IV; zbls2 (pie-1::LifeAct::RFP)	Zaidel-Bar Lab	RZB217
erm-1(mib15[erm-1::eGFP]) I	Boxem Lab	BOX213
Oligonucleotides		
See Table S4.2	N/A	N/A
Recombinant DNA		
Expression vector L4440	Bioscience/ Ahringer library	L4440
Plasmid pJA58	Fire Lab/ Addgene	CAS 59933
Plasmid pAC571	This study	pAC571
Plasmid pDC592	Cheerambathur Lab	pDC592
Plasmid pDD162	Goldstein Lab	pDD162
Expression vector for sma-1 ABD - pGEX-6P1_GST_TEV_sma-1_ABD (see STAR Methods for details)	This study	pAC543
Expression vector for sma-1 PH - pGEX-6P1_GST_TEV_sma-1_PH (see STAR Methods for details)	This study	pAC553
Software and algorithms		
ImageJ (Fiji)	Schindelin et al. 2012	https://imagej.net/software/fiji/ ; RRID: SCR_003070
GraphPad Prism	GraphPad Software	http://www.graphpad.com/ ; RRID: SCR_002798
Cytosim	Nédélec & Foethke, 2007	https://gitlab.com/JulioMBelmonte/cytosim
MagicPlotPro	Magicplot Systems, LLC	https://magicplot.com
MATLAB / Particle Image Velocimetry (PIV)	MathWorks	https://openresearchsoftware.metajnl.com/articles/10.5334/jors.bl/ ; RRID: SCR_001622

4.3.2. Experimental model and subject details

***C. elegans* strains**

The *C. elegans* strains used in this study are listed in the Key resources table. Strains were maintained at 20°C on nematode growth medium (NGM, for 1 L: 3 g NaCl, 17 g agar, and 2.5 g peptone, plus 1 mL of 1 M CaCl₂, 1 mL 5 mg/mL cholesterol in ethanol, 1 mL of 1 M MgSO₄ and 25 mL of 1 M KH₂PO₄ pH 6 after autoclaving at 110°C for 30 minutes and cooling down to 55°C) plates seeded with *Escherichia coli* OP50.

4.3.3. Method details

RNA interference

RNAi was performed either by feeding animals with *E. coli* HT115 bacteria expressing double stranded RNA (dsRNA) or by injecting animals with dsRNA.

To produce dsRNA for injection, primers with tails containing T3 and T7 RNA polymerase promoters were used to amplify the desired regions from N2 cDNA (Table S4.2). The PCR products were transcribed with T3 or T7 RNA polymerases (MEGAscript Transcription Kit; ThermoFischer Scientific). Transcription reactions were cleaned up (NucleoSpin RNA Clean-up, Macherey-Nagel) and diluted with 3 × soaking buffer (32.7 mM Na₂HPO₄, 16.5 mM KH₂PO₄, 6.3 mM NaCl, and 14.1 mM NH₄Cl). Injection of dsRNA at ~1 µg/µl was performed in L4-stage animals of strains GCP113 or GCP570, and the animals were incubated at 20°C for 48-54 hours prior to dissection for imaging. RNAi by injection was used to deplete ATN-1, PLST-1, FLN-1, UNC-70, UNC-59, ANI-1, and ERM-1. In the case of *fln-1(RNAi)*, dsRNAs fln-1#1 and fln-1#2 were mixed together to cover all isoforms.

For RNAi by feeding, we used the RNAi feeding vector L4440 transformed into *E. coli* HT115. L4440 vectors for RNAi of *cor-1*, *frm-1*, *unc-44* or *perm-1* were obtained from the Ahringer library (Source Bioscience) and sequenced to confirm the gene target. L4440 for *sma-1* RNAi was generated by inserting a *sma-1* fragment amplified from N2 cDNA (primers 5'-CCCCCGATATCGACACCCTTTCAAATG-3' and 5'-CCCCCGATATCCCTTCTGTTCCATCTCGTTTC-3') into the EcoRV restriction enzyme site. The bacteria were grown overnight at 37°C in 50 mL Luria broth medium containing 12.5 µg/ml tetracycline and 100 µg/ml ampicillin. The culture was centrifuged for 10 minutes

at 2,500 g. The cell pellet was resuspended in 2.5 mL Luria broth medium containing 12.5 µg/ml tetracycline, 100 µg/ml ampicillin, and 1 mM IPTG. 75 µl were used to inoculate NGM plates to which 100 µl of a 1:1:1 mix of 100 mg/ml ampicillin, 1 M IPTG, and 5 mg/ml tetracycline had been added 2 hours prior. The expression of double-stranded RNA was induced overnight at room temperature and in the dark. 25-30 L4-stage animals of strains GCP22, GCP113, GCP570, GCP646, GCP927 or GCP1170 were added to the plates and incubated at 20°C for 48-54 hours prior to dissection for imaging.

Generation of mutants using CRISPR/Cas9

Guide RNAs and repair templates used to edit the *C. elegans* genome, as well as diagnostic primers used to screen for positive editing events, are listed in Table S4.2. Modified regions were confirmed by sequencing. To remove potential off-target mutations, modified animals were subjected to six rounds of outcrossing with wild-type N2 animals. Other modifications (e.g., fluorescent markers) were subsequently introduced by mating.

For generation of *sma-1ΔPH*, *sma-1ΔABD#1* and *atn-1ΔABD*, we used a plasmid-based approach, in which a single guide RNA (sgRNA) and the Cas9 protein are co-expressed from pDD162 (Dickinson et al., 2013). Guide sequences were chosen using the online tools <https://zlab.bio/guide-design-resources> and <http://crispor.tefor.net/>. Single-stranded repair templates (Integrated DNA Technologies) included 50-60 bp homology regions flanking the Cas9 cleavage site and, when necessary, silent mutations to avoid repair template recognition by Cas9. For *sma-1ΔABD#1*, the intended modification was the removal of the entire ABD. However, the last 14 residues of the CH2 were retained. The sequence of the modified region is as follows: 5'-cagGTTTCGTGTTCCAACAAGTGGTGCACCACCAGTTCGCGCAGATGCCAATGGAACAGATCAAGACGAGTTCAATAATGAGACACTGTACTTTGAAAGATCACGCATTCGGACATCAATCATTACATATGTTTCCTTGTAATCATCATTTTGCAAAGCAAAGACTGAGATGACAGGAGCTCGACGTATTGCTAATgtaagtgttataagttcatg-3' (bases in lowercase indicate intron regions).

For *sma-1ΔSH3*, *sma-1ΔABD#2*, and *sma-1Δ11SR*, we injected a ribonucleoprotein (RNP) mix consisting of crRNA/tracrRNA (CRISPR/Cas9 design tool https://www.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN; Integrated DNA Technologies) and purified recombinant Cas9 protein. The repair template for *sma-1ΔABD#2* was partially single-stranded (assembled by overlap extension PCR; Dokshin et al., 2018) and consisted of 120-bp homology regions flanking the Cas9 cleavage site, silent mutations to avoid repair template recognition by Cas9, and the *sma-1* intron between

exons 3 and 4. For *sma-1ΔSH3* and *sma-1Δ11SR*, the single-stranded repair template included 50-bp homology regions flanking the Cas9 cleavage site. For *sma-1ΔABD#2*, the intended modification was the removal of the CH1 domain (part of exon 3 and exon 4) leaving the intron between exons 3 and 4 but not the intron between exons 4 and 5. However, sequencing of the modified region revealed that the first 17 residues of CH1 were retained (retains part of exon 3, entire intron between exons 3 and 4, and part of exon 5): 5'-

aaagttttaagatccgaaaaaaaactgagaacgtagaataatttaacagttcaattagcatcaagttcataatgatttttc
agGTTTCGTGTTCCAACAAGTGGTGCACCACCAGTTCGCGCAGATGCCAATGGAACAG
ATCAAGACGAGTTCAATAATGAGACACTGTACTTTGAAAGATCACGCATTCCGGACACT
GCAGGACGAACGTGTGCACATTCAGAAGAAGACGTTTCAAAAATGGTGCAACTCCTT
TCTTAATCGG- intron3/4 as in *sma-1* transcript R31.1a, Wormbase version WS279 -
GATGAGGAAGAGCGTGGAGAGCGAAAACATGCCAAAGATGCCTTGCTGTTGTGGTG
TCAGAGAAAAACAGCTGGATATCCAAATGTTTCGCATCGAAAACCTTCACTACAAGTTGG
-3'. For *sma-1Δ11SR* the intended modification was the removal of 13 spectrin-repeats,
however only 11 spectrin repeats were removed (mid spectrin repeat #14 to mid spectrin
repeat #25). The obtained sequence is as follows: 5'-
CGTGACGTTGACGAGTTTGAGCAATGGATGGCAGACAAAATGGCCAACATGCCACG
CTCACACGAACTGTACGAGATTGATCGCTTGAAGAAGCGTGCGGATGGACTTCTAGC
CAGAGAGCATCACGATGCAATGTCGATTGCTGCGAAACAACGCAAACTGGAAGCATT
GTTCCGAGACCTCTGCAGAGAATGTGCCAGACGACGAACTCAAATTGTTGATGCTTC
CAAATATCACAAGTTTGTAGACAAGCCGATGATTTGTCAGACTGGCTACGTGAGAAA
GAAAGGTCCGCATCAGCTGAGGATTACGGTCAAGATTTGGAGGATTGCCAACAAATT
ATTGAGCAGTTTGAATCTACAGTTTCGTGAATTGGCAGCTGCTGGAGAGCGTGTTGCT
GCTGTCCAACGTGCACAAGAAGATCTTCTACGAAGTGGACATCCATATGGAGCATCA
ATCACTGCAAAAGGCGCTGATGTTTCAGAGACTATGGACTCATGTCAATGAAGTGGCT
AATGAGAGAAAGCAAGCGTTGAATGGAGCTCGTCAAGTTCATCGATTTCGACCAAGAA
GCCGATCAGATATTGAATTGGTTACAAGATAAGGAAGCGACTGGAGTAGCAATGGAA
CAGGAAGATCTTTCGAGAGCTGATCTTGCTTCCGTGAA-3' (14 base pairs in italics
replaced the region removed from mid spectrin repeat #14 to mid spectrin repeat #25, and
did not cause a frameshift).

In all cases repair templates and pDD162 or RNPs were injected into gonads of young adult N2 hermaphrodites. To facilitate the identification of successfully injected animals, injection mixes also contained a repair template and pJA58 (a gift from Andrew Fire, Addgene plasmid # 59933; <http://addgene.org/59933>; RRID: Addgene_59933) or crRNA to

generate the R92C mutation in *dpy-10*, which causes a dominant roller phenotype in mutated F1 progeny (Arribere et al., 2014).

Tagging of endogenous SMA-1 and PLST-1

Guide RNAs and repair templates used, as well as diagnostic primers used to screen for positive editing events, are listed in Table S4.2. All modified regions were confirmed by sequencing.

To generate an endogenous fluorescent version of SMA-1, we used the self-complementing split GFP system Kamiyama et al., 2016. We inserted 6 copies of GFP11 in tandem downstream of the *sma-1* open reading frame using plasmid-based CRISPR/Cas9-mediated genome editing, as described above. The plasmid template used for homology recombination was pAC571, which was built using Gibson assembly (Gibson et al., 2009). The fragments used for pAC571 assembly were as follows: pBluescript backbone, amplified with primers 5'-CATGTGAGCAAAAGGCCAGC-3' and 5'-ATCGCCCTTCCCAACAGTTG-3'; 1 kb left homology region in the *sma-1* open reading frame, amplified from N2 genomic DNA (gDNA) with primers 5'-TCAGGCTGCGCAACTGTTGGGAAGGGCGATAGCTTTCTTCCCTCCGTC-3' and 5'-CTTTGAATGTTTGGATCCACGTTT**AAACAAGGATCCGAATCCTTTTCGTTCTG**-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9); 6xGFP11, amplified from pDC592 with primers 5'-**CTTGTTTAAACGTGGATCCAAACATTCAAAGGGAGGGAGGGCCGGCTCTG**-3' and 5'-GGTGATTCCAGCAGCGTTAACGTACTCAT-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9; bases in italics indicate part of the linker region); and 1 kb right homology region in the *sma-1* 3'UTR, amplified from N2 gDNA with primers 5'-ATGAGTACGTTAACGCTGCTGGAATCACCTAAATACGTCACCACACGCTGATCTTC-3' and 5'-CTGGCCTTTTGCTGGCCTTTTGCTCACATGCCATTTTGTGATTGCTC-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9). Animals expressing SMA-1::6xGFP11 were crossed with animals expressing GFP1-10 from the *mex-5* promoter (GCP794 strain). GCP794 was obtained by subjecting the strain OD4016 (kindly provided by Arshad Desai) to six rounds of outcrossing with N2 animals.

To generate an endogenous mCherry-tagged version of PLST-1 (PLST-1::mCherry), we generated a partially single-stranded repair template (hybrid template), as in Dokshin et al., 2018. The repair template consisted of 120-145-bp homology regions flanking the

Cas9 cleavage site, silent mutations to avoid repair template recognition by Cas9, and a 10 amino acid linker followed by the mCherry tag. The long fragment of the repair template was amplified with the primers: 5' **aaaatcg**tccaaaaccccaaattgttttcagGTGAAGCCAAAAATGGTGTGACAGTGTTCATGTCTAATGGCTCGTGACTATCTTCCCGATATGAAGCAAGGAGCAGCTTCTGCTCC**GATC**GT**CCCA**ATGAT**CAAT**GGAAATGAGGACCCTTGGAGGGTACCGGTAGAAAAAATGGTCTCAAAGGGTGAAGAAGATA-3' (145 bp of homology region in the *p/st-1* open reading frame; bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9; bases in italics correspond to the 10 amino acid linker, bases in lowercase indicate introns) and 5' aattttgtgagaaaaaagatggaaaaacgggaaaatagagatatatatggaggaaaattgtatttcgcgcgattttttcggcgggaaattcaagaaattcgaaattttgcagaaCTACTTATACAATTCATCCATGCCACC-3' (120 bp of homology region in the *p/st-1* 3' UTR; underlined bases in lowercase indicate 3'UTR region). The short fragment of the repair template was amplified with the following primers: 5' **GATCGTCCCA**ATGAT**CAAT**GGAAATGAGGACCCTTGGAGGGTACCGGTAGAAAAATGGTCTCAAAGGGTGAAGAAGATA-3' (bases in bold indicate silent mutations introduced to avoid repair template recognition by Cas9; bases in italic indicate the 10 amino acid linker) and 5' CTACTTATACAATTCATCCATGCCACC-3'. Animals expressing PLST-1::mCherry were subjected to six rounds of outcrossing with N2 animals.

Live imaging

Gravid hermaphrodites were dissected and one-cell embryos were mounted in a drop of M9 (86 mM NaCl, 42 mM Na₂HPO₄, 22 mM KH₂PO₄ and 1 mM MgSO₄) on 2% agarose pads overlaid with a coverslip. Live imaging was performed at 20°C.

Images in Figures 4.1, 4.3D, 4.4, 4.5, 4.6, 4.7, S4.1B, S4.1E, and S4.3–S4.6 were acquired on a spinning disk confocal system (Andor Revolution XD Confocal System; Andor Technology) with a confocal scanner unit (CSU-X1; Yokogawa Electric Corporation) mounted on an inverted microscope (Ti-E, Nikon) equipped with a 60x oil-immersion Plan-Apochromat objective (N.A. 1.4), extra 1.5x magnification, and solid-state lasers of 488 nm (50 mW) and 561 nm (50 mW). For image acquisition, an electron multiplication back-thinned charge coupled device camera (iXon Ultra 897; Andor Technology) was used. Acquisition parameters, shutters and focus were controlled by Andor iQ3 software. Images in Figures 3 and S7A were acquired on a similar system equipped with a confocal scanner unit (CSU-22; Yokogawa Electric Corporation), an iXon EM+ DU-897 (Andor Technology),

mounted on an inverted microscope (IX81; Olympus) equipped with a 100x oil-immersion UPLSAPO objective (N.A. 1.4), extra 2x magnification, and solid-state lasers of 488 nm (50 mW) and 561 nm (50 mW).

For cortical imaging, $7 \times 0.5 \mu\text{m}$ z stacks were collected every 5 s in embryos of strains GCP21, GCP22, GCP113, GCP570, GCP809, GCP831, GCP832, GCP838, GCP927, GCP991, GCP1014, GCP1036, GCP1163, GCP1164, GCP1169, GCP1170, GCP1176, GCP1187, GCP1207, GCP1208, RZB217, OD26, OD130 and GOU2936 (Figures 4.1A, 4.3, 4.4E, 4.5A, 4.5B, 4.6A, 4.6B, 4.6D, 4.6E, 4.7J, S4.5A, S4.6, and S4.7A). An image at the center of the embryo was also acquired to determine the timing of anaphase onset. For central plane imaging in Figures 4.1A, 4.2C, 4.5D, S4.1B, S4.1F, and S4.3A, $6 \times 1 \mu\text{m}$ z stacks were collected every 10 s in embryos of strains GCP21, GCP22, GCP113, GCP570, GCP646, GCP831, GCP832, GCP927, GCP991, OD26, OD130 and GOU3103. For fluorescence recovery after photobleaching (FRAP) experiments (Figures 4.6F–4.6H and S4.6), a FRAPPA photobleaching module (Andor Technology) placed between the spinning disk head and the microscope was used. Photobleaching was performed by 3 sweeps of a 405 nm laser with 100% power and 40 μs dwell time.

For measurement of total cytokinesis time, contractile ring diameter decrease, ring constriction rate and percentage of cytokinesis completion/failure in Figures 4.1C, 4.1D, 4.2A, 4.2D, 4.4C, 4.4D, 4.7I, and S4.2D, images were acquired with an epifluorescence microscope (Zeiss Axio Observer Z1) equipped with a 63x Plan-Apochromat objective (N.A. 1.4), a Colibri.2 LED light source, and an Orca Flash 4.0 camera (Hamamatsu). Acquisition parameters, shutters, and focus were controlled by Zen 2.3 software (Zeiss). $7 \times 1 \mu\text{m}$ z stacks were collected every 10 s in embryos of strains GCP113, GCP570, GCP809, GCP838, GCP1014 and GCP1036.

Image processing

Image processing and analysis was performed using Fiji (ImageJ; National Institutes of Health) or MATLAB (MathWorks). Z stacks taken on the cell cortex were projected using the maximum intensity projection tool. The equatorial region of the central plane was selected to make the kymographs displayed in Figures 4.2C and S4.1F, the equatorial region of the maximum projection of cortical planes was selected to make the kymographs displayed in Figures 4.5B, 4.6B, and S4.5A, and a 150×5 pixel rectangle positioned at the center of cortical plane was selected to make the kymographs in Figure S4.4D, using the Make Montage tool. Images in each figure panel are scaled equally, except for Figures 4.1A

and 4.3. Graph plotting, curve fitting and linear regressions were performed with Prism 8.0 (GraphPad Software) or MagicPlotPro 2.8.2.

Latrunculin A treatment

Acute treatment with Latrunculin A in Figures 4.6D–4.6H and S4.6 was performed in *perm-1(RNAi)* permeabilized embryos (Carvalho et al., 2011). 25–30 L4 animals were placed on a plate containing 0.005 mM isopropyl β -D-thiogalactopyranoside (IPTG) and *E. coli* HT115 bacteria expressing dsRNA against *perm-1*. After incubation for 14–18 hours at 20°C, adult hermaphrodites of strains GCP22 or GCP113 were dissected and permeabilized embryos were filmed in meiosis medium (25 mM HEPES, pH 7.4, 0.5 mg/ml inulin, 20% heat-inactivated fetal bovine serum, and 60% Leibowitz-15 medium) without compression. 10 μ M Latrunculin A (Sigma) was added at anaphase onset. At the end of each video, medium containing 33 μ M FM4-64 (Molecular Probes) was added to the imaging chamber to confirm that the imaged embryo was permeable.

Generation of antibodies against SMA-1

Two affinity-purified rabbit polyclonal antibodies against the actin-binding domain (SMA-1 ABD in Figures 4.4B and S4.1A) and pleckstrin homology domain (SMA-1 PH in Figures S4.2B and S4.2C) of SMA-1 were generated. To produce GST-tagged ABD, cDNA encoding amino acid residues 195-519 of SMA-1 (residue numbers as in isoform a, from transcript R31.1a, Wormbase version WS279) was cloned into the EcoRI and NotI restriction sites of pGEX-6P1 using primers 5'-CCCGAATTCATTTCCGGAGACAAACTCGG-3' and 5'-CCCGCGGCCGCTTCCTGTCTTTGAAGCTCGGC. To produce GST-tagged PH domain, cDNA encoding residues 3689-3898 of SMA-1 was cloned into the BamHI and NotI restriction sites of pGEX-6P1 using primers 5'-CCCGGATCCAGAAAGACTCAGGAAATCTCTC-3' and 5'-CCCGCGGCCGCTTAGTTGTACGCATACGACTTGAG-3'. GST::ABD and GST::PH were expressed in *E. coli* BL21 and Rosetta, respectively, purified and injected into rabbits at the in-house Animal Facility. Sera were affinity purified on a HiTrap N-hydroxysuccinimide column (GE Healthcare) against covalently coupled ABD and PH (TEV-cleaved with prescission protease in cleavage buffer: 50 mM HEPES pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% Tween 20, at 4°C overnight), aliquoted in 10% glycerol, snap-frozen and stored at –80°C.

Protein extracts and immunoblotting

For the immunoblots in Figures 4.4B, S4.1A, S4.2B, and S4.2C, protein extracts were prepared from 100 adult stage animals of strains N2, GCP808, AZ30, GCP986 and GCP564. Animals were collected in M9 medium with 0.1% Triton X-100 and washed three times in the same medium. The pellet of worms was resuspended in 100 μ l of SDS-PAGE sample buffer (250 mM Tris pH 6.8, 30% v/v glycerol, 8% w/v SDS, 200 mM DTT and 0.04% w/v bromophenol blue) and one-third of the volume of quartz sand (Sigma) was added. Tubes were subject to three 5-minute cycles of boiling at 95°C and vortexing, after which the quartz sand was pelleted and the supernatant recovered. Protein samples (20-30 μ l, equivalent to 20-30 animals) were resolved by SDS-PAGE (8% acrylamide gel) and transferred to a 0.2- μ m nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% non-fat dry milk in TBST (20 mM Tris, 140 mM NaCl, and 0.1% Tween, pH 7.6) and probed at 4°C overnight with 1 μ g/ml anti-SMA-1 ABD antibody or 1 μ g/ml anti-SMA-1 PH antibody and 1:5000 anti- α -tubulin antibody (DM1- α , Sigma). Membranes were washed three times with TBST, incubated with HRP-conjugated secondary antibodies (mouse anti-rabbit 1:5000 or goat anti-mouse 1:5000; Jackson ImmunoResearch) for 1 hour at room temperature, and washed again three times with TBST. Proteins were visualized by chemiluminescence using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and imaged in a ChemiDoc XRS+ System with Image Lab Software (Bio-Rad).

Immunofluorescence

For the immunofluorescence in Figure 4.3D, twelve gravid adult hermaphrodites were dissected in 5 μ l of M9 buffer on a poly-L-lysine coated slide. A 13 mm² round coverslip was placed over the M9, and slides were placed into a container with liquid nitrogen. After flicking away the coverslip, samples were fixed for 20 minutes in -20°C methanol. Samples were re-hydrated in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄), twice for 5 minutes, blocked with AbDil (PBS, 2% BSA, 0.1% Triton X-100) in a humid chamber for 30 minutes at room temperature, and incubated in AbDil with rabbit anti-SMA-1 PH antibody (1:1000) and anti- α -tubulin antibody (1:1000, DM1- α , Sigma), overnight at 4°C. After washing four times for 5 minutes in PBS, samples were incubated in AbDil with Alexa Fluor 488 anti-rabbit IgG (1:300; Jackson ImmunoResearch) and Alexa Fluor 594 goat anti-mouse IgG (1:300; Jackson ImmunoResearch) for 1 hour at room temperature. Samples were washed four times for 5 minutes in PBS and mounted in Prolong Gold (Invitrogen).

Computational simulations

We simulated the formation of cytokinetic rings using the Open Source engine Cytosim, which is capable of simulating the thermal environment of the cell as well as the forces generated by the interaction of biological fibers and binding proteins. We identified 4 essential components to simulate the assembly and maintenance of ring structures: F-actin, myosin motor minifilaments, and the crosslinkers plastin and β H-spectrin. Parameters that characterize each component are listed below (corresponding references are in Table S4.3).

Actin fibers were modeled as a series of geometrically polarized rigid segments connected by flexible hinges, with an overall rigidity $0.075 \text{ pN } \mu\text{m}^2$. Initially segments were $0.1 \text{ } \mu\text{m}$ long forming a nongrowing fiber of total length $1 \text{ } \mu\text{m}$, with a barbed and a pointed end. Actin dynamics are very complex and mostly mediated by auxiliary proteins such as formin, ADF/cofilin, capping proteins, and breakage events. We chose to model actin dynamics in simple terms, where each end of a fiber can stochastically switch between a growth state and a shrink state at fixed rates, and ends grow and shrink with pre-defined speeds. We prevented fibers from disappearing by defining a minimum length of $0.01 \text{ } \mu\text{m}$ where they remain inactive until a rescue event. When the depolymerizing end encounters a crosslinker, it has a 90% chance of continuing depolymerizing (and unbinding the crosslinker) and a 10% chance of stopping depolymerizing. Values were adjusted so that average fiber length remains roughly constant.

