Université de Montréal

Understanding the role of CAP proteins in the polarization process of *C. elegans*

par

Forum Bhanshali

Département de pathologie et biologie cellulaire

Faculté de médecine

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Understanding the role of CAP proteins in the polarization process of *C. elegans*

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Forum Bhanshali

a été évalué (e) par un jury composé des personnes suivantes:

Dr. Gilles Hickson président-rapporteur

Dr. Jean-Claude Labbé Directeur de recherche

Dr. Philippe Roux codirecteur

Dr. Christian Rocheleau membre de jury

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Résumé

La division cellulaire asymétrique est un processus crucial dans le développement des organismes multicellulaires puisqu'elle permet la génération de la diversité cellulaire. Les cellules qui se divisent de façon asymétrique doivent tout d'abord se polariser et correctement orienter leur fuseau mitotique pour ségréger des déterminants cellulaires en deux entités distinctes. L'embryon du nématode *C. elegans* est un modèle robuste et largement utilisé pour étudier la division cellulaire asymétrique. Dans cet embryon, le point d'entrée du spermatozoïde détermine l'axe de polarité antéro-postérieur. Suite à la fécondation, le cortex embryonnaire est uniformément contractile et un complexe conservé formé des protéines PAR-3, PAR-6 et PKC-3 (nommé complexe PAR-3 ci-dessous) est localisé sur l'ensemble du cortex. La complétion de la méiose maternelle induit une relaxation corticale au postétieur et un flux cortical vers l'antérieur de l'embryon. Ces contractions corticales asymétriques mènent à la formation d'un domaine antérieur contenant le complexe PAR-3, tandis que le cortex postérieur, dont le complexe PAR-3 s'est délocalisé, est enrichi avec les protéines PAR-2 et PAR-1. Par conséquent, les domaines formés par les protéines PAR définissent un pôle antérieur et un pôle postérieur dans l'embryon suite au remodelage du cytosquelette. Les protéines PAR-4 et PAR-5 restent localisées de façon uniforme dans l'embryon. Curieusement, les protéines PAR exercent une régulation par rétroaction sur la contractilité corticale. Il a été montré qu'une des protéines PAR récemment identifiée, PAR-5, est orthologue à la protéine adaptatrice 14-3-3 et joue un rôle important dans la contractilité corticale. En dépit de son rôle central dans la contractilité corticale et le processus de polarisation cellulaire, le mécanisme par lequel PAR-5 régule la contractilité corticale n'est pas bien compris. Le but de ce projet est de mieux comprendre comment PAR-5 et ses interacteurs contrôlent la régulation des contractions corticales et, de ce fait, la polarité cellulaire. Dans un essai de capture de la protéine GST (*GST pull-down*), nous avons identifié plusieurs nouveaux interacteurs de PAR-5. Parmi ceux-ci, nous avons trouvé CAP-2 (protéine de coiffage de l'actine), qui a été identifiée dans des expériences de capture de 14-3-3 dans trois systèmes modèles différents. CAP-2 est un hétérodimère des protéines CAP, qui sont impliquées dans la régulation de l'actine. Nous avons trouvé que la déplétion des protéines CAP par interférence à l'ARN dans

des vers de type sauvage mène à une augmentation létalité embryonnaire, ce qui suggère que ces protéines jouent un rôle important dans le développement embryonnaire. L'imagerie en temps réel d'embryons déplétés pour les protéines CAP montre qu'ils ont une diminution des contractions corticales avec un sillon de pseudoclivage mois stable, suggérant un défaut dans la régulation du cytosquelette d'actine-myosine. Ceci a également été confirmé par la diminution de la vitesse et du nombre de foci de NMY-2::GFP. En outre, ces embryons montrent une légère diminution de la taille du croissant cortical de PAR-2 lors de la phase d'établissement de la polarité. Les embryons déplétés en CAP-2 montrent également un retard dans la progression du cycle cellulaire, mais le lien entre ce phénotype et la régulation des contractions corticales reste à être précisé. La caractérisation des protéines CAP, des régulateurs du remodelage du cytosquelette, permettra d'améliorer notre compréhension des mécanismes qui sous-tendent l'établissement et le maintien de la polarité cellulaire, et donc la division cellulaire asymétrique.

Mots-clés: *C. elegans*, PAR- 5, la polarisation, protéine de coiffage de l'actine.

Abstract

Asymmetric cell division is a crucial step in organism development, as it allows the generation of cellular diversity. In order to achieve asymmetric division cells need to polarize their cell fate determinants and properly orient their mitotic spindle before division. The *C. elegans* embryo is a powerful and widely used model to study asymmetric cell division. In the embryo the sperm entry site determines the anterior-posterior axis of polarity. In the newly fertilized embryo, shortly after meiosis, the cortex is uniformly contractile and the conserved PAR-3/PAR-6/PKC-3 complex (hereafter referred to as the PAR-3 complex) is localised on the entire cortex. Entry of the sperm triggers posterior smoothening and anterior-directed cortical flows. Asymmetric cortical contractions result in the formation of an anterior domain containing the PAR-3 complex, while the posterior-pole cortex, depleted of the PAR-3 complex, is enriched in PAR-2 and PAR-1 proteins. Therefore PAR domains define an anterior and a posterior pole of the embryo in response to cytoskeleton remodelling. The PAR-4 and PAR-5 proteins remain localized uniformly throughout the embryo. Intriguingly, the PAR proteins exert a feedback regulation on cortical contractility. PAR-5, one of the lately identified PAR proteins, was shown to be an ortholog of the adaptor protein 14-3-3 and to play an important role in cortical contractility. Despite its central role in cortical contractility and henceforth the polarization process, little is known on how PAR-5 regulates cortical contractility. The aim of this project is to better understand the regulation of cortical contractions via the PAR-5 protein and its interactors, and how they control cell polarity. In a GST pull down assay we identified several new interactors of PAR-5. Among these we found CAP-2 (actin capping protein), which was also pulled down with 14-3-3 in three different model systems. CAP-2 has been implicated in actin regulation. Interestingly we found that depletion of CAP proteins by RNA interference in wild type worms results in increased embryonic lethality, suggesting an important role in embryonic development. Live imaging of embryos depleted of CAP proteins shows that these embryos have decreased cortical contractions with a less stable pseudo cleavage furrow, indicating a defect in the regulation of the actin-myosin cytoskeleton. This was further confirmed by the decreased velocity and the number of NMY-2::GFP foci in CAP depleted embryos. Furthermore, these embryos show mild decrease in PAR-2 domain size during the polarity establishment phase. *cap-2*(RNAi)

embryos also show a delay in cell cycle progression, however the role of the cell cycle delay in the regulation of cortical contractions has to be determined. The characterization of CAP proteins, which are cytoskeleton-remodeling regulators, will improve our understanding of the mechanisms underling the establishment and maintenance of cell polarity, and thereby asymmetric cell division.

Key words: *C. elegans*, PAR-5, polarization, actin-capping protein.

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C. elegans. : Caenorhabditis elegans

PSCF : Pseudeocleavage furrow

CF : Cleavage furrow

MT : Microtubules

MTOC : Microtubule organizing centre

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1. Introduction

1.1   Asymmetric cell division

The different cell types, carrying different function are formed from the single cell, or zygote as a result of the asymmetric cell division. Intrinsic and extrinsic cell fate determination is responsible for the cellular diversity. Cells using an extrinsic mechanism divide symmetrically to give rise to two identical daughter cells. In these systems, the different fate is then determined by the environment signal, which is received differently in one daughter cell compared to its sister. Using the intrisic mechanism, the cells establish asymmetry prior to division and divide according to this asymmetry. Cells undergoing asymmetric cell division segregate fate determinants according to the set polarity axis, which is further defined by the set of conserved proteins; the PAR proteins. The asymmetric inheritance of these determinants dictates the different cell fates of the two daughter cells (Betschinger and Knoblich 2004).

1.2   *C. elegans*

The free-living, soil nematode *C. elegans* is an excellent model organism for the genetic analysis of cell cycle regulation during embryonic development. These nematodes are about 1 mm in length and transparent, which make it easily accessible for light microscopy. The worms develop from eggs to fertile adults in about three days. The short reproductive life cycle of *C. elegans* makes it a powerful model organism (Wood, 1988). The worms have two sexes, hermaphrodites and males, consisting of a fixed number of somatic cells, 959 or 1031, respectively. Hermaphrodites reproduce by either mating or self-fertilization and lay about approximately 300 eggs during the reproductive life cycle. Males spontaneously arise by X-chromosome nondisjunction at meiosis at a low frequency. Most of the embryonic development occurs outside of the uterus. The first division of the early embryo is highly reproducible and takes about 30 minutes to complete after fertilization. The embryos are relatively large (about 50 µm), and therefore, embryonic development can be easily followed by time-lapse differential interference contrast microscopy.
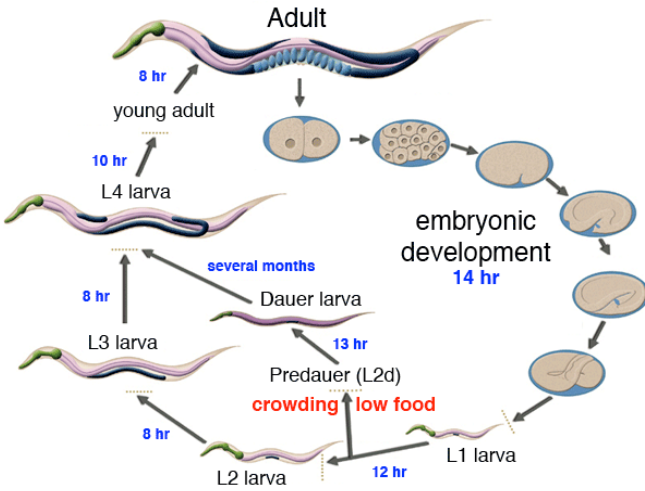
Figure 1: Image representing the life cycle of *C. elegans*.
Adapted from www.wormatlas.org

The effectiveness of RNA interference, as a tool to deplete cellular protein levels in *C. elegans* (Fire et al., 1998) has revolutionized the easiness to assess gene function (Fraser et al., 2000; Gonczy et al., 2000; Sonnichsen et al., 2005). In the past, many groups have performed a series of large-scale functional genomics analyses using RNAi to test additional genes required for early embryogenesis (Sonnichsen et al., 2005; Zipperlen et al., 2001). The phenotypic changes in the embryo are simple to follow, which provides an advantage for studying the polarization process in *C. elegans*: the nuclei and the centrosomes appear as cytoplasmic clearings within the mass of yolk granules in the embryo, which helps to determine the cell cycle stage. Changes in cortical activity, or ruffling, are easily observed and provide a marker of cortical polarity. Furthermore, tracking the movement of yolk granules allows an analysis of polarized cytoplasmic flows, a manifestation of polarity establishment. Advancements in the microscopic techniques such as time-lapse microscopy of fluorescent-tagged proteins allow the analysis of the role of proteins of interest in different processes within the embryo.

