Characterization of the RhoGAP proteins RGA-3 and RGA-4 and the centrosomal protein SAS-5 in the early *Caenorhabditis elegans* embryo

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Summary

The *Caenorhabditis elegans* embryo serves as a great tool to study cell biological processes like polarization and cell divison. The first cell division is unequal and cell polarization is dependant on the Rho GTPase regulated rearrangement of the cell cortex and the following localization of PAR proteins. The embryo serves also as a great model system to study centrosome duplication, which leads to the duplication of the centrioles provided by the sperm and their assembly into mature centrosomes during S phase of the one-cell stage embryo.

In this thesis, two novel Rho GTPase activation proteins (RhoGAPs), RGA-3 and RGA-4, were identified, constituting an essential part for development. Concomitant RNAi of RGA-3 and RGA-4 (*rga-3/4 (RNAi)*) resulted in a hyper-contractility phenotype with extensive membrane ruffling and furrowing of the zygote. These two RhoGAPs act redundantly in regulating the small GTPase RHO-1, which is essential for regulating the acto-myosin network during contractile polarization of the early *C. elegans* embryo. Simultaneous knock-down of *rho-1 (RNAi) and rga-3/4 (RNAi)* rescued the *rga-3/4* ruffling phenotype demonstrating that RHO-1 is the GTPase regulated by RGA-3/4. In contrast, triple knock-down of *rga-3/4* together with another small GTPase, CDC-42, which is involved in polarity maintenance in the embryo, did no rescue the *rga-3/4 (RNAi)* ruffling phenotype. Increased membrane ruffling was mainly observed at the anterior cortex of *rga-3/4 (RNAi)* embryos. Consistently, RHO-1 and its effector NMY-2 were enriched in these extra furrows in *rga-3/4 (RNAi)* embryos. Furthermore, the known Rho GEF ECT-2 and the Rho kinase LET-502 alleviated the membrane ruffling phenotype caused by *rga-3/4 (RNAi).*

As opposed to the third known RhoGAP of the early embryo, CYK-4, which is essential for posterior polarization and central spindle assembly, RGA-3/4 regulate anterior contractility of the early embryo. The RGAs play a role in regulating the acto-myosin network during cortical polarization, yet the initial establishment of polarity is not heavily affected in *rga-3/4 (RNAi)* embryos as indicated by the correct localization of the PAR proteins. However, the size of the anterior PAR-6 domain fluctuated more in *rga-3/4 (RNAi)* than in wild type. Over-expression of RGA-3/4 appears to be lethal in *C. elegans*, and no stable GFP::RGA-3 expressing line could be obtained, neither by injection nor microparticle bombardment.

RGA-3/4 do not only have a role in the one-cell stage embryo, they are also necessary for germ line development. Knock-down of RGA-3/4 in the background of the *let-502 (sb106)* mutant impaired the germ line development, a phenotype not observed for this

mutant by itself. This result indicates that both LET-502 and RGA-3/4 are required for gonadal function.

The second part of the thesis concerned the characterization of the centrosomal protein SAS-5. *sas-5 (RNAi)* resulted in a high penetrance of embryonic lethality. SAS-5 is a centrosomal protein and essential for centrosome duplication in *C. elegans*. Upon *sas-5 (RNAi)* the first cell division appeared to be unaffected. The two centrioles provided by the sperm were not duplicated, yet separated during S phase allowing the establishment of a bipolar spindle in the P0 cell. The centrosome duplication defect was obvious in the subsequent mitotic cycles and only mono-polar spindles were formed. As a consequence, nuclear morphology was strongly affected, but did not induce apoptosis.

Zusammenfassung

Der Nematode *Caenorhabditis elegans* eignet sich sehr gut um entwicklungsbiologische und zellbiologische Prozesse zu untersuchen. Zellbiologische Prozesse, die im Embryo erforscht werden, sind z.B. Polarität und Zellteilung. Der erste Schritt zu Polarisierung nach der Befruchtung ist die Rho GTPase abhängige Modulation des Zellkortex, auf die die asymmetrische Anordnung der PAR Proteine folgt. Für die Ausbildung der bipolaren mitotischen Spindel stellt das Spermium zwei Zentrosomen bereit, die in der ersten S Phase des Zellzyklus dupliziert werden und durch Rekrutierung maternaler Proteine zu reifen Zentrosomen werden.

Mit dieser Arbeit konnten zwei neue Rho GTPase aktivierende (GAPs) Proteine – RGA-3 und RGA-4- identifiziert werden, die im frühen Embryo für die Regulation der kleinen GTPase RHO-1 benötigt werden. RNAi von *rga-3* und *rga-4* (*rga-3/4 (RNAi*)) führte zu einem sogenannten Hyperkontraktilitätsphenotyp; am anterioren Kortex ist in *rga-3/4 (RNAi*) Embryonen eine erhöhte Zahl von Einfurchungen und höhere Kontraktilität als Wildtyp zu beobachten. RHO-1 ist notwendig zur Regulation des Aktin-Myosin-Skelettes im frühen Embryo und es akkumulierte an den in *rga-3/4 (RNAi*) vermehrt ausgebildeten Teilungsfurchen.

Die gleichzeitige Reduktion von *rho-1* und *rga-3/4* durch RNAi beseitigt den Hyperkontraktilitätsphänotyp und deutet darauf hin, dass RGA-3/4 die GTPase RHO-1 regulieren. Triple-RNAi der kleinen GTPase CDC-42, die für die Aufrechterhaltung der Polarität im frühen Embryo nowendig ist, zusammen mit *rga-3/4* verminderte diesen Hyperkontraktilitätsphänotyp nicht. Desweiteren zeigten *rga-3/4 (RNAi)* Embryonen eine Anreicherung von RHO-1 Effektorproteinen wie zum Beispiel NMY-2 in den vermehrt ausgebildeten Teilungsfruchen. Mit weiteren Triple-RNAi Experimenten konnte gezeigt werden, dass ECT-2, ein GEF für RHO-1, sowie die Effektorkinase LET-502 den Kontraktilitätsphenotyp von RGA-3/4 retteten. Im Gegensatz zu einem weiteren bekannten RhoGAP im frühen Embryo, CYK-4, das für Polarisierung und Bildung der zentralen Spindel notwendig ist, regulieren RGA-3 und RGA-4 somit redundant die Kontraktion am anterioren Kortex im frühen Embryo. Interessanterweise war die Etablierung der Polarisierung in *rga-3/4 (RNAi)* Embryonen nicht stark beeinträchtigt: PAR-6 lokalisierte am anterioren Kortex und PAR-2 am posterioren Kortex; die Grösse der PAR-6 Domäne fluktuierte jedoch stärker in *rga-3/4 (RNAi)* als im Wildtyp.

Die Ueberexpression von RGA-3/4 war lethal, und weder durch Injektion eines GFP::RGA-3 Konstruktes noch durch “microparticle bombardment” konnte eine stabile GFP Linie

erhalten werden. Außer im frühen Embryos spielten die beiden RhoGAPs auch in der Keimbahn eine wichtige Rolle: RNAi Ergebnisse zeigten, dass die Funktionen von RGA-3/4 und der Rho Kinase LET-502 in der Keimbahn genetisch verknüpft sind.

Neben der Identifizierung und Charakterisierung dieser beiden RhoGAP Proteine wurde ein weiteres Protein charakterisiert, das für den *C. elegans* embryo essentiell ist: SAS-5. *sas-5 (RNAi)* resultierte in einer sehr hohen embryonalen Lethalitätsrate. Antikörperfärbungen zeigten, dass SAS-5 ein zentrosomales Protein ist. Übereinstimmend mit der zentrosomalen Lokalisierung von SAS-5 konnte gezeigt werden, dass das Protein für die Duplikation der Zentrosomen in der Zelle notwendig ist. Einer mehr oder weniger normalen ersten Zellteilung in *sas-5 (RNAi)* Embryos, während derer die zwei paternalen Zentriolen die Ausbildung einer bipolaren Spindel ermöglichten, folgte aufgrund des Zentrosomen-Duplikationsdefekts in der AB und P1 Zelle die Ausbildung einer nur monopolaren Spindel. Damit war keine normale mitotische Teilung mehr möglich und ausgeprägte Kerndefekte waren die Folge. Die Kerndefekte in *sas-5 (RNAi)* führten jedoch nicht zu einem apoptotischen Sterben der Embryos.

I Introduction

It is fascinating to study early developmental processes of animals: a fertilized zygote is totipotent and possesses all information and components necessary to grow into an adult with different body parts, organs and cell types. To form this complex body structure, cells of a multi-cellular organism with the same genetic material must be able to adopt different cell fates. One evolutionarily conserved mechanism to achieve differentiation is the asymmetric division of a cell. Polarization leads to asymmetric protein and mRNA segregation and division yields two daughter cells with unequal cell fate determinants.

A well-established system to study cell polarization is the *C. elegans* embryo, where the very first cell division of the zygote (P0) results in two different daughter cells, AB and P1. Similarly, some immediate subsequent divisions are also unequal. In a forward genetic screen for embryonic lethal mutants, the conserved partitioning-defective (PAR) proteins were identified as being essential for polarization of the zygote (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Kemphues et al., 1988; Mains et al., 1990; Watts et al., 1996).

Apart from the classical genetic manipulations, which were and still are very important approaches for studying *C. elegans*, two important events in the mid 90s had a major impact on research in *C. elegans*: the sequencing of the worm genome (Kuwabara, 1997; Waterston and Sulston, 1995; Wilson, 1999) and the discovery of RNA-mediated interference (RNAi) (Fire et al., 1998; Guo and Kemphues, 1996; Montgomery and Fire, 1998; Tabara et al., 1998; Timmons and Fire, 1998). RNAi is a straightforward technique to introduce dsRNA directed against a gene of interest into the cells, which reduces its expression. This discovery has turned *C. elegans* into an attractive model organism for reverse genetics approaches and allows studying gene function on a genome-wide scale. In 2006, Craig Mello and Andrew Fire were honored with the Nobel Prize in Physiology for their discovery of RNA interference.

Several large-scale RNAi screens were performed after the completion of the worm genome sequence (Fraser et al., 2000; Gonczy et al., 2000; Kamath et al., 2003; Piano et al., 2000; Rual et al., 2004; Simmer et al., 2003; Sonnichsen et al., 2005). Two of these screens focused on identifying genes that are involved in the first mitotic cell division of the

embryo (Gonczy et al., 2000; Sonnichsen et al., 2005). In the first cell division screen in *C. elegans*, only chromosome III was analyzed (Gonczy et al., 2000). The second screen covered the whole genome and surprisingly, only 662 of the 19 075 targeted genes (3.5%) were shown to be essential for the first mitotic division (Sonnichsen et al., 2005). This is smaller than the fraction of genes shown to be necessary for cell division in budding yeast, where more than half of all genes (approx 5 900) are essential for division (Giaever et al., 2002). One has to keep in mind, that these two studies used different methods to study gene functions. The *C. elegans* study was performed using RNAi, whereas for the yeast work, gene deletions were analyzed. On the other hand, the unicellular lifestyle of yeast might have led to a tighter integration of the cell division cycle with cell homeostasis, while multi-cellular organisms can compensate for individual cells failing to proliferate.

Genes essential for cell division in *C. elegans* were classified according to their morphological phenotype. Half of the 662 genes displaying defects during the first cell cycle are involved in the cellular metabolism, e.g. maintaining osmotic integrity and tuning the general pace of development. The remaining 327 “real” cell division related genes were further grouped according to the defects observed in RNAi experiments: e.g. disturbed passage through meiosis (47 genes), cortical dynamics (19 genes), spindle assembly (9 genes), cytokinesis (15 genes) or asymmetry of division (12 genes) (Sonnichsen et al., 2005).

Despite the availability of genome-wide RNAi data, a deeper insight into the function of individual genes as well as their regulation is still lacking. Detailed *in vivo* characterization of the molecular interactions orchestrating the development of the early *C. elegans* embryo is required.

I.1. *C. elegans* as a model organism in cell biology

In 1965, Sydney Brenner[1] established the free soil-living nematode *Caenorhabditis elegans* as a model organism to study development and behavior. Nowadays, *C. elegans* is one of the most commonly used model organisms and can be manipulated through a broad spectrum of experimental techniques to study apoptosis, neurobiology and developmental or basic cell biological processes.

[1] Sydney Brenner, Robert Horvitz and John Sulston shared the Nobel Prize in Physiology in 2002 for their investigation of the *C.elegans* cell lineage and research on apoptosis in *C.elegans*.

I.1.1. The anatomy and life cycle of a worm

The approximately 1 mm long adult worm is transparent and can easily be cultivated in the laboratory on NGM agar plates seeded with *Escherichia coli* at 20 to 25°C (Brenner, 1974). The body plan of nematodes is quite simple: it consists of two concentric tubes, which are separated from each other by the liquid pseudocoelom. The outer tube contains the cutaneous muscle tube, the gonad, the excretory and secretory system and part of the nervous system. The inner one consists of pharynx, gut and the autonomous nervous system (Donald, 1997).

There are two sexes in *C. elegans,* hermaphrodites and males. Genetically, X0 males arise spontaneously at a low frequency of about 0.1% due to nondisjunction of chromosomes during meiosis. Nondisjunction and, consequently, the frequency of males rise under stress conditions. Usually, *C. elegans* reproduces through hermaphrodites by self-fertilization, but if males are present, mating is the preferred way of reproduction and enhances genetic variability. The fertile hermaphrodite first produces its sperm, which is stored in the spermatheca. A typical hermaphrodite produces more sperm than oocytes and typically gives rise to a progeny of about 250-300 (Donald, 1997).

Oocytes are fertilized in the spermatheca in the proximal part of the gonad. Afterwards embryogenesis begins in the uterus of the mother and continues after egg laying, which occurs about two hours after fertilization. Worms develop from eggs into fertile adults in about three days at 20°C, and the exact fate of each cell (the cell lineage) is known (Sulston et al., 1983). The life cycle of *C. elegans* starts with 14 hours of embryogenesis and embryos hatch as small L1 larvae with 558 cells. The following postembryonic development takes about 36 hours and leads through four larval stages, from L1 to L4, to adulthood. The adult hermaphrodite has 959 cells, whereas the adult male has 1032 cells. Adult worms live for about three weeks (Donald, 1997).

I.1.2. The *C. elegans* zygote as a system to study cell biology

The fact that the *C. elegans* zygote is relatively large has rendered it a very powerful system to answer cell biological questions. Embryos can be obtained in large numbers and embryogenesis can be investigated using a range of microscopic techniques (Schnabel et al., 1997). Simply monitoring the first cell division of an embryo by time-lapse differential interference contrast (DIC) microscopy reveals the most important events of the cell cycle (Figure 1 A, see chapter I.1.3.; (Schnabel et al., 1997; Wood, 1988). Furthermore, a large number of GFP-tagged proteins are available to study cell polarization and its coordination with cell-cycle events.