The contraction of actin fibers into a ring is driven by molecular motors designed to model non-muscle myosin II. The backbone of the motor is a $0.15 \text{ } \mu\text{m}$ long rigid solid. There are 4 binding heads on each end, equally spaced by $0.015 \text{ } \mu\text{m}$ starting from the ends of the solid for a total of 8 binding domains. Each head can bind to any actin fiber within a range of $0.01 \text{ } \mu\text{m}$ at a rate of 10 Hz and while bound can unbind at a rate of 0.3 Hz. While bound to an actin fiber, heads walk toward the barbed end of the fiber with a specified maximum speed which can be suppressed by the amount of drag force on the motor with a stall force of 4 pN. Maximum speed was chosen to match the proper time frame measured experimentally. Binding heads follow normal unbinding rules when reaching the end of fibers as opposed to immediately falling off. If the barbed end of an actin fiber attempts to grow or shrink while a motor head is attached, the motor head will remain attached to the end. Allowing for immediate myosin unbinding from depolymerizing barbed ends does not affect timing and success rate when all components are present, but slows down ring formation for all perturbed cases and leads to expulsion of most actin filaments from the ring midline (Figure S4.7D).

Plastin is a crosslinker consisting of two binding heads connected by a 0.012 μm long spring (Matsudaira et al., 1983) with stiffness 250 pN/ μm . If both heads are unbound then either head can bind any actin fiber within 0.01 μm at a binding rate of 10 Hz. While one head is bound, the second head can only bind actin segments closely aligned with the first segment. The maximum allowed angular difference between actin segments is 10 degrees. Bound heads unbind at a rate of 0.05 Hz. While attached to dynamic fibers, binding heads have a 10% chance of rescuing a depolymerizing fiber.

βH -spectrin has two heads like plastin but has an extended flexible body of 0.25 μm and no angular restriction while binding actin fibers. The backbone is modeled as a series of rigid segments of 0.05 μm in length, and a rigidity 3 orders of magnitude lower than that of our actin filaments ($7.5 \cdot 10^{-5}$ pN μm^2). At each end of the βH -spectrin fiber there is a binding head with identical binding range, binding rates and rescue behavior as plastin.

We modeled cytokinetic ring assembly similarly to the work of Bidone et al. 2017. We randomly placed all 4 components in a 10 μm by 4 μm rectangular space with periodic boundary conditions along the top and bottom edges. Increasing the “circumference” of our space by values higher than 4 μm did not change our results (Figure S4.7C). The initial horizontal range of our components is about 3 μm since we are concerned with the assembly of the ring itself and not the flux of material from the sides. Our space had background viscosity and temperature typical to those in cells of *C. elegans* embryos.

To quantify ring formation timing and success rate we created a metric M measuring the spatial distribution of myosin motors. This metric is defined by three calculations. We first created separate histograms of the horizontal and vertical positions of the center of masses of the motors. The exact binning method was unimportant, but the horizontal center bin should coincide with the ring midline since an ideal ring will concentrate all motors' horizontal position in the exact center. We then found the standard deviation of the bin counts in each direction and normalized by dividing by the largest possible deviation, representing a distribution with all motors in one bin. Since a ring should be localized in the horizontal but uniform in the vertical, we defined our metric as:

$$M = \underline{\sigma}_x * \left(1 - \underline{\sigma}_y \right) \quad (\text{Equation 1})$$

M ranges from 0 to 1 (Figure S4.7B). By comparing our metric to videos of simulations, we determined that a ring had successfully assembled if M exceeded a threshold value of 0.5.

To reproduce experimental results, we simulated four different configurations of components. All configurations had actin fibers and myosin motors since those are the

minimal components necessary to have a contractile network. We then simulated 100 trials with (1) no crosslinkers, (2) plastin only, (3) β H-spectrin only, and (4) both plastin and β H-spectrin. We determined a final ring success rate by the percentage of successful trials ($M > 0.5$).

4.3.4. Quantification and statistical analysis

Image quantifications

The total cytokinesis time in Figure 4.1 and C1 was obtained by determining the time elapsed between anaphase onset and the contractile ring reaching a diameter of 5 μ m, measured in the z-plane with the widest gap between the two tips of the ingressing cleavage furrow. Anaphase onset was defined by direct visualization of DNA, labeled with H2B::mCherry. Ring constriction rate was the slope of the linear region between ~75% and 10% furrow ingression.

To quantify the number of actin clusters and myosin puncta on the equatorial cortex in Figure 4.6C, an intensity threshold was applied (1500 gray values for NMY-2::mCherry and 2500 gray values for LifeAct::GFP) and myosin structures larger than 0.4 μ m and with a circularity of 0.5-1 and actin structures larger than 0.3 μ m and circularity of 0.3-1 were selected using the Analyze Particles tool (Fiji).

The percentage of cytokinesis success/failure in Figures 4.1C, 4.2A, 4.4C, 4.4D, 4.7I, S4.2D, C1 and Table C1 was assessed by counting the number of one-cell embryos in which furrowing completed/did not complete and dividing it by the total number of embryos filmed.

Deviation of F-actin bundles from vertical alignment at the equatorial cortex in Figure 4.5C was quantified in maximum projections of cortical planes in embryos expressing LifeAct::GFP (strains GCP22 and GCP927). The Directionality plugin for ImageJ was used in a 30 $\times$ 70 pixel box placed over the furrow region, using the local gradient orientation method. The average directionality (in degrees) was determined for each time point and the deviation from vertical alignment (in degrees) was calculated by subtracting the value of the obtained angles from 90°. Absolute numbers were plotted.

Spindle behavior in Figure S4.3B was determined by recording the distances between the plasma membrane and the anterior/posterior centrosomes, as well as between the two centrosomes over time after anaphase onset. All curves were aligned to the center of the centrosome-centrosome distance. Values were normalized to the length of the embryo at

metaphase, which was the distance between the outermost points of the embryo along the anterior-posterior axis at metaphase.

Actin cortical flows

Actin cortical flows were determined in maximum projections of cortical planes in embryos expressing LifeAct::GFP [strains GCP22 and GCP927 with and without *sma-1(RNAi)*]. Flows were analyzed between anaphase onset and the end of cytokinetic furrow ingression. Magnitude and direction of cortical flows were quantified using a Particle Image Velocimetry (PIV) MATLAB script (see Key resources table). A 216×128 pixel ($38.5 \times 22.8 \mu\text{m}$) region was applied to all embryos to exclude cortical curved peripheries. Two iterations were performed to calculate maximum particle displacement: a larger interrogation window of 64×64 pixels was used in the first iteration and a smaller interrogation window of 32×32 pixels was used in the second iteration. Consecutive interrogation windows overlapped by 50%. After flow field generation, the 216×128 pixel region was divided in two adjacent anterior and posterior regions: anterior cortex (1st-148th pixel; $0.0\text{--}26.3 \mu\text{m}$) and posterior cortex (149th-216th pixel; $26.3\text{--}38.4 \mu\text{m}$). Each velocity vector was resolved into a V_x component along the anterior-posterior axis and a V_y component along the dorsal-ventral axis. The mean V_x and V_y in the anterior and posterior cortex were estimated for each time point and plotted as a function of time to trace the cortical flow profiles in Figure S4.4.

FRAP

Quantification of NMY-2::GFP fluorescence signal in FRAP experiments in Figure 4.6F was performed in three different regions of interest (ROIs): ROI 1 – 10×13 pixel box in Figure S4.6A or 30×140 pixel box in Figure S6B, corresponding to the bleached region; ROI 2 - 300×200 pixel oval that covers most of the cortex and is used to correct for overall photobleaching of the sample resulting from acquisition and photobleaching sweeps; and ROI 3 – 20×20 pixel box, corresponding to the camera background measured outside the embryo. Mean fluorescence intensity in the three ROIs was determined over time, pre- and post-photobleaching. After background subtraction and photobleaching correction, the myosin signal in the photobleached region was normalized to the average signal in that region before photobleaching and plotted over time. Curve fitting with a one-phase association exponential equation was performed to extrapolate the half time of recovery and mobile fraction, using Prism 8.0 (GraphPad Software):

$$y=y_0+(Plateau-y_0) \cdot (1-e^{(-K \cdot x)}),$$

where y_0 is the fluorescence intensity right after photobleaching, Plateau is the fluorescence at infinite times, and K is the rate constant. The values of Plateau and K were obtained after fitting the curves with observed data to one phase association equation.

The half time of recovery was the time when the fluorescence intensity (y) equaled:

$$y=((Plateau-y_0)/2) + y_0$$

The mobile fraction, X_m , was calculated using the formula:

$$X_m=(plateau-y_0)/(y_{bleached}-y_0),$$

where $y_{bleached}$ denotes the average fluorescence intensity of 3 time points before photobleaching.

Statistical analysis

Statistical analysis was performed with Prism 8.0 software (GraphPad). Error bars represent 95% confidence interval of the SEM (95% CI), SEM (Figures 4.6C, S4.4B, and S4.4C), or SD (Figures 4.7F, 4.7G, and S4.7C). Statistical significance tests were performed using one-way ANOVA followed by Dunnett or Bonferroni's multiple comparison test, as indicated in the figure legends.

4.3.5. Data code and availability

Data reported in this paper will be shared by the lead contact upon request.

The code necessary to run the simulations is freely available and can be found at <https://gitlab.com/JulioMBelmonte/cytokinesis2021>. Further information regarding the computational aspects of this work should be directed to Julio Belmonte (jbelmon2@ncsu.edu).

Any additional information required to reanalyze the data reported in this work is available from the Lead Contact upon request.

4.4. Results

4.4.1. Multiple crosslinkers localize to the equatorial cell cortex and the tip of the ingressing cleavage furrow during cytokinesis

To assess localization dynamics of crosslinkers in one-cell *C. elegans* embryos undergoing their first cytokinesis, we used fluorescent versions of plastin (PLST-1::GFP), anillin (GFP::ANI-1), and septin (UNC-59::GFP) (Table S4.1). Cortical imaging allowed visualization of contractile ring assembly at the cell equator, and central plane imaging during furrow ingression offered a side view of the constricting contractile ring (Figure 4.1A). PLST-1::GFP formed linear bundles at the equatorial cortex and a meshwork with distinct puncta in the surrounding cortex. This localization pattern was similar to that of F-actin (LifeAct::GFP), as reported previously (Ding et al., 2017). By contrast, GFP::ANI-1 and UNC-59::GFP localized to cortical patches that were enriched in the equatorial region, similar to myosin (NMY-2::GFP). UNC-59::GFP was more prominent on the anterior cortex than GFP::ANI-1 and NMY-2::GFP. All probes were strongly enriched at the tip of the ingressing furrow during contractile ring constriction. These results illustrate that cytokinesis occurs in the presence of multiple crosslinkers, consistent with the idea that different types of crosslinkers jointly organize the actomyosin network.

4.4.2. A survey of nine conserved crosslinkers identifies plastin/PLST-1 as the only crosslinker whose depletion slows cytokinesis

To analyze crosslinker function during cytokinesis, we first identified *C. elegans* crosslinker orthologs that are highly conserved across species and have been demonstrated to crosslink F-actin *in vitro* (Brown et al., 2015; Goode et al., 1999; Jananji et al., 2017; Janji et al., 2006; Mavrakis et al., 2014; Meyer & Aebi, 1990; Nakamura et al., 2007): α -actinin (*atn-1*), plastin (*plst-1*), filamin (*fln-1*), β -spectrin (*unc-70*), β H-spectrin (*sma-1*), septins (*unc-59* and *unc-61*), anillin (*ani-1*), and coronin (*cor-1*). Cytokinesis completed successfully and with normal timing when ATN-1, FLN-1, SPC-1, UNC-59, UNC-61, ANI-1, and COR-1 were individually depleted by RNAi (Figures 4.1B–4.1D; note that *spc-1(RNAi)* also inhibits UNC-70 and SMA-1, the other spectrin complex subunits). Depletion of each crosslinker was validated as described in Figure S4.1. Depletion of PLST-1 slowed cytokinesis (278 ± 54 s versus 217 ± 29 s in controls), and 6% of *plst-1(RNAi)* embryos failed to complete furrowing (Figures 4.1C and 4.1D). Ring constriction rate (the slope of the linear region between ~75% and 10% furrow ingression) was not

affected after *plst-1(RNAi)* ($0.19 \pm 0.04 \mu\text{m/s}$ versus $0.19 \pm 0.02 \mu\text{m/s}$ in controls), confirming that PLST-1 acts primarily at the early stages of cytokinesis (ring assembly and furrow initiation) (Figure 4.2D; Ding et al., 2017; Leite et al., 2020). We conclude that among the nine conserved crosslinkers examined, only PLST-1 depletion impacts cytokinesis in the *C. elegans* one-cell embryo.

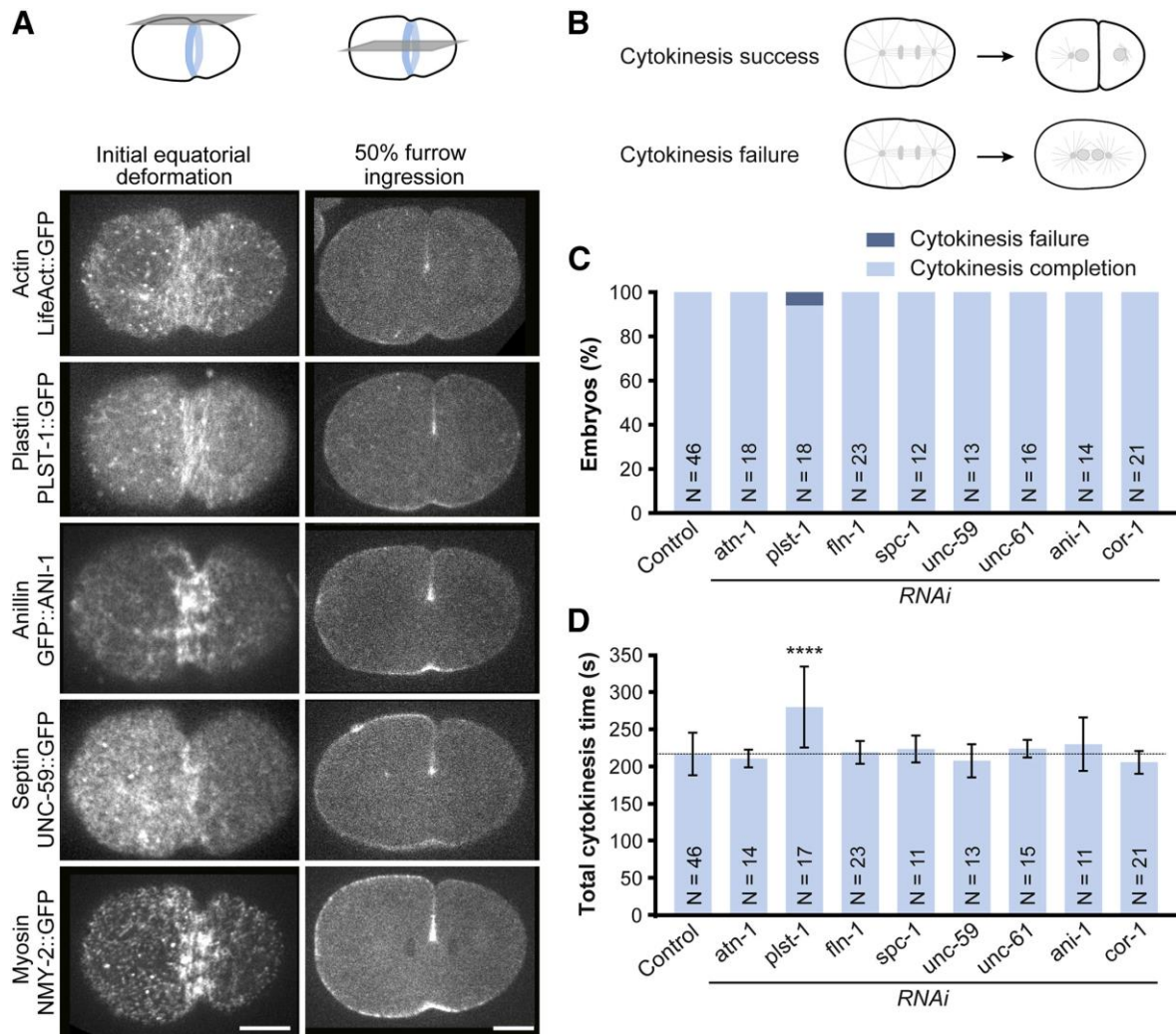


Figure 4.1. Cytokinesis completes when F-actin crosslinkers are depleted individually. (A) Images of the cortex (left) and central plane (right) in *C. elegans* one-cell embryos expressing fluorescent markers of actin, plastin, anillin, septins, and myosin at initial equatorial deformation and 50% furrow ingression, respectively. Scale bars, 10 μm . (B) Schematic illustrating cytokinesis completion or failure during the first embryonic division. (C) Percentage of one-cell embryos that complete/fail cytokinesis after RNAi-mediated depletion of crosslinkers. N indicates the number of embryos analyzed. (D) Total cytokinesis time (mean $\pm$ 95% CI), defined as the interval between anaphase onset and the contractile ring reaching a diameter of 5 μm (see STAR Methods for details), in embryos depleted of crosslinkers. N indicates the number of embryos analyzed. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test: **** $p \leq 0.0001$. See also Figure S4.1 and Tables S4.1 and S4.2.

4.4.3. Co-inhibition of PLST-1 and β H-spectrin/SMA-1 results in cytokinesis failure

To address potential cooperation between PLST-1 and other crosslinkers during cytokinesis, we combined the *plst-1(prt89)* mutant, which carries a premature stop in its open reading frame (Leite et al., 2020), with RNAi-mediated depletion of ANI-1, FLN-1, UNC-59, UNC-61, COR-1, or SPC-1 (Figure 4.2A). Additionally, we combined *plst-1(prt89)* with an *atn-1* mutant that lacks the actin-binding domain (*atn-1 Δ ABD*). We found that *spc-1(RNAi)* in *plst-1(prt89)* embryos was the only condition that significantly increased cytokinesis failure (86% failure in *plst-1(prt89);spc-1(RNAi)* versus 15% in *plst-1(prt89)*; Figures 4.2A and 4.2C; Video S1). Since the α -spectrin SPC-1 can form tetramers with either the conventional β -spectrin UNC-70 or the β H-spectrin SMA-1 (Figure 4.2B), we next performed *unc-70(RNAi)* or *sma-1(RNAi)* in *plst-1(prt89)* embryos. Similar to *plst-1(prt89);spc-1(RNAi)*, *plst-1(prt89);sma-1(RNAi)* caused cytokinesis failure in 87% of embryos, which were unable to form a cytokinetic furrow (Figures 4.2A–4.2D; Video S1). Consistent with the RNAi results, we were unable to generate viable double mutants carrying *plst-1(prt89)* and the null mutant *sma-1(ru18)* (McKeown et al., 1998). Homozygous *sma-1(ru18);plst-1(prt89)* animals that reached adulthood laid on average 2 embryos. 95% of the laid progeny died during embryogenesis and 5% arrested at the L1 stage. In contrast to *sma-1(RNAi);plst-1(prt89)*, *unc-70(RNAi);plst-1(prt89)* had no further adverse effects on cytokinesis beyond those observed in *plst-1(prt89)* alone (Figures 4.2A and 4.2C), which is in agreement with the minimal expression of UNC-70 in the early embryo (Jia et al., 2019; Moorthy et al., 2000). We conclude that SPC-1/SMA-1 α/β H-spectrin tetramers become essential for cytokinesis in the absence of PLST-1.

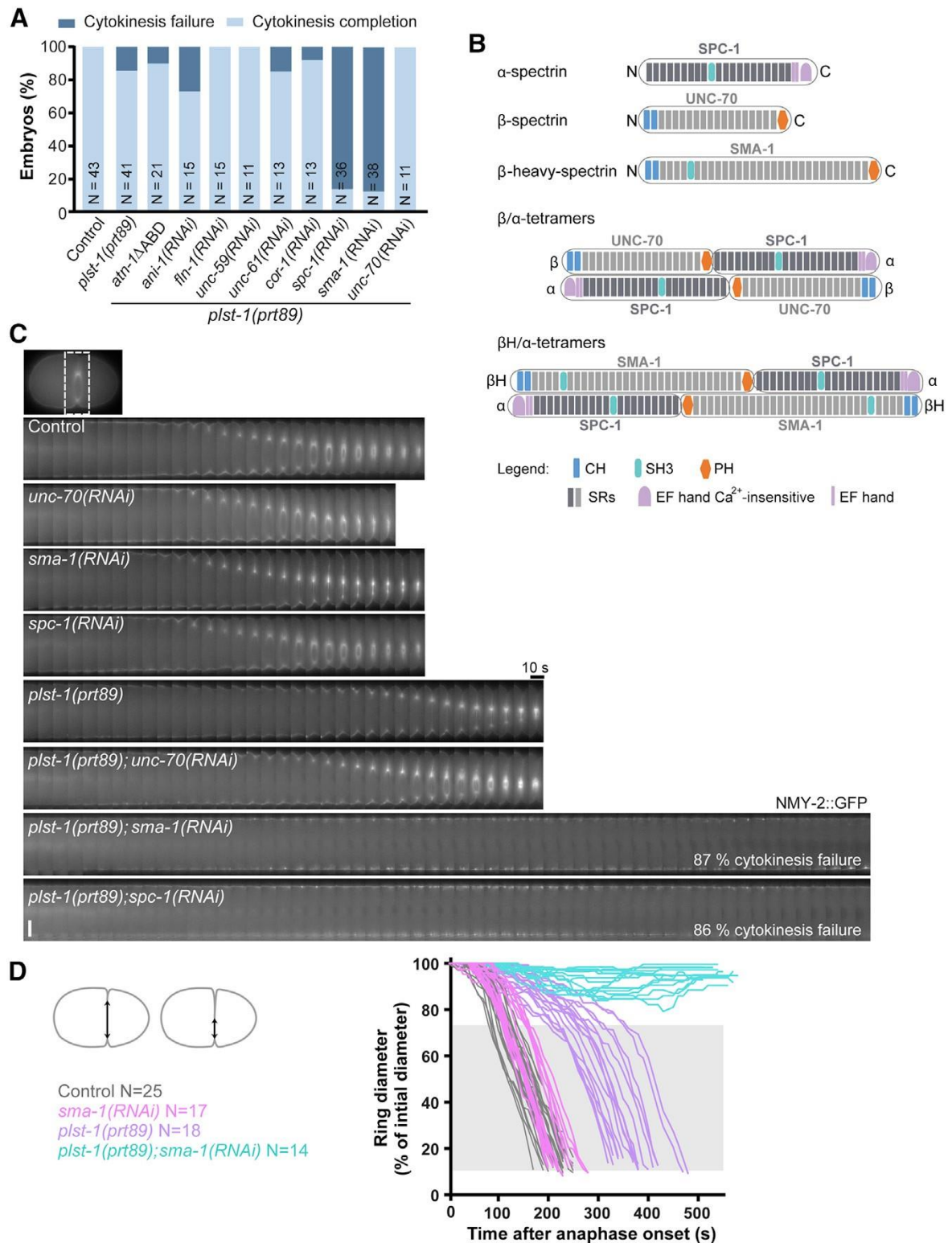


Figure 4.2. Inhibiting PLST-1 together with SPC-1 or SMA-1, but not UNC-70, results in cytokinesis failure. (A) Percentage of cytokinesis completion/failure in one-cell embryos after crosslinker co-inhibition. (B) Schematic illustrating the two types of spectrin tetramers: α -spectrin (SPC-1) pairs with either conventional β -spectrin (UNC-70) or β H-spectrin (SMA-1). CH, calponin homology domain; SH3, Src homology domain; PH, pleckstrin homology domain; SR, spectrin repeat. (C) Representative kymographs of the equatorial

region in one-cell embryos. The first frame corresponds to anaphase onset. Scale bar, 10 μ m. (D) Individual curves of contractile ring diameter over time after anaphase onset. Gray zone indicates the region corresponding to 75%–10% furrow ingression, which was used to calculate ring constriction rate. N indicates the number of embryos analyzed.

See also Video S1 and Tables S4.1 and S4.2.

4.4.4. PLST-1 and SMA-1 have distinct localization profiles at the cell cortex

To assess SMA-1 localization during cytokinesis, we generated a fluorescent version using the split GFP(spGFP) approach, in which the 11-stranded β -barrel structure of GFP is reconstituted from two separately expressed fragments: GFP1-10 (β strands 1–10) and GFP11 (β strand 11; Kamiyama et al., 2016). We inserted 6 tandem copies of GFP11 downstream of the *sma-1* open reading frame by genome editing and crossed animals expressing endogenous SMA-1::6xGFP11 with animals expressing transgenic GFP1-10 under the control of a germline promoter. Imaging of one-cell embryos revealed that the SMA-1::spGFP signal was significantly dimmer than that of PLST-1::GFP and formed small cortical dots that became enriched at the cell equator simultaneously with PLST-1::GFP (Figures 4.3A and S4.7A). While PLST-1::GFP localized to bundles similar to LifeAct::RFP (Figure 4.3A and signal overlap in Figure 4.3B), SMA-1::spGFP dots decorated those bundles but did not overlap with PLST-1::mCherry nor NMY-2::mKate2 (Figure 4.3B). The expression level and localization pattern of endogenous SPC-1::GFP was comparable to that of SMA-1::spGFP, and immunofluorescence with affinity-purified anti-SMA-1 antibody confirmed SMA-1's dotted distribution and equatorial accumulation during cytokinesis (Figures 4.3C and 4.3D). We conclude that SMA-1 is expressed at low levels in the one-cell embryo, that PLST-1 and SMA-1 have distinct localizations within the actomyosin network, and that, consistent with a previous report in mouse embryonic fibroblasts in interphase, spectrins and actomyosin distinctly organize in space (Ghisleni et al., 2020).