1.3   The role of PAR proteins in cell polarity

The first cell division of the *C. elegans* embryo is asymmetric, producing a larger anterior cell and a smaller posterior cell (Begasse and Hyman, 2011; Cowan and Hyman, 2004a; Munro

and Bowerman, 2009). A primary requirement of an asymmetric cell division to take place is the polarization of the parent cell. Polarization is the process by which cellular components are unequally distributed throughout the cell creating domains, which are biochemically, distinct. Depending on the cell type, polarization can define anterior/posterior, apical/basal, dorsal/ventral and other types of distinct regions. The *C. elegans* zygote undergoes anterior/posterior polarization. Establishment of polarized domains enables a cell to move in a certain direction, divide asymmetrically for further differentiation, and perform other important biological functions. The two critical requirements of successful asymmetric cell division in the *C. elegans* zygote are to make two cells with different volumes and to properly segregate cell fate determinants into one or the other half of the cell before the first division. The anterior cell will give rise to purely somatic tissue; while the posterior cell will reiterate asymmetric divisions to eventually give rise to somatic lineages and the germline of the adult organism. To develop into the germline, the posterior cell has to inherit germ cell factors, which are initially distributed throughout the oocyte during the first division.

The critical molecules for cell polarization are known as PAR (partitioning-defective) proteins, which were first identified in a genetic screen for maternal-effect mutations affecting asymmetric cell division (Kemphues et al., 1988). The proper segregation of PAR proteins at the anterior and posterior domains initiates asymmetry. Mutations in those genes produce many overlapping phenotypes, characterized by defects in membrane cleavage, cell cycle timing, and failure to properly segregate cell fate determinants. The homologs of PARs have been shown to perform similar roles in Drosophila and mammals, suggesting its universal requirement for the polarization process.

1.3.1 The PAR protein family

PAR proteins are required for two basic features of cell polarity: The asymmetric positioning of the mitotic spindle, which allows asymmetric cell division, and the asymmetric distribution of proteins and RNA for the specification of cell fates. Overall six genes encoding PAR proteins have been identified until now in *C. elegans*. PAR-1 and PAR-4 are serine-threonine kinases. PAR-5 is a member of the 14-3-3 family, and binds to phosphorylated serines and threonines. PAR-2 contains a RING finger domain. PAR-3 and PAR-6 contain PDZ domains,

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and, therefore, are suggested to act as scaffold proteins. The asymmetric distribution of these proteins in an embryo is critical for controlling cell polarity.
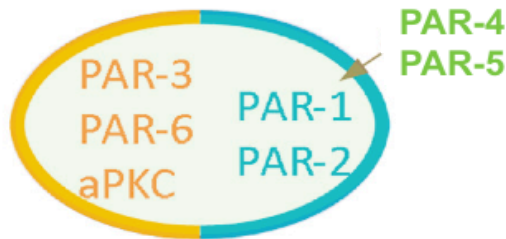
Figure 2: Image represents the localization of the PAR proteins after the establishment phase. Left side is an anterior pole of an embryo while the right side represents the posterior pole. PAR-4 and PAR-5 are found to be present throughout the cortex and the cytoplasm.

1.3.2 Polarity establishment in *C. elegans*

Polarization is subdivided into two distinct phases: establishment and maintenance. The *C. elegans* oocyte does not have a predetermined polarity. The *C. elegans* germ line is syncytial with germcell maturing along the distal to the proximal axis of the gonad (Hubbard et al., 2007). In the distal-most part, termed the proliferative zone, somatic DTC provides the stem cell like niche to maintain population of the mitotic cells while the cells towards the proximal region enter into meiosis (Joshi et al., 2010). The oocyte is ovulated from the proximal gonad. The development of an embryo starts when mature oocyte arrested in prophase of meiosis I gets fertilized as they pass through spermatheca to the uterus. This arrangement leads to the entry of sperm on the proximal side of the mature oocyte and the distal displacement of the oocyte pronucleus. Following fertilization the maternal genome is segregated by two rounds of meiosis and the maternally produced polar bodies are extruded opposite to the sperm entry position. Before the establishment of polarity, anterior PAR proteins occupy the whole cortex, while posterior PAR proteins are mostly in the cytoplasm. The sperm provides a microtubule-organizing center (MTOC) and a male pronucleus to the existing egg and the position of the sperm pronucleus determines the posterior pole in the one-cell embryo (Goldstein and Hird, 1996). At fertilization, the sperm MTOC provides a cue that excludes anterior PAR proteins from the cortex of the posterior pole; as a result, posterior PAR proteins accumulate at the

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posterior, thus initiating polarity establishment in the embryo. The nature of the initiating stimulus provided by the sperm MTOC is not completely understood, however, the weakening of actomyosin contractility at the posterior cortex is proposed to be a mechanism to initiate polarity. The break triggers a large-scale movement of the contractile actomyosin cortex, generating cortical flows towards the anterior, which further results in regression of the anterior PAR proteins toward the anterior side and concomitantly, the appearance of the posterior PAR proteins at the posterior cortex (Cowan and Hyman, 2004b; Cuenca et al., 2003; Munro et al., 2004).
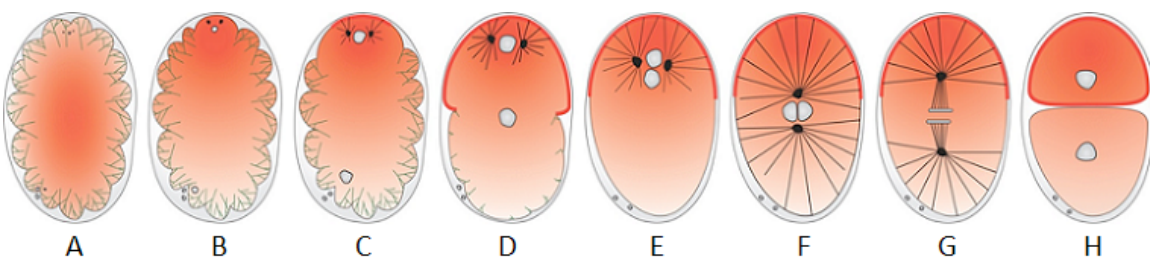
Figure 3: Image representing the different stages of polarity establishment.
(A) Before fertilization, the embryo exhibits uniform cortical ruffling. (B) Centrosomal cue bought by sperm causes destabilization of the actomyosin network at the posterior pole and results in the smoothening of the posterior end. (C) It generates the actomyosin flows towards anterior and the actomyosin network recedes from the posterior along with the anterior PAR proteins, PAR-3 and PAR-6 (D) The maternal pronucleus starts migrating towards posterior pole and the PAR-2 domain expands at posterior, which in turn recruits PAR-1 (E) Pseudo cleavage relaxes, pronuclei meet, and (F) move to the center of the embryo while microtubules form extensive cortical contacts. (G) Anaphase and (H) first asymmetrical cell division. Figure is adapted from the home page of the Wang lab, http://wanglab.lassp.cornell.edu/?page_id=210.

The anterior PAR-3, PAR-6 and aPKC proteins are positioned by a flow of actomyosin from the posterior (Cuenca et al., 2003; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996) whereas PAR-1 and PAR-2 localize to the posterior pole (Figure 2) ((Boyd et al., 1996; Cuenca et al., 2003; Guo and Kemphues, 1996).

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Both sets of proteins are then associated with the cortex, a thin contractile layer under the cell membrane composed of actin polymers crosslinked by the contractile protein myosin. However PAR-4 and PAR-5 are uniformly present at the cortex and in the cytoplasm (Figure 2) (Morton et al., 2002; Watts et al., 2000). Mutations in PAR genes disrupt their own asymmetry as well as many of the other developmental features mentioned above.

Recent studies have suggested another mechanism of polarity establishment, which relies on the initial enhancement of PAR-2, rather than depletion of anterior PAR proteins from the posterior cortex. According to these studies, partial depletion of *ect-2* or *mlc-4* eliminated cortical contractility and cortical flows but still PAR polarity was established, which was mediated by PAR-2. PAR-2-mediated partially redundant pathway exists to initiate polarity when the actomyosin network is compromised (Zonies et al., 2010). In this mechanism, PAR-2 binds to microtubules, and a high microtubule density near the cortex protects PAR-2 from phosphorylation by aPKC. This results in the initial recruitment of a few PAR-2 molecules to the cortex, and then additional recruitment facilitated by some positive feedback. PAR-2 then recruits PAR-1, which phosphorylates PAR-3. Phosphorylation results in the exclusion of PAR-3/aPKC complex from the cortex and expanding of PAR-1/PAR-2 domain. Late cortical flow displaces anterior PAR proteins from the posterior. Mutations in PAR-2 that affects microtubule-binding region delay PAR-2 loading by 30 s, presumably the time required for cortical flows to remove PKC-3. It is suggested that the primary function of the PAR-2 feedback loop is to maintain sufficient PAR-1 on the posterior cortex to ensure permanent exclusion of anterior PARs. PAR-2 dependent pathway might not be essential under normal circumstances where the cortical levels of anterior PAR proteins are already biased by the flows, which provides rapid and efficient response, further suggesting that PAR-2 and microtubules provides secondary mechanism to ensure polarization of the embryo (Cowan and Hyman, 2004b; Tsai and Ahringer, 2007; Sonneville and Gonczy, 2004). Once established, the anterior PAR proteins prevent the posterior PAR proteins from localizing anteriorly, and vice versa, which is termed as a maintenance phase. The maintenance phase is characterized by maintaining polarity due to mutual inhibition of anterior and posterior PAR proteins. The main component that is reported to play a role in this phase is PAR-2. PAR-2 on posterior pole restricts the PAR-6 to the anterior cortex by mutual exclusion. This is further been validated

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by the fact that in *par-2* mutant embryos, cortical PAR-6 can occupy the entire circumference of an embryo (Munro et al., 2004). CDC-42, a small G protein has been shown to be an important component as well. Cdc-42 is shown to interact with PAR-6 in the yeast two-hybrid assay and it has been theorized that CDC-42 may modulate the actomyosin network during the maintenance phase by preventing the flow of NMY-2 towards the posterior (reviewed in Munro and Bowerman, 2009; Willis et al., 2006; Marston and Goldstein, 2006). However, the precise mechanism by which this mutual exclusion is established and maintained is not fully understood.
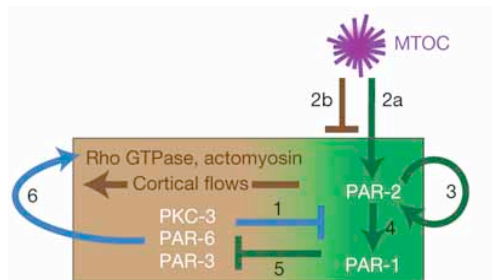
Figure 4: Image represents the schematic model proposed for the PAR-2 dependent microtubule-dependent polarization process for the establishment of the polarity.
Adapted from the paper (Motegi et al., 2011).

1.4 The role of actin during cell polarization

Extracellular or endogenous signals induce reorganization of the actin cytoskeleton, which leads to polarized cell morphology, and polarized distribution of downstream molecules. The actin cytoskeleton is a highly dynamic meshwork that provides mechanical support (Pollard and Borisy, 2003) and facilitates movement of molecules and organelles within the cell. Bundling of parallel actin filaments into cables stabilized by tropomyosins serves as tracks for myosin-V-mediated transport of vesicles, an essential process for cell polarization (Bretscher, 2003). The assembly of actin filaments and myosin into contractile filaments provides the force for cortical contraction and hence any change in the acto-myosin network could change the contractile properties of the cortex. The modulation of contractility is crucial in polarity establishment in *C. elegans* embryos. Cell polarization relies on the cue of the symmetry-breaking event to induce a reorganization of the actin cytoskeleton, leading to polarized

cellular domains and an asymmetric distribution of cytoskeletal function. Prior to polarization, the entire cortex undergoes uniform contractions. After polarization, half the cortex is contractile and the other half is noncontractile, which involves the change in its structural properties. Initial observations suggesting that the actin cytoskeleton is involved in establishing or maintaining asymmetry in the one-cell embryo were based on observations that the usage of F-actin inhibitors such as Latrunculin A and cytochalasin D leads to loss of polarity (Velarde et al., 2007). Other supporting evidence were that many other actin binding genes *nmy-2*, *mlc-4* and *pfn-1* also disrupt polarity (Velarde et al., 2007).