Since homologous recombination of chromosomes is a very rare event in *C. elegans*, the most common way of introducing trans-genes into the genome is injection of the desired trans-gene into the syncytial gonad. Thereby, an extra-chromosomal array is generated containing many copies of the trans-gene (Hope, 2000; Strange, 2006). Expressing trans-genes in the germline and the embryo is hampered by the silencing of repetitive sequences, including extra-chromosomal arrays (Seydoux et al., 1996) ("germ line silencing"), though. This problem can be circumvented by inserting trans-genes into the genome by microparticle bombardment (Praitis et al., 2001). Expression of genes in the germ line is most commonly driven using one of a small number of germline specific promoters (Mango et al., 1994; Mello et al., 1992; Tenenhaus et al., 2001).

RNA interference (RNAi) is the method of choice to study gene function in the early embryo (Fire et al., 1998; Montgomery and Fire, 1998; Tabara et al., 1998; Timmons and Fire, 1998). There are several different ways to induce RNAi, of which the injection of dsRNA into the gonad of L4 larvae is most efficient (Hope, 2000; Strange, 2006). Alternatively, the dsRNA can be provided within dsRNA expressing bacteria, which are fed to the worms, or by soaking *C. elegans* larvae in a buffer containing the dsRNA. These latter methods are especially powerful for whole genome analysis (Kamath and Ahringer, 2003). The RNAi phenotype can typically be observed after 12-24 hours. The knock-down efficiency of RNAi differs between tissues. By targeting the syncytial gonad, it is possible to deplete up to 95% of targeted essential gene products from the oocytes (Hyman, 2006). Similar results can be obtained in gut tissues. RNAi in the nervous system, on the other hand, is very inefficient.

I.1.3. The first *C. elegans* cell cycle

Upon fertilization of a *C. elegans* embryo, the zygote undergoes the following processes: completion of the female meiosis, S phase with the duplication of the paternally contributed centrioles and the first mitosis and cytokinesis; concomitantly, the zygote is polarized.

Oocytes are produced in the gonad of a hermaphrodite and remain arrested in prophase of meiosis I. As soon as a sperm enters the egg opposite to the arrested maternal nucleus, the maternal nucleus begins to complete meiosis (Figure 1, first picture in A and B). After each of the two meiotic cycles one polar body is extruded. During meiosis, the surface of the egg is not polarized yet. The whole cortex is contractile (Figure 1: B, third panel, first picture). The entry point of the sperm marks the future posterior pole of the embryo, and the sperm does not only provide the cue for initiation of meiotic progression and polarization but also provides two centrioles. The two paternally derived and posteriorly located centrioles are duplicated and recruit maternal proteins to build up two mature centrosomes for the first mitotic spindle (Donald, 1997).

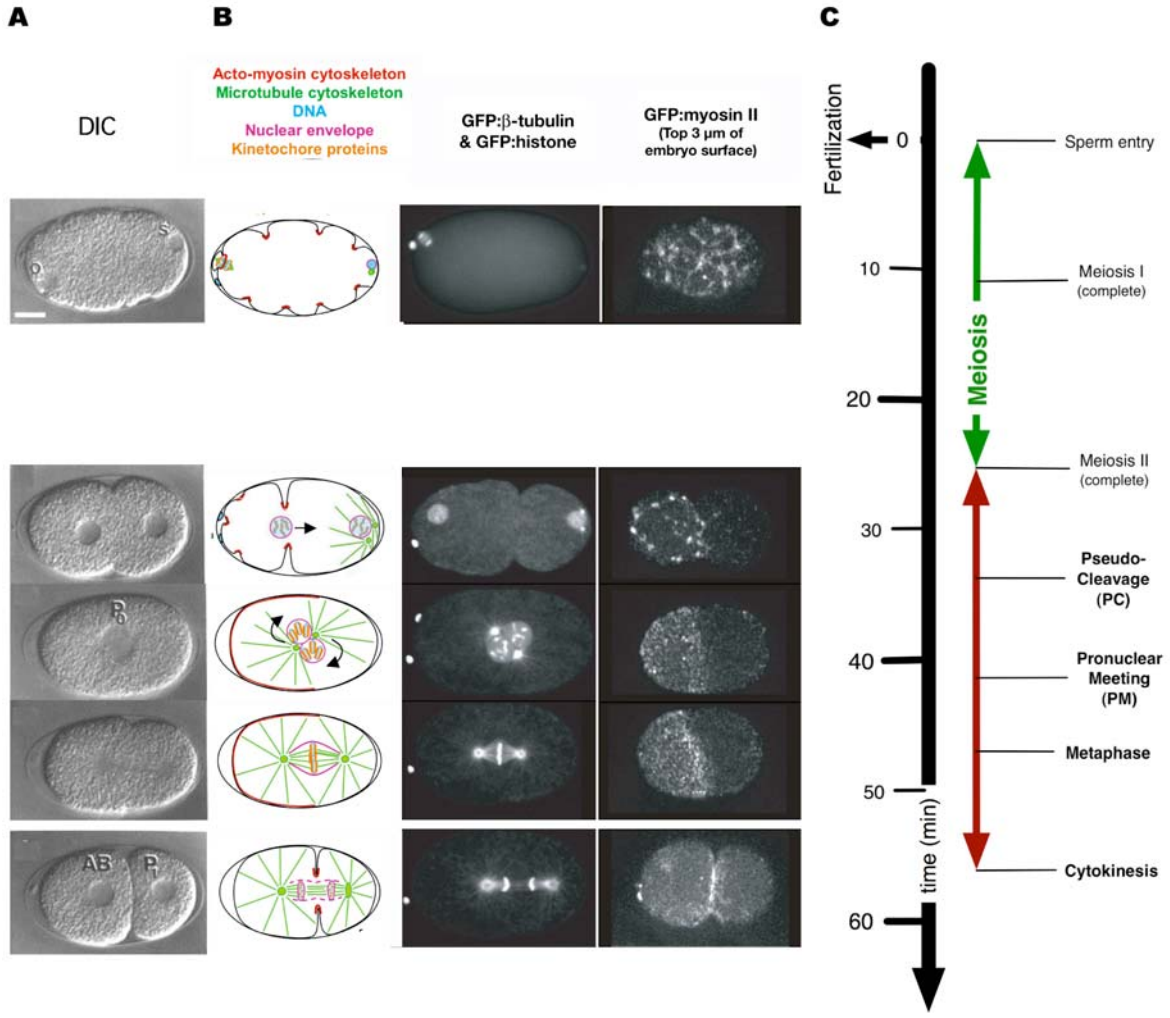

Figure 1: The characteristics of the first cell division in *C. elegans*.
(A) DIC pictures showing meiosis, pseudo-cleavage, pronuclear meeting and centration, metaphase and the first cytokinesis. s is the paternal nucleus, o is the maternal nucleus. Cytokinesis of the P0 cell leads to the large AB and a small P1 cell. (**B)** Schematic illustration (first panel), GFP: β-tubulin & GFP::histone (second panel) and GFP::myosin II (GFP::NMY-2) visualization (third panel). The second panel shows a schematic drawing of the acto-myosin network (red), microtubules (green), DNA (blue), the nuclear envelope (pink) and kinetochore proteins (orange) during the first cell division. Pictures of time-lapse *in vivo* data of the corresponding time points are shown in panel two and three. Here, GFP::histone&GFP::tubulin (second panel) and GFP::NMY-2 (third panel) behavior can be followed during the specific stages of the cell cycle. Generally, the acto-myosin network changes in the very first time; initially, the entire cortex is contractile (first picture) but with the cortical flow, contractile patches retract to the anterior cortex, leading to the so-called pseudo-cleavage (second picture). During this time, the paternal pronucleus recruits pericentriolar material (PCM) to built up to mature centrosomes (B, second panel second picture) indicated by the GFP-**β**-tubulin. After the meiotic divisions, the maternal pronucleus moves towards the posterior one. The maternal pronucleus meets the paternal one in the posterior hemisphere. Having met, they migrate back to the center of the cell, where they rotate. The nuclear envelope breaks down and the chromosomes condense (B, second panel, third picture). During metaphase (B, second panel, fourth picture), condensed chromosomes are aligned at the metaphase plate; during anaphase they are pulled towards their respective centrosome during in the future AB and small P1 cells (A, fifth picture; B fifth picture). (**C)** Time-line of the events during the first cell cycle. Times are in minutes. The scale bar in A is 10µm. Pictures in A: (Donald, 1997), in B: (Hyman, 2006).

After the completion of meiosis, the maternal pronucleus migrates towards the male pronucleus in the posterior part (Figure 1: second picture in A and B). Also initiated at the end of meiosis is the cortical and cytoplasmic rearrangement of the zygote (Hird and White, 1993). Cortical flow from the posterior male pronucleus towards the anterior female pronucleus takes place (Figure 2). Concurrently, a cytoplasmic flow is visible in the opposite direction from anterior to posterior part of the embryo (Figure 2). The interaction of the centrosomes provided by sperm with the cortex is believed to trigger the initiation of the cortical and cytoplasmic rearrangement (Cowan and Hyman, 2004); (Figure 2: cortex interactions).

The smoothening of the contractile cortex from the posterior pole outwards results in the establishment of a contractile anterior and a non-contractile posterior domain. Final establishment of these two domains culminates in the so-called “pseudo-cleavage”, where a furrow ingresses at the centre of the embryo, separating the contractile anterior cortex from the smooth posterior hemisphere (Figure 1: second picture in A and B; Figure 2) (Hyman, 2006). This difference in contractility is also indicated by the asymmetric distribution of the non-muscle-myosin II (NMY-2) to the anterior (Munro et al., 2004); (Figure 1: B second panel, third picture).

The reorganization of the cortex and the cytoplasm is accompanied by the asymmetric distribution of the cortical PAR proteins (Cowan and Hyman, 2007; Macara, 2004; Munro, 2006; Suzuki and Ohno, 2006). A conserved complex consisting of PAR-3, PAR-6 and the atypical protein kinase C (aPKC), first delocalized over the whole cortex, becomes restricted to the anterior cortex during polarization, similar to the cortical NMY-2 localization at this time (Figure 1, B third panel), (Cowan and Hyman, 2007). The anterior localization of PAR-3/6/aPKC allows the recruitment of the PAR-1 kinase and the PAR-2 protein to the posterior cortex. Following the establishment of cortical polarization, cytoplasmic factors like P granules, PIE-1, SKN-1 and MEX-5/6 are also distributed asymmetrically (Cuenca et al., 2003; Mango et al., 1994; Mello et al., 1992; Tenenhaus et al., 2001), (Figure 2: cytoplasmic and maintenance of polarization).

Upon polarity establishment the maternal meets paternal pronucleus in the posterior hemisphere. Both pronuclei migrate together towards the cell center, where they fuse and rotate (Figure 1: A and B third picture, Figure 2: Centration and Rotation). Then the nuclear envelope breaks down and the first mitotic spindle is assembled (Figure 1: A and B fourth picture). During anaphase, the condensed chromosomes are pulled to the different poles. Finally, the cytokinesis furrow ingresses (Figure 1: A and B fifth picture) during telophase. The asymmetric positioning of the spindle prior to anaphase is key to the size-difference of

the daughter cells after cytokinesis: a large AB and a small P1 cell are born (Figure 1, A and B fifth picture), (Hyman, 2006).

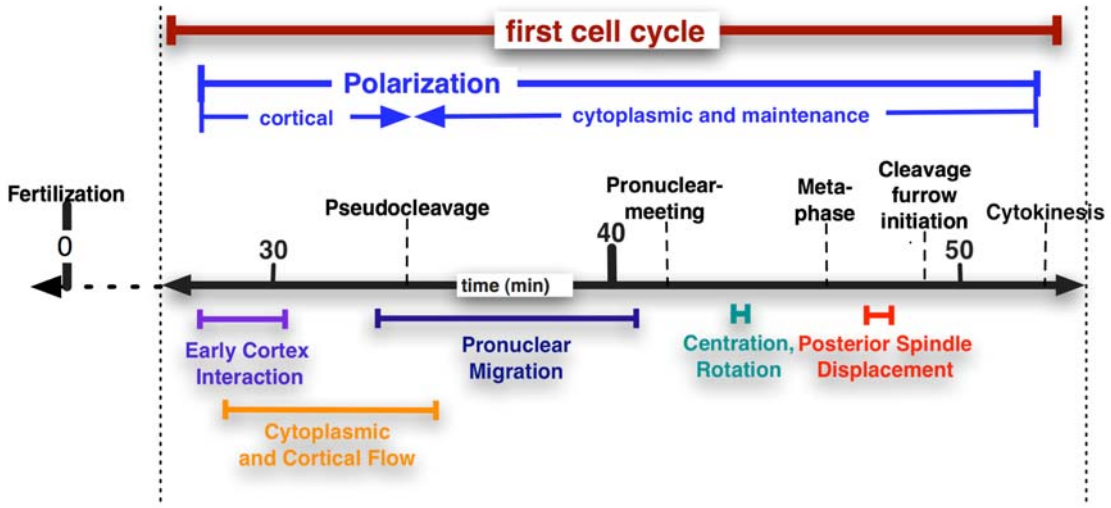

Figure 2: Detailed time-scale illustrating the events required for polarity establishment during the first mitotic cycle.
About 25 minutes after fertilization, the sperm-provided centrosome interacts with the posterior cortex to initiate polarization (purple) of the zygote. Initiation of polarization leads to a cytoplasmic flow from anterior to posterior as well as a cortical flow in the opposite direction (orange). About thirty-four minutes after fertilization, establishment of an anterior and posterior domain is completed at the pseudo-cleavage stage. Shortly before pseudo-cleavage, the maternal pronucleus begins to migrate towards the posterior paternal pronucleus (dark purple). They meet about 42 minutes after fertilization in the posterior part of the embryo, migrate back to the cell center together, fuse and rotate (green, approx 44 min). Then, the first mitoic spindle is assembled, displaced to the posterior (red) and cleavage furrow invagination is initiated (light green), giving rise to unequally large cells after cytokinesis. Time is in minutes relative to the fertilization of the embryo. Adapted from (Schneider and Bowerman, 2003).

I.2. Polarization of the *C. elegans* zygote

Cell polarization is important for diverse phenomena, many of which have been studied mainly in mammalian systems and in flies so far. Epithelial cells, for example, arrange themselves in parallel apical-basolateral orientation to form a physiological and mechanical barrier and to control tissue architecture (Rodriguez-Boulan and Nelson, 1989; Suzuki et al., 2004). Neutrophils detect chemoattractants and migrate towards their source. This orientated migration is mediated through G-protein coupled receptors that trigger polarized F-actin extensions at the leading edge (Li et al., 2003; Meili and Firtel, 2003; Xu et al., 2003). The most impressive example of a polarized cell is a neuron, where one pole of the cell, the extended axon, can be far away from the morphologically different cell soma with its dendrites. In many cases, initially unpolarized cells must break their symmetry to establish an axis of polarization. This process requires the reorganization of the cytoskeletal structures of the cell (Macara, 2004).