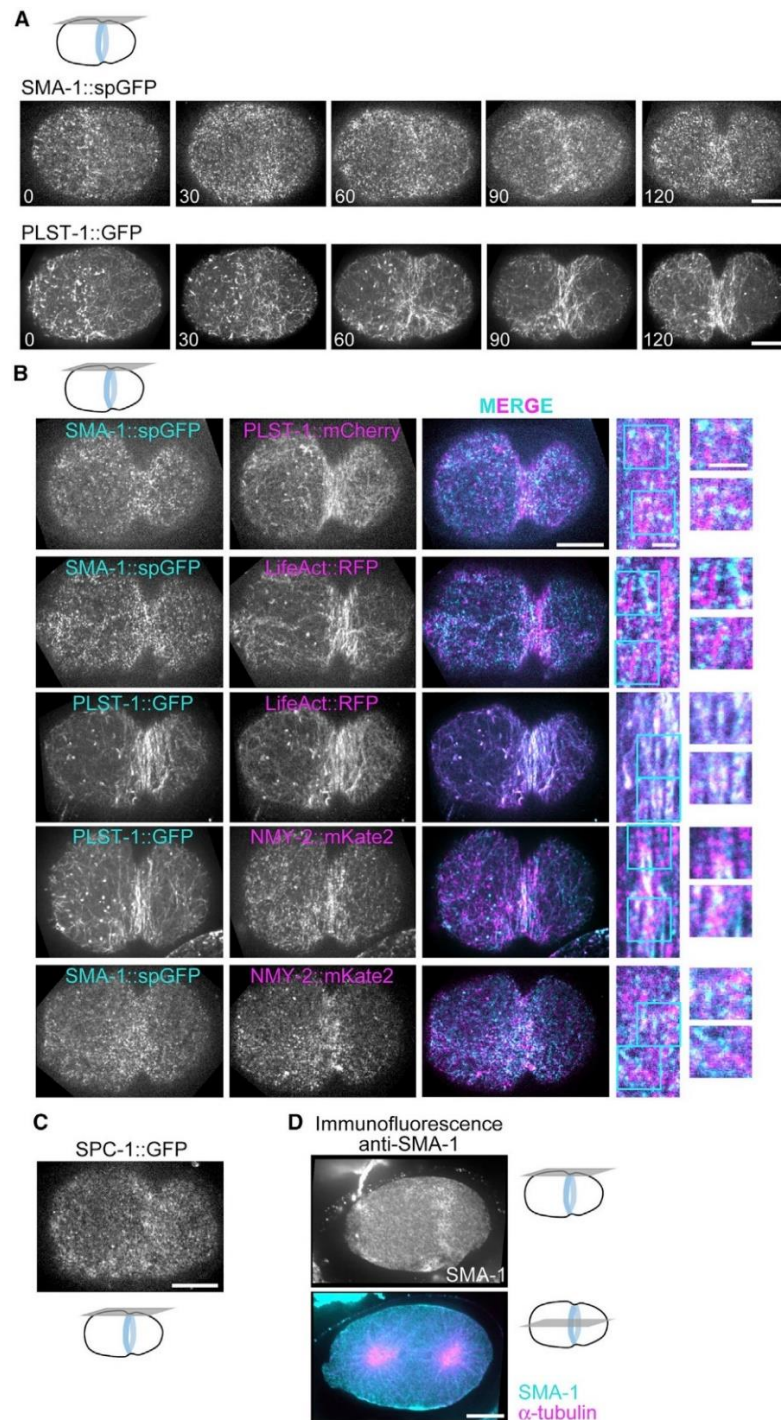


Figure 4.3. PLST-1 and SMA-1 show distinct profiles of localization on the cell cortex. (A) Images of the cortex in one-cell embryos expressing SMA-1::spGFP (splitGFP) or PLST-1::GFP. Numbers indicate the time in seconds after anaphase onset. Scale bars, 10 μ m. (B) Images of the cortex in one-cell embryos co-expressing either the two fluorescent crosslinkers or one of the fluorescent crosslinkers and fluorescent LifeAct or NMY-2. Scale bar, 10 μ m. Blow-ups of the equatorial region are shown on the right. Scale bars, 2 μ m. (C) Image of the cortex in a one-cell embryo expressing SPC-1::GFP. Scale bar, 10 μ m. (D) Images of the cortex and central plane of a fixed one-cell embryo stained with anti-SMA-1 and anti- α -tubulin antibodies. Scale bars, 10 μ m. See also Figure S4.7A and Tables S4.1 and S4.2.

4.4.5. Synergy between SMA-1 and PLST-1 does not require SMA-1's SH3 and PH domains

PLST-1 is a small protein whose only known function is F-actin crosslinking, whereas the much larger SMA-1 contains three functionally distinct domains: an N-terminal ABD, an Src homology-3 (SH3) domain, thought to be involved in signal transduction that is inserted between spectrin repeats in the N-terminal half, and a C-terminal PH domain, which mediates interactions with the plasma membrane. The remainder of the protein consists of spectrin repeats, which confer an elongated shape and flexibility, promote spectrin dimerization and tetramerization, and may mediate additional protein-protein interactions (Figures 4.2B and 4.4A; Machnicka et al., 2014). To define the contribution of SMA-1 domains to the observed synergy between SMA-1 and PLST-1, we used genome editing to delete the regions encoding the SH3 domain (Δ SH3) and the PH domain (Δ PH) from the *sma-1* locus (Figure 4.4A). *sma-1 Δ SH3* and *sma-1 Δ PH* animals were viable, grew to normal adult size, and were fully fertile. This contrasts with *sma-1(ru18)* animals, which are small and have a reduced brood size (McKeown et al., 1998). Immunoblots with anti-SMA-1 antibodies showed that the expression levels of SMA-1 Δ SH3 and SMA-1 Δ PH were similar to wild-type SMA-1, while no detectable SMA-1 was expressed in the *sma-1(ru18)* mutant (Figure 4.4B).

We next crossed the *sma-1* domain mutants with the *plst-1(prt89)* mutant and obtained homozygous viable double mutants. Imaging of one-cell embryos expressing NMY-2::GFP showed that the percentage of cytokinesis failure remained low in *plst-1(prt89);sma-1 Δ SH3* and *plst-1(prt89);sma-1 Δ PH* (19% and 0%, respectively, versus 8% in *plst-1(prt89)*; Figures 4.4C and 4.4D). Furthermore, cortical LifeAct::GFP and NMY-2::mCherry localization patterns in the single and double mutants were similar to those observed in controls and the *plst-1(prt89)* mutant, respectively (Figure 4.4E; Video S2). SMA-1 establishes interactions with the plasma membrane directly through its PH domain and indirectly through other membrane-localized proteins, namely, ankyrin, ezrin-radixin-moesin, and protein 4.1 (Machnicka et al., 2014). We therefore co-depleted ankyrin (UNC-44), ezrin-radixin-moesin (ERM-1), and protein 4.1 (FRM-1) by RNAi in *plst-1(prt89);sma-1 Δ PH* embryos. The efficiency of the triple RNAi was validated by confirming: signal disappearance in a strain that expresses ERM-1::GFP, delayed hatching due to FRM-1 depletion, and decreased brood size due to UNC-44 depletion (Choi et al., 2011; Ramalho et al., 2020; Skop et al., 2004). Cytokinesis successfully completed in 96% of triple-depleted *plst-1(prt89);sma-1 Δ PH* embryos (Figure 4.4D). We conclude that neither the SH3

nor the PH domain of SMA-1 is required for the synergistic action between SMA-1 and PLST-1 during cytokinesis, which completes even when 3 additional membrane anchoring proteins are co-depleted in *sma-1ΔPH* embryos. Although we cannot rule out the possibility that the SH3 domain may play a role in linking SMA-1 to the plasma membrane, these results suggest that cytokinesis failure in the absence of PLST-1 and SMA-1 is unlikely due to problems in anchoring the F-actin cortex to the plasma membrane.

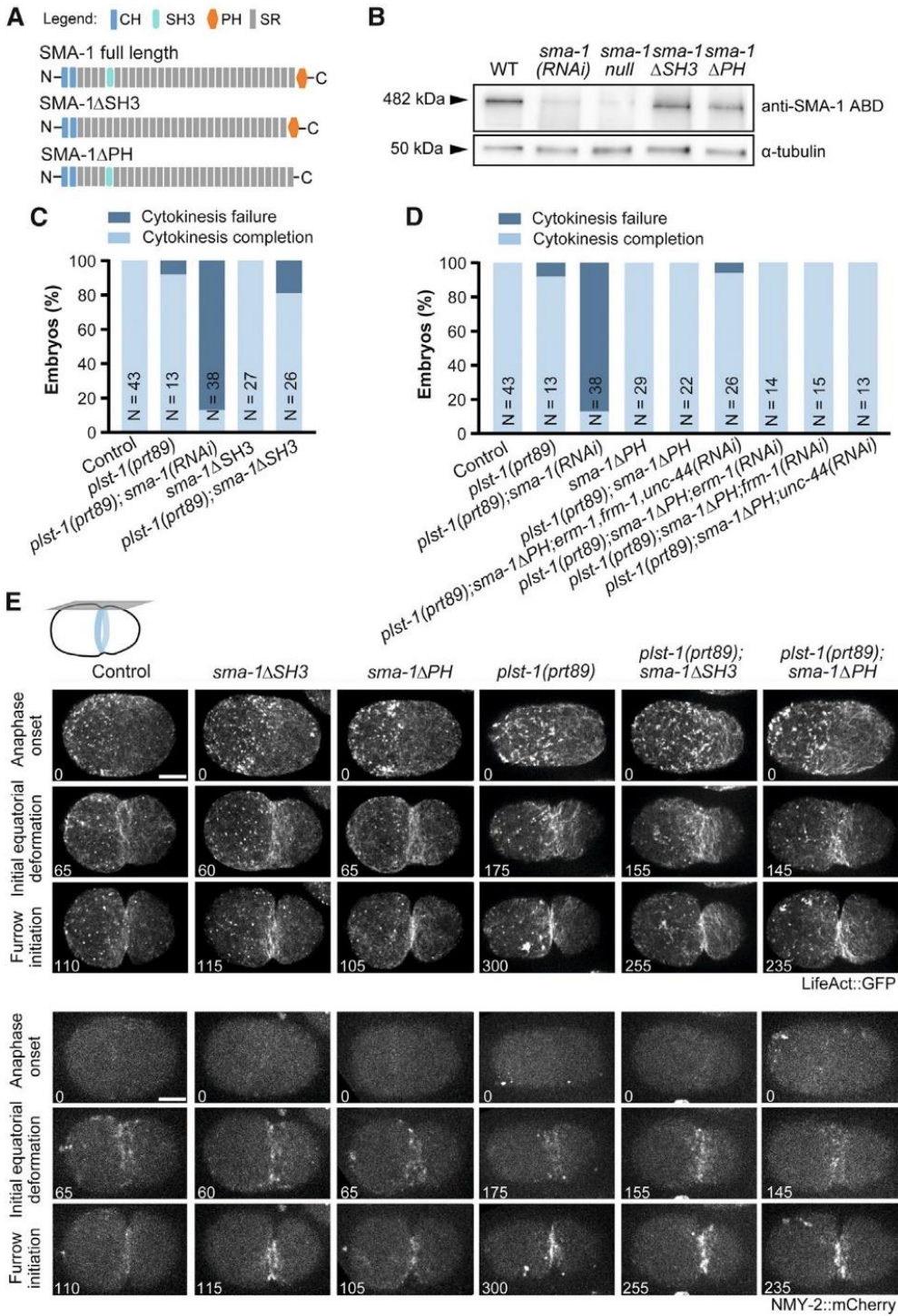


Figure 4.4. Synergy between SMA-1 and PLST-1 does not require SMA-1-mediated F-actin network anchoring to the plasma membrane or SMA-1 SH3 domain-mediated signaling. (A) Schematic of wild-type SMA-1, SMA-1 Δ SH3, and SMA-1 Δ PH. CH, calponin homology domain; SH3, Src homology domain; PH, pleckstrin homology domain; SR, spectrin repeat. (B) Immunoblots of adult lysate with an affinity-purified antibody raised against the actin-binding domain (ABD) of SMA-1. α -tubulin serves as the loading control. (C and D) Percentage of cytokinesis completion/failure in one-cell embryos. N indicates the number of embryos analyzed. (E) Images of the cortex in embryos expressing NMY-2::GFP. Numbers indicate the time in seconds after anaphase onset. Scale bars, 10 μ m. See also Video S2 and Tables S4.1 and S4.2.

4.4.6. Co-inhibition of PLST-1 and SMA-1 results in the collapse of equatorial F-actin bundles

We next asked whether PLST-1 and SMA-1 synergize through their F-actin crosslinking activities. To generate a SMA-1 mutant defective in actin-binding, we sought to delete its CH domains (CH1 and CH2). Although numerous attempts failed to produce precise full deletions, we succeeded in obtaining multiple partial deletions. We chose two for further characterization: SMA-1 Δ ABD#1, which only retains the last 14 residues of CH2, and SMA-1 Δ ABD#2, which retains the first 17 residues of CH1 and the entire CH2 (Figure S4.2A). Both mutants phenocopied the *sma-1(ru18)* mutant: adult animals were small and had a reduced brood size, and embryos completed cytokinesis successfully. Similar to genetic crosses with *sma-1(ru18)*, homozygous *sma-1* Δ ABD#1;*plst-1(prt89)* animals were unable to propagate, but in this case they laid more embryos (16 on average). These embryos did not hatch (91%) or arrested at the L1 stage (9%). Immunoblots with the antibody raised against the SMA-1 PH domain showed that protein levels were reduced in the two *sma-1* Δ ABD mutants (Figure S4.2B), which complicated the interpretation of the phenotype. We therefore asked whether partial RNAi-mediated depletion of SMA-1 in *plst-1(prt89)* embryos, to levels comparable to those in *sma-1* Δ ABD mutants, was per se sufficient to induce cytokinesis failure (Figure S4.2C). In contrast to penetrant *sma-1(RNAi)*, partial *sma-1(RNAi)* did not prevent cytokinesis in *plst-1(prt89)* one-cell embryos (Figure S4.2D). It is therefore likely that SMA-1's inability to bind actin contributes to the embryonic lethality in *plst-1(prt89);sma-1* Δ ABD mutants.

To examine how SMA-1 contributes to cortical F-actin dynamics during cytokinesis, we imaged LifeAct::GFP. In one-cell control embryos, approximately 70 s after anaphase onset, and coincident with the beginning of equatorial deformation, circumferentially aligned F-actin bundles could be observed in the division plane (Figure 4.5A). The localization pattern of F-actin in *sma-1(RNAi)* embryos was indistinguishable from that in controls

(Figures 4.5A and S4.5A). In *plst-1(prt89)* embryos, F-actin bundles took longer to align circumferentially (Figures 4.5A and 4.5C; Leite et al., 2020). In *plst-1(prt89);sma-1(RNAi)* embryos, equatorial F-actin bundles initially attempted to align circumferentially, similar to F-actin bundles in *plst-1(prt89)* embryos, but became increasingly disorganized around the time of equatorial deformation (Figures 4.5B and 4.5C). At this time, bright actin clusters started to appear in the equatorial region, and their number increased over time (Figures 4.5A, 4.5B, 4.6C, S4.5A). These bright F-actin clusters were never observed in control or *sma-1(RNAi)* embryos and were only rarely observed in *plst-1(prt89)* embryos (Figures 4.5A and 4.6C). Strikingly, equatorial F-actin bundles had completely disappeared by 312 ± 64 s after anaphase onset, such that only F-actin clusters remained. Eventually, the equatorial deformation regressed 382 ± 82 s after anaphase onset (Figure 4.5A; Video S3). We conclude that co-inhibition of PLST-1 and SMA-1 severely disrupts the F-actin network at the cell equator, resulting in cytokinesis failure.

When astral microtubules of the mitotic spindle pull on a weakened actomyosin cortex, plasma membrane and cortical invaginations form (Redemann et al., 2010). Consistent with F-actin network collapse and consequent weakening of the cortex, we observed numerous invaginations when imaging the central plane in *plst-1(prt89);sma-1(RNAi)* embryos (Figures 4.5D–4.5F). These invaginations were observed from anaphase onset and became more frequent ~ 300 s later (Figure 4.5E), just prior to F-actin bundle collapse (Figure 4.6C). *plst-1(prt89)* embryos also presented invaginations, but these were less pronounced and much less frequent, and their number decreased as cytokinesis progressed. Mitotic spindle morphology and positioning was identical in control, *plst-1(prt89)*, and *plst-1(prt89);sma-1(RNAi)* embryos expressing GFP:: β -tubulin (Figure S4.3), suggesting that the plasma membrane invaginations in *plst-1(prt89);sma-1(RNAi)* embryos were not caused by increased astral microtubule density or altered centrosome proximity to the cell cortex.

Directed F-actin cortical flows are a hallmark of early cytokinesis (Ding et al., 2017; Leite et al., 2020; Reymann et al., 2016). To assess whether perturbed F-actin cortical flows could contribute to cytokinesis failure in *plst-1(prt89);sma-1(RNAi)* embryos, we analyzed flows by Particle Image Velocimetry from anaphase onset in embryos expressing LifeAct::GFP (Figure S4.4). Posterior-anterior directed (X-component) flows in control embryos, which were particularly prominent on the posterior side, initiated before equatorial deformation and peaked shortly thereafter (Figures S4.4A and S4.4B). As described previously (Ding et al., 2017; Leite et al., 2020), F-actin cortical flows in *plst-1(prt89)* embryos were erratic and decreased in velocity. The flow profile in *plst-*

1(prt89);sma-1(RNAi) embryos was not further aggravated relative to that in *plst-1(prt89)* embryos. We also analyzed the Y-component flow profile and found it to be similar between *plst-1(prt89)* and *plst-1(prt89);sma-1(RNAi)* embryos (Figure S4.4C; Leite et al., 2020). These results imply that the failure to form a contractile ring in *plst-1(prt89);sma-1(RNAi)* embryos is not attributable to impaired F-actin flows.

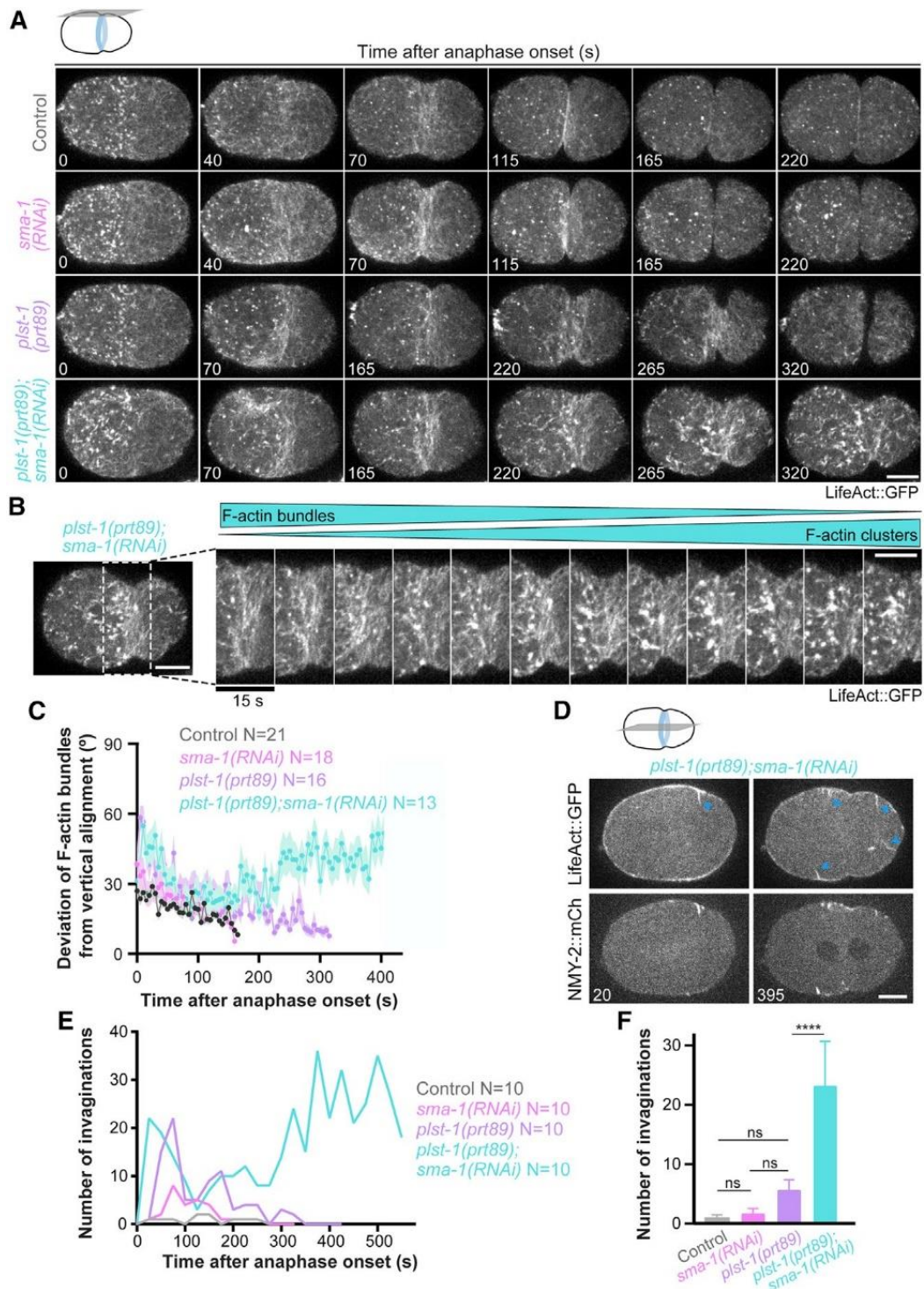


Figure 4.5. Co-inhibition of PLST-1 and SMA-1 results in collapse of F-actin bundles at the cell equator.

(A) Images of the cortex in one-cell embryos expressing LifeAct::GFP. Numbers indicate the time in seconds after anaphase onset. Scale bar, 10 μ m. (B) Images of the equatorial region in a *plst-1(prt89);sma-1(RNAi)* embryo expressing LifeAct::GFP. The first frame corresponds to the time at which a distinct F-actin equatorial band has formed. Scale bars, 10 μ m. (C) Deviation of F-actin bundles from vertical alignment at the equatorial cortex versus time after anaphase onset (mean $\pm$ SEM). (D) Images of the central plane in *plst-1(prt89);sma-1(RNAi)* embryos expressing LifeAct::GFP and NMY-2::mCherry. Numbers indicate the time in seconds after anaphase onset. Blue arrowheads point to membrane/cortex invaginations. Scale bar, 10 μ m. (E) Number of invaginations in the one-cell embryo during successive 25 s intervals after anaphase onset. For each time point the total number of invaginations observed in 10 embryos are plotted. (F) Number of cortical invaginations in the one-cell embryo (mean $\pm$ 95% CI; 10 embryos) in a 400 s interval starting at anaphase onset. Statistical significance was determined using one-way ANOVA followed by Bonferroni's multiple comparison: ****p $\leq$ 0.0001; ns, not significant (p > 0.05).

See also Figures S4.2 and S4.5, Video S3, and Table S4.1.

4.4.7. F-actin bundle collapse after co-inhibition of PLST-1 and SMA-1 coincides with the coalescence of myosin into large puncta

To further assess the status of the actomyosin network, we imaged the cortex of embryos co-expressing LifeAct::GFP and NMY-2::mCherry. Initially, the patchy equatorial NMY-2::mCherry distribution was similar in control and *plst-1(prt89);sma-1(RNAi)* embryos. However, NMY-2::mCherry patches in *plst-1(prt89);sma-1(RNAi)* embryos quickly gave way to large, bright puncta (Figures 4.6A–4.6C and S4.5A). The appearance of NMY-2::mCherry puncta (~115 s after anaphase onset) coincided with the appearance of F-actin clusters, and they frequently co-localized (Figures 4.6B and 4.6C). The number of bright NMY-2::mCherry puncta increased as F-actin bundles disappeared from the cell equator and reached a maximum 320 s after anaphase onset, shortly after the complete collapse of F-actin bundles (Figures 4.5A, 4.5B, and 4.6C). The remaining faint patches of NMY-2::mCherry disappeared along with the F-actin bundles. At this time (382 $\pm$ 82 s after anaphase onset) all cortical NMY-2::mCherry was in the form of bright puncta, and the cortex relaxed. The number of NMY-2::mCherry puncta decreased thereafter and all puncta eventually disappeared around 550 s after anaphase onset. We conclude that the disintegration of F-actin bundles at the cell equator of *plst-1(prt89);sma-1(RNAi)* embryos coincides with a re-distribution of myosin from faint patches to bright puncta.

4.4.8. Myosin localization dynamics after co-inhibition of PLST-1 and SMA-1 resemble those observed after drug-induced F-actin depolymerization

Myosin puncta are known to form at the cell surface in embryos treated with Latrunculin A (Michaux et al., 2018), a drug that inhibits F-actin polymerization by sequestering actin monomers (Leite et al., 2019). To assess the similarity between the myosin puncta in *p/st-1(prt89);sma-1(RNAi)* embryos and those observed after Latrunculin A treatment, we permeabilized one-cell embryos co-expressing Lifeact::GFP and NMY-2::mCherry and acutely treated them with 10 μ M Latrunculin A at anaphase onset (Carvalho et al., 2011). As previously described, Latrunculin A treatment resulted in depolymerization of the cortical F-actin network and failure of cytokinesis (Figure 4.6D). Concomitant with F-actin depolymerization, NMY-2::mCherry accumulated in bright puncta at the cell surface, especially at the cell equator where the contractile ring would be assembled. These NMY-2::mCherry puncta were undistinguishable in brightness, size, and shape from those we observed in *p/st-1(prt89);sma-1(RNAi)* embryos, and in both conditions puncta occasionally fused with one another (Figure 4.6E).