1.4.1 Contractile polarity

Another manifestation of anterior-posterior polarity is the establishment of "contractile polarity". At the end of meiosis, small transient cortical ruffles can be seen over the entire cortex. (Figure 3 A&B). Imaging studies using non-muscle myosin II heavy chain (NMY-2) fused to GFP revealed that initially a uniform contractile meshwork is formed. The ruffling ceases in the area where the centrosome became juxtaposed to the posterior cortex (Cheeks et al., 2004; Cowan and Hyman, 2004b; Cuenca et al., 2003; Munro et al., 2004) which is evident from the disassembly of the contractile meshwork in close vicinity to the posterior nucleus/centrosome complex (Munro et al., 2004). This depends on the regulated activity of the small GTPase Rho (RHO-1) (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). RHO-1 is activated by the activator guanine nucleotide exchange factor (GEF), ECT-2, and inactivated by the GTPase-activating protein (GAPs), RGA-3 and RGA-4 (Schonegg and Hyman, 2006). The asymmetry in contractile polarity is initiated by removing ECT-2 from the site of polarity establishment (Motegi and Sugimoto, 2006). RGA-3 and RGA-4 localises to the small cortical region in proximity to the pronucleus–centrosome complex (Jenkins et al., 2006), which results in the easing of contractions. The smooth cortical domain expands towards the anterior until approximately half the embryo is occupied: RHO-1 and ECT-2 follow the boundary of the shrinking contractile domain (Motegi and Sugimoto, 2006). This causes local weakening of the cortex and generation of the flow of the cytoplasm away from regions of low tension thus creating a tension gradient that propagates through the domain. The acto-myosin network, contractility, and the anterior PAR complex proceed to shrink in a coordinated manner until they occupy half of the embryo. A constriction called the

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pseudocleavage furrow separates the smooth posterior domain from the anterior domain, which remains contractile (Figure 3D) (Hirsh and Vanderslice, 1976; Strome, 1986). At this time, the maternal pronucleus migrates toward the paternal pronucleus. The pseudocleavage furrow relaxes as the pronuclei meet in the posterior half of the embryo.
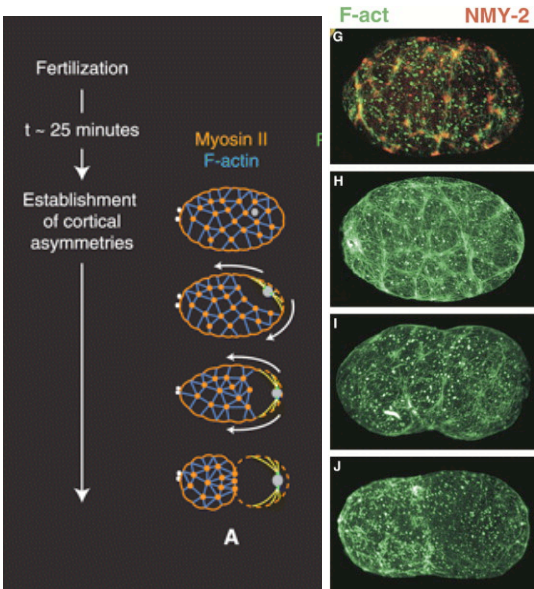
Figure 5: Schematic representation suggesting the movement of cortical acto-myosin flows away from the sperm MTOC towards anterior during the establishment of asymmetries.

The right panel shows the significant change in the structure of acto-myosin cytoskeleton during the establishment phase. The figure is adapted from the paper (Munro et al., 2004).

1.4.2 Cytoplasmic flows

Coincident with the establishment of contractile polarity, large cytoplasmic rearrangements are observed. A flow of cortical yolk granules begins at the posterior pole and moves along the cortex to the pseudocleavage furrow. Cytoplasmic flow directed to the posterior pole replenishes the yolk material that had moved away along the cortex previously (Cheeks et al., 2004; Golden, 2000; Hird and White, 1993). It helps with the distribution of cell fate determinants as well as organelles. Cytoplasmic flow is absent in embryos with abolished cortical contractions (Cuenca et al., 2003; Guo and Kemphues, 1996; Hird and White, 1993; Rappleye et al., 1999; Severson et al., 2002), indicating that acto-myosin contractility is implicated in generating cytoplasmic flows. The PAR proteins were shown to influence

cytoplasmic flows (Cheeks et al., 2004; Munro et al., 2004). In all *par* mutants, except *par-2*, the flows were abolished. However, how the PAR proteins achieve this mechanistically is not clear.

1.4.3 Asymmetric cell division

The pronuclei after meeting move together toward the middle and undergo rotation, such that the centrosomes orient themselves along the antero-posterior polarity axis (Figure 3F). In metaphase, the spindle is displaced towards the posterior of the embryo, giving rise to two daughter cells of different size called asymmetric cell division (Figure 3G&H) (McNally et al., 2010). Each AB and P1 decendents have distinct fates giving rise to different parts of the adult worm. During the second round of mitosis, AB and P1 divide at a different time, with the larger anterior AB cell dividing before the smaller posterior P1 cell, and their division axes are perpendicular to each other.

1.4.4 Relationship between contractile polarity and PAR polarity

The establishment of the contractile and the PAR domains correlates temporally and spatially. Various studies in the past (Cuenca et al., 2003; Munro et al., 2004) (Figure 3B), have suggested that one of the major mechanisms that exists for the establishment of PAR polarity depends on cortical actomyosin. Indeed, disruption of the acto-myosin cytoskeleton resulted in the loss of cortical contractility and mislocalization of the PAR proteins (Cuenca et al., 2003; Guo and Kemphues, 1996; Hill and Strome, 1990; Severson and Bowerman, 2003; Shelton et al., 1999). Depletion of myosin regulatory light chain (MLC-4) reduced the movement of both proteins and the flow of yolk granules to a similar extent and suggested that the anterior PAR proteins are transported somehow by the acto-myosin cytoskeleton to the anterior (Munro et al., 2004). However, the anteriorly-directed movements of NMY-2::GFP are nearly abolished in embryos lacking *par-3*, *par-4*, *par-5* or *par-6* function.

1.5 PAR-5 and polarity

As mentioned above, among the PAR proteins, PAR-3–PAR-6–aPKC complex localizes to the anterior embryonic cortex while PAR-1 and PAR-2 occupy the posterior part. The two domains do not intermix and are mutually exclusive. PAR-4 and PAR-5 remain symmetrically

localized throughout the first embryonic division and are both cortical and cytoplasmic. One of the less well-understood PAR proteins is PAR-5. PAR-5 is a 14-3-3 protein and thus a member of a protein family that is known to regulate numerous cellular processes. 14-3-3 proteins are an evolutionarily conserved family implicated in diverse cellular processes, such as apoptosis or cell cycle regulation, and are associated with pathologies such as cancer (Fig. 1A) (Porter et al., 2006; Tzivion et al., 2006). They bind mainly to serine phosphorylated motifs of other proteins and regulate their subcellular localizations, stability or activity. In mammals, there are seven 14-3-3 proteins corresponding to the isoforms encoded by individual genes (designated β, γ, ε, η, σ, τ or ζ). This redundancy has hindered the study of their cellular functions, and there is still little knowledge about the consequences of 14-3-3 misfunction at the organism level (Porter et al., 2006). *C. elegans* has two genes encoding 14-3-3 proteins, PAR-5 (also named FTT-1) and FTT-2. Both these proteins share 86% of the amino acid sequence identity. Despite this high identity, the expression pattern is distinct because only PAR-5 is expressed in the germline and one cell embryo (Wang and Shakes, 1997; Morton et al., 2002).
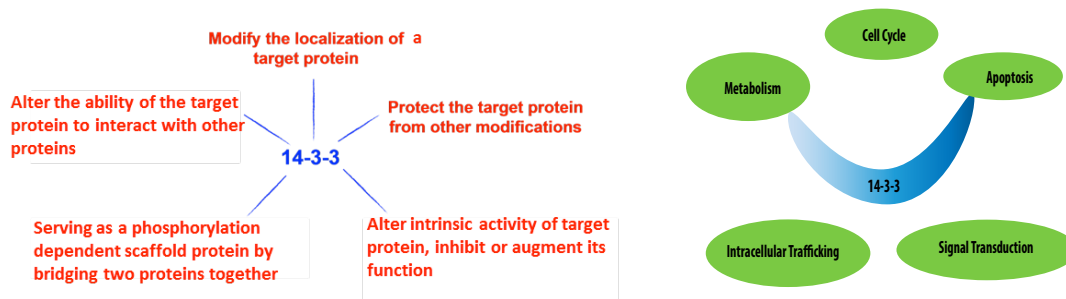
Figure 6: The schematics represent the different mode of action of 14-3-3 proteins and its known role in different biological processes.

In *C. elegans*, PAR-5 is associated with cell cycle control, DNA damage response, chromosome stability, nuclear export, and endocytosis. However, the role of PAR-5 in polarity is still not very clear. PAR-5 is required for the asymmetric localization of all the PAR proteins (except PAR-4). This has placed PAR-5 upstream of all other *par* genes. Animals homozygous for hypomorphic mutations in the 14-3-3 isoform encoded by *par-5* are viable but give rise to progeny with highly penetrant defects in the polarization of the A-P axis

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(Morton et al., 2002). The mutants of *par-5* show an overlapping of PAR-3 and PAR-2 cortical domains, suggesting its role in maintaining the distinct domains (Morton et al., 2002) (Cuenca et al., 2003). Though the exact mechanism of mutual exclusion mediated by PAR-5 is not clear, some mechanistic details could be extrapolated from studies in *Drosophila*, which suggest phosphorylation-mediated exclusion by PAR-5. In this system, the phosphorylation of PAR-1 by aPKC permits binding of 14-3-3, which both inhibits the PAR-1 kinase activity and blocks membrane binding (Hurov et al., 2004). Conversely, PAR-1 present at the lateral cortex of neuroblasts can phosphorylate any PAR-3 protein that diffuses down into its region. The phosphorylated PAR-3 binds PAR-5 and is released from the cell cortex, thereby preventing the spread of PAR-3 into the lateral domain occupied by PAR-1.
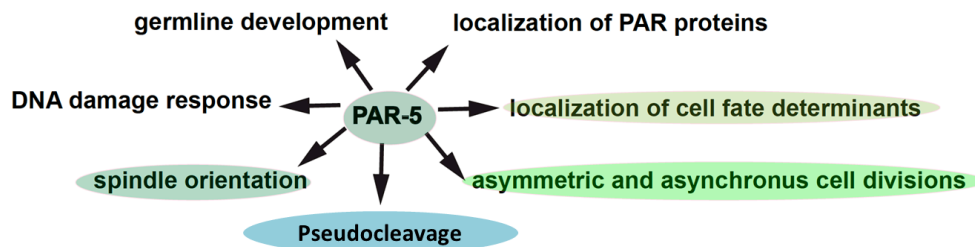
Figure 7: Schematic representing the role of *C. elegans* 14-3-3/PAR-5 in the various developmental process of a one cell embryo.