In the *C. elegans* embryo, the first step towards polarity is the subdivision of the cell cortex into distinct anterior and posterior cortical domains (Gonczy and Hyman, 1996), (Figure 1: GFP::NMY-2). The cell cortex, an acto-myosin-rich layer underneath the plasma membrane, provides both the mechanical stability and the capacity for force generation.

Shortly after the generation of different cortical domains in the *C. elegans* embryo, the different PAR proteins are recruited to their assigned cortical domain to mark and maintain the cellular polarity. Originally identified in worms, PARs have since been recognized as essential regulators in metazoans in general and many additional insights about their functions have been obtained from studies of mammalian cells or flies (Izumi et al., 1998; Nagai-Tamai et al., 2002; Rodriguez-Boulan and Nelson, 1989; Suzuki et al., 2004; Suzuki et al., 2001); (Benton and Johnston, 2003; Benton et al., 2002; Doerflinger et al., 2006; Huynh et al., 2001; Petronczki and Knoblich, 2001; Rolls et al., 2003; Schober et al., 1999; Yoshida et al., 2004; Zimyanin et al., 2007).

I.2.1. The acto-myosin network and its role and regulation in the early embryo

The first step in polarizing the *C. elegans* embryo is the establishment of the so-called cortical polarity (Figure 1 and Figure 2). Although a major component of a cell, little is known about the composition of the cortex.

I.2.1.1. Properties of the cortical acto-myosin cytoskeleton

Despite being a structural component of the cell providing and mechanical support, the actin meshwork underlying the plasma membrane is a highly dynamic network. Globular actin monomers (G-actin) are the building blocks of the long filamentous actin fibers (F-Actin) (Stossel, 1984). Actin filaments are polar and the ATP-dependent addition of further actin molecules is restricted to the „barbed“ end of an elongating filament. Many proteins bind to actin filaments and influence their dynamics, e.g. by capping, severing, crosslinking or bundling F-actin (Pollard and Cooper, 1986).

During cell division the acto-myosin machinery is recruited to the equatorial plane where it constricts the cleavage furrow during cytokinesis. Whether the network is assembled here *de novo* or if parts of the cortical actin meshwork are redeployed to form the contractile ring has not been elucidated, yet (Zhou and Wang, 2007).

Myosins constitute a large family of actin motor proteins, sharing homologous head and tail domains. Typically, the head domain interacts with actin filaments, while the tail is essential for interactions with cargo proteins. ATP-dependent conformational changes of myosin allow the molecule to slide along actin filaments and can lead to contractions, e.g. within the muscle sarcomere (Alberts, 2006). In the sarcomere, myosin-II, possessing two heavy and four light chains, is part of the highly organized acto-myosin array. Coordinated myosin movements mediate anti-parallel sliding of actin cables, and lead to uniform contraction or relaxation (Hyman, 2000 -a; Hyman, 2000 -b). The cortical acto-myosin meshwork is less ordered, yet the mechanism of force generation through interaction of actin and myosin molecules is in principle the same (Clarke and Spudich, 1977; Cowan and Hyman, 2007).

I.2.1.2. Cortical flow and contractions of the fertilized *C. elegans* embryo

In the *C. elegans* embryo, both NMY-2 and actin act upstream of the conserved PAR proteins in establishing polarity (Guo and Kemphues, 1996; Hill and Strome, 1990). The *nmy-2* gene encodes the myosin heavy chain of the non-muscle-myosin-II (Wormbase).

During the first 20-30 minutes after fertilization the cortex of a *C. elegans* one-cell embryo is highly contractile, and NMY-2 as well as the PAR3/6/aPKC complex is distributed uniformly throughout the cortex (Figure 1: first picture in A, B and C). After completion of meiosis the contractility of the cortex changes and the symmetry is broken (Figure 1, second panels in A and B, C). The actin cytoskeleton reorganizes and NMY-2 and PAR-3/6/PKC are enriched at the anterior pole, which continues to contract while the posterior pole becomes smooth (Hird and White, 1993; Munro et al., 2004; Seydoux, 2004)

Time-lapse video microscopy of GFP::NMY-2 revealed the acto-myosin network dynamics during polarity establishment. Cortical GFP::NMY-2 moves away from the sperm microtubule organizing center (MTOC) and towards the anterior pole (Munro et al., 2004; Seydoux, 2004); (Figure 1, B, third panel second picture). Ten minutes after meiosis the anterior enrichment of GFP::NMY-2 peaks and the pseudo-cleavage furrow is established (Figure 1: B, second picture, Figure 2). Visualizing actin dynamics *in vivo* confirmed these observations (Motegi et al., 2006) (Munro et al., 2004; Munro, 2006; Werner et al., 2007). Hence, it has been suggested that asymmetric contractions of the cortical acto-myosin meshwork drive the cortical flow and result in anterior cortical PAR-3/6/PKC localization (Motegi and Sugimoto, 2006; Munro et al., 2004; Schonegg and Hyman, 2006). These data are further supported by the knockdown phenotypes of NMY-2 or MLC-4, which encodes the regulatory light chain of the Myosin-II molecule (Wormbase). Knock-down of either of them abolishes contractile polarity establishment and therefore prevents the proper localization of the PAR proteins (Shelton et al., 1999).

I.2.2. The family of Rho GTPases and their regulation

The family of Rho GTPases plays important roles in relaying extra- or intracellular signals to the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005). Rho GTPases belong to an evolutionary conserved family of small, about 21 kDa proteins of the ras superfamily (Alberts et al, 2005). Like other small GTPases, Rho GTPases are molecular switches that exist in an active, GTP-bound form and an inactive, GDP-bound form. Upon GTP-binding they interact with and activate effector proteins (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005), (Figure 3).

The 3 best-characterized members of the Rho GTPase family are RhoA, Rac1 and Cdc42. Each of them is a member of a distinct subfamily of Rho GTPases. Rho GTPases were originally identified as regulators of the actin cytoskeleton controlling the formation and bundling of contractile acto-myosin filaments. RhoA promotes the assembly of contractile actin and myosin filaments into stress fibers. Rac1 favors the formation of actin-rich surface protrusions (lamellipodia), while Cdc42 activation induces finger-like membrane extensions (filopodia) (Jaffe and Hall, 2005). RhoA and Cdc42 have been implicated in polarity establishment: Cdc42 is required for the bud site selection in yeast (Nelson, 2003) as well as for polarization events in mammalian MDCK cells (Joberty et al., 2000) and *C. elegans* (Aceto et al., 2006; Gotta et al., 2001; Kay and Hunter, 2001). Morever, RHO-1, the *C. elegans* homolog of mammalian RhoA, has been shown to be essential for every contractility event in the embryo preceding polarization (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006).

Besides controlling the actin cytokeleton and polarization events, some Rho GTPases are also involved in microtubule turnover, gene expression and regulation of enzyme activity (Jaffe and Hall, 2005).

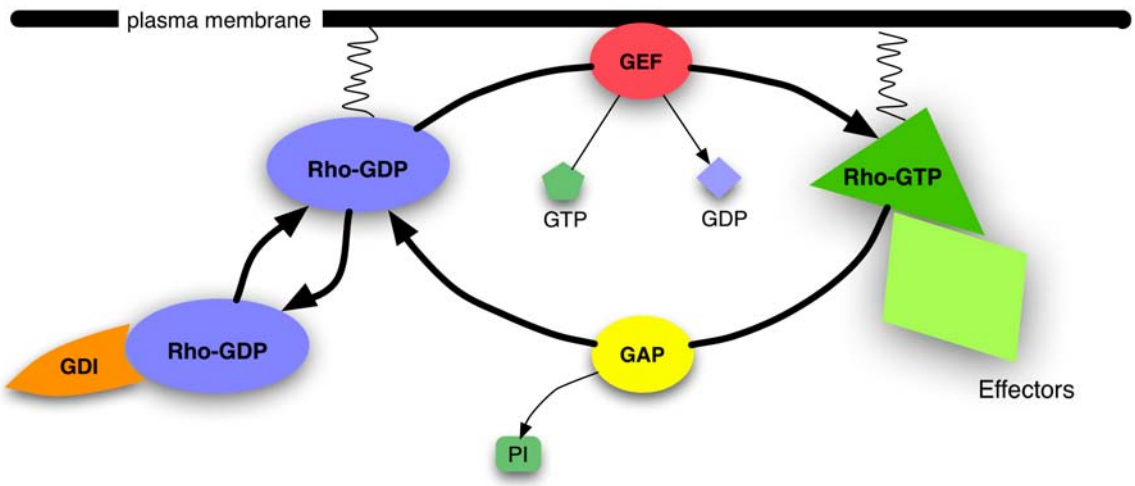

Figure 3: The GTP-GDP cycle of RhoGPTases.
RhoGTPases belong to the family of small GTP binding proteins, which are active in the GTP-bound form (green) and inactive in the GDP-bound form (blue). They are activated by GEFs (red), which catalyze the exchange from GDP to GTP. In the active form they are able to bind and activate effector proteins (light green). The hydrolysis of GTP to GDP, which is stimulated by a GAP (yellow), inactivates the RhoGTPase. Most of the RhoGTPases are anchored at the plasma membrane via a prenyl modification. They can be also sequestered into inactive complexes by GDIs (orange). Adapted from (Etienne-Manneville and Hall, 2002).

I.2.2.1. Rho GTPases in *C. elegans*

Eleven RhoGTPases have been predicted to be present in the *C. elegans* genome (Lundquist, 2006). So far, specific functions have been assigned to only 6 of them and only 2 of them, RHO-1 and CDC-42, seem to be essential in the P0 zygote (Kamath and Ahringer, 2003; Rual et al., 2004; Simmer et al., 2003; Sonnichsen et al., 2005).

I.2.2.1.1. RHO-1 is essential to generate contractions in the embryo

To date, no mutant allele for *rho-1* has been isolated from *C. elegans* (Lundquist, 2006). Instead, the *rho-1* loss-of-function phenotype has been analyzed using RNAi, which resulted in embryonic lethality with high penetrance (approx. 90-100%) (Sonnichsen et al., 2005). Furthermore, RHO-1 is essential for contractility of the early embryo during pseudo-

cleavage (Sonnichsen et al., 2005) and cytokinesis as well as for central spindle assembly (Jantsch-Plunger et al., 2000; Mishima et al., 2002).

Recently, two different groups provided additional insights into the role of RHO-1 at this early stage of development. Independently, Schonegg and Hyman (2006) and Motegi and Sugimoto (2006) showed that RHO-1 is essential for the establishment of polarity in the one-cell embryo. *rho-1 (RNAi)* lead to a complete absence of cortical contractions and a loss of polarity. Consistent with these findings, *rho-1 (RNAi)* resulted in a mis-localization of the NMY-2, which was dispersed over the entire cortex and never asymmetrically localized.

Moreover, since NMY-2 is upstream of the PAR proteins in the polarity cascad, the PAR proteins were not properly localized in *rho-1 (RNAi)* embryos. In agreement with a role in polarity establishment, RHO-1 itself is asymmetrically localized in the cortex in wild type embryos during polarization and its activation in the embryo seems to require the GEF ECT-2 (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006).

I.2.2.1.2. CDC-42 is essential for the maintenance of polarity in the embryo

Like RHO-1, CDC-42 is also essential for early *C. elegans* development and has a role in polarity. In contrast to RHO-1, however, it is not involved in cytokinesis or force generation in the P0 zygote. RNAi mediated knock-down of CDC-42 did not alter cortex contractility and polarity was established correctly, as indicated by the correct asymmetric localization of NMY-2 (Cowan and Hyman, 2007).

Instead, CDC-42 was required to maintain the localization of PAR-6 at the anterior cortex after its initial recruitment to the anterior cortex (Aceto et al., 2006; Gotta et al., 2001; Kay and Hunter, 2001). This observation in *cdc-42* (*RNAi*) knock-down embryos underlines the existence of distinct establishment and maintenance phases of PAR polarization in *C. elegans* (Cuenca et al., 2003).

Furthermore, a failure in spindle rotation after the first asymmetric cell division of P0 was observed (Gotta et al., 2001; Kay and Hunter, 2001) under these conditions. In wild-type embryos, the spindle of the P1 cell rotates by 90° with respect to the first cleavage plane. In contrast, in *cdc-42 (RNAi)* embryos both the P1 and the AB cell reorient their spindles. A similar polarity defect has also been observed in *par-6 (RNAi)* knock-down embryos (Gotta et al., 2001; Kay and Hunter, 2001). A direct interaction between CDC-42 and PAR-6 has been demonstrated through yeast-two-hybrid assays and CDC-42 itself localizes asymmetrically to the anterior cortex (Aceto et al., 2006; Beers and Kemphues, 2006; Kay and Hunter, 2001).

I.2.2.2. Regulation of Rho GTPases: GEFs and GAPs

Rho protein function is tightly regulated and a number of posttranslational modifications such as prenylation and phosphorylation have been identified (Etienne-Manneville and Hall, 2002). More importantly, GTPase activity is under the control of guanine-nucelotide-exchange factors (GEF), GTPase-activating proteins (GAPs) and GDP dissociation inhibitors (GDI) (Bos et al., 2007; Etienne-Manneville and Hall, 2002); Figure 3).

GEFs activate Rho proteins by catalyzing the nucleotide exchange of GDP by GTP (Cerione and Zheng, 1996; Zheng et al., 1996), GAPs inactivate the GTPases by stimulating their intrinsic GTPase activity (Hakoshima et al., 2003; Lamarche and Hall, 1994; Tcherkezian and Lamarche-vane, 2007) and GDIs sequester the inactive, GDP-bound form (Olofsson, 1999). GEFs and GAPs play important roles in the temporal and spatial control of Rho GTPase activity.

Most of the known GEFs and GAPs are multidomain proteins. GEFs regulating Rho family members contain either a DH-PH tandem domain or a recently identified DOCK domain, which is essential for GEF activity (Bos et al., 2007; Zheng et al., 1996). Similarly, RhoGAPs possess a conserved stretch of about 120-140 amino acids (Lamarche and Hall, 1994) directly involved in GTPase activation (Lamarche and Hall, 1994; Tcherkezian and Lamarche-vane, 2007).

I.2.2.2.1. RhoGEFs and RhoGAPs of *C. elegans*: ECT-2 and CYK-4

To date, more than 50 RhoGEFs and approximately 30 RhoGAPs have been identified through sequence analysis in the *C. elegans* genome. So far, no Rho GDI has been found (Lundquist, 2006).