We further examined the dynamics of myosin puncta by photobleaching regions of two different sizes at the cortical equator during contractile ring assembly in embryos expressing NMY-2::GFP (Figures S4.6A and S4.6B). Quantification of the NMY-2::GFP signal over time post-bleaching revealed a fast and full recovery in control embryos, with a half time of recovery of 23.3 ± 3.7 s. In contrast, recovery of the NMY-2::GFP signal was significantly slower in both Latrunculin A-treated and *p/st-1(prt89);sma-1(RNAi)* embryos, with a half time of recovery of 44.6 ± 5.4 s and 41.7 ± 5.5 s, respectively. Signal recovery occurs mostly from the cytoplasm in *p/st-1(prt89);sma-1(RNAi)* and Latrunculin A-treated embryos, as actomyosin cortical flows are essentially absent. In controls, it is possible that cortical flows contribute to signal recovery. We note, however, that the recovering signal at the cell equator does reach a plateau, which is not what would be expected if the bi-directional flows converging on the equator were driving recovery (in this case the signal should keep increasing). The mobile NMY-2::GFP fraction also decreased slightly in either condition (0.94 ± 0.05 and 0.93 ± 0.05 in Latrunculin A-treated and *p/st-1(prt89);sma-1(RNAi)* embryos, respectively; Figures 4.6F–4.6H). Thus, Latrunculin A treatment and *p/st-1(prt89);sma-1(RNAi)* have the same effect on cortical myosin turnover.

Collectively, our results support the idea that myosin puncta observed in the absence of PLST-1 and SMA-1 form as a consequence of F-actin bundle collapse at the cell equator. We conclude that the combined action of plastin and β H-spectrin is critical for maintenance of the cortical F-actin network that forms the contractile ring.

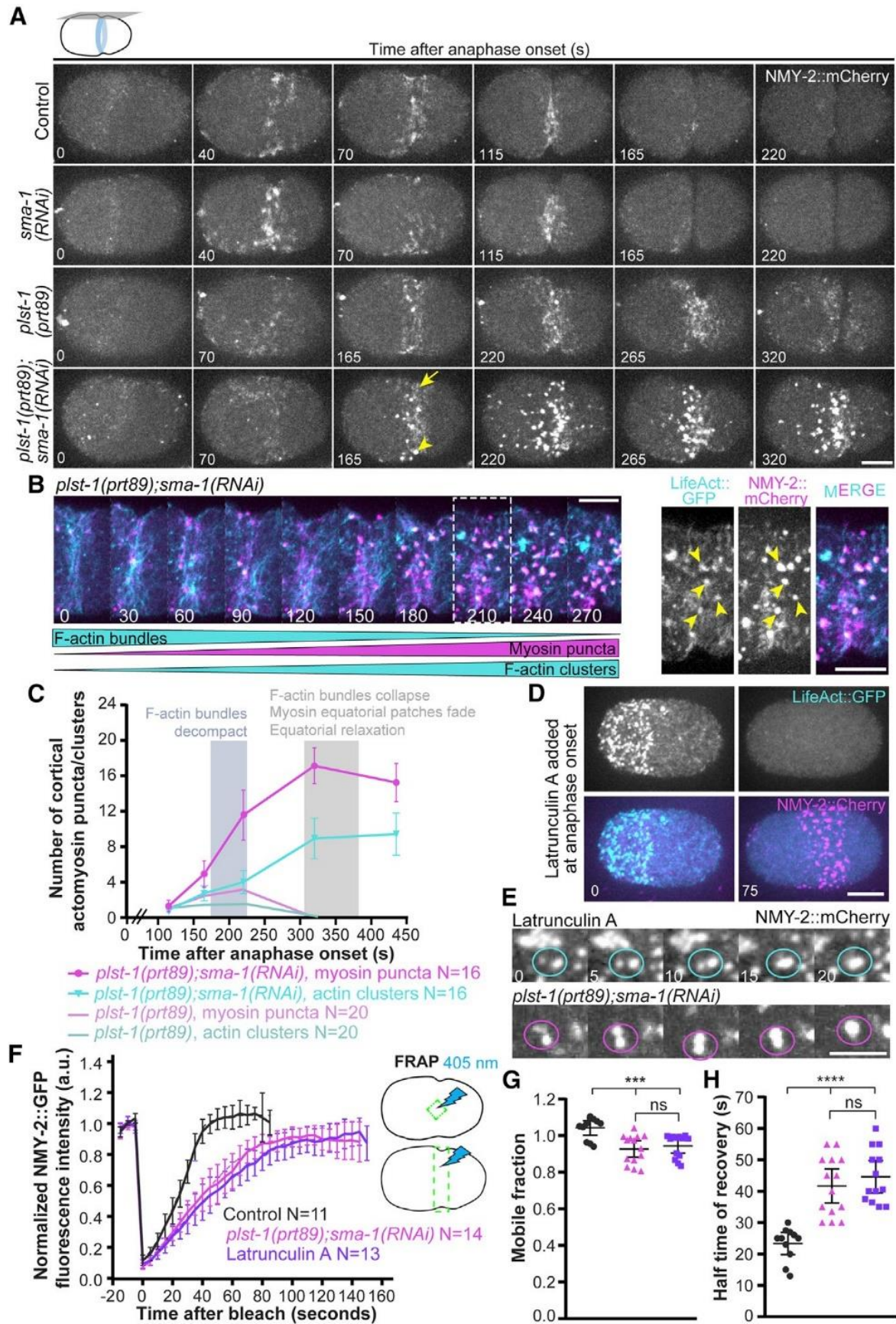


Figure 4.6. Co-inhibition of PLST-1 and SMA-1 leads to coalescence of equatorial myosin into large puncta, which behave identically to puncta that form after Latrunculin A treatment. (A) Images of the cortex in one-cell embryos expressing NMY-2::mCherry. Yellow arrows and arrowheads point to myosin patches and myosin puncta, respectively. Numbers indicate the time in seconds after anaphase onset. Scale bar, 10 μ m. (B) Images of the cortical equatorial region in a *plst-1(prt89);sma-1(RNAi)* embryo co-expressing NMY-2::mCherry and LifeAct::GFP. Numbers indicate the time in seconds after the initial enrichment of NMY-2::mCherry at the cell equator. A blow-up of the 210 s time point (outlined by a dashed line) is shown on the right. Arrowheads point to F-actin clusters and myosin puncta that co-localize. Scale bars, 10 μ m. (C) Quantification of the number of myosin puncta and F-actin clusters (mean $\pm$ 95% CI) over time after anaphase onset. N indicates the number of embryos analyzed. (D) Images of the cortex in one-cell embryos co-expressing LifeAct::GFP and NMY-2::mCherry after acute treatment with 10 μ M Latrunculin A. Numbers indicate the time in seconds after anaphase onset, when Latrunculin A was added. Scale bar, 10 μ m. (E) Cortical images of *plst-1(prt89);sma-1(RNAi)* and Latrunculin A-treated one-cell embryos expressing NMY-2::mCherry showing fusion of myosin puncta. Scale bar, 5 μ m. (F) Quantification of NMY-2::GFP fluorescence recovery after photobleaching (mean $\pm$ 95% CI). Values were normalized to the fluorescence before photobleaching. Results from photobleaching of the regions in the schematics on the right and of the regions shown in Figures S6A and S6B were combined. Dashed lines are the single exponential fitting curves from which the half time of recovery and mobile fraction were extrapolated. N indicates the number of bleaching events analyzed (one per embryo). (G and H) Quantification of the mobile NMY-2::GFP fraction (G) and the NMY-2::GFP half time of recovery (H) (mean $\pm$ 95% CI). Statistical significance was determined using one-way ANOVA followed by Bonferroni's multiple comparison test: ***p < 0.001; ****p < 0.0001; ns, not significant (p > 0.05). See also Figures S4.5 and S4.6, Video S3, and Table S4.1.

4.4.9. *In silico* simulations recapitulate contractile ring formation in the presence of a small and rigid and a large and flexible crosslinker

To gain insight into how plastin and β H-spectrin cooperate to stabilize the contractile ring, we developed an object/agent-based computational model for contractile ring formation using Cytosim. In our simulations, F-actin, myosin bipolar filaments, plastin, and β H-spectrin were individually represented in a 2D space that mimics the equatorial cortex of the *C. elegans* one-cell embryo (Figures 4.7A and 4.7B; Video S4; STAR Methods). F-actin was modeled as either static or dynamic; myosin filaments were modeled as rigid rods with heads that can independently bind and walk along F-actin and dwell at the barbed-end of filaments before unbinding (Wollrab et al., 2018); each crosslinker consisted of two heads that bind to different actin filaments, and a connecting backbone, which is short and rigid for plastin (12 nm), and long and flexible for β H-spectrin (estimated to be about 250 nm when stretched).

To test whether the combination of these 4 cytoskeletal elements could recreate contractile ring formation, we defined the metric *M* for successful ring assembly based on the distribution of the motors along the ring network (Figure S4.7B). *M* varied between one,

when motors move toward the middle of the simulated space horizontally and uniformly spread across the vertical parallel to the actin filaments, and zero, when the ring network ruptures because of connectivity loss, and the motors contract the network into clusters (Figure S4.7B; STAR Methods). By comparing our metric to videos of simulations, we determined that a ring has successfully formed if M exceeds a threshold value of 0.5. Using this threshold, we calibrated our simulations to the four scenarios: all 4 elements, no β H-spectrin, no plastin, and neither β H-spectrin nor plastin (no crosslinkers). Similar to our experimental results (Figure 4.2A), the success rate of ring formation was 100%, 98%, 92%, and 15% in the four scenarios, respectively (Figures 4.7C and S4.7E–S4.7H). In the 4 elements scenario, the calibrated system also recapitulated the time interval of contractile ring formation (average 55 s, range 20 s to 75 s; Figure 4.7D), which is similar to the interval between equatorial actin enrichment (~ 20 s after anaphase onset, Video S3) and equatorial deformation, when F-actin bundles are already aligned (Figure 4.5A). The calibration was possible when the number of plastin connectors was at least one order of magnitude higher than the number of β H-spectrins (Table S4.3), which is consistent with the higher signal of PLST-1::GFP relative to that of SMA-1::spGFP or SPC-1::GFP (Figure S4.7A). Changes in the polymerization and depolymerization rates of actin filament ends had little impact on timing of ring assembly or success rate in the 4-element scenario (Figure 4.7D).

Doubling the amount of plastin had adverse effects on the success rates, while halving its amount only affected simulations with no β H-spectrins. Changes in the amounts of β H-spectrins did not affect the success rate when all elements are present, but halving it did affect the success rate in the absence of plastin (Figure 4.7E). An excess of either crosslinker in the 4-element scenario slowed down ring formation times, which is in agreement with previous reports that relate increased network connectivity to decreased contractility (Ding et al., 2017; Ennomani et al., 2016). Interestingly, increasing plastin in the absence of spectrin or vice versa also led to an increase in ring formation time, indicating that one crosslinker does not compensate for the other. Decreasing the amount of either crosslinker to 75% or 50% sped up ring formation time (Figure 4.7F). The likely reason for this is that, as long as there is some level of F-actin connectivity, and in the absence of anchoring to a plasma membrane and of other actomyosin regulators that could provide drag, it is easier for motors to align the ring network.

The main difference between SMA-1 and UNC-70 structure is the number of spectrin repeats that confers different lengths to the two molecules. To test whether the length of SMA-1 is important for its function during cytokinesis, we simulated ring assembly in the presence of β H-spectrin that ranged from half to double its estimated length of 250 nm and

concluded that within this range β H-spectrin length had negligible effects on success rate and timing of ring formation (Figure 4.7G; Video S4). This is in agreement with results we obtained *in vivo* with a truncated version of SMA-1 that lacks 11 of its 29 spectrin repeats (SMA-1 Δ 11SR; Figure 4.7H). SMA-1 Δ 11SR is approximately the same length as UNC-70, which contains 17 spectrin repeats. Animals expressing SMA-1 Δ 11SR were viable, cytokinesis in one-cell embryos was normal, and expression of SMA-1 Δ 11SR in the *p/st-1(prt89)* background neither aggravated the time required for ring formation nor the success rate of cytokinesis (Figures 4.7I and 4.7J). We conclude that the difference in length between the two β -spectrins is not a critical parameter for SMA-1's cooperation with plastin.

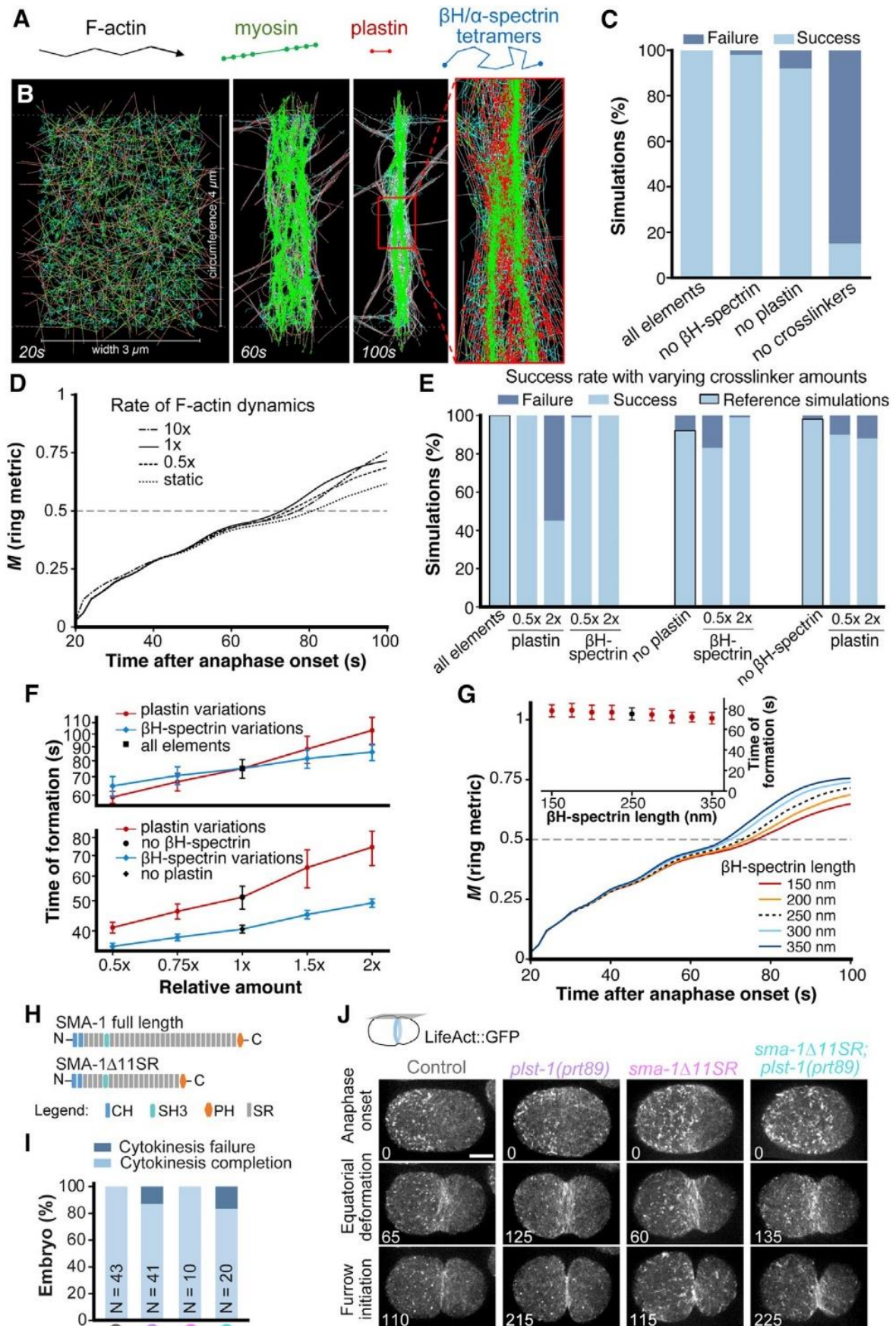


Figure 4.7. *In silico* simulations recapitulate contractile ring formation in the presence of a small and rigid and a large and flexible crosslinker.

(A) The 4 cytoskeletal elements of the simulations (not to scale). (B) Time series of ring formation for the reference simulation with all elements (white, actin fibers; green, myosin; red, plastin; blue, β H/ α -spectrin tetramer). A blow-up of the marked region is shown to highlight vertical F-actin alignment. (C) Success rate for different scenarios. (D) Ring metric over time for the scenario with all elements for different F-actin dynamics. The inset shows the success rate for each scenario as a function of different F-actin dynamics. (E) Success rate for higher (2 $\times$) and lower (0.5 $\times$) amounts of each crosslinker. Corresponding results with no crosslinker variation are shown with black borders for reference. (F) Time of ring formation $\pm$ SD as a function of changes in the amount of each crosslinker (measured for the successful cases). Top: changes in timing relative to simulations with all elements present. Bottom: changes in timing relative to simulations where the other crosslinker is absent. (G) Ring metric evolution for different lengths of β H-spectrin with all elements present. The inset shows the time of ring formation $\pm$ SD as a function of β H-spectrin length. (H) Schematic of wild-type SMA-1 and SMA-1 Δ 11SR. (I) Percentage of cytokinesis completion/failure in one-cell embryos. N indicates the number of embryos analyzed. (J) Images of the cortex in embryos expressing LifeAct::GFP. Numbers indicate the time in seconds after anaphase onset. Scale bar, 10 μ m.

See also Figure S4.7, Videos S2 and S4, and Tables S4.1–S4.3.

4.5. Discussion

Studies *in vitro* and theoretical approaches suggest that F-actin crosslinking is critical for tension propagation and contractility of actomyosin networks (Ding et al., 2017; Ennomani et al., 2016; Stam et al., 2017), yet the role of F-actin crosslinkers *in vivo* remains poorly understood. We determined the contributions of nine conserved crosslinkers to embryonic cytokinesis in *C. elegans* and found that none of them are essential when inhibited individually. Inhibition of the plastin homolog PLST-1, which has previously been implicated in cytokinesis, delays contractile ring assembly but does not affect the speed of ring closure (Ding et al., 2017; Leite et al., 2020). In the sensitized plastin inhibition background, the β H-spectrin SMA-1, but none of the other crosslinkers we examined, becomes essential for cytokinesis. Our findings indicate that interplay between plastin and β H/ α -spectrin occurs via their actin-binding activities and does not involve SMA-1's plasma membrane anchoring function. Thus, two types of F-actin crosslinker with distinct properties collaborate in actomyosin network organization during cytokinesis. The only previous hint that spectrins may have a role in animal cell division comes from work in *D. melanogaster* ovaries, where α -spectrin-deficient cysts were found to have fewer cells (de Cuevas et al., 1996). Of note, cell division in bacteria involves the spectrin-like protein EzrA, which may contribute to the condensation of the tubulin FtsZ (tubulin-homolog) filaments that compose the cytokinetic Z-ring (Squyres et al., 2021). However, EzrA is a small protein of 65 kD that only contains five spectrin-like repeats and lacks an ABD, implying a different function from that of SMA-1 that we report here.

The small (9–12 nm) and rigid plastin, whose main structural feature are two ABDs in tandem, organizes non-branched F-actin in tight, parallel bundles (Ding et al., 2017; Glenney et al., 1981; Matsudaira et al., 1983; Volkmann et al., 2001). By contrast, conventional spectrin tetramers are large and flexible (an estimated 80 nm when relaxed and 200 nm when extended; Byers & Branton, 1985; Beata Machnicka et al., 2014). β -spectrins contain up to 30 spectrin repeats linked by flexible loops. Unfolding of the spectrin repeats under shear stress allows for spectrin extension, which provides red blood cells with the ability to deform when crossing blood vessels (Machnicka et al., 2014). In axons, spectrins provide protection from mechanical stress, caused, for example, by animal movement in *C. elegans* (Hammarlund et al., 2007). In *C. elegans*, spectrins are also required for F-actin bundle organization and reinforcement during the cycles of stretching and contraction in the spermatheca, where oocytes enter to be fertilized before being expelled into the uterus (Wirshing & Cram, 2018), and spectrins help stabilize F-actin bundles in epidermal cells, allowing for their remodeling when muscle cells contract during embryo elongation (Lardennois et al., 2019). Whether β H-spectrin is under tension during cytokinesis and whether it offers mechanical protection to the cell equator remains to be investigated.

PLST-1 is clearly bundling equatorial F-actin, as its signal and that of LifeAct overlap. In contrast, SMA-1 appears to fill the spaces between F-actin bundles, which supports the idea that the two crosslinkers make distinct contributions to the stability of the contractile ring. Super-resolution microscopy will be required to better understand SMA-1's distribution within the actomyosin network. In our *in silico* model, successful ring formation requires that plastin is one order of magnitude more abundant than spectrin, and, while either crosslinker is sufficient for actomyosin ring formation, plastin must still be more abundant than spectrin in these scenarios. This requirement probably reflects the size difference between plastin and spectrin: the compact plastin can only crosslink actin filaments that are already in close proximity, whereas the approximately 20 times longer β H/ α -spectrin can crosslink filaments at much greater distance. Variations in the absolute amounts of β H/ α -spectrin had negligible effects in our simulations, but doubling plastin adversely affected ring formation success rate in the absence of β H/ α -spectrin. This is because an excess of plastin stiffens the network and stalls the motors, whereas too much spectrin, because of its length and flexibility, still allows the network to be remodeled.

An interesting open question is why the β H-spectrin SMA-1 is used for cytokinesis and not the conventional β -spectrin UNC-70, whose expression in the early embryo appears to be negligible (GFP-tagged endogenous expression is not detectable on the cortex (Jia et

al., 2019), and only a feeble signal is detected by immunofluorescence at the cell-cell boundary of the 2-cell embryo; Moorthy et al., 2000). The most obvious difference between β H-spectrin and conventional β -spectrin is length, but our simulations and *in vivo* results with truncated SMA-1, which contains only one more spectrin repeat than UNC-70, argue against this being an important factor. Another feature that distinguishes the two β -spectrins is the ability to respond to mechanical stress: β H-spectrin is mechanosensing in micropipette aspiration assays in S2 cells, whereas conventional β -spectrin is not (Duan et al., 2018). β H-spectrin's mechanosensing behavior explains its recruitment to the fusion site of myoblasts in *D. melanogaster* in response to the transient increase in protrusive forces by the attacking cell (Duan et al., 2018). At that site, β H/ α -spectrin keeps cell-adhesion molecules fenced and serves as a sieve to focus the protrusions of the attacking cell, facilitating the fusion event. In the context of cytokinesis, mechanosensing could potentially allow the β H-spectrin SMA-1 to detect tension at the cell equator that builds due to myosin recruitment, thereby facilitating local spectrin accumulation. If mechanosensing by β H-spectrin is important for cytokinesis, UNC-70 may not be able to functionally replace SMA-1. Unfortunately, we have not been able to test this, as efforts to force transgenic UNC-70 expression in the one-cell embryo have been unsuccessful, presumably due to the well-documented potency of silencing mechanisms that operate in the *C. elegans* germline.

Our analysis of myosin and F-actin dynamics shows that the equatorial actomyosin network progressively disintegrates in the absence of β H-spectrin and plastin. It is known that myosin can remodel isotropic F-actin meshworks into asters *in vitro* because myosin filaments dwell at F-actin plus ends, which results in plus-end clustering (Verkhovsky et al., 1997; Wollrab et al., 2018). Addition of α -actinin leads to the stabilization of asters, which become larger, interconnected, and able to move toward one another until they merge (Wollrab et al., 2018). In SMA-1 and PLST-1 co-inhibited embryos, it is possible that equatorial F-actin orientation becomes randomized and that the end-dwelling behavior of NMY-2 drives its coalescence into clusters of F-actin plus-ends. Lack of interconnectivity between these clusters would then prevent network contraction. In addition, F-actin itself is likely to become destabilized in the absence of β H-spectrin and plastin, since we show that the bright cortical myosin puncta resemble those that form after Latrunculin A treatment (Michaux et al., 2018). In this view, the roles of β H-spectrin and plastin during cytokinesis go beyond mere crosslinking. Several crosslinkers have been reported to stabilize F-actin *in vitro* by decreasing its depolymerization rate and, in some instances, by concomitantly increasing the elongation rate or decreasing access to the severing protein cofilin (Giganti et al., 2005; Loomis et al., 2003; Schmoller et al., 2011). There is also *in vivo* evidence for

F-actin stabilization by crosslinkers: plastin stabilizes actin patches in yeast, espin stabilizes and lengthens F-actin bundles in microvilli of epithelial cultured cells and in stereocilia in hair cells, and fascin and forked stabilize bristles in *D. melanogaster* (Loomis et al., 2003; Tilney et al., 2003; Young et al., 2004). During contractile ring assembly in cytokinesis, when formin-mediated F-actin elongation occurs concomitantly with alignment and compaction of F-actin bundles, a dual ability to crosslink and stabilize F-actin would appear to be advantageous. Following contractile ring assembly, however, contractile ring constriction requires F-actin depolymerization (Mendes Pinto et al., 2012; Osório et al., 2019). This raises the question of whether the F-actin crosslinking and stabilization activities of SMA-1 and PLST-1 might be regulated to facilitate F-actin depolymerization during ring constriction. In the future, it will be important to investigate how the cooperation between plastin and spectrin impacts F-actin network dynamics *in vitro*, including in the presence/absence of cofilin, which would allow further refinement of our *in silico* model.

Our findings highlight the importance of exploring how interplay between crosslinkers of variable size, flexibility, and mechanical properties contribute to actomyosin network contractility and stability. For cytokinesis, we envision the following sequence of events: equatorial plastin promotes myosin motor-driven F-actin alignment through F-actin bundling as well as stabilization, the latter possibly involving inhibition of F-actin depolymerization or restriction of cofilin access to F-actin. As tension builds at the cell equator because of continuous myosin recruitment, β H-spectrin accumulates locally due to its mechanosensing properties and helps protect the circumferential F-actin bundle array from mechanical stress. It will be interesting to test this model using additional engineered plastin and spectrin mutants and to investigate potential roles of the plastin/ β H-spectrin pair and other crosslinker combinations in actomyosin-dependent processes beyond cytokinesis.