PAR-5 is also believed to be a negative regulator of contractile polarity. In *par-5* mutant embryos, the cortical flows are compromised and they show hypercontractility, a phenotype that is not common among other polarity-defective mutants; therefore, the hypercontractility cannot be a consequence of the polarity defect. Depletion of PAR-5/14-3-3 by RNAi results in multiple furrows and increased contractility during both pseudocleavage and cytokinesis in one-cell *C. elegans* embryos (Morton et al., 2002). Its role in contractile polarity and PAR polarity has placed PAR-5 upstream of other PAR proteins.

We began our work by characterizing the targets of PAR-5 important for contractile polarity, to understand the mechanistic detail by which PAR-5 regulates polarity. To identify the

interactors, we performed glutathione S-transferase pull-down assays with a PAR-5 fusion protein followed by LC-MS/MS (Figure 8). This approach allowed the identification of 535 unique *C. elegans* PAR-5 protein interactors. We compared these with the proteins that were pulled down as 14-3-3 interactors in *Drosophila* and human cells and found 20 proteins common to all three systems. To functionally validate the interactors, we performed an RNAi-based screen to determine if any of the identified interactors could modulate the change in acto-myosin contractility, by acquiring time-lapse movies of pseudocleavage in wild-type and *par-5*(RNAi) embryos. By assessing the stability and persistence of the pseudocleavage furrow (Result section-6.1), we found that knockdown of the actin binding proteins CAP-1 and CAP-2 (a protein found in our pull down) resulted in embryos that exhibited decreased cortical contractility and produced a less stable pseudocleavage furrow in otherwise hypercontractile *par-5* mutants. As the actin cytoskeleton typically mediates the cortical organization of PAR proteins, we hypothesized that CAP may control *C. elegans* embryonic polarity through its role in modulating actin cytoskeleton, in a PAR-5 dependent or independent manner.

Figure 8: Schematic representing the protocol used for the extraction of protein from the total population of the worms for the Mass spectrometry analysis along with the suitable negative control.

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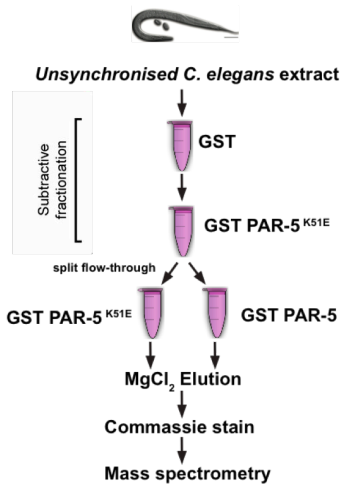| C. elegans gene	Human Ortholog	Drosophila ortholog	GO: Biological Process
pas-7	PSMA3	Prosalpha7	proteolysis
cap-2	CAPZB	cpb	cellular process; cellular component organization
pyp-1	PPA2	Nurf-38	polyphosphate catabolic process
eef-2	EEF2	Ef2b	translation;regulation of translation
atp-2	ATP5B	ATPsyn-beta	respiratory electron transport chain; purine nucleobase metabolic process;cation transport
pbs-3	PSMB3	Prosbeta3	proteolysis
tag-320	PDIA6	CaBP1	protein folding;cellular protein modification process
pdi-2	P4HB	prtp	protein folding;cellular protein modification process
rpt-1	PSMC2	Rpt1	proteolysis
rpt-5	PSMC3	Rpt5	proteolysis
rpn-2	PSMD1	Rpn2	proteolysis;cell cycle;regulation of catalytic activity
pas-1	PSMA6	Prosalpha1	proteolysis
tsn-1	SND1	Tudor-SN	transcription from RNA polymerase II promoter
rack-1	GNB2L1	Rack1	cellular process;protein targeting
ech-6	ECHS1	CG6543	coenzyme metabolism; vitamin biosynthesis; carbohydrate metabolism; fatty acid beta-oxidation
rpl-6	RPL6	RpL6	translation
rab-11.1	RAB11B	Rip11	protein transport, small GTPase mediated signal transduction
cdc-48.1	VCP	TER94	ER-associated misfolded protein catabolic process, embryogenesis
got-1.2	GOT1	Cat	biosynthetic process, cellular amino acid metabolic process
eef-1B.1	EEF1D	Ef1beta	translation elongation

Table 1: Table representing the 20 common interactors of 14-3-3 that were pulled down in the MS analysis of three different model organisms.

1.6 Properties of actin cytoskeleton and its regulation by actin-binding proteins

Actin is one of the most abundant and evolutionary conserved proteins in cells. It exists as a globular monomer called G-actin and as a filamentous helical polymer called F-actin, which is a linear chain of G-actin subunits (Unlu et al., 2014). The actin filament is a polar structure and is formed by head to tail polymerization of G-actin (Alberts et al., Molecular Biology of the cell, 4th edition). Dynamic microfilament reorganization is essential for various developmental stages of first cell *C. elegans* embryo (Ganguly et al., 2011). Actin filament length can be influenced by a number of actin-binding proteins that serve as nucleators, actin-severing proteins or actin-capping proteins (Pollard and Borisy, 2003; Pollard and Cooper, 2009). In mammalian cells, actin nucleators initiate new actin filaments (Firat-Karalar and Welch, 2011). Actin filament growth starts with the formation of an actin dimer, a step, which is extremely unfavorable (Pollard and Cooper, 1986; Pollard, 1986). The addition of a third actin monomer to form a trimer makes the complex more stable, and trimer formation allows subsequent binding of additional actin monomers leading to an elongating filament.

Regulation of the nucleation step is critical for controlling the initiation of actin polymerization and involves several regulators including the Arp2/3 complex and profilin (Cooper and Schafer, 2000). Each monomer binds an ATP molecule that is hydrolyzed following polymerization. This creates polarity in the actin filament. The "new" (barbed) end contains ATP-bound monomers, the neighboring part of the filament is composed of monomers containing ADP and unreleased phosphate (ADP-Pi) and the "old" end or pointed end contains ADP-bound monomers from which the phosphate has been released. Actin monomers assemble much more rapidly at the barbed end, compared to the pointed end. Many proteins bind to actin filaments and influence the dynamics or state. Various nucleation-promoting factors, including junction-mediating and regulatory protein JMY (Sun et al., 2011), N-WASP (Yi et al., 2011) and WAVE2 (Sun et al., 2011), activate the Arp2/3 complex and help to form new branched actin filaments (Pasic et al., 2008; Dominguez, 2009). In addition to actin nucleators, other actin-binding proteins cap, depolymerize, elongate and bundle actin filaments, and are therefore crucial for actin dynamics in various cell types (Pollard and Cooper, 2009). Capping proteins (e.g. CAPZ, gelsolin) for instance bind to the barbed end and prevent further elongation (Jo et al., 2015). Severing proteins (e.g. ADF/cofilin) cause fragmentation of actin filaments. Crosslinking proteins (e.g. α-actinin, fimbrin) and bundling proteins (e.g villin) organize actin filaments into parallel bundles or into branched networks, depending on the cellular context (Pollard et al., 2000; Revenu et al., 2004). This indicates the importance of various actin-binding proteins in Arp2/3-mediated actin reorganization.

1.6.1 Capping protein: a highly conserved heterodimer and regulator of the actin cytoskeleton

One of the crucial regulators of the actin cytoskeleton behaviour is a heterodimeric CAP protein, composed of the α- subunit of 32–36 kDa and a β- subunit of 28–32 kDa (Eckert et al., 2012). CAP protein is found in nearly all eukaryotic organisms, where the structure and amino acid sequence of actin are evolutionary conserved. α- and β- subunits are extensively intertwined in a mushroom-shaped structure and the heterodimer acts as a single protein in terms of its physical properties (Cooper et al., 2008). *In vitro*, CAP binds to barbed ends of F-actin with 1:1 stoichiometry, with an affinity in the subnanomolar range and thus stabilize the

filament by blocking the addition and loss of actin subunits. In the sarcomere of striated muscle cells, CAP is an essential component of the Z-disk (leading to its other name CapZ), where it caps the barbed ends of actin-based thin filaments (Edwards et al., 2014). In non-muscle cells, CAP is important for the assembly of cortical actin and for cases of actin-based motility, such as the formation of membrane protrusions at the leading edge of migrating cells (Cossart el., 2000).

Structural and biochemical studies have led to a capping model in which CAP interacts with the barbed end through two main surfaces (Kim et al., 2010). The top surface of the mushroom-shaped CAP heterodimer is proposed to interact at the interface between the two terminal actin subunits of the barbed end, while another surface comprised of the C-terminal of the β-subunit, known as the β-tentacle binds to the last actin subunit of the F-actin filament (Valladares et al., 2010; Cooper et al., 2008; Edwards et al., 2014).

CAP is required for the ARP2/3 complex-mediated assembly of dendritic actin filament networks, as shown by *in vitro* and *in vivo* studies (Wear et al., 2004). According to this model, nucleator complex ARP2/3 first results in the generation of a branched filament by forming the new daughter filaments on the sides of mother filaments (Wear et al., 2004; Volkmann et al., 2014). The new filaments grow for a while before getting capped by CAP protein. This model points out that the main function of F- actin capping is to ensure that the contractile network consist of short filaments with a high density of branches, which in turn could provide the mechanical stiffness to the membrane (Edwards et al., 2014). Thus, CAP protein indirectly controls branch density (Edwards et al., 2014). Furthermore, another proposed role for capping is to prevent the growth of non-productive barbed ends and, in turn, maintain the available pool of actin subunits for filament elongation at productive locations (Dominguez et al., 2009; Edwards et al., 2014).

Various biochemical and cellular experiments have shown that capping protein is essential for various actin-mediated processes such as cell shape and cell migration (Fan et al., 2011; Mejillano et al., 2004; Pappas et al., 2008). The knockdown of capping protein in hippocampal neurons impairs dendritic spine formation and alters the morphology of dendritic spines (Fan

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et al., 2011). In *Drosophila*, CAP is important for development and morphogenesis, loss-of-function mutations in the ß subunit are lethal at an early larval stage (Hopmann et al., 1996; Ogienko et al., 2013). Reducing the function of CAP function, in the *Drosophila* bristles, results in the disorganization of the actin bundles with the development of the bristles with an abnormal shape (Frank et al., 2006). In mammalian cells, CAP depletion leads to the explosive formation of filopodia, rather than lamellipodia (Mejillano et al., 2004). These results highlight the importance and varied functions of this protein in the formation of actin-based structures and in actin filament dynamics.
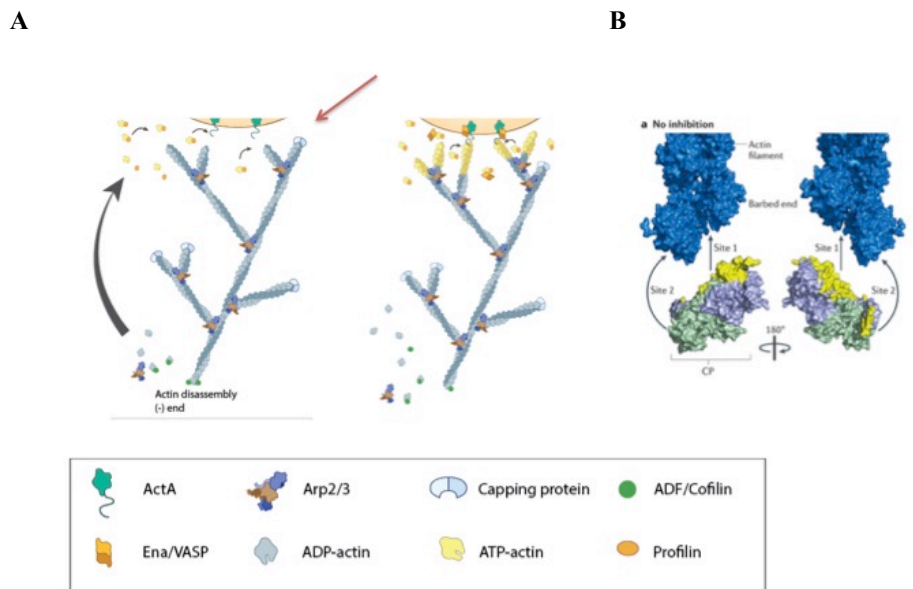
Figure 9: Proposed mechanism of the actin-binding protein and their structural representation. A) The left side represents the schematics of the binding of CAP protein to the barbed end of the F-actin, which in turn prevents the addition and loss of actin monomers. Right side depicts the situation when the CAP protein is removed from the barbed end, which results in the further addition of G-actin to the filament.