Little is known about the regulation of the RhoGTPases during early *C. elegans* development and only two RhoGEFs have been characterized to date in more detail in *C.elegans*, ECT-2 and UNC-73 (Morita et al., 2005; Steven et al., 2005). ECT-2 is needed both early and late during development and its early loss-of-function phenotype resembles that of *rho-1 (RNAi)*. *ect-2 (RNAi)* results in multinucleated one-cell embryos due to a defect specifically in cytokinesis (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006; Sonnichsen et al., 2005; Yuce et al., 2005). In addition, ECT-2 is necessary for P cell

migration after hatching (Morita et al., 2005). The 12 epidermal P cells migrate to the ventral midline in the L1 stage, where their daughter cells give rise to motoneurons.

Similarly, UNC-73 is also required for P cell migration (Steven et al., 2005) and acts as a GEF for RHO-1 during this process. In contrast, it does not seem to be required during embryogenesis (Kubiseski et al., 2003; Spencer et al., 2001; Steven et al., 2005). In addition UNC-73 can act as a GEF for two other GTPases, RAC-1 and CED-10, during vulva development (Kishore and Sundaram, 2002).

Several negative regulators of RHO-1 signaling have been identified in *C. elegans.* The RhoGAPs RGA-1 and RGA-2 are involved in morphogenesis (Diogon et al., 2007). RGA-2 shows *in vitro* GAP activity towards RHO-1 and also regulates the Rho-associated kinase (ROCK) LET-502 during embryonic elongation (Diogon et al., 2007). Another GAP, SYD-1, is required in the nervous system to define axonal identity (Hallam et al., 2002; Whited and Garrity, 2002).

The investigation of the RhoGAP CYK-4 during early embryogenesis has contributed most to our current understanding of RHO-1 regulation at this stage of development. CYK-4 is a member of the centralspindlin complex involved in late stages of cytokinesis (Jantsch-Plunger et al., 2000; Mishima and Glotzer, 2003; Mishima et al., 2002; Piekny et al., 2005). Both *cyk-4 (RNAi)* and genetic mutations of the *cyk-4* locus cause a high frequency of embryonic lethality. Embryos initiate cytokinesis during the first cell division but the cleavage furrow regresses immediately before abscission, giving rise to multinucleated one-cell embryos (Jantsch-Plunger et al., 2000). *In vitro*, CYK-4 can act as GAP for RHO-1 as well as CDC-42 and RAC-1 (Jantsch-Plunger et al., 2000). More recently, CYK-4 has also been implicated in the establishment of cortical polarity in the embryo (Jenkins et al., 2006). CYK-4 is contributed both maternally and paternally; the paternal pool may inactivate RHO-1 specifically at the posterior cortex and contribute to breaking the symmetry of the egg (Jenkins et al., 2006).

I.2.2.3. RhoGTPase effectors: Formins, Arp2/3 and the Rho kinase

Activation of Rho GTPases can lead to increased actin polymerization (Jaffe and Hall, 2005). Different families of Rho GTPases use different effectors to regulate the assembly of distinct actin structures, Rho employs formins, while Rac and Cdc42 initiate actin nucleation through the Arp2/3 complex (Jaffe and Hall, 2005).

Formins are activated by binding directly to Rho and actin and promote the linear elongation of filaments at barbed ends (Goode and Eck, 2007). The initiation of branched

actin networks by Rac and Cdc42 through the Arp2/3 complex is a two-step process. Cdc42 and Rac activate the structurally related WASP (for Cdc42) and WAVE (for Rac) proteins, which subsequently activate Arp2/3. In addition to WASP, Cdc42 also targets a second effector, Toca-1, to trigger Arp2/3 activation (Higgs and Pollard, 2001; Jaffe and Hall, 2005; Pollard, 2007).

In *C. elegans*, the Arp2/3 complex seems to be dispensable for the establishment of the a-p axis in the embryo and the first embryonic cleavage. In contrast, the formin CYK-1 and the actin binding protein PFN-1 (a homolog of vertebrate cofilin) are essential for these two processes (Severson et al., 2002).

Rho-associated Ser/Thr kinases (ROCKs) are among the most intensively studied Rho effectors. In mammalian fibroblasts two Rho kinases (ROKalpha and p160 ROCK) promote the formation of stress fibers and focal contacts (Leung et al., 1996; Manser et al., 1995). Furthermore, Rho kinases are necessary for the contraction of smooth muscles (Borman et al., 2002; Endo et al., 2004; Wooldridge et al., 2004). ROCKs both activate myosin light chain (MLC) and inhibit the action of the counteracting phosphatase (Borman et al., 2002; Endo et al., 2004; Wooldridge et al., 2004).

The *C. elegans* ROCK LET-502 regulates the epidermal cell shape changes that drive elongation of the embryo by counteracting the MLC phosphatase MEL-11 (Piekny et al., 2003; Piekny et al., 2000; Wissmann et al., 1997). Isolation of various *let-502* alleles provided *in vivo* evidence that ROCK and MLC compete to regulate actin-mediated cell constrictions in *C. elegans*. Loss of *mel-11* function results in embryonic arrest due to hypercontraction during elongation (Piekny and Mains, 2002; Piekny et al., 2000). The *let-502* mutant phenotype, in contrast, includes a loss of elongation again indicating antagonistic roles of MEL-11 and LET-502. In addition to elongation, other morphogenetic events in the early embryo seem to be regulated by LET-502, as some *let-502* alleles exhibit additional defects in pseudocleavage formation and cytokinesis (Piekny and Mains, 2002).

I.2.3. PAR protein mediated polarization of a cell

Loss of PAR proteins in *C. elegans* results in symmetrical cleavages giving rise to equally sized daughter cells. Subsequent development is aberrant and embryos arrest as amorphous masses of differentiated cells (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Kemphues et al., 1988; Mains et al., 1990; Watts et al., 1996).

Six partitioning defective (*par)* genes, *par-1* to *par-6*, have been identified in *C. elegans*, and orthologous proteins have been found in other species for five of them (Gomes and Bowerman, 2002; Kemphues, 2000; Kemphues et al., 1988). In addition to the *par* genes, the atypical protein kinase C (aPKC) is a component of the anterior PAR complex and the interaction between PAR-6 and PKC is conserved throughout metazoans. Although the *par* genes are involved in the same process, mutations in individual loci give rise to distinct loss-of-function phenotypes (Donald, 1997).

The PAR-3/6/aPKC complex is involved in various types of polarity. It marks the anterior pole of in *C. elegans* embryos (Cheng et al., 1995; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Nance et al., 2003; Watts et al., 1996) and *Drosophila* oocytes (Huynh et al., 2001; Pellettieri and Seydoux, 2002). Furthermore, the complex is required for establishment and maintenance of apical-basal polarity in *Drosophila* and mammalian epithelial cells (Izumi et al., 1998; Suzuki et al., 2004; Suzuki et al., 2001). In addition, PARs are also essential for polarization of neurons, e.g. during *Drosophila* neuroblast development (Petronczki and Knoblich, 2001; Rolls et al., 2003; Schober et al., 1999).

I.2.3.1. The conserved PAR-3/6/PKC complex covers the anterior cortex

Prior to the fertilization of a *C. elegans* oocyte, PAR-3, PAR-6 and aPKC are uniformly distributed throughout the cortex (oocytes are not polar). After establishment of the anterior-posterior axis in the embryo, cortical flow transfers the anterior PAR proteins to the anterior 50-60% of the cortex (Etemad-Moghadam et al., 1995; Tabuse et al., 1998; Watts et al., 1996). In *C. elegans* the loss of any one member of the complex leads to the loss of the other two members from the cortex, resulting in a uniform distribution of posterior PAR proteins over the entire cortex (Cuenca et al., 2003) and an equal first cleavage (Schneider and Bowerman, 2003).

The correct apical localization of the PAR-3/6/aPKC complex is also required for accurate asymmetric positioning of the mitotic spindle in mammals (Ahringer, 2003; Suzuki and Ohno, 2006). In flies, loss-of-function of any member of the PAR comples results in symmetric cleavages and mis-localization of cell fate determinants in neuroblasts (Huynh et al., 2001; Petronczki and Knoblich, 2001; Schober et al., 1999).

PAR-6 appears to be the scaffolding protein in the PAR-3/6/aPKC complex. Its CRIB domain establishes a connection to CDC-42 (Aceto et al., 2006; Beers and Kemphues, 2006; Kay and Hunter, 2001), while the N-terminal PDZ and PB1 domains of PAR-6 bind to aPKC, while a PDZ-PDZ domain interaction establishes contact with PAR-3 (Hung and Kemphues, 1999; Suzuki and Ohno, 2006).

The kinase activity of aPKC regulates the localization of PAR-complex members. In *C. elegans* PAR-2 and PAR-1 phosphorylation by aPKC prevents their cortical localization (Hao et al., 2006).

I.2.3.2. PAR-1 and PAR-2 are located a the posterior cortex

While the PAR-3/6/aPKC complex is restricted to the anterior cortex in the *C. elegans* embryo, PAR-2 and PAR-1 occupy the posterior cortex (Boyd et al., 1996; Cheng et al., 1995; Guo and Kemphues, 1995; Levitan et al., 1994). The anterior and posterior PAR proteins are mutually exclusive and the anterior PAR complex prevents PAR-2 from associating with the cortex (Cheng et al., 1995). PAR-1, in turn, depends on PAR-2 for its cortical localization at the posterior cortex (Boyd et al., 1996).

PAR-2 is the only PAR protein for which no ortholog has been found in other species than *Caenorhabditis* (Hao et al., 2006; Levitan et al., 1994). GFP::PAR-2 is enriched on the posterior cortex during its smoothening, when polarity is established. PAR-2 possesses a RING finger domain, wich is often associated with E3 ubiquitin ligase activity. The presence of an additional UBA (ubiqitin associated domain) also links it to this pathway (Hao et al., 2006; Levitan et al., 1994). However, this E3 ubiquitin ligase activity has not been demonstrated, yet.

The *par-1* gene encodes an evolutionarily conserved Ser/Thr kinase (Guo and Kemphues, 1995). In *Drosophila* oocytes and epithelial cells PAR-1 phosphorylates PAR-3 at two sites thereby disrupting its interaction with aPKC (Benton and Johnston, 2002; Benton and Johnston, 2003). In addition, PAR-1 phosphorylates the asymmetrically localized Oskar protein in fly oocytes, thereby stabilizing it at the posterior pole of the egg (Riechmann et al., 2002).

Both PAR-5 and PAR-4 have been detected throughout the whole cortex and in the cytoplasm. PAR-5 is a 14-3-3 protein acting upstream of PAR-1 (Benton et al., 2002; Morton et al., 2002) and may have a role in separating the anterior and posterior cortical PAR domains (Morton et al., 2002). Furthermore, PAR-5 is required for the correct segregation of the PIE-1 transcription factor to the posterior pole (Lo et al., 2004).

Like PAR-1, PAR-4 also encodes a putative Ser/Thr Kinase (Morton et al., 1992; Watts et al., 2000). Loss of *par-4* weakens the cytoplasmic streaming and surprisingly, causes all cell divisions after the first asymmetric cleavage to be symmetric. It may therefore be required for asymmetric segregation of soluble cell fate determinant (Morton et al., 1992; Watts et al., 2000).

I.3. Structure and function of centrosomes

During the interphase of the cell cycle, microtubules (MTs) play a major role in determining cell shape, polarity and motility. During mitosis they reorganize to form the bipolar spindle, which is important for the faithful separation of the sister chromatids.

Microtubules are generally nucleated from a specific intracellular location known as the microtubule-organizing center (MTOC). Long microtubules consist of cylindrical protofilaments, built from α- and β-tubulin dimers. γ-tubulin is not incorporated into filaments but has a specialized role in microtubule growth and is located at the MTOC. γ-tubulin and its accessory proteins form the so-called γ-tubulin-ring-complex (γ-TuRC), which mediates tubulin nucleation. Similar to actin filaments, microtubules are polar and grow only at their plus end from each MTOC outwards. The minus end points towards or remains anchored at the MTOC. In organism or cells that lack centrosomes (higher plants and e.g. mouse oocytes), bipolar spindles can form through centrosome-independent mechanisms, only mediated by γ-tubulin and motor proteins (Alberts, 2006).

In most animal cells, there is a single, well-defined MTOC called the centrosome. From this central organelle, the cytoplasmic microtubules emanate in a star-like, “astral” conformation. A centrosome is composed of two centrioles - a pair of cylindrical structures arranged perpendicular to each other, which are embedded in a fibrous protein matrix known as the pericentriolar material (PCM) (Alberts, 2006). The tiny barrel-shaped centrioles are structurally related to basal bodies, which in turn are essential for the formation of cilia and flagella (Machesky and Bornens, 2003; O'Toole et al., 2003).

The bipolar mitotic spindle, through which the DNA is segregated, is organized by two centrosomes. A mitotic cell must therefore have exactly two centrosomes and strict control of centrosome number is crucial for accurate chromosome segregation. In the first phase of the new cell cycle, G1, proliferating cells possess a single centrosome with two centrioles and PCM. This centrosome duplicates during S phase allowing for the formation of a proper bipolar spindle during mitosis (Figure 4). Any defect in the control of centrosome number results in the formation of mono- or multipolar spindles, severely hampering accurate chromosome segregation and very often yielding cell death.

I.3.1. The composition of a centrosome

A pair of centrioles constitutes the core of each centrosome. Vertebrate centrioles are barrel-shaped microtubule-based structures of approx. 175 nm in diamteter and approx. 400 nm in length (Susan, 2007). Centrioles are composed of stable microtubule arrays organized in a 9-fold radial symmetry. While vertebrate centriolar microtubule arrays usually consist of triplet microtubules, *Drosophila* microtubules are doublets in some tissues and triplets in others (Callaini et al., 1997; Moritz et al., 1995; Moritz et al., 1998). In *C. elegans*, finally, centriolar microtubules are singlets (Ward et al., 1981), (Figure 4).

The PCM surrounding the centrioles has been visualized as a fibrous lattice. The human centrosome contains over 100 different proteins (Andersen et al., 2003), including components required for microtubule nucleation like γ-tubulin and associated proteins (the γ-TuRC complex), which are also found in fungal spindle pole bodies (Moritz et al., 1995; Moritz et al., 1998). Other PCM components are less conserved but many of them harbor predicted coiled-coil domains suggesting that they perform scaffolding functions, e.g. for the recruitment of cell cycle regulatory proteins.

I.3.2. The centriole duplication pathway

In ciliated epithelial cells and male gametes of lower plants centrioles are generated *de novo* as (Nigg, 2007). However, if parental centrioles are already present in the cell, centrioles generation by semi-conservative duplication is preferred and an old parental centriole serves as template for a new one (Nigg, 2007). This mechanism tightly couples the number of novel centrioles to the number of parental centrioles (Marshall, 2001).