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4.7. Supplemental Information

4.7.1. Supporting figures and tables

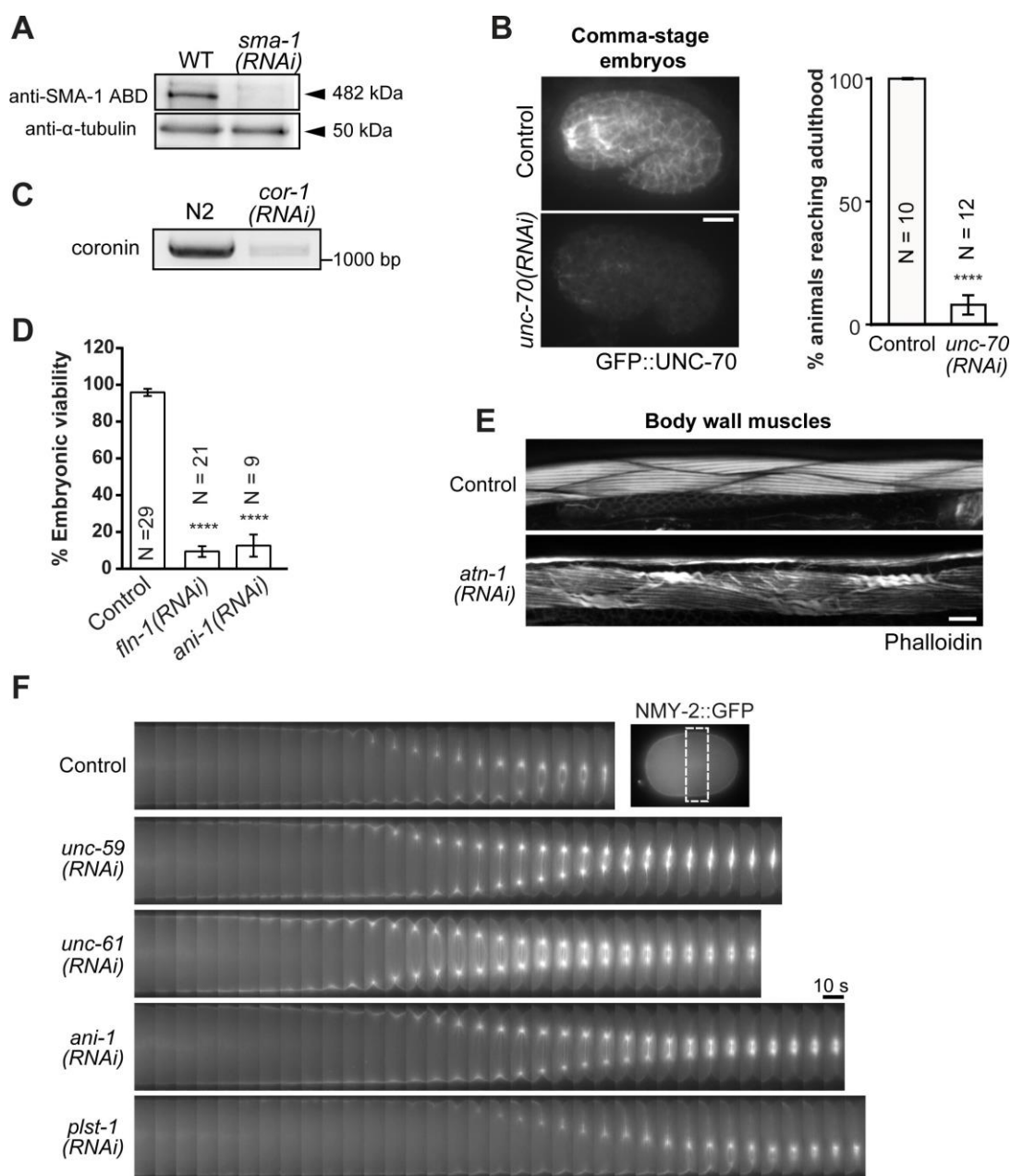


Figure S4.1. Validation of RNAi efficiency. Related to Figure 4.1. (A) Immunoblot of adult *C. elegans* lysate with an antibody raised against the actin-binding domain (ABD) of SMA-1, showing efficient depletion of SMA-1 after *sma-1*(RNAi). WT is wild-type. α -tubulin serves as the loading control. (B) (left) Stills of comma-stage embryos expressing endogenous UNC-70::GFP before and after *unc-70*(RNAi). Scale bar, 10 μ m. (right) Percentage (mean $\pm$ 95% CI) of *unc-70*(RNAi) animals that develop to adulthood. Most *unc-70*(RNAi) animals arrest at the L1 stage, consistent with a previous study [S1]. Statistical significance was determined using

unpaired two-tailed Student t-test: **** $p \leq 0.0001$. (C) RT-PCR for *cor-1* in N2 controls and after *cor-1(RNAi)* using primers Fw 5'-CGTCGACAAGGACTATCCA-3' and Rv 5'-GGAGCCAAAGTGCTCAAA-3' that amplify the 3 isoforms. Method was described previously [S2]. (D) Percentage of embryonic viability (mean $\pm$ 95% CI) in N2 controls, after *fln-1(RNAi)*, and *ani-1(RNAi)*, consistent with previous studies [S3,S4]. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test: **** $p \leq 0.0001$. (E) Images of body wall muscles stained with phalloidin. *atn-1(RNAi)* results in curled F-actin fibers, as expected from previous study [S5]. Method was described previously [S6]. Scale bar, 10 μ m. (F) Images of the equatorial region in dividing one-cell embryos expressing NMY-2::GFP. The first frame corresponds to anaphase onset. Symmetric furrow closure confirms efficient RNAi-mediated depletion of UNC-59, UNC-61 and ANI-1, as described previously [S7]. Prolonged cytokinesis confirms efficient RNAi-mediated depletion of PLST-1. Scale bar, 10 μ m.

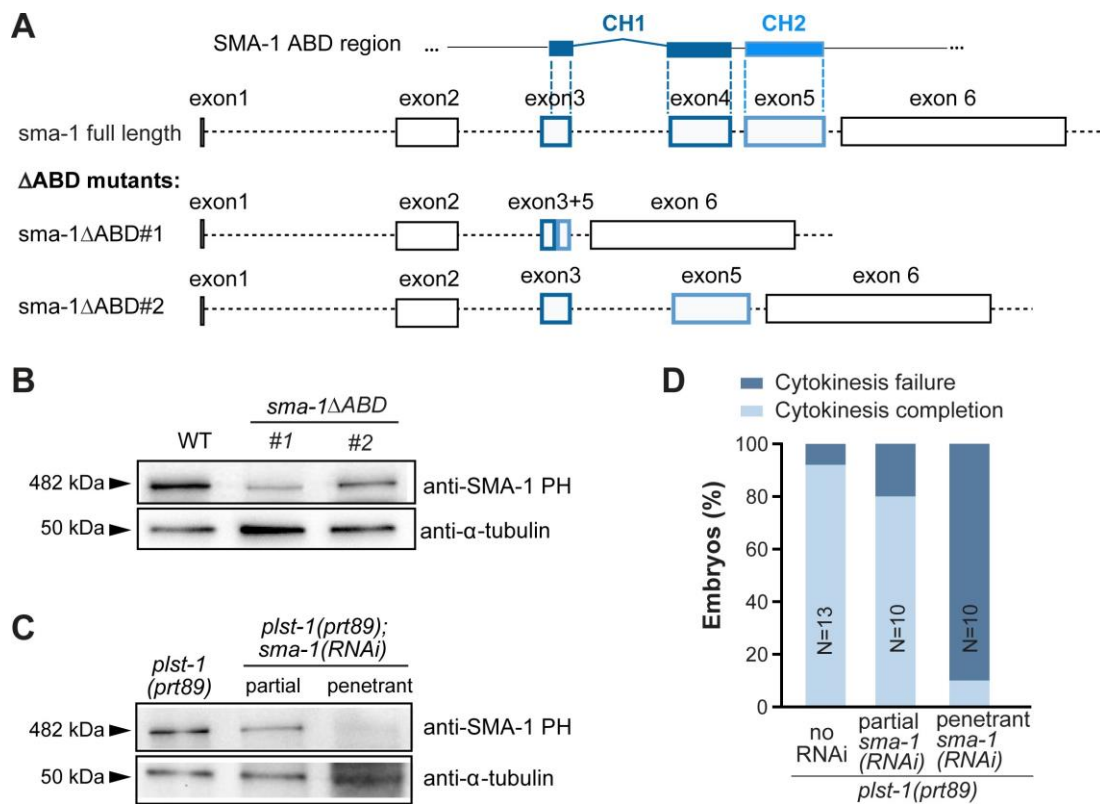


Figure S4.2. SMA-1 deletion mutants that lack most of the actin binding domain are expressed at lower levels but this is unlikely to explain the cytokinesis failure in the *plst-1(prt89)* background. Related to Figure 4.4. (A) Schematic of the first 6 exons and 5 introns of the *sma-1* gene in wild-type and after editing to obtain ABD deletions (Δ ABD). Part of exon 3 and exon 4 encode the first CH domain (CH1), and exon 5 encodes the second CH domain (CH2). SMA1 Δ ABD#1 lacks most of exon 3, the entire exon 4, and most of exon 5. SMA1 Δ ABD#2 lacks exon 4. (B) Immunoblot of adult *C. elegans* lysate with an affinity-purified anti-SMA-1 antibody raised against the PH domain, showing SMA-1 levels in animals expressing the two different SMA1 Δ ABD versions. α -tubulin serves as the loading control. (C) Anti-SMA-1 immunoblot of adult *C. elegans* lysate, showing protein levels in *plst-1(prt89)* animals after partial or penetrant *sma-1(RNAi)*. α -tubulin serves as the loading control. (D) Percentage of cytokinesis completion/failure in one-cell *plst-1(prt89)* embryos after partial and penetrant depletion of SMA-1. N indicates the number of embryos analyzed.

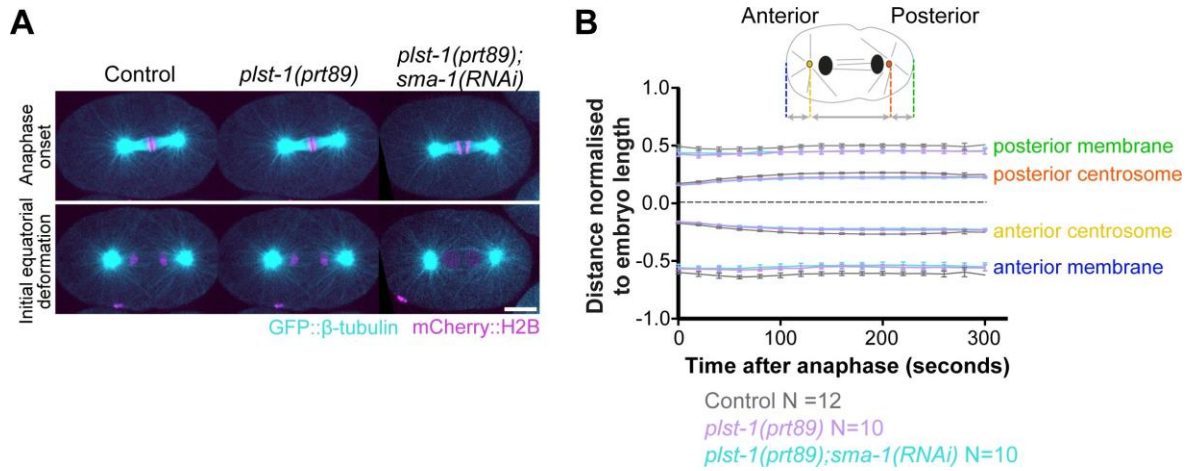


Figure S4.3. Mitotic spindle morphology and positioning is normal in *plst-1(prt89)* and *plst-1(prt89);sma1(RNAi)* embryos. Related to Figure 4.5. (A) Images of the central plane in one-cell embryos co-expressing GFP::β-tubulin and mCherry::histone H2B at anaphase onset and equatorial shallow deformation. Scale bar, 10 μm. (B) Centrosome positioning (mean ± 95% CI) along the anterior-posterior axis of the embryo over time after anaphase onset. Dashed line indicates the midpoint between the two centrosomes. N indicates the number of embryos analyzed.

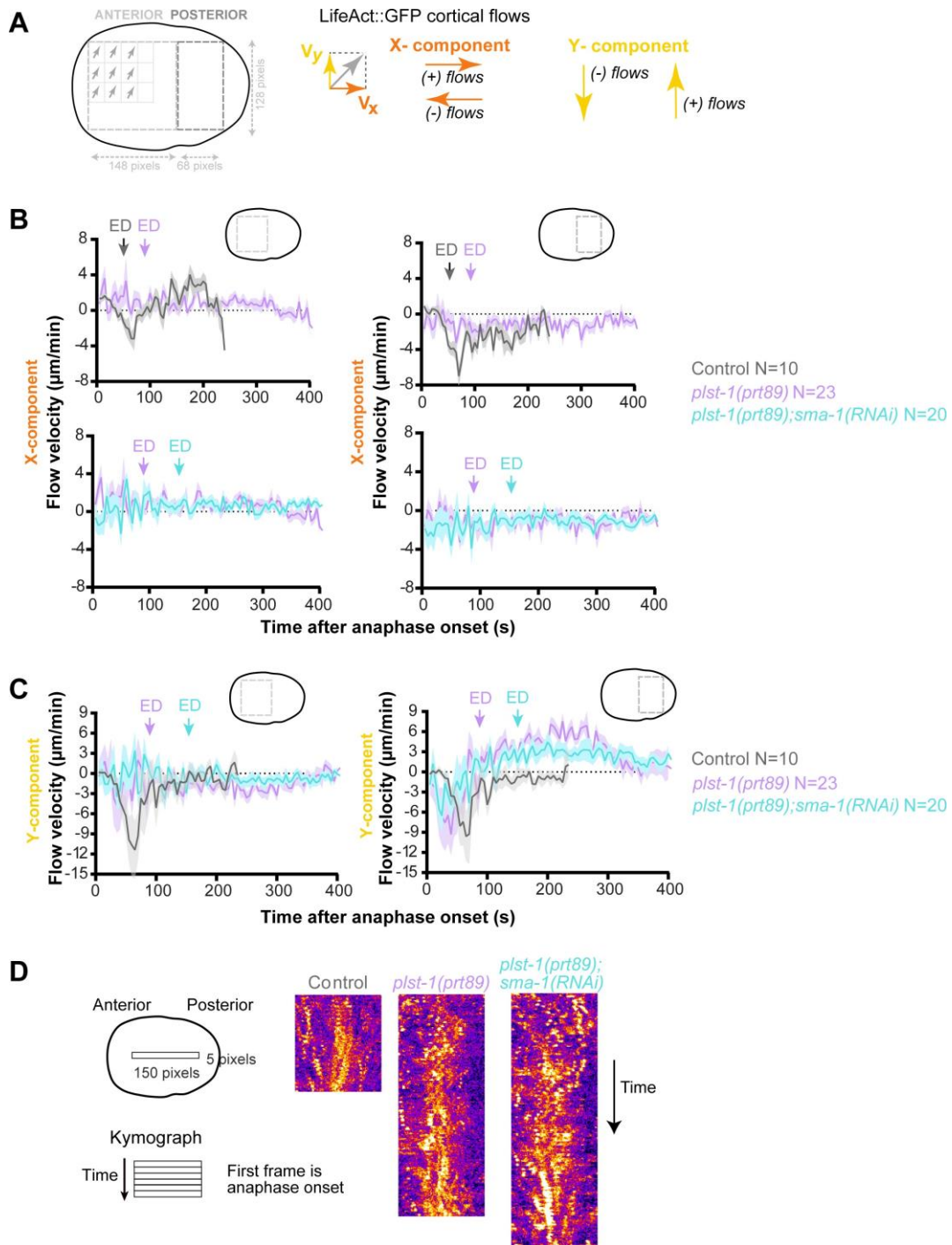


Figure S4.4. Failure of cytokinesis in *plst-1(prt89);sma-1(RNAi)* embryos is not due to perturbed F-actin cortical flows. Related to Figure 4.5. (A) Schematic illustrating the analysis of cortical F-actin flows by particle image velocimetry. X- and Y-components of the velocity vectors in the anterior or posterior regions of the embryo were averaged for each time point after anaphase onset. For the X-component, negative velocity values indicate posterior-anterior oriented flows and positive velocity values indicate anterior-posterior oriented flows. (B,C) X-component (B) or Y-component (C) flow velocities (mean $\pm$ SEM) at anterior (left) and posterior (right) cortex over time after anaphase onset in embryos expressing LifeAct::GFP. ED, initial equatorial deformation. Posterior-anterior directed flows in control embryos were particularly prominent on the posterior side, initiated

before equatorial deformation, and peaked shortly thereafter (panel B, top right). F-actin cortical flows in *plst-1(prt89)* and *plst1(prt89);sma-1(RNAi)* embryos were erratic and decreased in velocity (panel B, bottom right). N indicates the number of embryos analyzed. (D) Kymographs of the region indicated in the schematic on the left showing LifeAct::GFP cortical flows along the longitudinal axis in one-cell embryos, starting at anaphase onset.

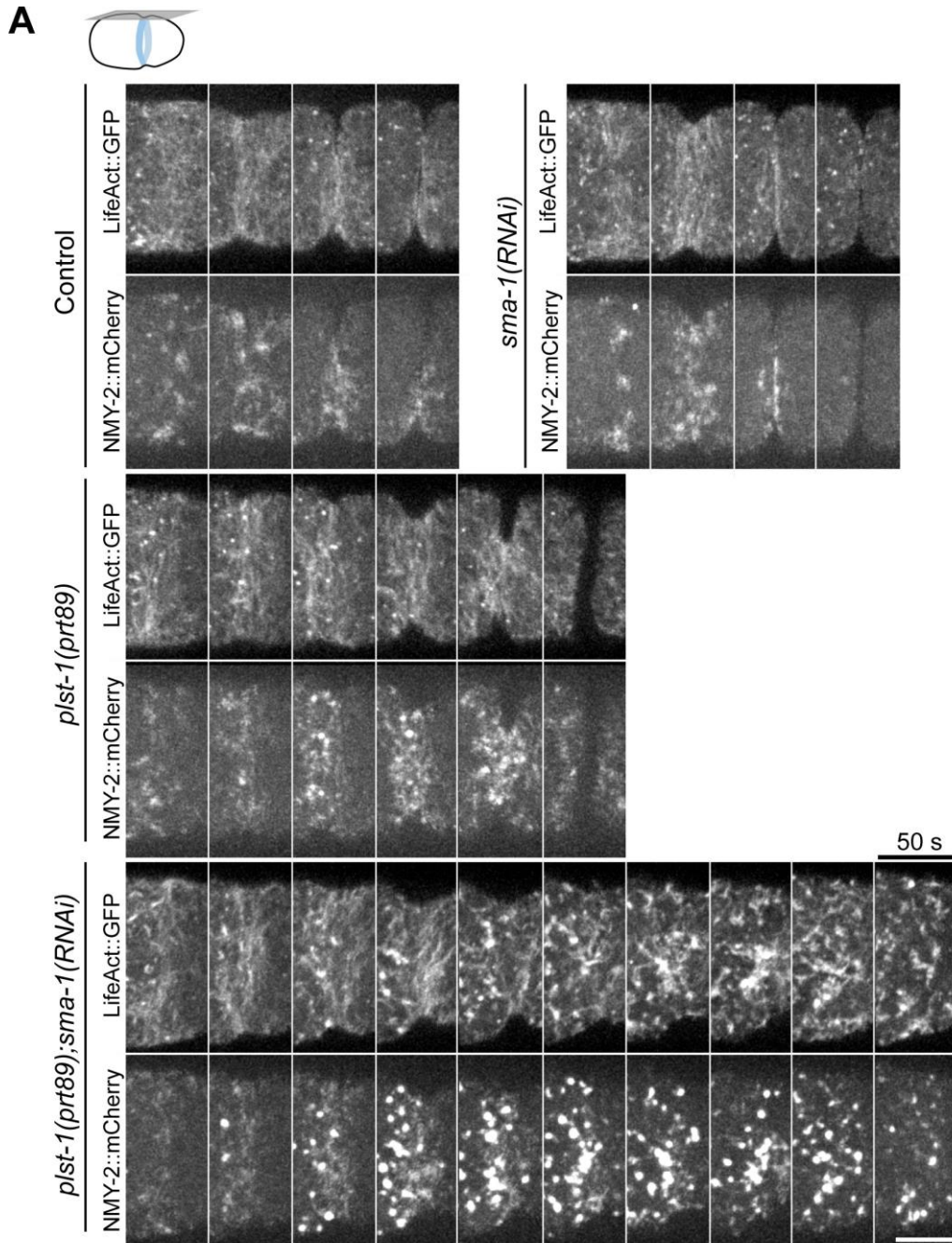


Figure S4.5. Myosin coalescence into bright puncta in *plst-1(prt89);sma-1(RNAi)* embryos coincides with F-actin cluster formation. Related to Figures 4.5 and 4.6. (A) Images of the equatorial cortical region in one-cell embryos co-expressing NMY-2::mCherry and LifeAct::GFP. First frame corresponds to the initial enrichment of NMY-2::mCherry at the cell equator. Scale bar, 10 μ m.

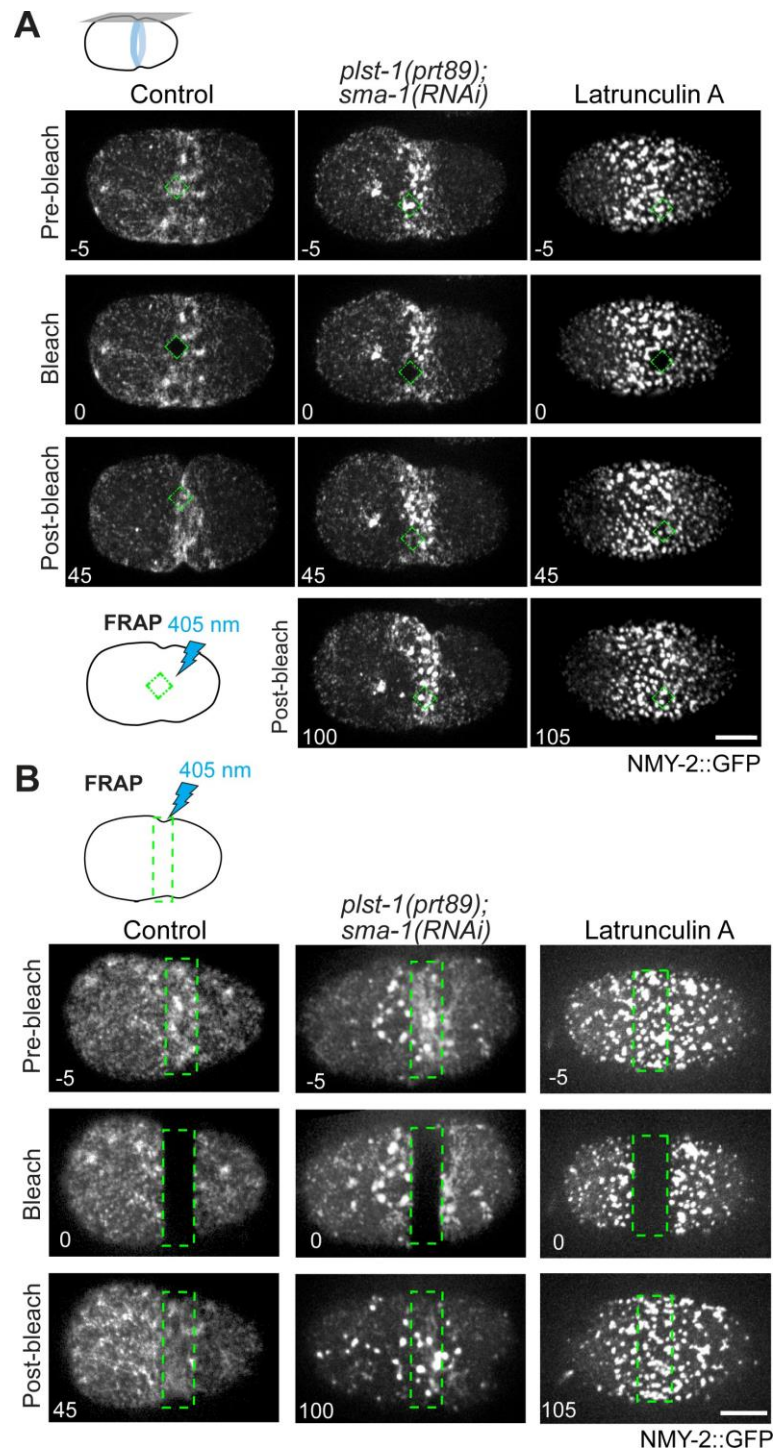


Figure S4.6. Fluorescence recovery after photobleaching of NMY-2::GFP at the cell equator. Related to Figure 4.6. (A,B) Images of the cortex in *plst-1(prt89);sma-1(RNAi)* and Latrunculin A-treated one-cell embryos expressing NMY-2::GFP just before and after photobleaching of a portion (A) or the entire (B) cell equator at initial equatorial deformation, when myosin puncta start accumulating at the cell equator in *plst-1(prt89);sma-1(RNAi)* embryos. Numbers indicate the time in seconds relative to the photobleaching event. Schematics show the photobleached regions. Scale bars, 10 μ m.