B) Structural representation of the interaction of CAP protein to the F-actin through two different surfaces. This figure is adapted from the paper of (Pollard and Cooper, 2009).

Not many studies have been done regarding the role of CAP proteins in *C. elegans*. In *C. elegans*, capping protein is encoded by the genes *cap-1* (α subunit) and *cap-2* (β subunit). In

the germline, partial depletion of CAP proteins dramatically increased the apparent level of F-actin in wild-type animals, where the F-actin lining the rachis of the gonad was thickened and ruffled. In addition to its role in the actin cytoskeleton, CAP-1 and CAP-2 were found to be the homologs of two dynactin complex components in *C. elegans*, where dynactin is a multi-subunit complex required for dynein function and enhancement of dynein processivity along microtubules. CAP-2 was shown to have a role in pronuclear migration (Le Bot et al., 2003). Recently it had been shown that actin capping protein is a novel regulator of MT stability that functions by antagonizing formin mDIa1 activity toward actin filaments, which in turn enhances its activity towards MT in NIH3T3 cells (Bartolini et al., 2012). Another study in developing axons suggested that β subunit of CAP protein could interact with microtubules and regulate growth cone morphology and neurite outgrowth (Davis et al., 2009). Since it was shown earlier that in *C. elegans*, CAP-2 has a role in a number of microtubule dependent events (Le Bot et al., 2003), there might be a possibility that CAP-2 could be mediating the cross talk between actin and the microtubule cytoskeleton in the *C. elegans* embryos. As mentioned previously, microtubules can stimulate the self-organization of PAR proteins that is independent of actin dynamics, there is an interesting possibility that CAP proteins could be mediating the cross talk between two pathways to ensure the polarization process to be robust.

1.7 Hypothesis

We hypothesize that Capping proteins are involved in regulating the acto-myosin contractility in *C. elegans*

1.8   Aim

In order to understand polarity establishment in the *C. elegans* embryo, it is important to understand the interplay between the acto-myosin cytoskeleton and the polarity markers, the PAR proteins. We started this work with an aim to identify novel genes with an essential role in the first cell cycle of the *C. elegans* embryo. From a large amount of data generated in GST pull down assays, candidate genes were selected for a detailed analysis based using functional assay. We identified CAP-2 with a potential role in contractile polarity in the *C. elegans* embryo. We decided to pursue our work further with the CAP proteins, since the regulation of

actin filaments is particularly important for its dynamic assembly that could control acto-myosin contractility. For that, actin needs to be accurately incorporated with proper orientations and uniform lengths. Actin filament dynamics must be differentially controlled to support rapid actin reorganization and turnover during polarization process by an actin-regulatory system. The idea is that the termination of actin elongation by capping protein could be playing an essential role in Arp2/3-driven actin dynamics during polarization process. However, the role of capping protein in the asymmetrical division and more specifically into the polarization process of *C. elegans* embryos is not known. Thus, the detailed understanding of the actin regulatory proteins, such as CAP could be important in investigating the basic mechanism of contractile polarity.

The objective of this thesis was to address several questions concerning the biological role of the CAP proteins in the early *C. elegans* embryo, and more specifically which process is disturbed by RNAi depletion of CAP-1 and CAP-2. What are the molecular functions of CAP protein at the one-cell stage? Does CAP protein modulate microtubule dependent events, as would be predicted from the published data available with the studies in other model systems? And furthermore, if their function is linked to controlling the acto-myosin and microtubule networks in the embryo, how can they be integrated into the known regulatory network governing polarity and asymmetric cell division?

2. Materials and Methods

2.1 Worm strains and Growth

C. elegans strains were maintained on Nematode Growth Medium (NGM) agar plates and grown on a lawn of E. coli strain OP50 as a food source as described (Brenner, 1974). NGM agar was prepared by autoclaving 3% (w/v) NaCl, 2.5% (w/v) peptone, 1.7% (w/v) agar and 5 μg/ml cholesterol in 390 ml H_2O, along with $CaCl_2$ and $MgSO_4$ to a final concentration of 1 mM and potassium phosphate buffer (KPO_4, pH 6.0) to a final concentration of 25 mM. NGM agar was seeded with 0.1 ml OP50 liquid culture and incubated overnight at the room temperature to allow growth and the plates were then stored at 4°C NGM agar plates. Every alternative day, 3 to 5 adult hermaphrodites are transferred to a new feeding plate for its maintenance. All the experiments are performed at 20°C.

The following strains were used for the experiments: N2 [wild-type, Bristol]; TH120 (mCherry–PAR-6 and GFP–PAR-2); JJ1473 (NMY-2–GFP; (Nance et al., 2003)). N2 was maintained at 20°C and GFP and m-Cherry transgenic strains were maintained at 25°C to optimize transgene expression.

2.1.1 Freezing and recovery of *C. elegans* stocks

A plate containing freshly starved animals, with lots of L1 and L2 larvae was washed off with 0.1 M NaCl in a small volume and transferred to a freezing vial with 85% glycerol and NaCl in an equal volume. The vial was placed in -80°C freezer for its storage (Brenner, 1974). For the recovery of frozen worms, one vial of the frozen worm stock was removed from the freezing vial and placed on a fresh feeding plate on room temperature, until it gets dried and then were stocked at the respective temperature, suitable for its growth. After 2 to 5 days, thawed worms were recovered and transferred to a fresh feeding plate.

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2.2 RNA-mediated interference (RNAi) by feeding

Feeding double stranded RNA expressing bacteria to *C. elegans* is a commonly used method to induce RNAi: A fragment corresponding to the gene of interest is inserted into the feeding vector (L4440) via TA cloning between two T7 promoters in an inverted orientation. The vector is transformed into E. coli strain (HT115(DE3)), having IPTG based inducible expression of T7 polymerase (Kamath and Ahringer, 2003) (Timmons and Fire, 1998). Bacterial strains that we have used in our experiments to inactivate the gene of interest were obtained from the library designed by the Ahringer lab (Fraser et al., 2000). Bacteria containing a plasmid with DNA from the candidate gene were streaked out on LB plates with 50μg/ml ampicillin. A single colony was inoculated into 5 ml LB media with 50μg/ml ampicillin and was allowed to grow overnight on shaking condition. The plasmid was extracted, following day and the sequence of the insert was validated by sequencing using the L4440 seq primer at our genomics platform at IRIC.

For preparation of the plates, RNAi feeding bacteria were inoculated in an over night culture (1 ml LB + 50μg/ml ampicillin) at 37°C with shaking overnight. These cultures were diluted 1:100 in 1 mL LB/amp and were grown for three hours before being seeded onto nematode growth plates supplemented with carbenicillin and IPTG for induction to produce double-stranded RNA (Kamath et al., 2001). The plates were then allowed to dry room temperature over night. The plates were then seeded with L4-stage hermaphrodites and incubated at 20°C, depending on the experiment for 24 hours (Arur et al., 2009). The L4440 RNAi vector alone was used as a control.

2.3 Microscopy

DIC or fluorescence microscopy was performed using established protocols (Sulston et al., 1983). Gravid hermaphrodites were transferred to a drop of egg buffer on a depression slide and dissected by a surgical blade to release the embryos. Embryos were collected by the pasteur pipette bulb coupled to the capillary and were then added to freshly made 2% agarose pads on slides. The slide was then covered with the coverslip along with a drop of egg buffer

to prevent the slides from drying out during imaging, and the coverslip was sealed with liquid Vaseline.

Alternatively, for experiments with NMY-2::GFP strain, the coverslip was coated with 20 µl of 0.01% poly-L-lysine solution. Worms were dissected in 10 µl of egg buffer and embryos were pressed gently onto the sticky poly-L-lysine coat using an eyelash glued to a toothpick. On a glass slide, the coverslip was inverted to create an imaging chamber.

2.3.1 Image acquisition

Intensity measurement of the cortical NMY-2::GFP and the velocity of the flows were measured using Swept Confocal microsocopy. Images were acquired on a Nikon SFC microscope (Nikon and Prairie Technologies, Madison, WI, USA) using the 45 µm pinhole setting. Samples were illuminated with 488 nm, 10–30% power and fluorescent light was collected by a 100×/1.4 NA Plan-Apochromat objective to acquire 16 confocal sections (separated by 0.5 µm) at 10 second intervals. Time-lapse Differential Interference Contrast (DIC) microscopy was done using Zeiss Axioimager microscope, using 63×/1.4 oil Plan Apochromat objective. For time-lapse experiments, to answer the questions about the pseudocleavage furrow stability, images were captured at 5 second intervals. To look at the localization of PAR proteins, the time lapse movies were taken at the interval of 10 seconds. The establishment and maintenance phase measurements were taken during the stable period between the end of nuclear envelope breakdown and anaphase onset. All images were taken at the embryo midplane defined as the focal plane with a maximum cross-sectional area. Image analysis was performed using ImageJ software.

2.4 Image analysis

2.4.1 Calculation of pseudocleavage furrow position

 The position of pseudocleavage furrow was measured as a distance along the long axis of the embryo. The distance was standardized to the total length of the embryo and was expressed as a percentage of embryo length. 0% indicates posterior pole.

2.4.2 Counting of NMY-2::GFP foci and its velocity

To count the cortical NMY-2::GFP foci, we have used the method described by Xiong et al., 2011. The cytoplasmic background fluorescence was removed from the images using the ImageJ plug-in 'Subtract Background' (with a 'Rolling ball radius' at 50.0 pixels for NMY::GFP) before converting the processed images into binary images. Subsequently the binary images were used for counting of the cortical NMY-2::GFP foci using the ImageJ plug-in 'Analyze Particles' (Figure 12A).

To measure the velocity of the NMY-2 foci, Image J software was used. Three kymographs were generated using a line tool for each embryo. The line was drawn from the anterior to the posterior pole of the embryos and the line was positioned on each side and one in the center of the embryo (Figure 11A). Ten most posterior foci present in these kymographs were used for quantitation. The velocity of flows was calculated by reporting the distance traveled by these foci over time.