During template-mediated duplication the two parental centrioles lose their special orthogonal arrangement and move slightly away from each other. Then a small daughter centriole forms perpendicular to each parental centriole (Figure 4), elongates and forms a complete centrosome (Figure 4). Finally, the PCM is divided and the two resulting centrosomes separate (Leidel and Gonczy, 2005).

Upon fertilization, the sperm of most animal species contributes not only one complement of chromosomes but also one or two centrioles. Mature centrosomes are reconstituted by recruitment of proteins stored in the egg (Delattre and Gonczy, 2004).

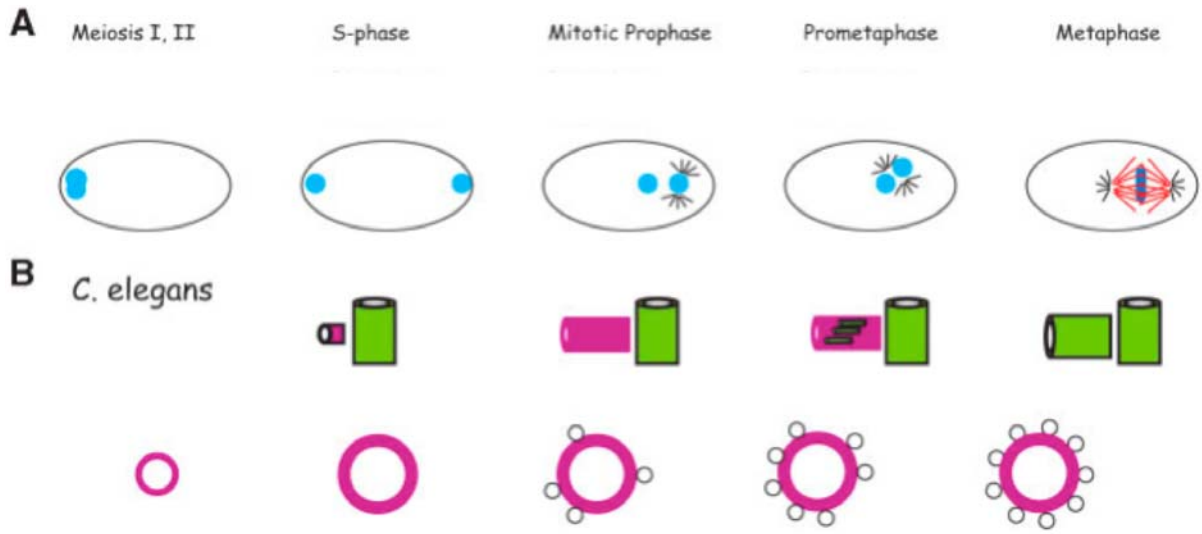

Figure 4: Centriole duplication in the *C. elegans* one-cell stage.
(A) Timing of the cell cycle events during centriole duplication. Black lines indicate the microtubule asters and red lines indicate the spindle microtubules. In blue are the maternal and paternal pronuclei. (B) Centriole events in *C. elegans* as uncovered by cryo-electron microscopy (Pelletier 2006). The parental centriole is shown in green. The newly formed tube is shown in magenta. Microtubules are shown in green in longitudinal sections and in black in cross-sectional analysis.
A daughter centriole is formed during the first mitotic cell division in *C. elegans* perpendicular to both paternally contributed centrioles. Centriole duplication is initiated during S phase by recruiting maternal proteins. During Prophase, the daughter centriole is already established, giving rise to a functional centrosome during metaphase of the first mitosis. From (Susan, 2007).

I.3.2.1. Centriole assembly in *C. elegans*

By morphological criteria centrioles in *C. elegans* are less complex than in mammalian cells. Each is ca. 150 nm by 100 nm in size and comprises nine singlet microtubules. The oocyte is devoid of centrioles but contains a large store of maternally contributed products and the sperm contributes two required centrioles (Hyman, 2006).

In recent years, several studies have investigated the centrosome duplication cycle in the *C. elegans* embryo (Delattre et al., 2006; Delattre et al., 2004; Leidel et al., 2005). EMS mutagenesis screens and RNAi screens have identified five centrosomal/centriolar associated proteins in *C. elegans* essential for centriole duplication: ZYG-1, SAS-4, SAS-5,

SAS-6 and SPD-2 (Dammermann et al., 2004; Dammermann et al., 2004; Delattre et al., 2004; Hamill et al., 2002; Kemp et al., 2004; Leidel and Gonczy, 2003; O'Connell et al., 2001; Pelletier et al., 2006; Pelletier et al., 2004). While SAS-4, SAS-6 and SPD-2 have human homologs, SAS-5 and ZYG-1 seem to be unique to *C. elegans* (Susan, 2007).

The kinase ZYG-1 was the first centrosomal protein identified in *C. elegans* (O`Connell et al., 2001). It is already required during spermatogenesis and the mature sperm from *zyg-1* mutants contains only a single centriole. Consequently, only one functional centrosome is formed in the zygote and the first mitosis fails during cytokinesis, giving rise to a tetraploid cell. Though centrosome duplication and bipolar spindle formation during the second cell cycle appear normal, the abnormal DNA content causes lethality (O`Connell et al., 2001).

When *zyg-1* is depleted maternally, the sperm provides two cenrioles. However, centriole duplication is blocked. Nevertheless, the centrioles recruit PCM material, separate from each and form of a functional bipolar mitotic spindle. After an apparently succesful first cell division, however, each cell contains a single centriole that cannot be duplicated and further cell cycles fail (O`Connell et al., 2001).

When *zyg-1* is depleted maternally and paternally, the zygote contains only one centriole, which cannot be duplicated. The monopolar spindle that forms resembles the phenotype seen with paternal depletion (Leidel and Gonczy, 2005; O'Connell et al., 2001).

Three *sas* (spindle assembly defective) genes were identified in being essential for centrosome duplication in *C. elegans*: *sas-4, sas-5* and *sas-6.*

sas-4 and *sas-6* were identified in large-scale RNAi screens and further characterized based on RNAi experiments (Dammermann et al., 2004; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gonczy, 2003; Strnad et al., 2007). RNAi of both genes yields an intact bipolar spindle in the P0 zygote, but only monopolar spindles assemble in each blastomere at the two-cell stage. This phenotype resembles the one of maternally depleted *zyg-1*, and therefore, a role for SAS-4 and SAS-6 in centriole duplication has been proposed (Dammermann et al., 2004; Kirkham et al., 2003; Leidel et al., 2005). SAS-4 and SAS-6 are both coiled-coil proteins and enriched in a tiny dot at the center of the centrosome throughout the cell cycle, suggesting a centriolar localization (Leidel et al., 2005; Leidel and Gonczy, 2003). *sas-5* is the third *sas* gene in *C. elegans* required for centrosome duplication (Delattre et al., 2004). It was identified in a forward genetic screen and also characterized by RNAi (Delattre et al., 2004) (Schmutz and Spang, 2005). It encodes a putative coiled-coil domain protein and is provided both maternally and paternally. SAS-5 is localized at the centrosome, more specifically at the centrioles (Delattre et al., 2004).

FRAP experiments using GFP::SAS-4 and GFP::SAS-6 revealed that SAS-4 and SAS-6 are recruited to the centriole only once per cell cycle. While SAS-4 associates only with the daughter centriol during duplication, SAS-6 is incorporated into both parental and daughter centrioles (Leidel et al., 2005; Leidel and Gonczy, 2003). Similarly to SAS-4 and SAS-6, SAS-5 shuttles between the centrosome and the cytosol (Delattre et al., 2004). In addition SAS-5 interacts with SAS-6 in a yeast-tweo-hybrid experiment, indicating that they may be part of the same complex (Delattre et al., 2004).

Another component of the centrosome in *C. elegans*, SPD-2, is unique in its role since it is localized to the centriole and the PCM and is essential for both the centriole and PCM assembly (Kemp et al., 2004; Pelletier et al., 2004).

The prevailing view how centrosomes assemble predict that centrioles recruit PCM components from the cytoplasm to build a mature centrosome. However, PCM components also play a role in centriole formation. After inactivation of γ-tubulin or the PCM protein SPD-5, GFP::SAS-4 fails to be incorporated into the centrosome in 50% of the embryos indicating a partial failure in centrosome duplication in *C. elegans* (Dammermann et al., 2004). In contrast, knock-down of the Aurora Kinase AIR-1, which is required for the maturation of the centrosome, does not impair GFP::SAS-4 incorporation into the centrosome. Taken together, these results suggested that the PCM plays a role in efficient centriole duplication (Dammermann et al., 2004).

A simple model has been proposed to explain the role of the factors described above thus in centrosome duplication. First, SPD-2 binds to the parental centrioles. Shortly afterwards, ZYG-1 is recruited. The relocation of SAS-5 and SAS-6 to the centrioles coincides with a drop in ZYG-1 level, allowing the recruitment of SAS-4 (Leidel and Gonczy, 2005). Despite this reconstruction of their temporal and spatial recruitment to the centrioles, the molecular function of these proteins has remained elusive.

I.4 AIM

The aim of this study was to identify novel genes with an essential role in the first cell cycle of the *C. elegans* embryo. From the large amount of phenotypic data generated in high-throughput RNAi screens, candidate genes were selected for a detailed analysis. The possibility to use RNAi to down-regulate these genes alone and in combination provided the opportunity to study their role at different stages of polarization and cell division.

Small GTPases are important for cell polarity and their activity is tightly controlled, e.g. through GTP/GDP exchange factors (GEFs) or GTPase activating proteins (GAPs). One of selected candidates, *rga-3*, encodes a putative RhoGAP protein and has high sequence similarity to another locus in the *C. elegans* genome, *rga-4*. The aim of the first part of this thesis was to address several questions concerning the biological role of these two predicted RhoGAPs. What is the role of these two genes in the early embryo and, more specifically, which process is disturbed in *rga-3* and *rga-4 (RNAi)?* What are the molecular functions of RGA-3/4 at the one-cell stage and which GTPases do they regulate? Do RGA-3/4 really modulate to actin dynamics, as would be predicted from their conserved RhoGAP domains? And if their function is linked to controlling the acto-myosin network in the embryo, how can they be integrated into the known regulatory network governing polarity and asymmetric cell division?

Similarly, the second part of the thesis contains the characterization of another gene essential for development of the early embryo, *sas-5*. *sas-5 (RNAi)* had also been shown to be embryonic lethal in large-scale RNAi screens, yet the particular cellular function of *sas-5* had remained elusive. Sequence analysis alone was not instructive, as SAS-5 contains no other predicted structural elements than putative coiled-coil domains and has no known ortholog outside *Caenorhabditis*. The most important questions were therefore: Where is SAS-5 required in the one-cell stage embryo, does it localize to a specific sub-cellular domain, what is its molecular function and what is the primary defect that causes *sas-5 (RNAi)* lethality?

To address these questions, a set of GFP reporter strains was used to visualize different cell organelles and processes, e.g. to follow nuclear division with a histone::GFP reporter or the mitotic spindle with a tubulin::GFP expressing line.

II RESULTS

II.1. Functions of the novel RhoGAP proteins RGA-3 and RGA-4 in the germ line and in the early embryo of *C. elegans*

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Functions of the novel RhoGAP proteins RGA-3 and RGA-4 in the germ line and in the early embryo of *C. elegans*

Cornelia Schmutz, Julia Stevens and Anne Spang*

We have identified two redundant GTPase activating proteins (GAPs) – RGA-3 and RGA-4 – that regulate Rho GTPase function at the plasma membrane in early *Caenorhabditis elegans* embryos. Knockdown of both RhoGAPs resulted in extensive membrane ruffling, furrowing and pronounced pseudo-cleavages. In addition, the non-muscle myosin NMY-2 and RHO-1 accumulated on the cortex at sites of ruffling. RGA-3 and RGA-4 are GAPs for RHO-1, but most probably not for CDC-42, because only RHO-1 was epistatic to the two GAPs, and the GAPs had no obvious influence on CDC-42 function. Furthermore, knockdown of either the RHO-1 effector, LET-502, or the exchange factor for RHO-1, ECT-2, alleviated the membrane-ruffling phenotype caused by simultaneous knockdown of both RGA-3 and RGA-4 [*rga-3/4 (RNAi)*]. GFP::PAR-6 and GFP::PAR-2 were localized at the anterior and posterior part of the early *C. elegans* embryo, respectively showing that *rga-3/4 (RNAi)* did not interfere with polarity establishment. Most importantly, upon simultaneous knockdown of RGA-3, RGA-4 and the third RhoGAP present in the early embryo, CYK-4, NMY-2 spread over the entire cortex and GFP::PAR-2 localization at the posterior cortex was greatly diminished. These results indicate that the functions of CYK-4 are temporally and spatially distinct from RGA-3 and RGA-4 (RGA-3/4). RGA-3/4 and CYK-4 also play different roles in controlling LET-502 activation in the germ line, because *rga-3/4 (RNAi)*, but not *cyk-4 (RNAi)*, aggravated the *let-502(sb106)* phenotype. We propose that RGA-3/4 and CYK-4 control with which effector molecules RHO-1 interacts at particular sites at the cortex in the zygote and in the germ line.

KEY WORDS: RhoGAP, *C. elegans*, Acto-myosin, Early embryo, Germ line

INTRODUCTION

Asymmetric cell division is a general mechanism by which to generate two non-identical daughter cells. One of the most-studied systems is the first asymmetric division of the *Caenorhabditis elegans* embryo (Cowan and Hyman, 2004a; Gönczy et al., 2001; Schneider and Bowerman, 2003). After fertilization, the egg completes the meiotic divisions and extrudes two polar bodies. During the meiosis events, right after sperm entry, the entire cortex of the embryo is highly contractile. The sperm initiates cortical and cytoplasmic movements, which are actin-dependent. These streamings are essential for the establishment of polarity. How they are controlled, however, has remained largely elusive. Upon movement of the maternal pronucleus towards the paternal pronucleus, the posterior part of the cortex smoothens, which is supposedly controlled by the centrosomes brought along by the sperm (Cheeks et al., 2004; Cowan and Hyman, 2004b; Munro et al., 2004). Posterior smoothening and anterior ruffling of the cortex lead to a membrane invagination, called pseudo-cleavage. The two pronuclei meet in the posterior part of the embryo and migrate to towards the middle of the embryo, where mitosis is initiated. Upon centration, cortical ruffling activity ceases, and resumes during anaphase of the first mitosis. After cytokinesis, the larger anterior AB cell and the smaller P1 cell prepare the next division cycle, which is again asymmetric.