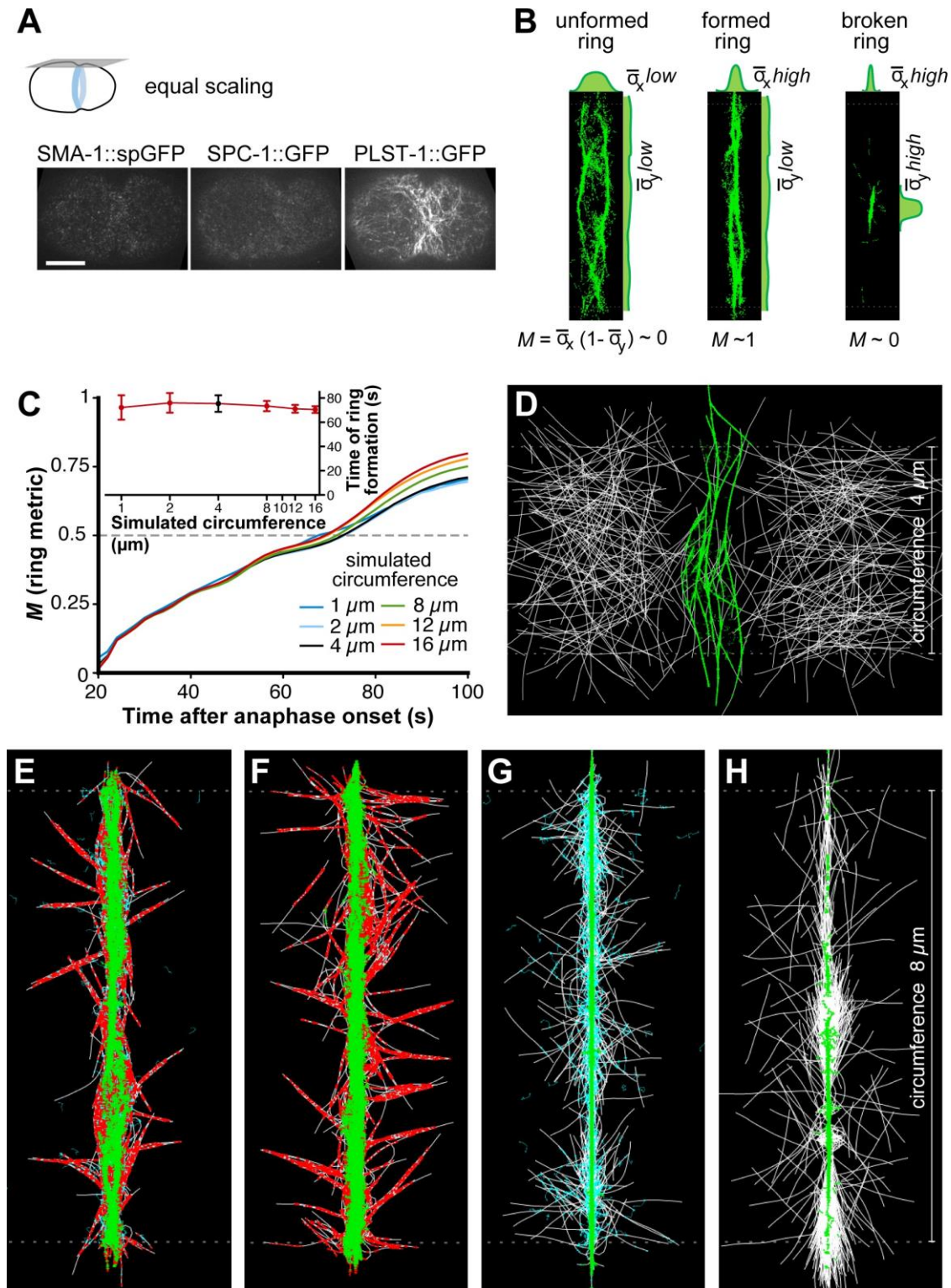


Figure S4.7. Relative expression levels of GFP-tagged SMA-1 and PLST-1 in the early embryo, simulation metric calculation, and scaling and effects of no end-dwelling on depolymerizing ends. Related to Figures 4.3 and 4.7. (A) Equally scaled images of the cortex in one-cell embryos expressing SMA-1::spGFP, SPC-1::GFP or PLST-1::GFP. Scale bar, 10 μm . (B) Simulation snapshots showing only myosin motors for simulations that (from left to right): fail to form a compact ring (double amount of plastin); successfully form a

ring (reference simulation, same as last panel in Figure 4.7B); experience ring rupture (scenario with no plastins). Next to all snapshots are sketch plots that illustrate the measurements of x and y that are used for the ring metric (eq. 1). (C) Use of longer or shorter lengths of simulated circumference does not affect simulation results. Main plot: ring metric evolution for different circumference lengths. Inset: timing of ring formation or different circumference lengths $\pm$ SD. (D) Allowing motors to immediately fall off from depolymerizing barbed ends, instead of end-dwelling, leads to the gradual removal of F-actin from the ring midline and the ring forms with only a few F-actin fibers. (E-H) Simulation snapshots at 100 s after anaphase for scenarios with (E) all elements, (F) no spectrin, (G) no plastin, and (H) no plastin and no spectrin. Color legend: white - actin fibers, green - myosin, red - plastin, and cyan - β H/ α -spectrin tetramer.

Table S4.1. Summary of fluorescent tagged proteins used in this study. Related to Figures 4.1-4.7.

Fluorescent probe (expressed from transgene or endogenous locus; found in which strains)	Method used for strain generation	Test of functionality	Reference
LifeAct::GFP (transgene; GCP22, GCP927, GCP1163, GCP1164, GCP1169, GCP1170, GCP1176, GCP1187)	Bombardment	Normal cytokinesis timing in 1-cell embryo. Normal strain growth.	[S8]
LifeAct::RFP (transgene; GCP1207)	Bombardment	Normal cytokinesis timing in 1-cell embryo. Normal strain growth.	[S9]
PLST-1::GFP (endogenous locus; GCP831, GCP832, RZB217)	CRISPR/Cas9 ^a	Normal cytokinesis timing in 1-cell embryo. Normal strain growth.	[S8]
NMY-2::GFP (endogenous locus; GCP113 and GCP570)	CRISPR/Cas9	Normal cytokinesis timing in 1-cell embryo. Normal strain growth.	[S8]
NMY-2::mCherry (transgene; GCP22, GCP927, GCP1163, GCP1164, GCP1169, GCP1170, GCP1176, GCP1187)	MosSci, Chromosome II ^b	NMY-2::mCherry is able to replace endogenous <i>nmy-2</i> . Normal strain growth.	[S10]
GFP::ANI-1 (transgene; OD130)	Bombardment	Normal strain growth. Pattern of localization of GFP::ANI-1 identical to that observed in strain MDX29, where the endogenous locus	[S11]

		of <i>ani-1</i> is tagged with mNeonGreen (Rehain-Bell et al., 2017).	
UNC-59::GFP (transgene; OD121)	Bombardment	UNC-59::GFP expression under the control of the <i>pie-1</i> regulatory sequences rescues the localization of UNC-61 (the other septin with which UNC-59 oligomerizes)	[S12]
SMA-1::splitGFP (endogenous locus; GCP991, GCP1207, GCP1208)	CRISPR/Cas9 ^a	Normal strain growth.	This study
SPC-1::GFP (endogenous locus; GOU2936)	CRISPR/Cas9	Normal strain growth.	[S13]

^a CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9

^b MosSci - Mos1 mediated Single Copy Insertion

Table S4.2. List of oligonucleotides used in this study. Related to Figures 4.1-4.4 and 4.7.

Oligonucleotide purpose	Oligonucleotide sequence (5'-3')
For production of dsRNA against <i>ani-1</i> (Y49E10.19)_Fwd	taatacgactcactataggTCAAACCTCAATGGAGAGGACAA -3' <i>T7 promoter sequence is in lowercase</i>
For production of dsRNA against <i>ani-1</i> (Y49E10.19)_Rev	aattaaccctcactaaaggCATTTGTGCTTCAAATTCCTCAC <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against <i>atn-1</i> (W04D2.1)_Fwd	aattaaccctcactaaaggCAAAGCTCGAGGACTACCG <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against <i>atn-1</i> (W04D2.1)_Rev	taatacgactcactataggAGCTGCGAATCTCTTCTTGG <i>T7 promoter sequence is in lowercase</i>
For production of dsRNA against <i>fln-1</i> #1 (Y66H1B.2)_Fwd	aattaaccctcactaaaggCCGATTTCCTCACTTCACTTCC <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against <i>fln-1</i> #1 (Y66H1B.2)_Rev	taatacgactcactataggGACTTCACCAGCAACCTTAAC <i>T7 promoter sequence is in lowercase</i>
For production of dsRNA against <i>fln-1</i> #2 (Y66H1B.2)_Fwd	aattaaccctcactaaaggTGGATGGGCAACTGTTTATG <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against <i>fln-1</i> #2 (Y66H1B.2)_Rev	taatacgactcactataggGAGCTCCTCCTCAGAGAAT <i>T7 promoter sequence is in lowercase</i>

For production of dsRNA against plst-1 (Y104H12BR.1)_Fwd	aattaaccctcactaaaggACTCGGAGTCCATGGAAATG <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against plst-1 (Y104H12BR.1)_Rev	taatacgactcactataggCACCATTTTGGCTTCACCT <i>T7 promoter sequence is in lowercase</i>
For production of dsRNA against unc-59 (W09C5.2)_Fwd	taatacgactcactataggCGTGAAACTCGTGGAACA <i>T7 promoter sequence is in lowercase</i>
For production of dsRNA against unc-59 (W09C5.2)_Rev	aattaaccctcactaaaggTTGTGGTGGAGTTCAACGTG <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against unc-61 (Y50E8A.4)_Fwd	taatacgactcactataggAGCTGTCTGAAGCTGGATTTC <i>T7 promoter sequence is in lowercase</i>
For production of dsRNA against unc-61 (Y50E8A.4)_Rev	aattaaccctcactaaaggACGGCTGAACTCGTCTTGAT <i>T3 promoter sequence is in lowercase</i>
For production of dsRNA against unc-70 (K11C4.3)_Fwd	aattaaccctcactaaaggCACTGTCTTGAGAACGTTGAG <i>T3 promoter sequence is in lowercase</i>

Table S4.3. Cytosim parameters used in simulations (where parameters were varied, base values are shown in parentheses). Related to Figures 4.7 and S4.7.

Parameter	Symbol	Value Range (standard value)
Actin Rigidity	μ_{actin}	0.075 pN μm^2 [S14]
Actin Segmentation Length	l_{actin}	0.1 μm
Actin Fiber Length (initial)	L_{actin}	1 μm [S15]
Growing Speed of Barbed End	v_{b-}	0 - 0.8 $\mu\text{m/s}$ (0.08 $\mu\text{m/s}$) [S16]
Shrinking Speed of Barbed End	$v_{b/}$	0 - 0.56 $\mu\text{m/s}$ (0.056 $\mu\text{m/s}$) [S16]
Growing Speed of Pointed End	v_{p-}	0 $\mu\text{m/s}$
Shrinking Speed of Pointed End	$v_{p/}$	0 - 0.14, $\mu\text{m/s}$ (0.014 $\mu\text{m/s}$) [S16]
Switch Rate to Depolymerization	r_c	8 Hz
Switch Rate to Polymerization	r_r	4 Hz
Backbone Length of Myosin	L_{motor}	0.15 μm [S17]
Number of Binding Heads of Myosin	n	8
Binding Range of Motor Head	d_{bind}	0.01 μm

Chapter IV – Plastin and spectrin cooperate to stabilize the actomyosin cortex during cytokinesis

Binding Rate of Motor Head	b_{motor}	10 Hz [S18]
Unbinding Rate of Motor Head	u_{motor}	0.3 Hz [S18]
Maximum Motor Speed	v_0	0.1 $\mu\text{m/s}$ [S19]
Stall Force	f_0	4 pN [S20]
Backbone Resting Length of Plastin	$l_{plastin}$	0.012 μm [S21]
Spring Stiffness	k	250 pN/ μm
Binding Range of Plastin	d_{bind}	0.01 μm
Binding Rate of Plastin	$b_{plastin}$	10 Hz
Unbinding Rate of Plastin	$u_{plastin}$	0.5 Hz
Maximum Allowed Angular Difference	$\Delta\theta_{max}$	10 degrees
Rescue Probability of Plastin	$r_{plastin}$	0.1
$\beta\text{H-Spectrin Rigidity}$	$\mu_{spectrin}$	7.5*10 ⁻⁵ pN μm^2 [S22]
$\beta\text{H-Spectrin Segmentation Length}$	$l_{spectrin}$	0.05 μm
$\beta\text{H-Spectrin Backbone Length}$	$L_{spectrin}$	0.15-0.35 μm (0.2 μm)
Binding Range of Binding Head	d_{bind}	0.01 μm
Binding Rate of Binding Head	$b_{spectrin}$	10 Hz
Unbinding Rate of Binding Head	$u_{spectrin}$	0.5 Hz
Rescue Probability of $\beta\text{H-Spectrin}$	$r_{spectrin}$	0.1
Width (Non Periodic Boundary)	x_0	10 μm
Height (Periodic Boundary)	y_0	1-16 μm (4 μm)
Initial Component Horizontal Range	x_i	3 μm
Background Viscosity	ν	1 pN s / μm^2 [S23]
Background Temperature	kT	0.0042 pN μm
Fiber Count	NF	94-1,500 (375)
Motor Count	NM	260-4,168 (1,042)
Plastin Count	NP	0-120,000 (30,000)
$\beta\text{H-spectrin Count}$	NS	0-5,600 (1,400)

4.7.2. Supplemental Videos and Legends

Supplemental Videos are available at: <https://doi.org/10.1016/j.cub.2021.09.055>

Video S1. Co-inhibition of PLST-1 and SMA-1 results in cytokinesis failure

Time-lapse series of the central plane in dividing one-cell embryos expressing NMY-2::GFP. First time point corresponds to anaphase onset (0 s) and frames are 10 seconds apart. Frame rate is 6 frames per second and time stamp is in seconds. Scale bar, 10 μ m.

Video S2. SMA-1 mutant versions lacking the PH or the SH3 domains do not aggravate the cytokinesis phenotype in *plst-1(prt89)* embryos

Time-lapse series of the cell cortex in dividing one-cell embryos co-expressing LifeAct::GFP and NMY-2::mCherry. Cortical images are maximum intensity projections of seven z-sections 0.5 μ m apart acquired every 5 seconds from anaphase onset (0 s). Frame rate is 5 frames per second and time stamp is in seconds. Scale bar, 10 μ m.

Video S3. PLST-1 and SMA-1 cooperate to stabilize the actomyosin cortex during cytokinesis

Time-lapse series of the cell cortex in dividing one-cell embryos co-expressing LifeAct::GFP and NMY-2::mCherry. Cortical images are maximum intensity projections of seven z-sections 0.5 μ m apart acquired every 5 seconds from anaphase onset (0 s). Frame rate is 5 frames per second and time stamp is in seconds. Scale bar, 10 μ m.

Video S4. A truncated SMA-1 version with 18 instead of 29 spectrin repeats supports cytokinesis in the absence of PLST-1

Time-lapse series of the cortical plane in dividing one-cell embryos co-expressing LifeAct::GFP and NMY-2::mCherry. Cortical images are maximum intensity projections of seven z-sections 0.5 μ m apart acquired every 5 seconds from anaphase onset (0 s). Frame rate is 5 frames per second and time stamp is in seconds. Scale bar, 10 μ m.

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Complementary Results (unpublished)

Besides testing the contribution of each individual crosslinker to cytokinesis by using RNAi-mediated depletions (Figures 4.1C-D), I also tested the same using the available crosslinker mutants, previously characterized in other contexts (Chapter III). Consistently, I observed that PLST-1 was the crosslinker that most affected cytokinesis, as only *p/st-1(prt89)* embryos present a prolonged and sometimes failed cytokinesis [215 ± 26 s in control vs 370 ± 60 s in *p/st-1(prt89)* for total cytokinesis time; 0 % in control vs 13 % in *p/st-1(prt89)* embryos for cytokinesis failure; Figure C1]. Also consistent with the RNAi experiments, none of the other crosslinker mutants affected cytokinesis success and total cytokinesis time was only slightly prolonged in *fln-1* mutant embryos (Figure C1).

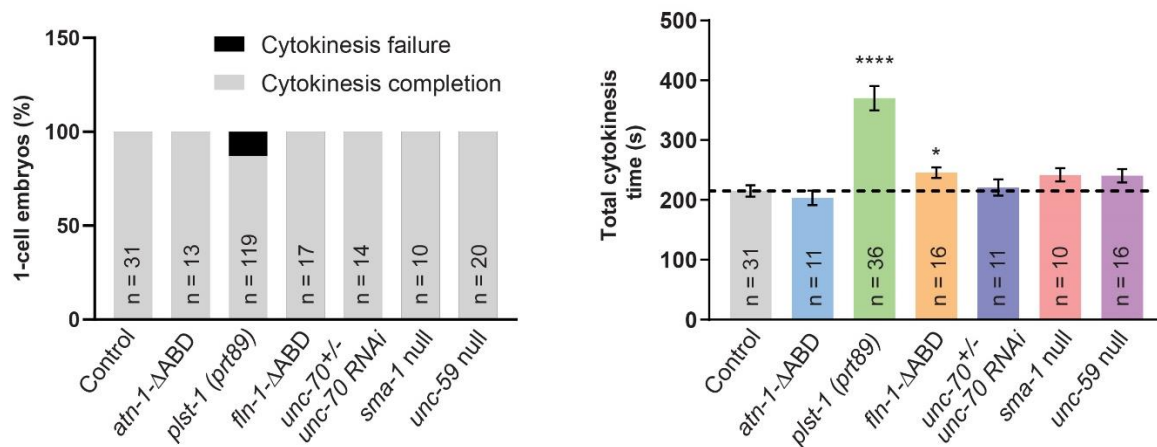


Figure C1. Crosslinker mutants, similarly to the RNAi depletions, reveal that PLST-1 is the crosslinker that most impacts cytokinesis. (Left) Percentage of one-cell embryos that complete/fail cytokinesis in embryos of the depicted phenotypes. (Right) Total cytokinesis time (mean $\pm$ 95% CI), defined as the interval between anaphase onset and the contractile ring reaching a diameter of 5 μ m (see STAR Methods in section 4.3 for details), in embryos of the depicted genotypes. N indicates the number of embryos analyzed. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test: * $p < 0.05$, **** $p \leq 0.0001$.

Additionally, besides the co-inhibition of PLST-1 and other crosslinkers already described in Figure 4.2A of this Chapter, other inhibition combinations were tested, either using double RNAi-mediated depletions, single RNAi-mediated depletions on the background of crosslinker mutants or double crosslinker mutants. Results are compiled in Table C1. Although the number of embryos analysed needs to be further increased for some combinations (and the penetrance of the double RNAi-mediated depletions needs to be

validated), so far the results indicate that none of the tested co-inhibitions results in a significant increase in the percentage of failed cytokinesis.

Table C1. Co-inhibition of two crosslinkers and percentage of cytokinesis failure.

Co-inhibition methods: double mutant of *atn-1* and *plst-1*; single mutant of *atn-1*, *plst-1*, *unc-59* or *sma-1* with other crosslinkers depleted by RNAi; double RNAi of *fln-1;unc-70*, *unc-70;ani-1*, *unc-70;unc-61*, *unc-70;cor-1*, *ani-1;unc-61*, *ani-1;cor-1*, *unc-61;cor-1*. N is the number of embryos analysed.

CROSSLINKERS	ATN-1	PLST-1	FLN-1	UNC-70	SMA-1	ANI-1	UNC-59	UNC-61	COR-1
ATN-1		N=21	N=15	N=4					
PLST-1			N=15	N=11	N=38 86%	N=15 27%	N=11	N=13 15%	N=13
FLN-1				N=8			N=5		
UNC-70					N=18	N=4	N=5	N=9	N=9
SMA-1						N=11	N=6	N=8	N=13
ANI-1							N=12	N=6	N=11
UNC-59								N=10	N=7
UNC-61									N=11
COR-1									

Not tested
 No or low % of cytokinesis failure (0-9%)
 Mild % of cytokinesis failure (10-45%)
 High % of cytokinesis failure (>46%)

CHAPTER V

**ALPHA-ACTININ CAN REPLACE PLASTIN DURING
CYTOKINESIS IN ONE-CELL *C. ELEGANS* EMBRYOS**

5. ALPHA-ACTININ CAN REPLACE PLASTIN DURING CYTOKINESIS IN ONE-CELL *C. ELEGANS* EMBRYOS

5.1. Abstract

Different-sized crosslinkers are expressed within the same cell, promoting the formation of distinct actomyosin network architectures necessary for fundamental processes, such as cell division or motility. Nevertheless, the importance of the size of crosslinkers during actomyosin-dependent processes *in vivo* is not explored. Cytokinesis in *C. elegans* also occurs in the presence of multiple crosslinkers of distinct sizes. Plastin (PLST-1) and β H-spectrin (SMA-1), one small and one large crosslinker, respectively, cooperate during cytokinesis by interconnecting and stabilizing the F-actin network and ensuring a successful division. In this Chapter, we set out to determine whether another crosslinker of relatively small size, alpha-actinin ATN-1, could replace PLST-1 or SMA-1 functions during cytokinesis of the *C. elegans* one-cell embryos. Transgenic expression of ATN-1 selectively compensated for the loss of PLST-1, but not for the loss of SMA-1, and fully rescued cytokinesis failure in embryos co-inhibited of PLST-1 and SMA-1 by replacing the smaller crosslinker's functions. This work indicates that crosslinkers of similar sizes can compensate for one another and provides new insights into the relevance of crosslinkers' size for proper functioning of actomyosin networks during cytokinesis.

5.2. Introduction

Actomyosin networks are populated by several different-sized crosslinkers. It is known that crosslinkers size, as well as F-actin binding kinetics and concentration, are determinants for the formation of F-actin networks with different organizations and mechanical properties, as stiffness or elasticity (Ahmed & Betz, 2015; Lieleg et al., 2010; Wagner et al., 2006; Wachsstock et al., 1994; Scheff et al., 2021). The type and mechanical properties of an actomyosin network are important to ensure the proper behavior of the cell undergoing dynamic processes. One of the most fascinating cellular processes to study the behavior of an F-actin network is cytokinesis, where there is the formation of an equatorial contractile actomyosin ring composed of bundles of F-actin intercalated by myosin bipolar filaments and interconnected by crosslinkers. The interconnectivity of the network by crosslinkers is particularly important to allow the propagation of tension. In an interconnected network, the force exerted by the embedded myosin motors on F-actin generates contraction that is effectively propagated, allowing the network to deform as a whole (Belmonte et al., 2017; Ehrlicher et al., 2015; Ennomani et al., 2016; Weirich et al.,

2021). Nevertheless, much little is known about how the crosslinkers' biophysical characteristics affect the actomyosin network during cytokinesis *in vivo*.

It has been reported that crosslinkers with different sizes can be sorted to different sites or types of network. *In vitro* studies reported that the distance imposed by a crosslinker's length is a spontaneous segregating factor, as crosslinkers with similar sizes were shown to bind to the same filaments of actin and set their bundling spacing. The spacing between the bundles then limited the binding of other crosslinkers of different sizes, revealing a size-dependent competitive bundling mechanism (Winkelman et al., 2016; Weirich et al., 2021). Recently, a study in mammalian cells showed that the large crosslinker spectrin occupies spatially distinct cell cortical territories to those occupied by F-actin bundles in protrusions or contractile structures, which are usually populated by smaller crosslinkers (Ghisleni et al., 2020). In the previous chapter, we observed that several crosslinkers of different sizes localize to the equatorial cortex and contractile ring of one-cell *C. elegans* embryos undergoing cytokinesis. Of those, plastin (PLST-1), a rigid and small-sized crosslinker of ~12 nm that forms tight parallel or antiparallel F-actin bundles, was the only crosslinker that mildly impacted cytokinesis (Ding et al., 2017; Leite et al., 2020; Matsudaira et al., 1983; Skau et al., 2011; Chapter IV). Its inhibition prolonged cytokinesis completion and, in a small percentage of embryos, resulted in cytokinesis failure. Co-inhibition of plastin and other crosslinkers led us to uncover a synergy between PLST-1 and the long and flexible crosslinker β -heavy (β H)-spectrin (SMA-1). In contrast to single inhibitions, co-inhibition of plastin and β H-spectrin resulted in cytokinesis failure in 87 % of the embryos (Chapter IV, Figure 4.2). Conventional β -spectrin and β H-spectrin tetramerize with α -spectrin to form large and flexible F-actin networks. The α/β -spectrin tetramers are about 80 nm at resting but can reach 200 nm when stretched, thus the tetramers formed by α/β H-spectrin are expected to be even longer (Pan et al., 2018; Shen et al., 1986). Acknowledging the evident difference in length and in the type of network formed by these two crosslinkers, we were driven to understand whether other crosslinkers of similar sizes would perform the same functions.

α -actinin is a well-conserved relatively small and rigid crosslinker that measures ~35 nm in length. Both α -actinin and plastin form close F-actin parallel or antiparallel bundles *in vitro* (Hampton et al., 2007; Meyer & Aebi, 1990; Wachsstock et al., 1993; Matsudaira et al., 1983; Skau et al., 2011). In *C. elegans*, ATN-1 is the closest crosslinker to PLST-1 both in terms of size and type of F-actin bundles it forms and therefore, it might perform similar functions as PLST-1.

In the present study, combining the powerful genetics and visual accessibility of *C. elegans*, we assessed whether the forced expression of the small and rigid crosslinker ATN-1 during cytokinesis in one-cell embryos could selectively replace PLST-1. We aimed to

gain new insights into the importance of the crosslinkers' size to their specific contribution for cytokinesis.

5.3. Methods

5.3.1. *C. elegans* strains

The *C. elegans* strains used in this study are listed in Table 5.1. Strains were maintained at 16 °C or 20 °C on nematode growth medium (NGM) plates seeded with OP50 *E. coli*, as described in Chapter III - Methods.