2.4.3 Quantifying the size of PAR-2 and PAR-6 cortical domains

To measure the PAR-2 and PAR-6 domain, during establishment and maintenance phase, ImageJ software was used with a method already established in the lab (Chartier et al., 2011). A 10 pixel-thick line was drawn on the whole perimeter of the embryos, using free line tool and the corresponding mean fluorescence intensity profiles were plotted. The mean fluorescence intensity values were interpolated as the percentage of the total perimeter for each embryo. In all cases 0%=anterior pole and 100%=posterior pole. Intensities were represented as the percentage of maximum intensity along the perimeter. The length of domains was calculated as the length of embryo perimeter with intensity values superior to a 70% threshold.

2.4.4 Calculation of male pronuclear migration distance

Male pronuclear migration distance was measured at pronuclear meeting and when their adjacent membranes form a straight line, using the line tool in Image J. To calculate the

distance, we divided the distance travelled by male pronucleus from the adjacent membrane to the tip of the pronuclear membrane divided by the total length of an embryo.

3. Results

3.1 CAP proteins regulates the stability of the pseudocleavage furrow ingression

In wild-type one-celled embryos a constriction of the cortex named the pseudocleavage begins approximately 200 seconds before pronuclear meeting and recedes by the time of pronuclear meeting. In the embryos, depleted of CAP by RNAi, the pseudocleavage relaxed much earlier compared to the control embryos (Figure 10A). In embryos depleted of CAP-1, pseudocleavage relaxed 40 seconds earlier, while in CAP-2 depleted embryos, pseudocleavage relaxed approximately 45 seconds earlier as measured as the duration between pronuclear meeting to the pseudocleavage relaxation, compared to WT (Figure 10A and Table 2). We even measured the depth of the pseudocleavage ingression, where we found that in wild-type embryos, the average size of the maximal ingression was approximately 40% of the total embryo width, while in the mutants the depth of the pseudocleavage furrow ingression was reduced, with *cap-1* (RNAi) showed the maximal constriction of 20% and *cap-2* (RNAi) showed the maximal constriction of 22% respectively (Figure 10B). Together these results suggested that the depletion of CAP proteins affected the stability of the furrow in the one-celled embryos.
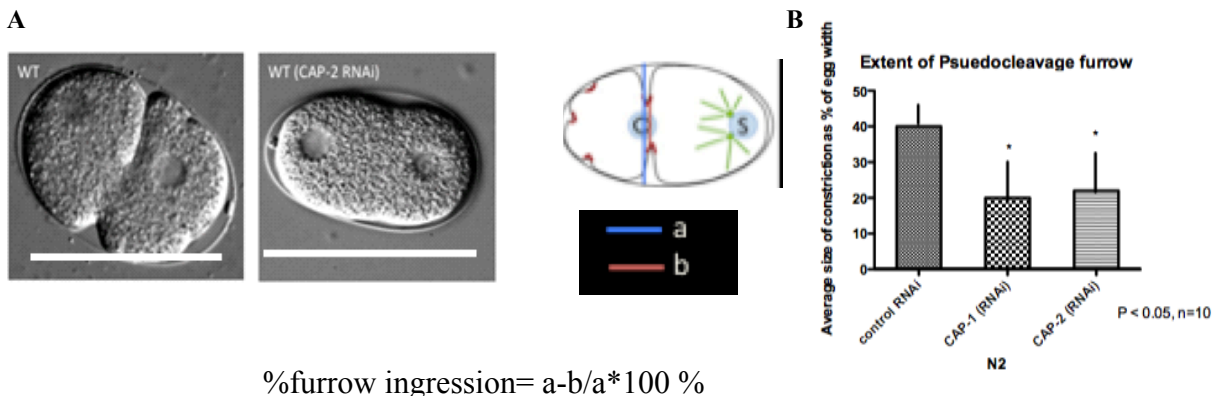
A

B

%furrow ingression= a-b/a*100 %

Figure 10: CAP is required for the stability of pseudeocleavage furrow and the postion of the pseudocleavage furrow.

A) A representative image suggesting that the *cap-2* (RNAi) embryos have less stable pseudocleavage furrow compared to the controls. The image is taken at the time of maximal pseudocleavage constriction.

B) Graph representing the average pseudo-cleavage width relative to the average width of the one-cell embryo in control and *cap-1* (RNAi) and *cap-2* (RNAi) embryos. In all panels error bars represent SD with significance of $p < 0.05$.

The placement of the PSCF was affected, where in control embryos, PSCF were placed at placed at $49 \pm 2\%$ (n=8) of embryo length away from the posterior pole. However in *cap-1* (RNAi) embryos, PSCF moves $43 \pm 1.7\%$ (n=10) and in *cap-2* (RNAi) embryos, PCSF moved $44 \pm 1.9\%$ of embryo length away from the posterior pole (Table 2).

RNAi	n	Position of Pseudocleavage furrow (% egg length from posterior)	Duration between Pronuclear meeting to pseudocleavage relaxation
Wild type	8	49%±2	5s±1.8
CAP-1	10	43%±1.7	-40s±5
CAP-2	9	44%±1.8	-42s±4.2

Table 2: Table representing the quantification of phenotypes after *cap-1* and *cap-2* (RNAi) on various aspect of the pseudocleavage furrow.

3.2 CAP-2 may regulate the acto-myosin cytoskeleton during polarity establishment.

To investigate in more detail the requirement of CAP protein in cortical contractility, a transgenic strain expressing NMY-2::GFP was used to monitor the organization and dynamics of myosin by time-lapse microscopy. In early control embryos, NMY-2::GFP forms first a dynamic network throughout the entire cortex consisting of foci clusters interconnected by small filaments (Figure 5 and Figure 11A). As mentioned before, in close vicinity to the posterior cortex and from the cue delivered by the male pronuclei, the NMY-2::GFP network begins to disassemble and coincidently segregates towards the anterior half (Munro et al.,

2004). During this process, the NMY-2::GFP foci clusters become concentrated into an anterior cap, while the posterior half becomes devoid of detectable NMY-2::GFP foci.

A

B

C
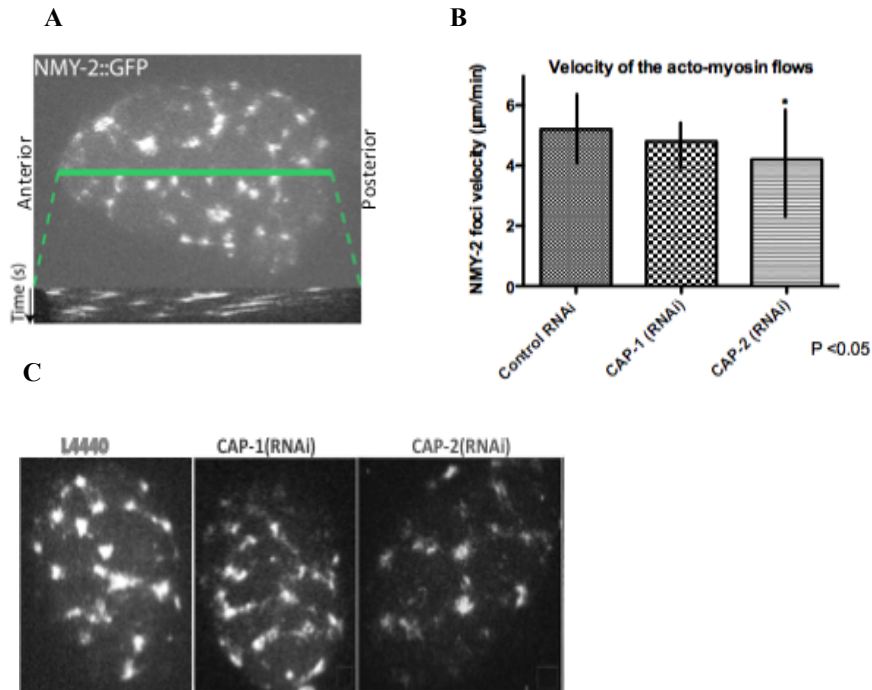
Figure 11: Velocity of the acto-myosin flows are reduced in the CAP-2 depleted embryos.

A) Kymographs were generated by drawing the line from anterior to posterior axis to measure the velocity of the flows.

B) Quantification of the velocities ± SD of NMY-2::GFP foci calculated from kymographs at the posterior of wild-type (5.2 ± 1.6 μm/min, n = 8), *cap-1* (RNAi) (4.8 ± 06 μm/min, n = 7, N.S), and *cap-2* (RNAi) (4.2 ± 1.6 μm/min, n = 7, P<0.05) embryos. Ten individual foci were quantified per embryo.

C) Representative epifluorescent images along the anteroposterior axis.

We observed that reducing the function of CAP-2 by RNAi altered the NMY-2::GFP organization, where the number of foci in the early embryos were fewer compared to WT (as evident from the representative image, Figure 11C) as well as the NMY-2::GFP foci appeared smaller in *cap-2* (RNAi) (not quantified) (Figure 11C). CAP-2 had an average of 29 foci

compared to 45 in wildtype (Figure 12B). Moreover on measuring the velocity of the NMY-2::GFP flows, we observed that these foci segregated to the anterior pole at a slower rate (average velocity 4.2 μm/min, n=7) in a *cap-2* (RNAi) embryo compated to WT (5.2 μm/min, n=8) (Figure 11B). However no significant defect was observed in the velocity or an organization of NMY-2, on depletion of CAP-1 protein.
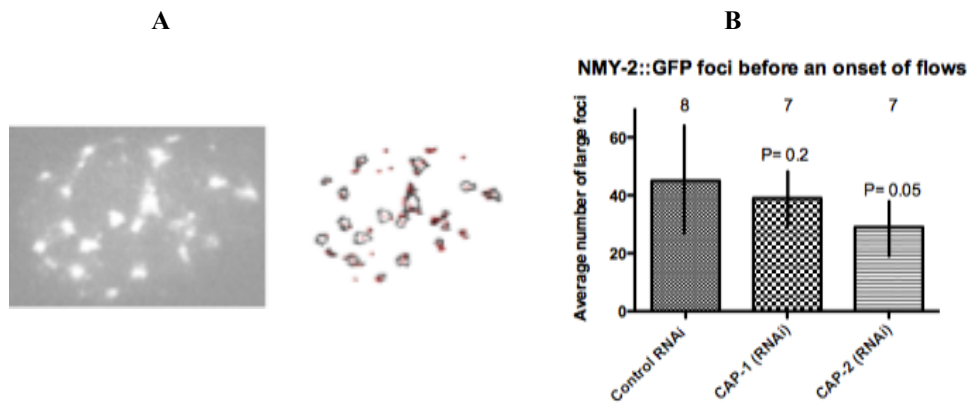
Figure 12: Number of acto-myosin focis are reduced in CAP-2 depleted embyros.

A) Schematic representation of the method used to count the foci using Image J software by applying an appropriate threshold.

B) Quantification of the number of NMY-2 GFP foci calculated using Image J, with wild type embryos had an average of 45 foci, *cap-1* (RNAi) (39 foci, NS) while *cap-2* (RNAi) (29 foci, P=0.05).

Together with the changed number of large NMY-2::GFP puncta, and the slower dynamics of anterior movement of NMY-2::GFP to the anterior, these results suggest that CAP-2 may regulate the acto-myosin cytoskeleton during polarity establishment.