The PAR proteins play a crucial role in the setup and maintenance of polarity right after fertilization (Cuenca et al., 2003). Whereas the PAR-3–PAR-6–aPKC complex determines anterior polarity, PAR-1 and PAR-2 occupy the posterior part. The two domains do not intermix and are mutually exclusive. PAR-5 is a 14-3-3 protein that seems to be essential to maintain the two different domains (Kemphues, 2000).

Contractility in most systems involves the acto-myosin system, which is controlled by GTP-binding proteins of the Rho superfamily (Etienne-Manneville and Hall, 2002; Glotzer, 2005). Rho GTPases are molecular switches that exist in an activated, GTP-bound form and an inactive GDP-bound conformation. The switch between the two conformations is achieved by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Bernards, 2003; Schmidt and Hall, 2002). In their activated state, the GTPases can interact with and signal to downstream effectors, which in turn control a variety of cell processes. For example, effectors of RhoA (RHO-1 in *C. elegans*), such as ROCK1 (LET-502) regulate the acto-myosin system. RhoGAPs are particularly interesting proteins, because they often contain multiple functional motifs in addition to the GAP domain that might regulate localization or GAP activity, or might recruit other signalling proteins (Moon and Zheng, 2003). They outnumber RhoGTPases by 4:1 in humans (and 5:1 in *C. elegans*), so, in principle, the action of a given RhoGTPase could be regulated by at least four different RhoGAPs in different spatial and temporal patterns. Their versatility is exemplified by the ARAP proteins, which contain both a RhoGAP domain and an ArfGAP domain, and can thus serve as converging signalling platforms (Krugmann et al., 2002; Miura et al., 2002).

In oocytes, the acto-myosin network is distributed over the entire cortex. Upon fertilization, due to cortical and cytoplasmic rearrangements, the acto-myosin network becomes restricted to the anterior cortex, giving rise to contractions in the anterior region of the embryo and a smooth posterior part. Local contractility in the posterior region appears to be inhibited by a signal from the centrosome (Cowan and Hyman, 2004b; Munro et al., 2004). Interestingly, a more recent study provides evidence for the RhoGAP CYK-4 being the signal for establishing the posterior PAR-2 domain and causing the retraction of the acto-

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myosin system to the anterior region of the embryo (Jenkins et al., 2006). How contraction is controlled in the anterior embryo, however, remains elusive.

We investigated the role of two RhoGAP proteins, RGA-3 and RGA-4, in the early *C. elegans* embryo and in the germ line. Knockdown of the two RhoGAPs by RNA interference (RNAi) resulted in hyper-contractility of the anterior cortex in the zygote. Although no major defects were detected in polarity establishment, we observed fluctuations in the size of the PAR-6 domain at the anterior cortex. RGA-3 and RGA-4 (RGA-3/4) have overlapping functions in the early embryo, because concomitant knockdown of both proteins yielded the strongest phenotype. RGA-3/4 are GAPs for RHO-1, because RHO-1, but not CDC-42, was epistatic to RGA-3/4. The only other characterized RhoGAP in the early embryo, CYK-4, has non-redundant functions with RGA-3/4, because the phenotypes of the triple knockdown were additive. Furthermore, *rga-3/4* RNAi treatment [*rga-3/4(RNAi)*] enhanced the gonadal defects in *let-502(sb106)* mutants, whereas *cyk-4(RNAi)* had no effect. RGA-3/4 are required to control the activation of the *C. elegans* ROCK LET-502 in the early embryo. We provide evidence that the specificity of RHO-1 is determined by the differential use of RhoGAPs during early *C. elegans* development.

MATERIALS AND METHODS

General methods and strains

C. elegans was cultured and maintained as described previously (Brenner, 1974) on NGM medium at 20°C unless noted otherwise. The *C. elegans* strains N2 and the alleles *let-502(ok1283)* and *let-502(sb106)* (Piekny et al., 2000) were used for time-lapse studies and for the counting of embryonic lethality rates. Transgenic GFP-expressing lines used in this study are listed below:

The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC): strain AZ212, which expresses a GFP::H2B histone fusion in the germ line (Praitis et al., 2001); KK866, a strain with GFP::PAR-2 (Wallenfang and Seydoux, 2000); JJ1473, a strain expressing NMY-2::GFP (Munro et al., 2004); and WH204, a strain expressing GFP::β-tubulin (Strome et al., 2001). JH1512, a strain bearing GFP::PAR-6 fusion (Cuenca et al., 2003) was a gift from Geraldine Seydoux (John Hopkins University Medical School, Baltimore, USA), and SA115, a strain expressing GFP::RHO-1 (Motegi and Sugimoto, 2006), was a gift from Fumio Motegi (RIKEN, Kobe, Japan).

RNAi experiments

For RNAi-by-feeding experiments, plasmid L4440, containing the desired sequence (see Table 1) was transformed into the *Escherichia coli* strain HT115 (Timmons et al., 2001). NGM plates containing 0.5-2.0 mM IPTG and 25 μg/ml carbenicillin were inoculated with transformed HT115 bacteria. The expression of double-stranded RNA (dsRNA) was induced for 6-9 hours at room temperature on plate. Subsequently, L1, dauer larvae or L3 larvae were transferred to these plates. Animals were cultured at 15, 20 or 23°C and their progeny were analyzed.

For RNAi-by-injection experiments, *C. elegans* genomic or cDNA was used as a template to PCR-amplify the desired sequence (Table 1). PCR products were used as templates for in vitro transcription using T7 polymerase (Promega). dsRNA was produced according to the manufacturer's protocol (Promega), purified by phenol/chloroform extraction and resuspended in 20 μl DEPC-H_2O. dsRNA was injected into the gonad of young adult worms, which were subsequently incubated at 20°C. The progeny of injected animals was analyzed.

For RNAi-by-soaking experiments, L3 larvae were incubated for 24 hours in dsRNA solution in a wet chamber. The animals were transferred onto an agar plate seeded with OP50 bacteria for recovery. The progeny was analyzed.

Live-embryo imaging

Embryos were mounted in a drop of M9 buffer ('hanging drop' method) and covered with a cover slip. Embryos were imaged with a Zeiss Axioplan 2 microscope equipped with a Zeiss Axio Cam MRm camera (Carl Zeiss, Aalen Oberkochen, Germany) and a Plan Apochromat 63×/NA1.40 objective. Zeiss Axiovision 3.1 software was used to control hardware and to acquire and process images. Confocal images were captured with a Leica confocal microscope TCS SP2 (Leica, Bensheim, Germany) and an HCX PL APO 63×/1.32-0.6 oil objective. Laser intensities were adjusted to avoid any defects in the development of the embryo. Images were collected at 20-second or 45-second intervals over a period of 15-35 minutes and processed using Adobe Photoshop 7.0.

Table 1. RNAi experiments

Gene targeted	Sequence (5′–3′)	RNAi method
K09H11.3 (RhoGAP)	GTTACACTCTTGGAGAGGTATGCTC TGGACTCTTGGTTCTCTTCATATTC	Feeding, injection
K09H11.3 (middle)	CGAGAAATAACGGTAGTGGAAAGT GAGATTGGTTCTAGGAGACGATGT	Feeding
K09H11.3 (3 UTR)	GTTTAGTGCGATCCAGAGTCAAT TGCATTGAAGTTAAGAGATGGGTA	Feeding
Y75B7AL.4 (middle)	CGAGAATGTAAAATCCAGAAACG TTGGAGTGAGATTGGTTCT	Feeding
cyk-4 (Kamath et al., 2003)	TGGTTTGTCCTGGTGTTTGA ACGGTTTTCACGCATTTTTC	Feeding
cyk-4	GGTCGCAAGAAGCTGGCAAT TGGTTTGTCCTGGTGTTTGA	Feeding, injection
rho-1	ATTATGTTGCCGACATTGAAGTT CATGCACTTGCTCTTCTTTTTCT	Feeding, injection
cdc-42 (Kamath et al., 2003)	TTCTTCGATAATTATTGCTCCCA AACGACGACGAAAATGTTAAAGA	Feeding
cdc-42	AGATGGAGCTGTCGGTAAAACT TTCTTCTTCTTCTCCTGTTGTGG	Feeding, injection
let-502 (Kamath et al., 2003)	GCATTATCTCGATCACGGGT ATTTGAACTCCGACCGAATG	Feeding
nmy-2 (Kamath et al., 2003)	TCCGAGAAGTGAAGCGATTT TATGCAGAACGTCTCAACCG	Feeding
ect-2 (Kamath et al., 2003)	CTCTGAATTTCTGCCAAAGCC GGCAAAGAAATCCGATTCAA	Feeding, injection

The guide strands (top) and anti-guide stands (bottom) are shown.

Table 2. Penetrance of the ruffling phenotype and embryonic lethality in early embryos

	Ruffling phenotype (%)	Embryonic lethality rate (%)
rga-3/4(RNAi)	62.3 (*n*=131)	82.10
rga-3(RNAi)	26.5 (*n*=159)	9.30
rga-3 3′UTR(RNAi)	24.0 (*n*=54)	4.24
rga-4(RNAi)	42.2 (*n*=90)	6.36
cyk-4(RNAi)	2.8 (*n*=106)	22.75
Control	6.6 (*n*=106)	1.40

The penetrance of the ruffling phenotype was determined by imaging embryos from the one- to two-cell stage. *n* gives the number of embryos analyzed for each category. Control refers to embryos from mothers fed with empty pDT7 vector. Images of the representative embryos for each RNAi are shown in Fig. 2. The embryonic lethality rate was determined by counting the offspring of animals subjected to RNAi treatment in parallel experiments to those used for the determination of penetrance of the ruffling phenotype.

Quantification of the domain sizes of PAR-2 and PAR-6

ImageJ was used to measure the PAR-2 and PAR-6 domains in wild-type and RNAi-treated embryos. Pictures of embryos – from the beginning of polarization until reaching the two-cell stage – were acquired in the Nomarski and the GFP channel. For quantification, the length of the embryo was measured by drawing a line from the anterior to the posterior of the embryo with ImageJ; this line was set to 100%. The length of the respective PAR domain was measured along this line by using the cortical GFP localizations. The ratio between the length of the PAR domain and the length of the entire embryo was determined.

Quantification of GFP::NMY and GFP::RHO-1 signals

To quantify the GFP signals in wild-type and *rga-3/4(RNAi)* embryos, either confocal or epifluorescence pictures with the same exposure times were subjected to analysis with ImageJ. The length of NMY-2::GFP patches in the embryo before pronuclear meeting was determined by drawing cortical lines corresponding to the contractile patches with the highest fluorescence signal. The length of these lines was measured.

For quantification of the RHO-1::GFP data, the cortical fluorescence signal was measured. The ratio of the signal between the contractile cortex at the ingressing furrow and the non-contractile cortex was determined.

DAPI and rhodamin-phalloidin staining of gonads

Young adult worms were cut open to release the gonads in a depression slide and gonads were fixed with 3.7% formaldehyde for 10 minutes at room temperature. After washing with egg buffer, incubation in 0.4% BSA, 0.1% Tween-20 for 10 minutes, followed by another washing step, the gonads were stained with rhodamin-phalloidin (Molecular Probes, 0.01 U/μl) and DAPI (Boehringer Mannheim, 0.5 μg/ml) in a wet chamber for 30 minutes at room temperature. Gonads were mounted onto an agarose pad and examined with a Zeiss Axioplan 2 microscope using a 40× Axioplan objective as described above.

RESULTS

Two RhoGAP proteins are required to control membrane ruffling in the early *C. elegans* embryo

Upon fertilization, the *C. elegans* zygote segregates specific proteins and RNAs to opposite poles to provide asymmetry and polarity for the subsequent cell division (Schneider and Bowerman, 2003). This polarity establishment involves cytoplasmic streaming and cortical contractions. The nature of the membrane dynamics and the cortical contractions upon fertilization are poorly understood. Therefore, we undertook a candidate approach and screened databases for genes that influenced membrane dynamics, especially cortical movement and membrane ruffling in the fertilized *C. elegans* oocyte. RNAi of K09H11.3 resulted in a pronounced increase in membrane ruffling and ectopic furrowing after fertilization, and increased pseudo-cleavage (Fig. 1A,B; compare Movies 1 and 2 in the supplementary material), similar to phenotypes that had been reported previously (Kamath et al., 2003; Simmer et al., 2003; Sonnichsen et al., 2005). The membrane dynamics in the RNAi embryos were strongly increased compared with wild type and the pseudo-cleavage was sometimes shifted towards the anterior pole. Upon RNAi, the number of ruffles increased at least two- to threefold over wild type (Fig. 1B). The depth of the ruffles was also increased [*rga-3/4 (RNAi)*: 6.32±2.0

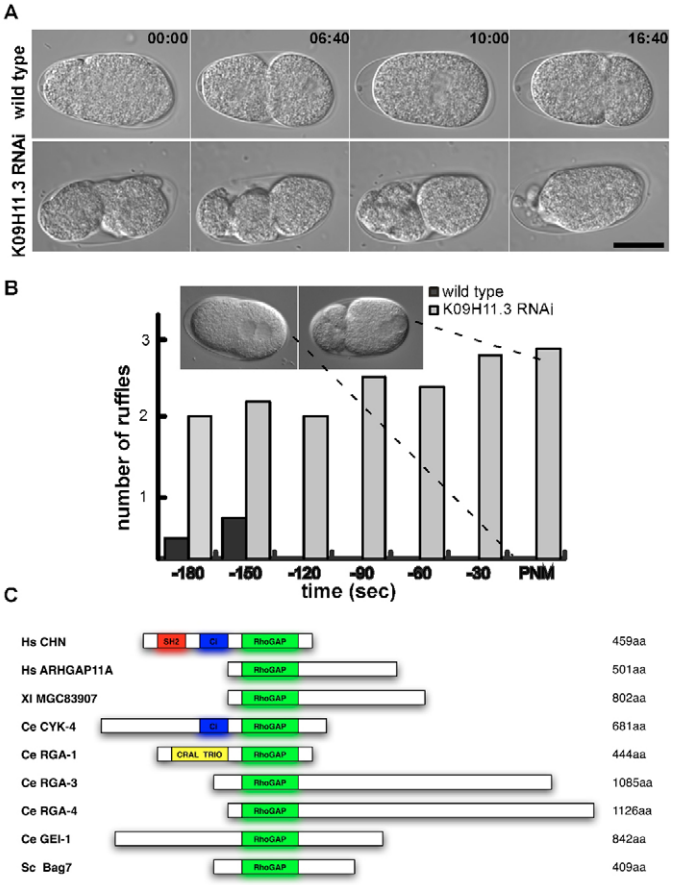

Fig. 1. The RhoGAP protein K09H11.3 is required to control membrane ruffling in the early *C. elegans* embryo. (**A**) Pictures from time-lapse studies of wild-type and K09H11.3 RNAi-treated embryos. K09H11.3 RNAi treatment was performed by feeding dsRNA-expressing bacteria targeting the RhoGAP domain containing the N-terminus of the K09H11.3 gene (*rga-3*). Embryos are grown at 20°C and mounted for microscopy: anterior ruffling is more pronounced in the RNAi-treated embryo (lower panel) than in the wild-type embryo (upper panel). This exaggerated ruffling also persists longer in the RNAi-treated embryo than in wild type. (**B**) Quantification of ruffles in embryos up to pronuclear meeting (PNM) in wild type (*n*=8) and after K09H11.3 RNAi treatment (*n*=13). The ruffles were counted from time-lapse movies of individual embryos. The average number of ruffles per embryo at a given time point is indicated. (**C**) Schematic drawing of different RhoGAP-domain-containing proteins in humans (hs), *Xenopus laevis* (Xl), *C. elegans* (Ce) and *Saccharomyces cerevisiae* (Sc). The RhoGAP domain (green) is present in most RhoGAPs in the more C-terminal part of the protein. RGA-3 and RGA-4 belong to a family of uncharacterized RhoGAP-domain proteins, which carry the GAP domain at the N-terminus of the protein. The SH2 domain, the C1 domain and the CRAL/Trio domain are depicted in red, blue and yellow, respectively. Scale bar: 20 μm.