5.3.2. Generation of transgenic strain ATN-1::GFP*

* This strain was generated by other lab members.

The *atn-1* transgene fused to GFP in strain GCP339 was generated by Mos1-mediated single-copy transgene insertion (MosSCI) onto chromosome II, tTi5605 (Frøkjær-Jensen et al., 2008; Frøkjær-Jensen et al., 2012). The *mex-5* promoter region (0.55 kb; primer Rv CTGGAAGATTGCGTTCGGTC), *atn-1* ORF (primers Fw CAGAGAGGTGACCGAACGCAATCTTCCAGATGGTCGACTACTACCAACAG and Rv GAAAAGTTCTTCTCCTTTACTCATCTGCAGTTGTGAATAAAGACTACGAC) and *tbb-2* 3'UTR (0.33 kb; primer Fw ATGCAAGATCCTTTCAAGCATTCC) were cloned as overlapping fragments from genomic DNA. GFP was cloned from pAC93 with the primers Fw CTGCAGATGAGTAAAGGAGAAGAACTTTTCACTGG and Rv GAAGGGAATGCTTGAAAGGATCTTGCATCTGCAGTTATGCACCCTTTTC. All fragments were assembled with pCFJ151 backbone through Gibson assembly (Gibson et al., 2009). Single-copy transgene insertions were generated by injecting a mixture of target plasmid pAC310, plasmid with transposase pCFJ601 and plasmids carrying the selection markers pCFJ90, pCFJ104 and pGH8 into the strain EG6699, as described previously (Frøkjær-Jensen et al., 2008; Frøkjær-Jensen et al., 2012). Transgene integrations were confirmed by PCR of regions spanning each side of the insertion, amplification and digestion with restriction enzymes were made to confirm the correct size and band pattern of the entire genomic locus and fluorescence microscopy was used to confirm the expression of ATN-1::GFP.

5.3.3. RNA interference

RNAi was performed by feeding hermaphrodites with bacteria that express dsRNA, as previously described in Chapter III. L4440 (Addgene_1654) carrying the *sma-1* sequence was built by cloning a *sma-1* cDNA fragment amplified with the primers Forward CCCCCGATATCGACACCCTTTCAAATG and Reverse CCCCCGATATCCCTTCTGTTCCATCTCGTTTC in from N2 cDNA into the EcoRV restriction enzyme site. L4440 vector was transformed into HT115 *E. coli* and these bacteria were used to prepare RNAi plates as described in Chapter III. L4-stage animals of strains GCP339, GCP570 and GCP987 were fed with dsRNA and incubated at 20 °C for 48-54 hours prior to dissection for imaging.

5.3.4. Live-imaging

Gravid hermaphrodites were dissected and one-cell embryos were mounted in a drop of M9 (86 mM NaCl, 42 mM Na₂HPO₄, 22 mM KH₂PO₄ and 1 mM MgSO₄) on 2 % agarose pads overlaid with a coverslip. Live imaging was performed at 20 °C.

Images were acquired on a spinning disk confocal system (Andor Revolution XD Confocal System; Andor Technology) with a confocal scanner unit (CSU-X1; Yokogawa Electric Corporation) mounted on an inverted microscope (Ti-E, Nikon) equipped with a 60x oil-immersion Plan-Apochromat objective (N.A. 1.4) and solid-state lasers of 488 nm (50 mW) and 561 nm (50 mW). For image acquisition, an electron multiplication back-thinned charge coupled device camera (iXon Ultra 897; Andor Technology) was used. Acquisition parameters, shutters and focus were controlled by Andor iQ3 software.

For cortical imaging, 7 x 0.5 µm z-stacks were collected every 5 s in embryos of strains GCP22, GCP339, GCP832 and GCP987 (Figures 5.1D, 5.2A and 5.3B). An image at the center of the embryo was also acquired to determine the timing of anaphase onset. For central plane imaging in Figures 5.1A-C, 5.2A, 5.3C, 6 x 1 µm z-stacks were collected every 10 s in embryos of strains GCP339, GCP987 and COP1506.

For measurement of total cytokinesis time, ring assembly, the percentage of initial ring diameter and percentage of cytokinesis completion/failure in Figures 5.1E-F, 5.2B-D, 5.3A and 5.3D-E images were acquired with an epifluorescence microscope (Zeiss Axio Observer Z1) equipped with a 63x Plan-Apochromat objective (N.A. 1.4), a Colibri.2 LED light source, and an Orca Flash 4.0 camera (Hamamatsu). Acquisition parameters, shutters, and focus were controlled by Zen 2.3 software (Zeiss). 7 x 1 µm z-stacks were collected every 10 s in embryos of strains GCP113, GCP339, GCP570 and GCP987.

5.3.5. Image processing and analysis

Image processing and analysis was performed using Fiji (ImageJ; National Institutes of Health; Schindelin et al., 2012). Z-stacks taken on the cell cortex were projected using the maximum intensity projection tool. The equatorial region of the central plane was selected to make the kymographs displayed in Figures 5.1C and 5.3C using the Make Montage tool. Images in each figure panel were scaled equally, except for Figure 5.1D. Graph plotting was performed with Prism 9.0 (GraphPad Software).

The total cytokinesis time in Figures 5.1E and 5.2B and 5.3D was obtained by determining the time (in seconds) elapsed between anaphase onset and the contractile ring reaching a diameter of 5 μ m, measured in the z-plane with the widest gap between the two tips of the ingressing cleavage furrow. Anaphase onset was defined by direct visualization of DNA, labelled with H2B::mCherry in GCP113 and GCP570, or indirectly by visualization of regions devoid of ATN-1::GFP signal in GCP339 and GCP987.

Ring assembly time in Figure 5.2C was defined as the interval, in seconds, between anaphase onset and the formation of a double-membrane at the cleavage furrow site.

The percentage of ring diameter in Figures 5.1F, 5.2D and 5.3E was calculated by measuring the diameter between both sides of the equatorial embryo, at the contractile ring site, over time, starting at anaphase onset. Values were normalized to the % of the initial diameter (100%) and the mean curves for each condition were plotted.

The percentage of cytokinesis success or failure in Figure 5.3A was assessed by counting the number of one-cell embryos in which furrowing completed or did not complete, respectively, and dividing it by the total number of embryos filmed.

5.3.6. Statistical analysis

Statistical analysis was performed with Prism 9.0 software (GraphPad). Error bars represent 95 % confidence interval of the SEM (95 % CI). Statistical significance tests were performed using unpaired two-tailed t-test with Welch's correction or one-way ANOVA followed by Bonferroni's multiple comparison test, as indicated in the figure legends.

Table 5.1. List of *C. elegans* strains used in this study.

Name	Genotype	Source/ reference
N2	Ancestral	<i>Caenorhabditis</i> Genetics Center

Chapter V – alpha-actinin can replace plastin during cytokinesis in one-cell *C. elegans* embryos

GCP22	ItIs157 [pAC16;pie-1/LifeAct::GFP; unc-119 (+)]; unc-119(ed3) III (?); prtSi2[pAC71;Pnmy-2:nmy-2:reencoded::mCherry::StrepTagII::3'UTR-nmy-2; cb-unc-119(+)]II	Our lab (Osório et al., 2019)
GCP113	nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	Our lab (Silva et al., 2016)
GCP339	unc-119(ed3)III; prtSi118[pAC309; 0.55 kb mex-5p::Atn-1::GFP::StrepTagII::0.33 kb tbb-2 3'UTR; cb-unc-119(+)] II	This thesis
GCP570	plst-1(prt89) IV; nmy-2(cp13[nmy-2::GFP+LoxP]) I; unc-119(ed3) III (?); ItIs37 [pAA64; pie-1/mCherry::his-58; unc-119 (+)]	This thesis (Chapter IV)
GCP832	[plst-1(msn190[plst-1::GFP])] IV; ItIs37 [pAA64; pie-1/mCherry::his-58 +unc-119(+); unc-119(ed3) III (?)]	This thesis (Chapter IV)
GCP987	plst-1 [(prt89)] IV; unc-119(ed3) III; prtSi118[pAC309; 0.55 kb mex-5p::atn-1::GFP::StrepTagII::0.33 kb tbb-2 3'UTR; cb-unc-119(+)] II	This study
COP1506	atn-1(knu478 - degron::mkate2 unc-119); unc-119(ed3) III	Zaidel-Bar Lab
EG6699	ttTi5605 II; unc-119(ed3) III; oxEx1578	<i>Caenorhabditis</i> Genetics Center

* unc-119(ed3) III (?) - ed3 allele was present in one of the paternal strains, but its presence was not checked after the genetic cross

Table 5.2. List of plasmids used in this study.

Plasmid	Source	Reference	Use
L4440	Addgene	RRID:Addgene_1654	RNAi by feeding
pCFJ151	Addgene	RRID:Addgene_19330	Generation of MosSCI strain ATN-1::GFP
pCFJ601	Addgene	RRID:Addgene_34874	MosSCI strain ATN-1::GFP co-injection marker
pCFJ90	Addgene	RRID:Addgene_19327	MosSCI strain ATN-1::GFP co-injection marker
pCFJ104	Addgene	RRID:Addgene_19328	MosSCI strain ATN-1::GFP co-injection marker

pGH8	Addgene	RRID:Addgene_19359	MosSCI strain ATN-1::GFP co-injection marker
pCFJ151-Pmex-5 0.55kb::atn-1::GFP-streptag::3'tbb-2 0.33kb	This study	pAC310	Generation of ATN-1::GFP strain (GCP399)

5.4. Results

5.4.1. Transgenic expression of small crosslinker α -actinin ATN-1 in the early *C. elegans* embryo slows down cytokinesis

In our previous work, we observed that cytokinesis occurs in the presence of multiple crosslinkers in one-cell embryos of *C. elegans*, such as PLST-1, SMA-1, ANI-1 and UNC-59. Using an endogenous mKate2-tagged version of ATN-1 (ATN-1::mKate2), we observed that this crosslinker is not expressed in early embryos of *C. elegans* (Figure 5.1A). In order to understand if the expression of an additional crosslinker in one-cell embryos would impact the F-actin network behavior during cytokinesis *in vivo*, we generated a strain expressing a transgene of ATN-1 tagged with GFP under a germline promoter, to force the expression of ATN-1 in the early embryo (ATN-1::GFP). In this strain, ATN-1::GFP was successfully expressed in one-cell embryos (Figure 5.1B) and localized both to the cell cortex and in the contractile ring throughout cytokinesis (Figures 5.1C-D). Its localization pattern was very similar to that of LifeAct::GFP and the crosslinker PLST-1::GFP, building up an anterior cap of foci at anaphase onset and then accumulating at the equatorial region in linear bundles during ring assembly and constriction (Figure 5.1D). Because of the presence of an extra crosslinker, this presumably represents a situation in which an increased concentration of crosslinkers exists during cytokinesis. To further analyze the impact of the forced expression of ATN-1::GFP in cytokinesis we measured cytokinesis timing. We observed that embryos expressing ATN-1::GFP took longer to complete cytokinesis when compared to control embryos expressing NMY-2::GFP (232 ± 17 s in controls vs 275 ± 18 s in *atn-1::gfp* embryos, Figure 5.1E). That is because the ring constriction stage was slower in embryos expressing ATN-1::GFP when compared to controls (Figure 5.1F). The initial phase of cytokinesis when the ring assembly takes place was similar between controls and embryos expressing ATN-1::GFP (125 ± 13 s in controls vs 125 ± 12 s in *atn-1::gfp* embryos; Figures 5.1F and 5.2C). These results demonstrate that the forced expression of ATN-1::GFP in one-cell embryos slows cytokinesis ring constriction, possibly due to an excessively interconnected actomyosin network.

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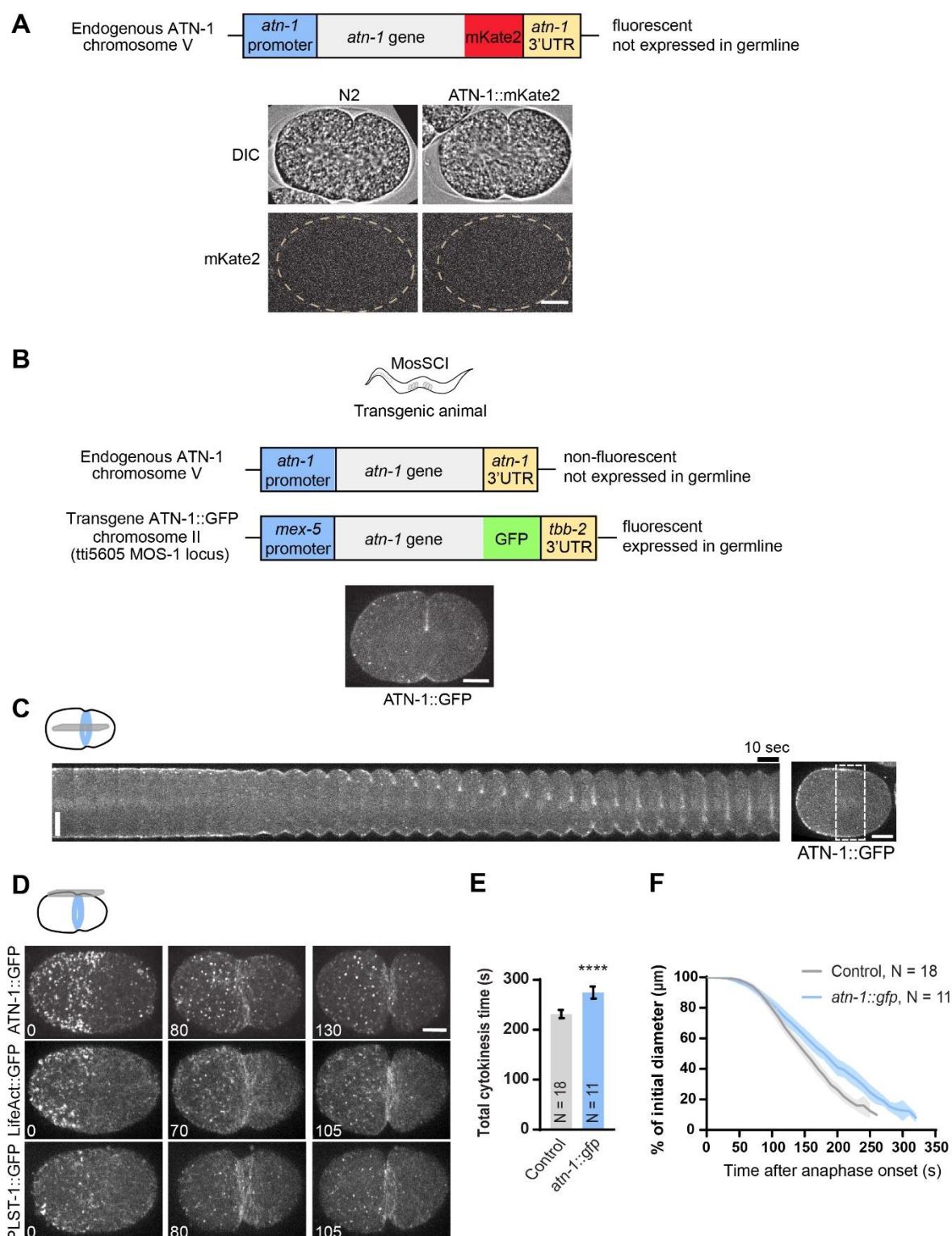


Figure 5.1. Transgenic expression of the small crosslinker α -actinin ATN-1 in the early *C. elegans* embryo slows down cytokinesis. (A) Schematic of the endogenous *atn-1* loci fused to mKate2 (top). Differential interference contrast images (DIC) and fluorescence images of the central plane of N2 and ATN-1::mKate2 one-cell embryos (bottom). (B) Schematic of endogenous *atn-1* and transgenic *atn-1* fused to GFP under the germline-specific *mex-5* promoter and *tbb-2* 3'UTR; transgene was introduced in single copy on

chromosome II using MosSCI (top). Representative fluorescence image of the central plane of *atn-1::gfp* dividing one-cell embryos (bottom). (C) Representative kymograph of the equatorial central plane of ATN-1::GFP in one-cell embryos undergoing cytokinesis. The first frame corresponds to anaphase onset. (D) Images of the cortex in one-cell embryos expressing ATN-1::GFP, LifeAct::GFP or PLST-1::GFP. Numbers indicate the time in seconds after anaphase onset. (E) Total cytokinesis time (mean $\pm$ 95 % CI; see methods for details), in control or *atn-1::gfp* embryos. (F) Mean curves (mean $\pm$ 95 % CI) of contractile ring diameter over time after anaphase onset in control and *atn-1::gfp* embryos. N is the number of embryos analyzed. Statistical significance was determined using an unpaired two-tailed t-test with Welch's correction: ****P $\leq$ 0.0001. Scale bars, 10 μ m.

5.4.2. ATN-1 can replace PLST-1 during cytokinesis

Next, we asked whether ectopic expression of ATN-1 could replace PLST-1 in early embryos of *C. elegans*. To test this, we crossed *atn-1::gfp* transgenic animals with *plst-1(prt89)* animals. Looking at the cell cortex of one-cell embryos during cytokinesis, we observed that in *plst-1(prt89)* embryos, ATN-1::GFP levels were increased in the equatorial region. Similarly, ATN-1::GFP levels were increased in the contractile ring at the tip of the closing furrow (Figure 5.2A). This result indicates that the absence of PLST-1 leads to an increased recruitment of ATN-1::GFP to the cortical layer and to the contractile ring during cytokinesis, possibly as a compensatory mechanism.

Conversely, depletion of the large crosslinker SMA-1 did not impact the recruitment or localization of ATN-1::GFP (Figure 5.2A). This indicates that ATN-1::GFP increased levels selectively occur when plastin (another small crosslinker) is absent.

We next measured the cytokinesis kinetics in *plst-1(prt89)* embryos expressing ATN-1::GFP. We had previously shown that *plst-1(prt89)* embryos had a significantly increased total cytokinesis time when compared to controls, due to prolonged ring assembly (Leite et al., 2020; Chapter IV). Interestingly, we observed that ATN-1::GFP expression rescued the prolonged ring assembly time in *plst-1(prt89)* embryos, which became similar to that of control (dashed grey line) and ATN-1::GFP-expressing embryos [295 ± 41 s in *plst-1(prt89)* vs 144 ± 26 s in *atn-1::gfp;plst-1(prt89)* vs 125 ± 12 s in *atn-1::gfp*; Figure 5.2C]. The total time of cytokinesis was similar in embryos expressing ATN-1::GFP and in *atn-1::gfp;plst-1(prt89)* embryos [379 ± 42 s in *plst-1(prt89)* vs 281 ± 34 s in *atn-1::gfp;plst-1(prt89)* vs 275 ± 18 s in *atn-1::gfp*; Figure 5.2B]. That is because ring constriction in *atn-1::gfp;plst-1(prt89)* embryos was still slower than that of control embryos and became similar to that of *atn-1::gfp* embryos (Figure 5.2D). This means that the absence of plastin did not rescue the slow ring constriction observed in embryos co-expressing ATN-1::GFP and PLST-1. The reason why the constriction of *atn-1::gfp;plst-1(prt89)* is slower than that of control embryos that do not express ATN-1::GFP will need further investigation but it may be due to the overcompensation of ATN-1::GFP observed in *plst-1(prt89)* embryos, possibly maintaining an overly interconnected (and less contractile) network (Figure 5.2A). Alternatively, ATN-1

is not an ideal crosslinker for ring constriction and consequently slows it down. Of note, several other characteristic phenotypes described in *plst-1* mutants, such as the lack of pseudocleavage formation during polarity establishment (a transient cleavage furrow that occurs before the first zygotic mitosis), cortical relaxation throughout mitosis, and the low percentage of cytokinesis failure (Ding et al., 2017; Chapter IV), were all rescued and never observed in *plst-1(prt89)* embryos expressing ATN-1::GFP. Altogether, we conclude that ATN-1 expression in the one-cell embryo is able to largely substitute PLST-1 during cytokinesis.

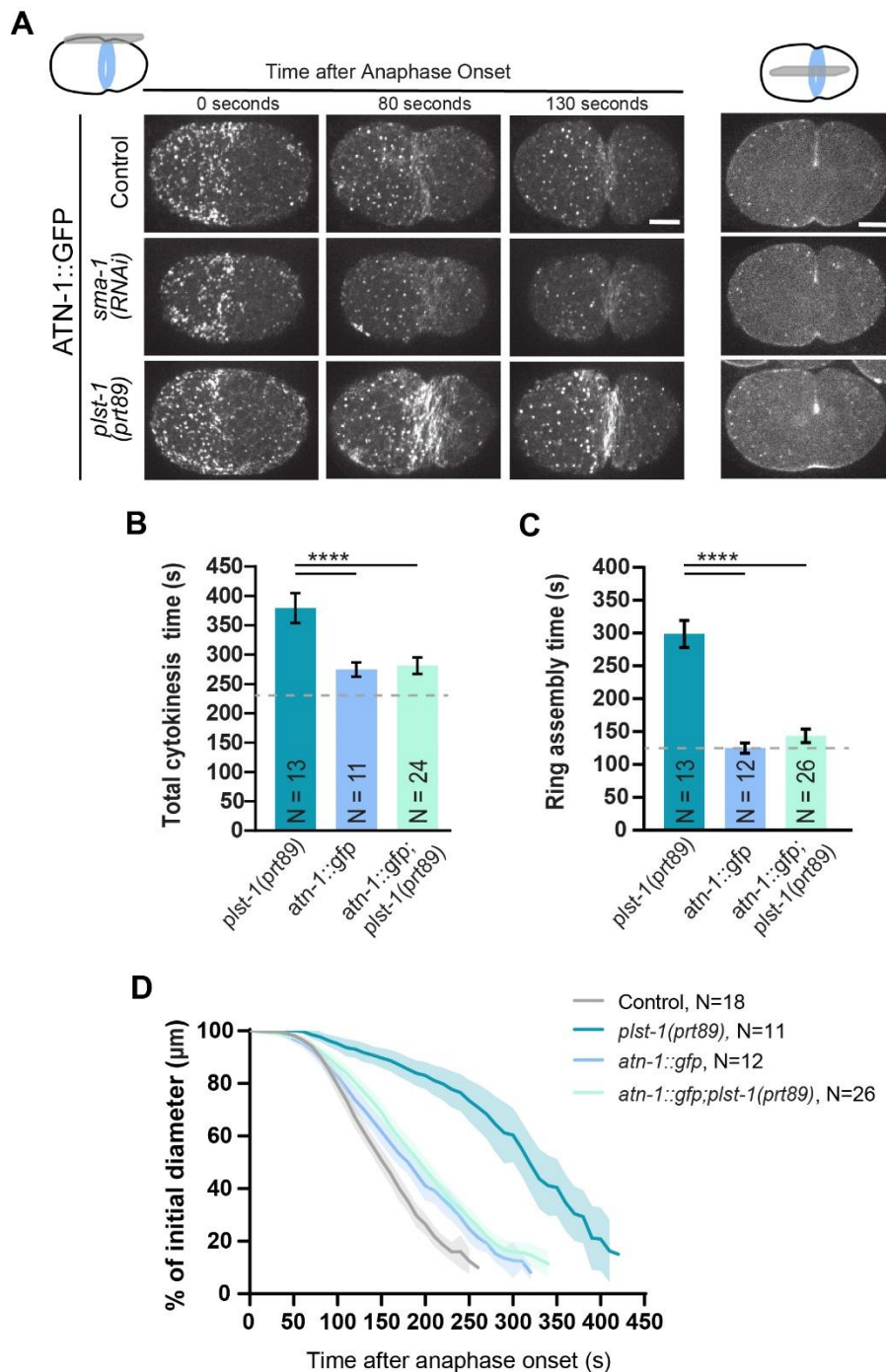


Figure 5.2. ATN-1 can replace PLST-1 during cytokinesis. (A) Images of the cortex (left) and central plane (right) in one-cell embryos of the depicted genotypes expressing ATN-1::GFP. Central plane images were acquired at ~50 % of ring ingression. (B) Total cytokinesis time (mean $\pm$ 95 % CI), defined as the interval between anaphase onset and the contractile ring reaching a diameter of 5 μ m (see methods for details), in embryos of the depicted genotypes. (C) Ring assembly time (mean $\pm$ 95% CI), defined as the interval between anaphase onset and the formation of a double-membrane at the cleavage furrow site. (D) Mean curves (mean $\pm$ 95% CI) of contractile ring diameter over time after anaphase onset in embryos of the depicted genotypes. Grey dashed lines represent the mean of control embryos not expressing ATN-1::GFP. N is the number of embryos analyzed. Statistical significance was determined using one-way ANOVA followed by Bonferroni's multiple comparison test; ****P $\leq$ 0.0001. Scale bars, 10 μ m.

5.4.3. Expression of ATN-1::GFP rescues cytokinesis failure in *plst-1(prt89);sma-1(RNAi)* embryos

We next aimed to understand whether expression of ATN-1::GFP was sufficient to enable cytokinesis in *plst-1(prt89);sma-1(RNAi)* embryos. Strikingly, we observed that the expression of ATN-1::GFP completely rescued the cytokinesis failure of embryos co-inhibited of PLST-1 and SMA-1 [87 % cytokinesis failure in *plst-1(prt89);sma-1(RNAi)* embryos vs 0 % cytokinesis failure in *atn-1::gfp;plst-1(prt89);sma-1(RNAi)* embryos; Figure 5.3A]. Also consistent with our previous results, ATN-1 expression levels were still increased in *atn-1::gfp;plst-1(prt89);sma-1(RNAi)* embryos when compared to control *atn-1::gfp* embryos (Figure 5.3B).