3.3 Depletion of CAP-2 mildly affects the size of PAR-2 domain

The movement of NMY-2 to the anterior pole recruits the anterior PAR proteins towards anterior (Munro et al., 2004). Therefore, changes in NMY-2 and actin dynamics could alter the establishment of anterior/posterior polarity through the PARs. Upon recruitment by NMY-2, PAR-6 becomes enriched at the cortex in the anterior half of the embryo and disappears from

the posterior half by the time of pronuclear meeting. To investigate whether the reduced contractile activity affects PAR localization in *cap-1* and *cap-2* (RNAi) embryos, time-lapse images of GFP::PAR-2 and mcherry::PAR-6 were made, with measurements taken at the establishment phase and maintenance phase (Figure 13A).

Embryos depleted of both CAP-1 and CAP-2 by RNAi exhibited mildly smaller PAR-2 posterior domain of approximately 42% embryo length, n=5 compared to the wildtype with the 49% of embryo length during the establishment phase (Figure 13B, right panel). The PAR-2 domain size was found to be further corrected and repositioned to wild type animals to match the site of cell division during the maintenance phase (Figure 13B, right panel). However we have not measured the rate at which PAR-2 expanded during division, or if the smaller size of the PAR-2 domain during establishment phase corresponds with the decreased velocity of NMY-2. Neverthless the analysis of *cap* (RNAi) embryos suggests that both CAP-1 and CAP-2 is required in some way to establish the boundary between the posterior and the anterior PAR domains. We show that CAP-1 behaved similarly to CAP-2 with respect to its effect on the domain size, but only CAP-2 has the effect on the flows. It is possible that both CAP-1 and CAP-2 could be involved in the mechanism of polarity establishment, which is independent to its role in the cortical flows.

A

Establishment phase: N2(L4440 RNAi)

Anterior   Posterior

0%   50%
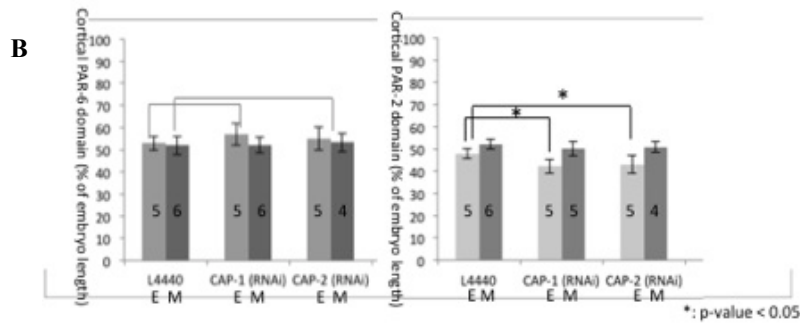(anterior)   100%   Maintenance phase: N2(L4440 RNAi)
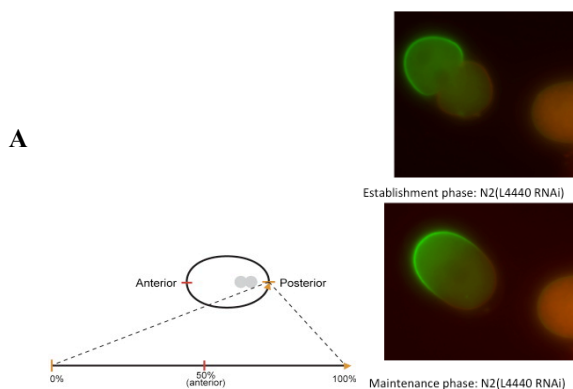
Figure 13: Size of PAR-2 domain is mildly decreased by the depletion of CAP proteins.

A) Schematic representation of the method used to quantify the PAR-2 and PAR-6 domains and the right panels shows the time at when the quantifications were made.

B) Quantification of the mean percentage ± SD of cortical perimeter occupied by 70% of the maximal PAR-2::GFP and PAR-6::mcherry intensity. 'E' and 'M' represents establishement and Maintenance phase respectively. *p < 0.05 (Student's t test).

Moreover, when maternal expression of CAP-1 and CAP-2 was inhibited by the feeding RNAi method, the 40% and 70% of embryos did not hatch respectively, indicating that these genes are essential for embryonic development.

3.4 Events in the zygote thought to depend on microtubules are delayed by the loss of CAP-2 protein.

We found a couple of papers in the literature suggesting that CAP-2 could influence the microtubule cytoskeleton as it has been shown in the flies and in mice (Bartolini et al., 2012; Davis et al., 2009). To further validate that, we looked at the early zygotic events which are thought to be dependent on microtubules.

As mentioned previously, in the one cell embryos of the wild-type animals, the male pronucleus localizes to the posterior end, whereas the female pronucleus from the opposite side migrates toward the male pronucleus (Fig. 3D, E). The male pronucleus simultaneously begins a migration towards the anterior pole to meet the female pronucleus. It has been

previously demonstrated that the movements of the nucleus depend on the length of astral microtubules (Tsai and Ahringer, 2007). Any defect in the distance traveled by the male pronucleus, relative to the posterior membrane would suggest microtubule defects. In control embryos, the male and female pronuclei meet at the posterior, at approximately 70% of the emrbyo length (Figure 14A&B). In *cap-2* (RNAi) embryos, the migration of the female pronucleus occurred normally towards posterior pole (Figure 14A&B). However, the male pronucleus showed a varied change in the distance of migration from the posterior cortex towards anterior. This was observed in approximately 30% of the *cap-2* (RNAi) embryos and the effect was not fully penetrant. Such defects have been reported before for mutants in genes such as dynein, which is the minus-end directed microtubule motor, where the migrations of both the pronuclei were affected (O'Connell et al., 2000; Schmidt et al., 2005).
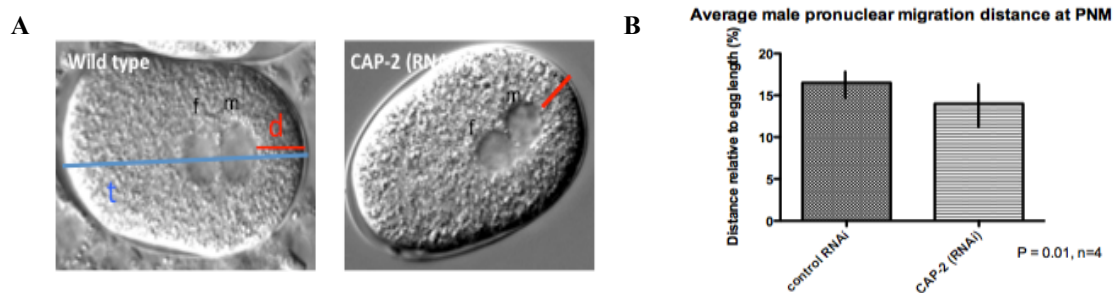
Figure 14: Migration of the male pronuclei is affected in CAP-2 depleted embryos.

A) DIC images of one-cell embryos for control and *cap-2* (RNAi) are shown at the stage of the pronuclear meeting. The female and male pronuclei are marked by f and m, respectively. Blue bar indicates the total distance between anterior and posterior pole while the red bars indicate the distance of male pronuclear migration away from the posterior pole.

B) Graph representing the quantification of the male pronuclear migration distance relative to the total length of the one-cell embryo at the pronuclear meeting. Error bars indicate SD; P=0.01.

Male and female pronuclei from posterior pole, then travel together to the center of the one-cell embryo, in a process known as centration. Simultaneously, the pronuclei undergo a rotation along the anterior-posterior axis that permits the first cell division to be asymmetrical

(White and Strome, 1996). These events of centration and rotation have been demonstrated before to be dependent on the astral microtubules, that extends from the centrosome and interact with motors at the cortex (Cowan and Hyman, 2004b) along with GOA-1/GPA-1 function and NMY-2 (Goulding et al., 2007). Any change in the length and the stability of microtubules could affect the nuclear centrosomal rotation.

To further analyze the function of CAP-2 in the nuclear centrosomal rotation, we investigated the phenotypes of *cap-2* (RNAi) embryos using DIC microscopy. In wild-type embryos, during the pronuclear centrosomal rotation, both the pronuclei rotated and aligned to 90 degrees along the anteroposterior axis, in a way that the first division is asymmetrical in nature (Figure 15, left panel). However in the CAP-2 depleted embryos, the pronuclei moved to the center of the embryo but had significant defects in the angle of rotation (Fig.15 A and B). Four out of six embryos, failed to fully rotate during the time of centration and in those embryos, the rotation was delayed and occurred simultaneously with nuclear envelope breakdown.

After NEBD, we even observed a significant defect in the assembly of the bipolar spindle in one cell embryo, where a few of the CAP depleted embryos showed smaller and misaligned spindle (Figure 16A). Few had a defect in the oscillatory properties of the spindle (data not shown). In addition, 2/10 *cap-2* (RNAi) embryos often had multiple female pronuclei indicating defects of meiotic divisions.

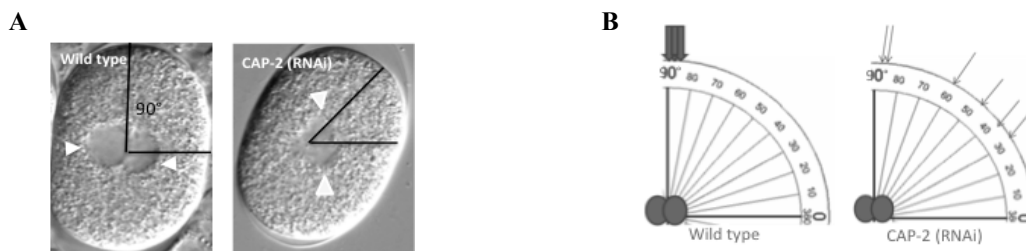Figure 15: Pronuclear centration/rotation is affected in CAP-2 depleted embryos.

A) DIC images of one-cell embryos are shown at the stage of pronuclear centration/rotation.

B) In wild-type embryos, during the pronuclear centration, pronuclei rotated and aligned to 90 degrees for the anterior posterior placement of the spindle. In contrast, the pronuclear rotation

was found to be unsynchronised with pronuclear centration in *cap-2* (RNAi) embryos. The arrowmark represents the angle alignment of pronuclear midplane relative to the A/P axis.

We even observed the significant delay in the cell cycle of CAP-2 depleted embryos compared to wild type. In the wild type embryos, centration occurs by 140 s after pronuclear meeting and the nuclear-centrosomal rotation began as soon as the both pronuclei met, while in CAP-2 depleted embryos centration occurs on average 400s later and the nuclear-centrosomal rotation often occurred after centration (Figure 16B). In wild type embryos, Nuclear envelope breakdown (NEBD), usually occurs within 110 seconds of centration. However in CAP-2 depleted embryos, the nucelar-centrosomal rotation was accompanied by NEBD (Figure 16B). The time taken for the first furrow cleavage to take place was delayed by apporximately 110 seconds in CAP-2 depleted embryos compared to WT. We observed the symmetrical placement of the cleavage in 1/6 of the observed CAP-2 depleted embryos.

A
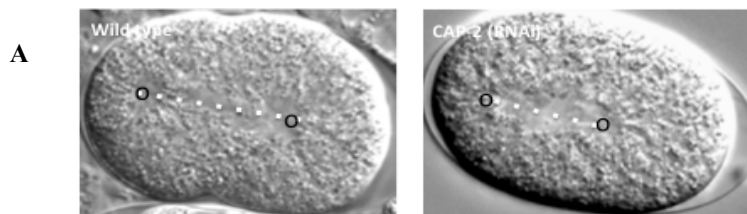
Figure 16: Defect in the size of the bipolar spindles and the developmental timing were observed in CAP-2 depleted embryos compared to wild type.