μm; wild type: 3.24±1.57 μm). Most importantly, ruffles formed until pronuclear meeting, which was not the case in wild type (Fig. 1B). Sequence analysis revealed, however, that another gene, Y75B7AL.4, which is 81% identical to K09H11.3 on the amino acid level, was probably also reduced upon RNAi treatment, because our RNAi construct – like those used in other screens – targeted the highly conserved 5′ region of the K09H11.3 transcript (see Fig. S1 in the supplementary material). Both genes encode putative RhoGAP proteins that accelerate GTP hydrolysis on members of the Rho family, namely on RHO, CDC42 and RAC (Fig. 1C). K09H11.3 and Y75B7AL.4 were named RGA-3 and RGA-4 for RhoGAP-3 and RhoGAP-4, respectively. RGA-1 and RGA-2 are RhoGAPs expressed in epithelial cells and are the worm orthologues of mammalian RhoGAP1 (ARHGAP1) and ARHGAP20, respectively (Jenna et al., 2005; Schwarz et al., 2006). RGA-3 and RGA-4 belong to a subfamily of RhoGAPs with their GAP domain located at the N-terminus. Other members of this family comprise the hitherto uncharacterized mammalian ARHGAP11A and the amphibian MGC83907 (Fig. 1C). Both RGA-3 and RGA-4 are expressed in the germ line and in the early embryo (NEXTDB Version 4.0). Expression seems to cease for both genes around the 100- to 200-cell stage. These are not the only RhoGAPs expressed at this early stage: CYK-4 is essential for cytokinesis and polarity establishment in the P0 cell (Jantsch-Plunger et al., 2000; Jenkins et al., 2006). RNAi treatment of CYK-4 generated multinucleated cells because of cytokinesis failure (Jantsch-Plunger et al., 2000) and cortical anterior markers were distributed over the entire cortex (Jenkins et al., 2006).

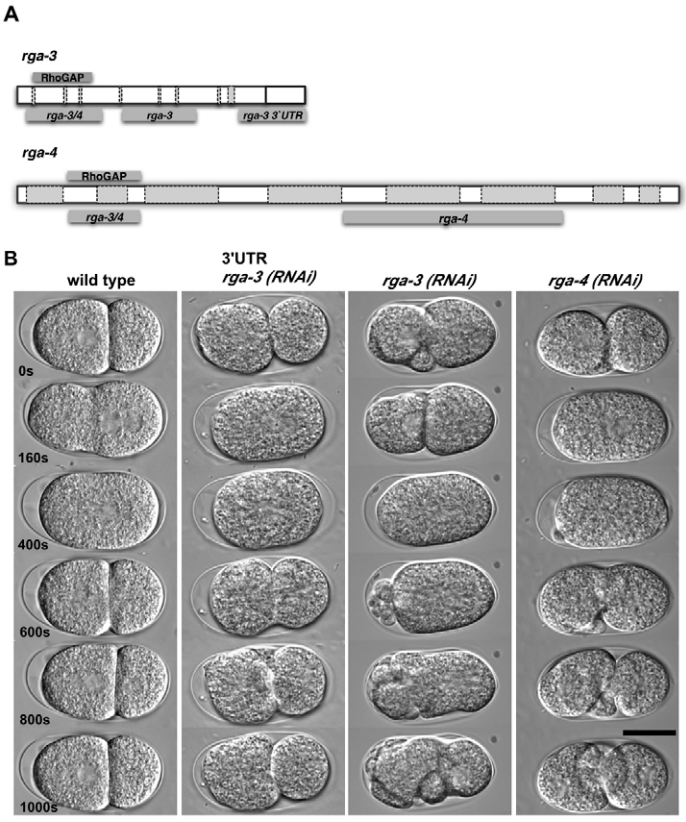

Fig. 2. Knockdown of either RGA-3 or RGA-4 leads to a hyper-contractile cortex. (**A**) Schematic drawing of the RNAi constructs used: for *rga-3*, apart from the initially cloned N-terminal sequence, constructs matching the middle part and to the 3′UTR of the gene are chosen; for *rga-4*, an RNAi targeting the middle part of the gene is also created. Exons are indicated in white stretches; introns in yellow. Introns are bigger in the Y75B7AL.4 gene (*rga-4*) compared to K09H11.3 (*rga-3*). (**B**) Pictures of Nomarski time-lapse studies after feeding with bacteria expressing dsRNA of the different RNAi constructs. For all constructs, a membrane-ruffling phenotype was observed. s, seconds. Scale bar: 20 μm.

RGA-3 and RGA-4 have overlapping functions in the early *C. elegans* embryo

To decipher the contribution of RGA-3 and RGA-4 to the membrane-ruffling phenotype, we constructed different RNAi probes specifically targeting either RGA-3 or RGA-4 (Fig. 2A). Knockdown of the individual genes caused, in both cases, cortical hyper contractility, albeit with a lower penetrance (Table 2, Fig. 2B). Thus, RGA-3 and RGA-4 have overlapping functions and, given the phenotype of the RNAi-treated worms, both RhoGAPs are involved in inactivation of a Rho family GTPase, most probably at the plasma membrane. The contraction phenotypes were additive and the loss of both GAPs resulted in embryonic lethality, indicating that these two proteins are at least partially redundant. By contrast, another early-expressed RhoGAP, CYK-4, either plays less of a role or is not involved in the same process, because no anterior ectopic furrowing or invaginations were observed when this GAP was knocked down, although cytokinesis failures were detected (Tables 2 and 4). Given the redundant function of RGA-3 and RGA-4, the N-terminal construct, which targets both proteins and resulted in the highest penetrance, was therefore used for all subsequent experiments.

Loss of RHO-1 function rescues the ruffling phenotype of *rga-3/4(RNAi)*

Which Rho GTPase is regulated by RGA-3/4? Lack of RGA-3/4 GAP activity leads to sustained activation of its target GTP-binding protein. In the early embryo, two GTPases of the Rho family show severe phenotypes when knocked down by RNAi: RHO-1 (the orthologue of mammalian RhoA) and CDC-42. Both GTPases have been reported to control actin dynamics in numerous different cell types and organisms (Narumiya and Yasuda, 2006; Raftopoulou and Hall, 2004). In the early *C. elegans* embryo, however, they seem to also be essential for the establishment and maintenance of polarity (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006; Gotta et al., 2001; Kay and Hunter, 2001). Upon knockdown of the specific GTPase regulated by the RhoGAPs, the hyper-contractility phenotype should be rescued, because the GTPase would no longer be present to hyper-activate the acto-myosin network, which performs the contractions in the early embryo. By contrast, if the GTPase were not involved in the regulation of the acto-myosin system at the anterior cortex, loss of this specific GTPase activity would have no impact on the ectopic furrowing and membrane ruffling. Whereas RNAi of RHO-1 rescued the extensive-ruffling phenotype caused by knockdown of RGA-3/4, reducing the levels of CDC-42 had no effects on the extent of invaginations and contractions (Fig. 3A,B, Movies 3-6, Table 3). CDC-42 levels were reduced in the triple-RNAi experiment, because polarity defects were observed as described for *cdc42(RNAi)* (Gotta et al., 2001; Kay and Hunter, 2001). More importantly, no cortical contractions were observed at all upon *rho-1(RNAi)*, indicating that RHO-1 is responsible for the regulation of the acto-myosin network at the anterior cortex. RHO-1 has been implicated in the control of all contractions at the cortex (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). Our results strongly indicate that RGA-3/4 act as RhoGAPs on RHO-1 and that they control contractility at the anterior cortex.

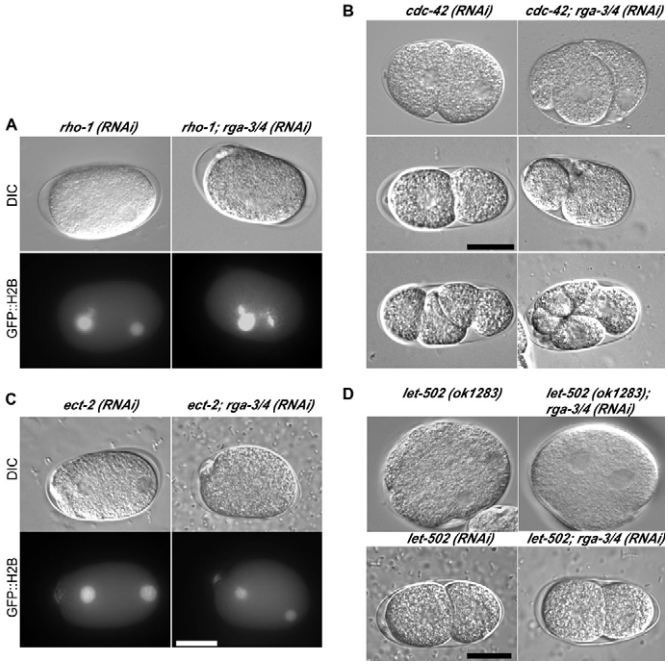

Fig. 3. RGA-3 and RGA-4 are RhoGAPs for RHO-1 and not for CDC-42. (**A-D**) Epistasis RNAi experiments of *rho-1* (A), *cdc-42* (B), *ect-2* (C) or *let-502* (D) with or without *rga-3* and *rga-4* (*rga-3/4*) were performed by feeding with the RNAi construct in question either alone, or by mixing together the same amounts of bacteria containing the *rga-3/4* construct or RNAi construct being studied and feeding these at the same time. In the case of LET-502, epistasis experiments were also performed with the *let-502(ok1283)* allele, which is a knockout of LET-502. (A) Concomitant knockdown of RGA-3/4 and RHO-1 led to a catastrophic one-cell arrest, similar *to rho-1* RNAi treatment [*rho-1(RNAi)*]. (B) *cdc-42(RNAi)* did not rescue the *rga-3/4(RNAi)* phenotype. The knockdown of the RhoGEF ECT-2 (C) and the Rho-associated kinase LET-502 (D) rescued the *rga-3/4(RNAi)* phenotype. Nuclei were visualized with GFP::H2B. Scale bars: 20 μm.

To provide further evidence in support of this hypothesis, we simultaneously knocked down the GEF for RHO-1, ECT-2, with RGA-3/4 (Fig. 3C, Table 3). Again, the membrane-ruffling phenotype was rescued and contractility was greatly reduced even when compared to wild type. Without its GEF, RHO-1 cannot be activated and thus the concomitant loss of the GAPs has no impact on the phenotype. Finally, we knocked down the RHO-1-associated kinase (ROCK), which, in *C. elegans*, is encoded by *let-502* and relays the signal of RHO-1 to the acto-myosin network (Piekny and Mains, 2002). As predicted, co-knockdown of ROCK and RGA-3/4 rescued the cortical hyper-contractility caused by the loss of RGA-3/4 function. Furthermore, knockdown of RGA-3/4 in *let-502*$^{-/-}$ animals gave a similar result (Fig. 3D, and see Movies 7 and 8 in the supplementary material).

GFP::RHO-1 is enriched at the plasma membrane in *rga-3/4(RNAi)* embryos

To determine the effect of silencing GAP expression on the subcellular distribution of the Rho GTPase, we localized GFP::RHO-1 in wild-type and *rga-3/4(RNAi)* embryos. In wild type, RHO-1 was mostly cytoplasmic but was also visible at the cortex. Upon loss of RGA-3/4, GFP::RHO-1 was enriched at the plasma membrane, more precisely in the ruffles and furrows (Fig. 4). We determined the ratio of the concentration of RHO-1 at a given length at the contractile and at the non-contractile cortex in wild-type and *rga-3/4(RNAi)* embryos. The ratio in the RNAi-treated embryos was higher (1.55±0.21; n=11) compared with wild type (1.34±0.12; n=9; P<0.05). Given the increased number and persistence of ruffles in the knockdown, this ratio even underscores the enrichment of GFP::RHO-1 at the plasma membrane in *rga-3/4(RNAi)* zygotes. This result is exactly what one would expect for a GTPase that can no longer be deactivated and cannot stop signalling downstream. Therefore, the enrichment of GFP::RHO-1 in the ectopic cleavage furrows at the plasma membrane substantiates our finding that RGA-3/4 act on RHO-1. Taken together, these experiments strongly indicate that RGA-3/4 are novel RhoGAPs for RHO-1 during early embryonic development and that they regulate contractility at the anterior cortex.

Table 3. Quantification of phenotypes of the epistasis experiments

	Cytokinesis (*rho-1*, *ect-2* and *nmy-2*) or polarity (*cdc-42*) defective (%)	Exaggerated ruffling (%)
rho-1(RNAi)	66 (n=39)	nd
rho-1;rga-3/4(RNAi)	66 (n=39)	12.0 (n=39)
ect-2(RNAi)	80 (n=30)	nd
ect-2;rga-3/4(RNAi)	48 (n=45)	8.0 (n=45)
cdc-42(RNAi)	32 (n=31)	nd
cdc-42;rga-3/4(RNAi)	7 (n=27)	59.0 (n=27)
nmy-2(RNAi)	54 (n=31)	nd
nmy-2;rga-3/4(RNAi)	41 (n=46)	8.8 (n=46)

The phenotype in RNAi experiments was determined by analyzing F1 embryos derived from mothers subjected to RNAi. Cytokinesis failure was used as a read-out for *rho-1*, *ect-2* and *nmy-2* RNA treatment (*RNAi*) phenotypes, whereas polarity defects (as judged by parallel cleavages of AB and P1, see Fig. 3C) was counted as a read-out for the *cdc-42(RNAi)* phenotype. Exaggerated ruffling was judged as shown in Fig. 2. RNAi was performed either by injection or feeding. n is the number of embryos per condition analyzed. nd, not determined.