In terms of kinetics, we observed that *atn-1::gfp;plst-1(prt89);sma-1(RNAi)* embryos completed cytokinesis in similar time as *atn-1::gfp* embryos, *atn-1::gfp;sma-1(RNAi)* and *atn-1::gfp;plst-1(prt89)* embryos [275 $\pm$ 18 s in *atn-1::gfp* vs 251 $\pm$ 21 s in *atn-1::gfp;sma-1(RNAi)* vs 281 $\pm$ 34 s in *atn-1::gfp;plst-1(prt89)* vs 252 $\pm$ 26 s in *atn-1::gfp;plst-1(prt89);sma-1(RNAi)* embryos; Figures 5.3C and 5.3D]. This suggests that the increased expression of another small crosslinker is sufficient to overcome the lack of PLST-1 in the absence of SMA-1. The *atn-1::gfp;plst-1(prt89);sma-1(RNAi)* showed a tendency to constrict slightly faster than *atn-1::gfp* embryos (Figure 5.3E), which might be indicative that the removal of another crosslinker may facilitate constriction, supporting the hypothesis that embryos expressing both ATN-1 and PLST-1 or increased levels of ATN-1 have slower ring constriction due to an excess of interconnectivity.

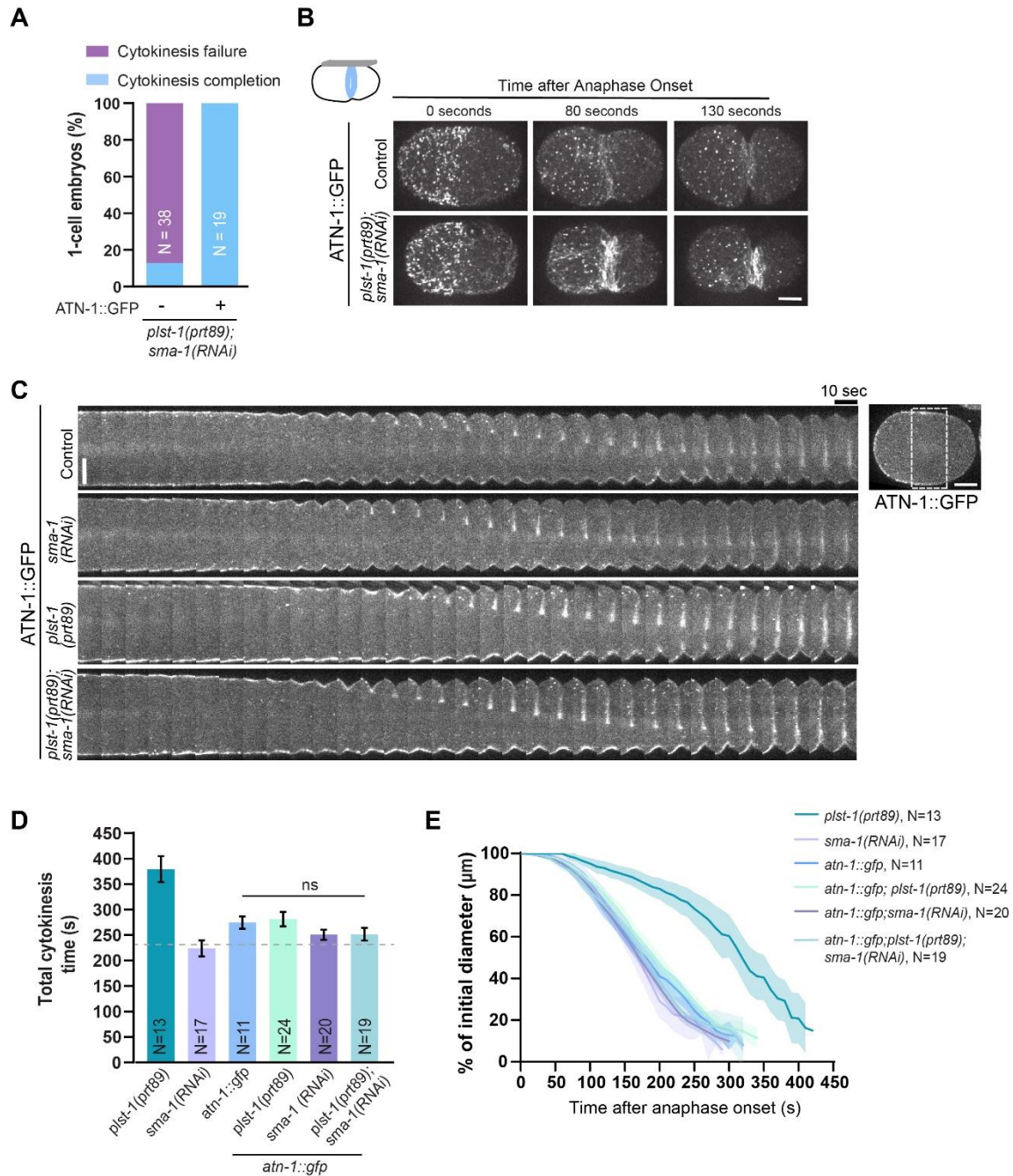


Figure 5.3. Expression of ATN-1::GFP rescues cytokinesis failure in *plst-1(prt89);sma-1(RNAi)* embryos.

(A) Percentage of cytokinesis completion/failure in one-cell embryos of *plst-1(prt89);sma-1(RNAi)* expressing or not expressing ATN-1::GFP. (B) Images of the cortex in one-cell embryos of the control or *plst-1(prt89);sma-1(RNAi)* expressing ATN-1::GFP (C) Representative kymographs of the equatorial central plane of one-cell embryos undergoing cytokinesis of the depicted genotypes expressing ATN-1::GFP. The first frame corresponds to anaphase onset. (D) Total cytokinesis time (mean $\pm$ 95 % CI), defined as the interval between anaphase onset and the contractile ring reaching a diameter of 5 μm (see methods for details), in embryos of the depicted genotypes expressing ATN-1::GFP. (E) Mean curves (mean $\pm$ 95% CI) of contractile ring diameter over time after anaphase onset in embryos of the depicted genotypes. Grey dashed line represents the mean of control embryos not expressing ATN-1::GFP. N is the number of embryos analyzed. Statistical significance was determined using one-way ANOVA followed by Bonferroni's multiple comparison test; ns, not significant. Scale bars, 10 μm .

5.5. Discussion

In vitro and theoretical models postulate that crosslinkers' intrinsic characteristics, such as size and F-actin binding kinetics, are important to modulate the mechanical properties and behavior of the F-actin networks. However, the importance of crosslinkers' size during *in vivo* processes is not explored.

In Chapter IV, we reported that β H-spectrin and plastin, two crosslinkers of very distinct sizes cooperate for a successful cytokinesis. Although SMA-1 per se is not important for cytokinesis [null *sma-1(ru18)* or *sma-1(RNAi)* embryos show no problems in cytokinesis], it becomes essential when PLST-1 is inhibited. Yet, these two crosslinkers are unlikely to perform redundant functions as they have distinct localization patterns. Moreover, SMA-1 is ~20 times longer than PLST-1 and crosslinks F-actin at larger distances, while PLST-1 is compact and crosslinks neighboring F-actin into tight bundles. We then reasoned that if crosslinkers play specific functions within a certain context according to their size, it is possible that other crosslinkers of similar sizes could perform similar functions.

In this chapter we set out to determine whether one crosslinker with similar size to PLST-1 could replace PLST-1 functions during cytokinesis of *C. elegans* one-cell embryos. Apart from PLST-1 and SMA-1, other well-known and conserved F-actin crosslinkers in *C. elegans* are α -actinin (ATN-1), conventional β -spectrin (UNC-70), filamin (FLN-1), anillin (ANI-1), septins (UNC-59 and UNC-61) and coronin (COR-1). Considering these crosslinkers' sizes and characteristics, α -actinin ATN-1 and β -spectrin UNC-70 are the closest to PLST-1 and SMA-1, respectively.

α -actinin is a well-conserved relatively small and rigid crosslinker of ~35 nm. Its canonical structure comprised one actin-binding domain (ABD) composed of two calponin-homology (CH) regions in the N-terminus, followed by four spectrin-repeats (SRs) and two pairs of EF-hand motifs in the C-terminus (Meyer & Aebi, 1990; Taylor & Taylor, 1993). α -actinin shares similar ABDs and/or SRs with other crosslinkers, such as spectrins, plastin, filamin or dynacortin, all of which belong to the spectrin superfamily of proteins (Broderick & Winder, 2005; Dubreuil et al., 1991). Due to its size and characteristics, α -actinin preferentially forms F-actin parallel or antiparallel bundles (Wachsstock et al., 1993; Hampton et al., 2007; Meyer & Aebi, 1990). Despite being ~3-fold the size of plastin (~12 nm), α -actinin is still considered a small-to-intermediate sized crosslinker, and the closest related to plastin in *C. elegans*.

Our findings revealed that the forced expression of ATN-1::GFP as a transgene in the one-cell embryo where it is natively not detected, slows down cytokinesis. It is likely that the presence of an additional small crosslinker in this process leads to excessive bundling and

interconnectivity of the F-actin network and thus restrains the motors activity, stiffens the network and creates more resistance to cell deformation. Consistent with this hypothesis, both theoretical, *in vivo*, and *in vitro* data postulate that excessive interconnectivity does not allow for effective contractility of F-actin networks (Descovich et al., 2018; Ding et al., 2017; Ennomani et al., 2016; Li et al., 2016; Reymann et al., 2012). In fact, in dividing NRK cells, α -actinin overexpression slowed down or impeded cytokinesis (Mukhina et al., 2007). Recent studies have also shown that the stiffness and rigidity of a crosslinked network directly affects the magnitude of myosin forces (Weirich et al., 2021).

Then, it was observed that the expression of ATN-1::GFP in *plst-1(prt89)* mutants could largely replace PLST-1 functions during cytokinesis. *plst-1(prt89)* embryos presumably present a loosely connected and less contractile cortex that suppresses the formation of the stereotypical pseudocleavage during zygote polarization and a drastic delay in cytokinesis completion (Ding et al., 2017; Chapter IV). These phenotypes of *plst-1(prt89)* were fully rescued in *atn-1::gfp;plst-1(prt89)* embryos. Moreover, ATN-1::GFP levels increased in the background of *plst-1(prt89)* but not in *sma-1(RNAi)* depleted embryos. This strongly indicates that ATN-1::GFP selectively compensated for the inhibition of the other small crosslinker, PLST-1, but not for the lack of the large crosslinker, SMA-1, implying a size-related selective compensation. In line with this, *S. pombe* co-express α -actinin and plastin and in this case the two crosslinkers have been shown to be redundant, as knockouts of both proteins fail cytokinesis and are not viable (Wu et al., 2001). In *C. elegans*, *atn-1* null or *atn-1(RNAi)* embryos do not aggravate the phenotypes of *plst-1* mutants, but this is explained by the fact that ATN-1 is not expressed in the early embryos, as we report here (Ding et al., 2017; Chapter III). Nevertheless, these new findings provide an indication that similar-sized crosslinkers may play overlapping functions in physiological contexts where they are both expressed.

Finally, ATN-1::GFP expression was sufficient to rescue the cytokinesis failure in *plst-1(prt89);sma-1(RNAi)*. ATN-1::GFP expression was increased in the co-inhibited embryos in a similar manner to the single *plst-1(prt89)* embryos.

We were eager to know whether UNC-70 could replace SMA-1 functions. UNC-70, the other β -spectrin subunit, is closely related to SMA-1, as it contains identical structural domains, also forms tetramers with the α -spectrin SPC-1 subunit and builds similar types of F-actin networks as SMA-1. The major structural difference between them relies on the number of spectrin-repeats (SRs) that they contain. In *C. elegans*, UNC-70 is composed of 17 SRs while SMA-1 contains 29 SRs. Still, UNC-70 is considered a very large crosslinker when compared to ATN-1 or PLST-1 and a good candidate to replace SMA-1. This hypothesis could not be directly assessed because we were unable to force its expression in the germline, where it has almost undetectable levels, presumably due to well-

documented germline silencing effects (Jia et al., 2019; Moorthy et al., 2000; Chapter IV). Nevertheless, consistent with this hypothesis, we had previously observed in Chapter IV that a smaller SMA-1 mutant that mimics the size of the UNC-70 (*sma-1 Δ 11SRs*) still allowed cytokinesis to occur in the background of *plst-1(prt89)* embryos, thus it is possible that UNC-70 may replace SMA-1 in PLST-1 and SMA-1 co-inhibitions. Contrasting to *atn-1::gfp;plst-1(prt89)* embryos, *plst-1(prt89);sma-1 Δ 11SRs* embryos still presented all the phenotypes of the single *plst-1* mutant, suggesting that the large crosslinkers can selectively replace the large but not the small crosslinkers' function. Despite the similarities between SMA Δ 11SRs and UNC-70, which represent large crosslinkers that form a functional spectrin cytoskeleton and build large flexible F-actin networks, we cannot disregard the possibility that SMA-1 and smaller-SMA-1 may have a relevant mechanosensing function but UNC-70 may not, as other β H- and β -spectrins in *D. melanogaster* S2 cells (Duan et al., 2018).

From the collective data we have so far in this Chapter, we conclude that the increased expression of another small crosslinker ATN-1 is sufficient to overcome the lack of PLST-1, in the presence or absence of SMA-1. This provides new evidence of crosslinkers redundancy *in vivo*. Other cooperations between a small and a large crosslinker are likely to exist. This highlights the importance of the crosslinkers' size in their ability to perform specific functions *in vivo*. Overall, the work developed here provides valuable progress in the understanding of how crosslinkers' biophysical characteristics, as their size, are relevant for the actomyosin networks functioning in cytokinesis.

In the future, further experiments are necessary to complete this study. For instance, we aim to test if another large crosslinker with presumable mechanosensing properties, the *C. elegans* filamin orthologue FLN-1, which seems not to be expressed in early embryos (DeMaso et al., 2011; Kovacevic & Cram, 2010), can replace SMA-1 during cytokinesis. Interestingly, a strain expressing a transgenic full length GFP::FLN-1A under the endogenous promoter and 3'UTR revealed that FLN-1A has a punctate profile that only partially colocalizes with F-actin bundles in the spermatheca and uterus (Kovacevic & Cram, 2010), which is similar to the punctate localization pattern of SMA-1::spGFP in one-cell embryos (Chapter IV, Figure 4.3). It would also be interesting to reduce SMA-1 size even more, in order to understand whether there is a minimal size for it to be able to allow cytokinesis completion in the absence of PLST-1.

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CHAPTER VI

CONCLUDING REMARKS AND PERSPECTIVES

6. CONCLUDING REMARKS AND PERSPECTIVES

My doctoral project consisted in the *in vivo* characterization of a subclass of actin binding proteins, the F-actin crosslinkers.

Crosslinkers mediate the essential interconnection between the filaments of actin that compose an actomyosin network. Actomyosin networks are contractile three-dimensional viscoelastic materials comprising crosslinked F-actin, class II myosin motors, and F-actin and myosin regulators. These networks are a main component of the cytoskeleton of the cell and are involved in essential cellular mechanical processes. In animal cells, the conformations acquired by actomyosin networks are dynamic and adaptable in response to intrinsic or environmental stimuli, so that cells can constrict at the equatorial region to divide, can generate lamellipodia, filopodia and stress fibers to migrate and can contribute to large-scale processes, such as tissue morphogenesis during animal development and muscle contraction during breathing or locomotion (Blanchoin et al. 2014; Munjal and Lecuit 2014). From a molecular point of view to a tissue-scale effect, an actomyosin contractile system is a fine-tuned orchestra. As several pathological conditions, like cancer, neurodegeneration and embryonic malformations (Blanchoin et al. 2014) are known to stem from dysregulation of actomyosin networks, there is an incessant demand for understanding the underlying mechanisms that lead to disease.

Although current data show that crosslinkers localize to virtually any actomyosin-dependent cellular process, studies of the functionalities of multiple crosslinkers in the same context *in vivo* were missing. Also, whether the simultaneous expression of several crosslinkers reflects functional redundancy or a necessary diversification of functionalities was underexplored.

The major goal of this project was to get a more comprehensive understanding on what crosslinkers co-exist and how they contribute, individually or in cooperation, to a range of actomyosin-dependent cellular processes *in vivo*. Focusing on the process of embryonic cytokinesis, the function of a set of well-conserved crosslinkers in one-cell *C. elegans* embryos undergoing their first cell division was evaluated. Additionally, the contribution of the selected set of crosslinkers in two other actomyosin-dependent contexts in this animal was explored: the reproductive system, where oocytes and sperm are formed, and the body wall muscles, which are responsible for animal locomotion and work similarly to vertebrate skeletal muscles.

Crosslinkers diversity is necessary

Throughout the work developed in this thesis, crosslinkers were shown to be involved in the organization of actomyosin networks in a tissue-dependent manner. Some crosslinkers are shown to be more impactful during cytokinesis, such as plastin and β H-spectrin, in organizing the oocyte-producing gonad, such as plastin, anillin and filamin, or in defining skeletal muscle structure, such as α -actinin. These findings complement previous reports in *C. elegans* and, adding to their other reported roles in different organisms (for instance of filamin's signaling functions or anillin's role in organizing the actomyosin cytoskeleton in epithelial cell-cell junctions), highlight the relevance of crosslinkers' diversity and specificity *in vivo*.

The diversity of crosslinkers in the same cell can be advantageous to tune F-actin network dynamics and mechanics in order to optimize specific cell behaviors. At the same time, some redundancy may exist: loss-of-function of some crosslinkers known to be expressed in a specific tissue led to no or only mild phenotypes. Whether two or more crosslinkers are redundant or synergize, requires careful analysis of individual function and localization profiles. In the context of early embryonic cytokinesis, we found that the simultaneous inhibition of two crosslinkers, plastin and β H-spectrin, was lethal. Yet, our data indicate that the two crosslinkers are not redundant: their biophysical properties are distinct and they present different patterns of localization. Although we already tested several other combinations to co-inhibit more than one crosslinker, many more should be tried to clarify the existence of redundancy or synergies in different tissues, taking into account the crosslinker expression profiles and biophysical properties.

To complete this work, we will continue to explore the contribution of each crosslinker in more actomyosin-dependent processes, such as neurons' mechanical robustness, distal tip cell migration and vulval anchor cell invasion through the basement membranes. These processes are readily accessible through the use of specific dyes or fluorescent probes. Although there are transgenic strains carrying constructs of the *fln-1* promoter tagged with GFP or expressing the full-length FLN-1A::GFP under the endogenous promoter and 3'UTR, these strains show no FLN-1 expression in the germline, leaving unclear whether FLN-1 is not expressed in embryos or if this results from the silencing of repetitive extrachromosomal arrays in the germline (Kovacevic and Cram 2010; DeMaso et al. 2011). To overcome this issue, it would be of value to generate an endogenously tagged version of filamin FLN-1 by CRISPR/Cas9, and possibly uncover new contexts where it acts. We cannot disregard the fact that FLN-2, other filamin isogene that was not tested in this project due to low structural conservation to vertebrates and lack of a dimerization domain, also has an ABD. Nevertheless, FLN-2 ABD contains three CH domains, instead of the two

present in FLN-1, being the third more divergent. Additionally, the regulatory region that links CH1 and CH2 is absent in FLN-2, suggesting that the actin binding activity of this filamin may be regulated differently (DeMaso et al. 2011). It could still be that FLN-2 plays a role during cytokinesis, for example. Thus, removal of both FLN-1 and FLN-2 is also an interesting complement to this work.

Novel interplay of crosslinkers during cytokinesis

One of the most fascinating cellular processes that depend on actomyosin contractility is cytokinesis. Similarly to other organisms, cytokinesis in early embryos of *C. elegans* is an intrinsically complex and a highly spatially and temporally regulated process, which relies on very well conserved mechanisms and proteins.

Depending on the crosslinker, the F-actin networks can be arranged into a myriad of architectures, from disordered meshworks to ordered tightly packed bundles (Lieleg, Claessens, and Bausch 2010; Blanchoin et al. 2014), both of which are present in cells during cytokinesis: the equatorial contractile ring is formed by an array of bundled F-actin integrated in and surrounded by a dense criss-cross meshwork of F-actin. Therefore, despite current *in vitro* and *in silico* models only taking into consideration a single crosslinker (usually alpha-actinin), it is not unexpected that different crosslinkers co-exist during cytokinesis and might even interplay or act redundantly.

In the work presented in this thesis, it was observed that cytokinesis occurs in the presence of multiple crosslinkers and, interestingly, a completely novel interplay between the small crosslinker plastin and the large crosslinker β H-spectrin was uncovered. Although plastin has been previously implicated in cytokinesis, this represents the first evidence that spectrins are involved in cytokinesis of animal cells.

Due to their size differences and no overlapping localization at the cell cortex during cytokinesis, it is unlikely that these two crosslinkers play redundant functions. Instead, we found that the two crosslinkers together cooperate to maintain the actomyosin network, especially at the equatorial region of the cell, ensuring the formation of a functional contractile ring. Inhibition of both plastin and β H-spectrin resulted in the collapse of the F-actin bundles and coalescence of myosin into puncta, culminating in cytokinesis failure. Myosin puncta formation and behavior were similar to the myosin puncta that is formed after drug-induced F-actin depolymerization, implying an important F-actin stabilization role for these crosslinkers. Importantly, our data indicate that this synergy is mediated by the actin-binding/crosslinking functions of the two crosslinkers rather than by the plasma membrane F-actin anchoring or SH3-mediated signaling functions of β H-spectrin, evidencing the importance of maintaining an optimal amount of interconnectivity during cytokinesis.

This work highlights plastin as a major modulator of actomyosin network contractility and stability, and spectrin as a new player on cytokinesis which acts as a lifeguard that stabilizes F-actin and maintains its interconnectivity when the system is compromised by the lack of other crosslinker. Overall, we concluded that crosslinkers of different sizes cooperate not only to organize but also to stabilize actomyosin networks *in vivo*.

In collaboration with the Belmonte's laboratory at North Carolina State University (NCSU), we validated our *in vivo* data *in silico* by computational modeling considering a simplified network that mimicked the equatorial cell cortex and that was able to compact into a contractile ring. The four elements involved were F-actin, myosin, a small/rigid crosslinker (plastin) and a large/flexible crosslinker (β H-spectrin). By simulating four distinct scenarios with the four elements (no plastin, no β H-spectrin and neither plastin nor β H-spectrin), we were able to recapitulate the success/failure of cytokinesis that we observed *in vivo*, as long as plastin was more abundant than β H-spectrin.

Future work in human tissue culture cells will provide valuable information about the conservation of this synergy. If conserved, this may shed new insight into the mechanisms underlying polyploidy, a hallmark of cancer. Finally, a systematic analysis across a variety of systems showing which crosslinkers are present within the contractile ring or at the surrounding cortex, as well as their relative abundances, would also be necessary to have a definitive overview of the individual roles of crosslinkers during cytokinesis. This collective knowledge will be essential for the global understanding of cytokinesis biomechanics.

Does size matter?

Crosslinkers are important modulators of the mechanical properties of F-actin networks (Lieleg, Claessens, and Bausch 2010). The observation that two different-sized crosslinkers cooperate for a successful cytokinesis led us to consider size as a relevant feature on the basis of this synergy. Indeed, our studies show that replacing the endogenous smaller crosslinker, plastin, by a transgenic small crosslinker, α -actinin (fused to GFP, ATN-1::GFP), in one-cell embryos, not only rescued the contractility during early embryonic polarity establishment and the timing to assemble the contractile ring during the first embryonic cytokinesis in plastin mutant embryos, but also rescued the cytokinesis failure phenotype in embryos lacking both plastin and β H-spectrin [*plst-1(prt89);sma-1(RNAi)*]. Moreover, ATN-1::GFP levels were selectively increased in *plst-1(prt89)* embryos but not in *sma-1(RNAi)* embryos, supporting the idea that the small crosslinker ATN-1::GFP was able to compensate for the small crosslinker PLST-1 but not for the large crosslinker SMA-1.

This work highlights that the crosslinker size is a major determinant of their function, providing new evidence that crosslinkers of similar size can play similar functions *in vivo*.

This represents valuable information on the understanding of how crosslinkers' biophysical characteristics shape the actomyosin networks' mechanics and behavior, and further emphasizes the importance of considering the contribution of different types of crosslinkers during cytokinesis in both simulated and *in vivo* studies.

In the future, it will be interesting to test if another large crosslinker, such as filamin, also seemingly missing in *C. elegans* one-cell embryos, can replace β H-spectrin functions during cytokinesis. Filamin falls in the large crosslinker category since it is ~160 nm long when extended. However, it has significant structural differences from the spectrins, since it forms V-shaped dimers instead of elongated tetramers and has a rod-domain composed of immunoglobulin-like repeats instead of SRs. Thus, filamin is expected to form a type of F-actin network that is a bit different from that formed by spectrins, but likely similarly loose and flexible.

Moreover, we aim to generate another strain expressing transgenic ATN-1 without a fluorescent tag, in order to understand whether the slightly slower ring constriction observed in *atn-1::gfp* embryos, when compared to wild-type embryos, is either due to excessive interconnectivity (both PLST-1 and ATN-1 are present) and/or less efficient performance of ATN-1 during ring constriction, or if it occurs as a result of the presence of the GFP tag, which may compromise the process. If ATN-1::GFP proves to be fully functional, linescan quantification of the ATN-1::GFP levels in *plst-1(prt89)* embryos will also be performed in order to confirm the significance of the increase in its levels compared to controls'.

Final considerations

This comprehensive *in vivo* study validates data obtained *in vitro* and extends the knowledge on F-actin crosslinking proteins. It defines specific contributions conferred by each crosslinker, unveils novel cooperative roles and interplay between them, and enlightens the importance of their structural and biophysical properties to modulate the actomyosin network behavior, in a more integrative manner.

Therefore, this work provides new insights into the molecular mechanisms that give rise to the impressive functional diversity of the actin cytoskeleton and may help to shed light into the role of perturbed crosslinkers in a variety of human disorders.

I believe that the work developed in this thesis is of significant interest to the scientific community and represents a valuable advance for the development and cell division fields.

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