A) DIC image of the one cell embryo representing the size of the bipolar spindle.

B) The average time lapse between the developmental events is indicated in seconds. The timing of the following developmental events was determined at 22 °C: from pronuclear meeting (PNM) to nuclear-centrosomal rotation onto the anterior/posterior axis (NC Rotation), from PNM to pronuclear centration, from centration to nuclear envelope breakdown (NEBD) and from NEBD to completion of cytokinesis of the one-cell embryo.

Developmental timing of one-cell embryo

B
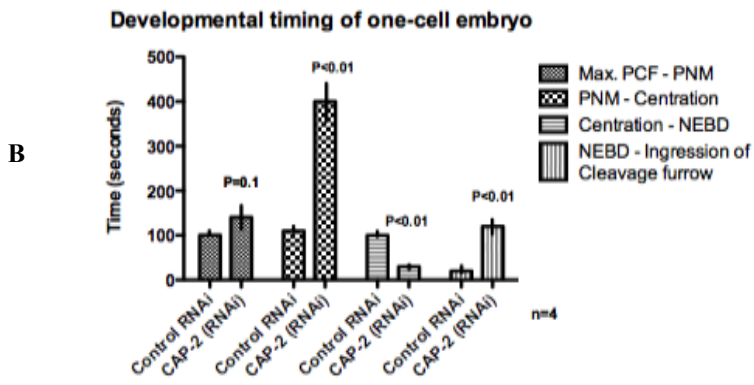
These observed phenotypes which are defects in the pronuclear migration, delayed centration and nuclear-centrosomal rotation, defects in the oscillatory features and the size of the bipolar spindles and the defect in oocyte meiosis give the preliminary hint that CAP-2 is required for microtubule-mediated events. However, these phenotypes do not necessarily separate it from its link to the actin cytoskeleton and thus future direct experiments will be needed to answer if the CAP-2 regulates microtubule cytoskeleton, independently of its well-established role in F-actin stabilization.

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4. Discussion

In this study, we have used the *C. elegans* one cell embryos to understand the role of CAP proteins in *C. elegans* polarization process. We found that CAP-2 plays a role in the various early developmental processes of an embryo, namely cortical contractility, polarity establishment, nuclear migrations and cell cycle progression. These findings are compatible with a role for CAP-2 in the regulation of both the actin and microtubule cytoskeletons.

4.1 CAP-2 and contractility

Here we report that CAP-2 depleted embryos showed decreased stability of the pseudocleavage furrow. Moreover, there is a significant delay in the clearing of the actomyosin contractility from the posterior zone suggesting that CAP-2 contributes to the dynamics of actomyosin contractility. However CAP-1, another CAP protein though had an effect on the stabilization of the pseudocleavage furrow but had no significant effect on the velocity of the flows. This observation could be explained by two possibilities: 1) Since, in all our experiments, we have used RNAi feeding method to deplete the protein, it is possible that the depletion of CAP-1 by RNAi was not efficient to produce the phenotype as we have not measured the amount of protein depleted in our worms after the knockdown. This possibility could be addressed by measuring the amount of protein by blotting against anti-CAP-1 after the knockdown procedure. 2) Another intriguing possibility is that even though CAP-1 and CAP-2 are obligate heterodimers (Pollard and Cooper, 2009), CAP-2 has a role independent of CAP-1 in regulating acto-myosin contractility. This theory could be supported by various facts, such as only knockdown of CAP-2 is lethal in fly embryos and not CAP-1 and we had only found CAP-2 in our screen. However, this possibility could be addressed by depleting both proteins by injecting double stranded RNA in mothers. Any additional effect imparted by CAP-1 can be addressed by looking at the additional modulation of the phenotypes associated with the various events. This will even help to address the fact that we have observed mild defects using feeding method. Many people in the past have reported that capping proteins bind and stabilize the barbed plus end of actin finalemts, regulating the rate of assembly and disassembly and this depends on the critical concentration of G-actin at any given time (Gil-Krzewska et al., 2010). According to this mechanism, when the concentration of G-actin

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levels are low, depletion or removal of CAP protein should result in the reduction of F-actin levels and when G-actin levels are high, depletion of CAP protein is expected to increase F-actin levels (Gil-Krzewska et al., 2010). In this paper, when CAP proteins were depleted using RNAi, animals show severe ruffling and thickening of the rachis in the *C. elegans* gonad, which suggests increased contractility. However many studies in the past using different model organisms have given a hint about decreased contractility on the loss of CAP proteins. Since CAP proteins were proposed to play a dual role, one intriguing possibility is that the critical concentration of G actin is low in the one cell embryo and hence depleting the CAP proteins is resulting in the severing of the F-actin filaments in *C. elegans*, which in turn reduces contractility by reducing the stiffness of the membrane. This hypothesis could be addressed by measuring the concentration of G/F actin in the one-celled embryos.

4.2 CAP-2 and polarity establishment

We report the mild decrease in the size of the PAR-2 domain upon depletion of CAP proteins at establishment phase, which was corrected during maintenance phase. This correction could be dependent on CDC-42 mediated late flows, as have been reported previously in the literature or it is because of the activation of the PAR-2 mediated symmetry breaking mechanism that is dependent on the microtubules. According to (Motegi et al., 2011), the strain in which cortical flows are abolished*, ect-2 (ax751)*, could still establish polarity, through the microtubule organizing center (MTOC) and the establishment of polarity through contractility independent pathway is delayed relatively compared to the wild type. We have not yet measured if the delayed expansion of the PAR boundaries coincides with the decreased velocity of the cortical flows. The later experiment could address a very interesting question and the second hypothesis of our project, whether CAP proteins regulate the polarity establishment which is dependent on microtubules and PAR-2. However, only a very mild effect on the size of the domain might be possible due to the fact that the depletion of CAP protein is not sufficient enough to see the effect. This experiment will have to be repeated after injecting the mothers with the double stranded RNA and by taking the movies from the beginning of the flows to the end of the establishment phase.

4.3 CAP-2 and microtubule cytoskeleton

Many events that have been reported previously to be dependent on the microtubules are affected by the loss of CAP-2. However, it often becomes difficult to separate the role of both actin and microtubule cytoskeletons due to their remarkable connections in various events. It has been shown before that the migration distance of male pronuclei from the posterior membrane is solely dependent on the microtubules, which were affected by the loss of CAP-2 (Tsai and Ahringer, 2007). We have performed the simultaneous experiment with the small number of CAP-1 depleted embryos (results not shown), but we did not find any significant effect of CAP-1 on these events. This might suggest that CAP-2 only has role on microtubule cytoskeleton and not CAP-1. This is further supported by two papers available in the literature : (Bartolini et al., 2012) have reported that capping protein promote stability of microtubules by antagonizing the function of mDIa1 in flies, while (Davis et al., 2009) showed that ß-tubulin binds to Capzb2 and this decreases the rate and extent of tubulin polymerization *in vivo*. However further direct experiments will be needed to validate our hypothesis if CAP-2 has a role in the microtubule dependent polarization pathway in the *C. elegans* embryo. One intriguing possibility is that this is the conserved property of other actin binding proteins as well, as it has been shown before that Arp-2/3, an actin nucleator that has a role in nuclear migrations (Xiong et al., 2011). We know that microtubule dependent machinery for the polarity establishment is the redundant mechanism available in the embryos. Modulating the activity of the conserved actin binding proteins might be responsible for the activation of the redundant mechanism for the polarity establishment. It will be interesting to see if the mechanism remains conserved in other organisms such as flies and humans for polarity establishment, considering the fact that polarity establishment mechanism is highly conserved and so are the actin binding proteins such as CAP-2.

4.4 CAP-2 and PAR-5

We had found CAP-2 protein as an interactor of PAR-5/14-3-3 in our pull down. This interaction was found to be common in three different model systems including flies and HEK-293 cells. However, we have not yet validated this interaction using other means such as immuno precipitation experiments. A 14-3-3 binding motif was found to be present on the

CAP-2 protein at a low stringency using scansite. Does PAR-5 regulate CAP-2, and, in turn, cross-talk between actin and the microtubule cytoskeleton for the establishment of the polarity is a very interesting question? Though the role of PAR-5 in regulating the acto-myosin cortex is already established but this could explore the possible role of PAR-5 in regulating microtubule cytoskeleton. Keeping in consideration that 3 out of 6 PAR proteins are kinases, it is a question worthwhile to explore. It will be even interesting to address the upstream regulators of this pathway as well.

Conclusions and Future perspectives

We conclude that CAP-2 plays a role in the acto-myosin contractility in the one cell embryo of *C. elegans*, as evident from its effect on pseudocleavage relaxation and the reduced velocity of the myosin flows during the polarization phase. We have picked up the hint that CAP-2 could be playing a role in the establishment of polarity in the one-celled embryos. These observations in *C. elegans* coincide with the known role of CAP in regulating the acto-myosin contractility in other organisms. However to the best of our knowledge, we are the first to show the role of CAP proteins in the polarity establishment of *C. elegans* embryos. Another interesting possibility that we have touched upon is the role of CAP-2 in regulating the microtubule cytoskeleton. We have got this preliminary hint from the fact that some of the events that are presumed to be dependent on the microtubules are affected by the depletion of CAP-2 along with the cell cycle. Our results confirm the role of CAP-2 in the regulation of actin cytoskeleton and the possible role of CAP-2 in the regulation of microtubule cytoskeleton.

In the future, to address if CAP-2 has role in the microtubule polymerization and/or stabilization, independent of its role in F- actin stabilization, we would like to perform more direct assays. One of the ways would be to use the GFP::ß-tubulin strain and to check whether depletion of CAP-2 has any effect on the centrosomal microtubule density and their organization in *cap-2* RNAi embryos compared to WT. This would be measured by quantifying the size of the area of high MT accumulation around the centrosomes by taking Z-stacks at the time of the pronuclear meeting.

To answer if CAP-2 regulates the redundant mechanism for symmetry breaking, which is dependent on microtubules and PAR-2, we would deplete CAP-2 in mCherry::PAR-6; GFP::PAR-2; *ect-2(ax751)* strain. This strain has been shown before to have no acto-myosin flows (Motegi et al., 2011), where the polarization is established by an expansion of PAR-2 domain, a mechanism dependent on its interaction with microtubules. To answer this question, we would observe if the loading of PAR-2 is modulated in this strain on depletion of CAP-2 compared to controls. We will look at the dynamics of PAR-2 loading at the microtubule-

dense core of the MTOC and whether PAR-2 domain correlates with the site of MTOC/cortex contact. If CAP-2 depletion would interfere with microtubule nucleation, than we could expect the zygotes with variation in the size of PAR-2 domain along with the variation in the dynamics of loading in the mCherry::PAR-6; GFP::PAR-2; *ect-2(ax751)* strain compared to controls. Another question that we would like to address is if CAP-2 directly interacts with microtubules. To answer that we would use the more direct approach such as microtubules sedimentation assay.

To understand the mechanism of CAP-2 in regulating the acto-myosin contractility in the one-celled embryos, we would like to perform laser ablation experiment in WT and CAP-2 depleted embryos and we will observe the rate of addition of actin monomers to the filament. This would give us approximate idea of the critical concentration of G-actin and henceforth mechanism of CAP proteins in regulating the acto-myosin contractility.

In our screen, we have identified more than 500 proteins, as interactors of PAR-5. It would be worthwhile to perform functional assays for their additional role in the polarization process.

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