RGA-3/4 function is not required for proper cytokinesis

RHO-1 and CDC-42 are both involved in cytokinesis. Therefore, by co-staining early embryos with the lipophilic dye FM4-64 and GFP::H2B, we investigated whether *rga-3/4(RNAi)* causes cytokinesis defects. FM4-64 served as a marker for the plasma membrane in these experiments. *rga-3/4(RNAi)* resulted in a moderate cytokinesis defect (Table 4 and see Fig. S2 in the supplementary material). Upon feeding, approximately 20% of the *rga-3/4(RNAi)* embryos showed multinucleated and anucleated cells. We observed cytokinesis defects, which were most probably a result of extra furrowing, that led to small anucleate cells (see Fig. S2 in the supplementary material). However, injection of RGA-3/4 dsRNA yielded in a higher level of over-pronounced cleavages and less cellularization defects (Table 4). Therefore, we concluded that failure in cytokinesis upon *rga-3/4(RNAi)* is most likely a secondary effect and that RGA-3/4 play no major role in cytokinesis.

The non-muscle myosin NMY-2 is enriched at the anterior cortex after *rga-3/4(RNAi)*

The extensive contractility and ruffling caused by loss of RGA-3/4 function implied a role for actin and the non-muscle myosin NMY-2 (Guo and Kemphues, 1996) in this process. To test whether NMY-2 acts downstream of RGA-3/4-dependent RHO-1, we first knocked down both NMY-2 and RGA-3/4 (Fig. 5A). The ruffling phenotype of *rga-3/4(RNAi)* was rescued by the simultaneous loss of NMY-2

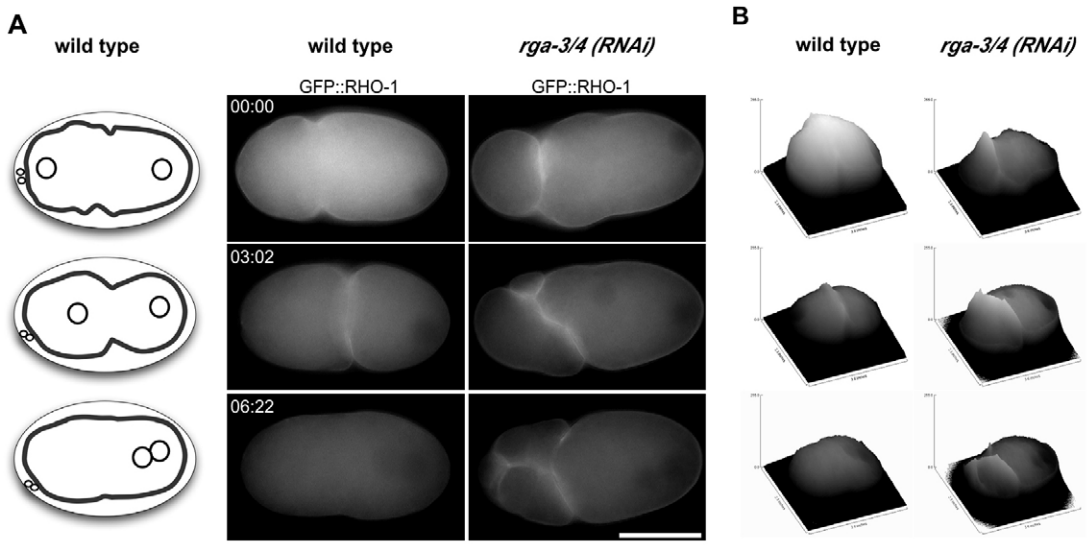

Fig. 4. GFP::RHO-1 accumulates at ingressing furrows in *rga-3/4(RNAi)* embryos. (**A**) Pictures from time lapses showing GFP::RHO-1 in wild-type or *rga-3/4(RNAi)* embryos. The different stages are depicted in the cartoon. (**B**) Visualization of GFP intensities in the respective embryos using ImageJ. Scale bar: 20 μm.

and RGA-3/4 function, confirming that indeed the contractile machinery in the early embryo is hyper-activated in the absence of the GAPs RGA-3/4. Hence, NMY-2::GFP should be enriched at the anterior cortex in *rga-3/4(RNAi)* embryos, which was what we observed: NMY-2::GFP was concentrated in invaginations and ectopic cleavages in the anterior part of *rga-3/4(RNAi)* embryos as well as in the pseudo-cleavage (Fig. 5B,C). Moreover, NMY-2::GFP covered a large extent of the anterior cortex, unlike the NMY-2::GFP speckles detected in wild-type embryos before pronuclear meeting (Fig. 5B-D). The NMY-2 patches at the cortex were at least twice as long in *rga-3/4(RNAi)* than in wild-type embryos (Fig. 5D). The concentration of NMY-2::GFP was also strongly increased in the cleavage furrow of the first cell division after RGA-3/4 knockdown (data not shown). Thus, the inability of the embryo to deactivate RHO-1 at the anterior cortex results in hyper-activation and aberrant recruitment of the acto-myosin system to the cortex.

Onset of polarity occurs normally in *rga-3/4(RNAi)* embryos

After fertilization and meiosis II of the female pronucleus, the entire cortex of the embryo is contractile. Via the cortical and cytoplasmic streaming, ruffling later becomes restricted to the anterior part of the embryo. These contractions and ruffles are thought to be important for the establishment of polarity in the early embryo. In a subset of *rga-3/4(RNAi)* embryos, extensive ruffling and furrowing was not restricted to the anterior part and seemed to extend also towards the posterior region of the embryo. Therefore, we wanted to determine whether polarity establishment occurred normally upon depletion of RGA-3/4. First, we tested whether posterior polarity was established in *rga-3/4(RNAi)* embryos in the same manner as in wild type. Despite the extensive ruffling, the polarity establishment seemed to be mostly correct and we did not detect a significant difference in the posterior cortex area upon *rga-3/4(RNAi)* compared to wild type, as judged by the localization of the posterior marker GFP::PAR-2 (Fig. 6A,C). Because the ruffling in wild type occurs at the anterior cortex, we next tested the localization of GFP::PAR-6 in *rga-3/4(RNAi)* embryos. PAR-6 normally localizes together with PAR-3 and the atypical PKC, PKC-3, to the anterior cortex (Hung and Kemphues, 1999). Again, the onset of polarity was unperturbed. However, the GFP::PAR-6 signal seemed to be restricted to the hyper-contractile region and within the cleavage furrow (Fig. 6B and see Movie 9 in the supplementary material).

Taken together, our data indicate that there is no primary defect in polarity onset and maintenance per se. However, we observed greater fluctuations in the anterior-domain size as signified by GFP::PAR-6, indicating that the stable posterior domain prevented overshooting of the anterior domain into the posterior part of the embryo (Fig. 6C). Conversely, knockdown of the other GAP, CYK-4, caused a polarity defect, because GFP::PAR-6 and NMY-2::GFP could be detected over the entire cortex and GFP::PAR-2 was mostly cytoplasmic (Jenkins et al., 2006) (Fig. 7, Table 4 and see Movie 10 in the supplementary material). The fact that knockdown of RGA-

Table 4. Quantification of phenotypes after RhoGAP RNAi

	Cytokinesis defective		
	Cellularization failure (%)	Over-pronounced cleavages (%)	Polarity defective (%)
cyk-4(RNAi)	94 (*n*=18)	0 (*n*=18)	65.0 (*n*=20)
rga-3/4(RNAi)	0 (*n*=20)	65 (*n*=20)	18.5 (*n*=27)
cyk-4;rga-3/4(RNAi)	54 (*n*=33)	42 (*n*=33)	70.1 (*n*=24)
Control	0 (*n*=18)	0 (*n*=18)	0.0 (*n*=18)

The phenotype was determined by analyzing F1 embryos derived from mothers subjected to RNAi. Control represents wild-type embryos from mothers fed with OP50 bacteria. Cytokinesis failure was classified into either furrow-regressing failure ('cellularization failure') and therefore arrest as multinucleated embryos or 'over-pronounced cleavages' as indicated by extra furrows built during first cell division (Fig. 2; 1000s). The polarity phenotype was scored by the localization of PAR-2 and PAR-6 after the first mitosis of the embryo. *n* is the number of analyzed embryos. RNAi was performed by injection or soaking.

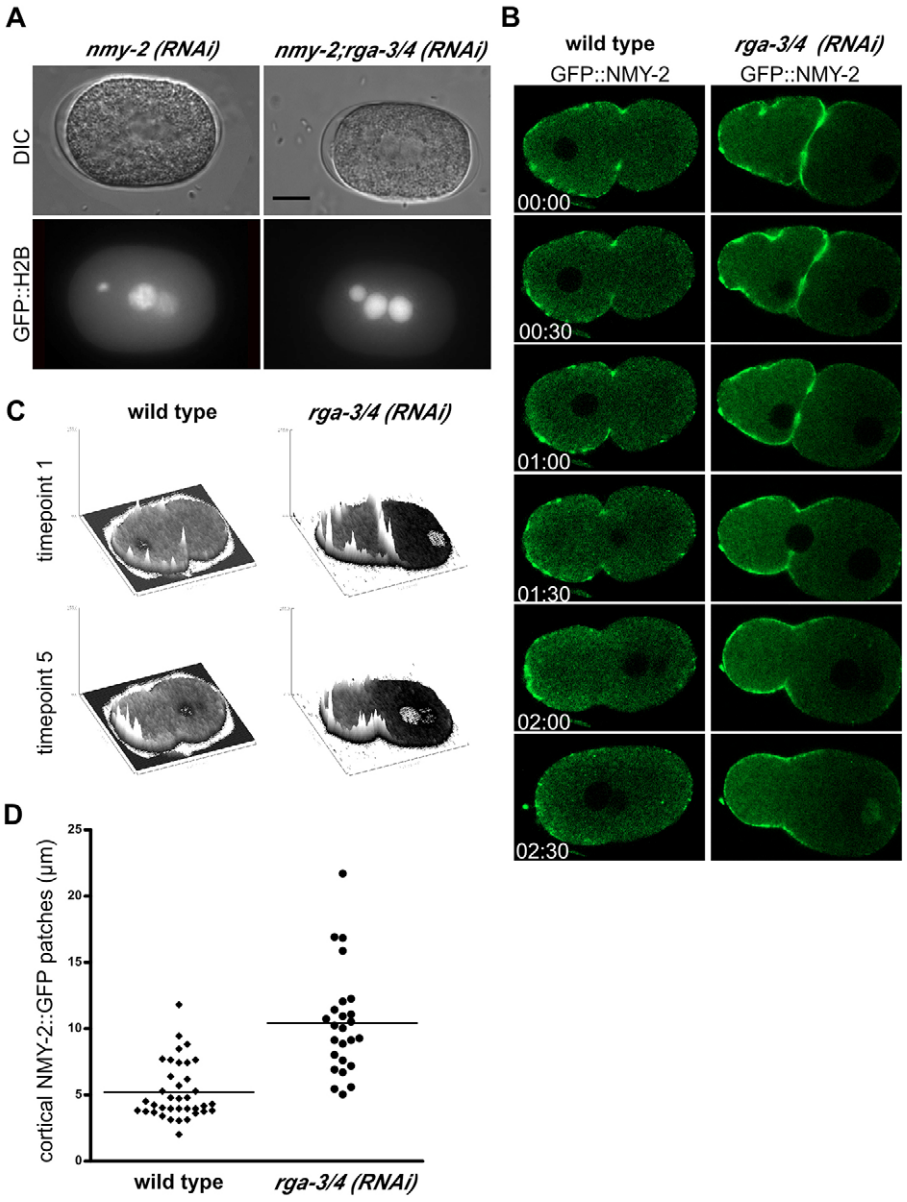

Fig. 5. Hyper-contractility in *rga-3/4(RNAi)* embryos is dependent on NMY-2, and NMY-2::GFP is enriched at cortical membrane ruffles in *rga-3/4(RNAi)* embryos. (**A**) *nmy-2(RNAi)* rescues the membrane-ruffling defect of *rga-3/4(RNAi)*. All three proteins were knocked down at the same time by feeding. (**B**) Time-lapse images of NMY-2::GFP behaviour in wild-type and *rga-3/4(RNAi)* embryos. NMY-2::GFP is strongly enriched in the pseudo-cleavage furrow and at the anterior cortex after *rga-3/4(RNAi)*. (**C**) Quantification of the fluorescence intensity of NMY-2::GFP using ImageJ. (**D**) Quantification of the length of the NMY-2::GFP domains at the plasma membrane in wild-type (n=7) and *rga-3/4(RNAi)* (n=9) zygotes. Scale bar: 20 µm.

3/4 did not cause any major polarity defects suggests that the GAPs fulfil different functions in the early embryo. If this were the case, the phenotypes caused by the loss of the three GAPs should lead to extended membrane ruffling and to a polarity defect. When we performed simultaneous knockdown of the three GAPs, we observed a hyper-contractile cortex [caused by the *rga-3/4(RNAi)*], and NMY-2::GFP was spread over the entire cortex and GFP::PAR-2 was cytoplasmic, indicating a loss of polarity [caused by the *cyk-4(RNAi)*] (Fig. 7). Furthermore, we detected a strong cytokinesis defect unlike the effect we observed upon *rga-3/4(RNAi)* (Table 4). Hence, CYK-4 and RGA-3/4 appear to fulfil different functions in the P0 cell.

A role for RGA-3/4 in the germ line

To gain insight into the subcellular localization of RGA-3, we expressed a GFP::RGA-3 fusion in the germ line under the control of the *pie-1* promoter. The expression of GFP::RGA-3 seemed to be toxic, because it was very difficult to obtain and maintain transgenic lines. Furthermore, the transmission of the extrachromosomal array was very poor, the transgenic worms had a reduced brood size and only a few offsprings contained the transgene. Given these difficulties, it was impossible to analyze the early embryonic phenotype in a quantitative manner. However, in adult transgenic animals, we observed robust defects in gonad proliferation and migration, whereas the gonad architecture seemed to be unaffected (see Fig. S3 in the supplementary material, data not shown). Gonad arms were too long and crossed from one side of the body to the other. GFP::RGA-3 was barely detectable in the gonad, which might be due to low expression because of germline silencing of the transgene.

We inferred from our studies in the zygote that RGA-3/4 are involved in the control of the *C. elegans* ROCK, LET-502, and given the phenotype of the expression of GFP::RGA-3, we tested the effect of RGA-3/4 RNAi treatment on the temperature-sensitive (ts) *let-502(sb106)* mutant in the germ line. Although *let-502*$^{-/-}$ animals are sterile and have severe gonadal defects, such as unusually large spaces devoid of nuclei and actin (‘holes’), or nuclei fallen into the rachis, the ts mutant *let-*

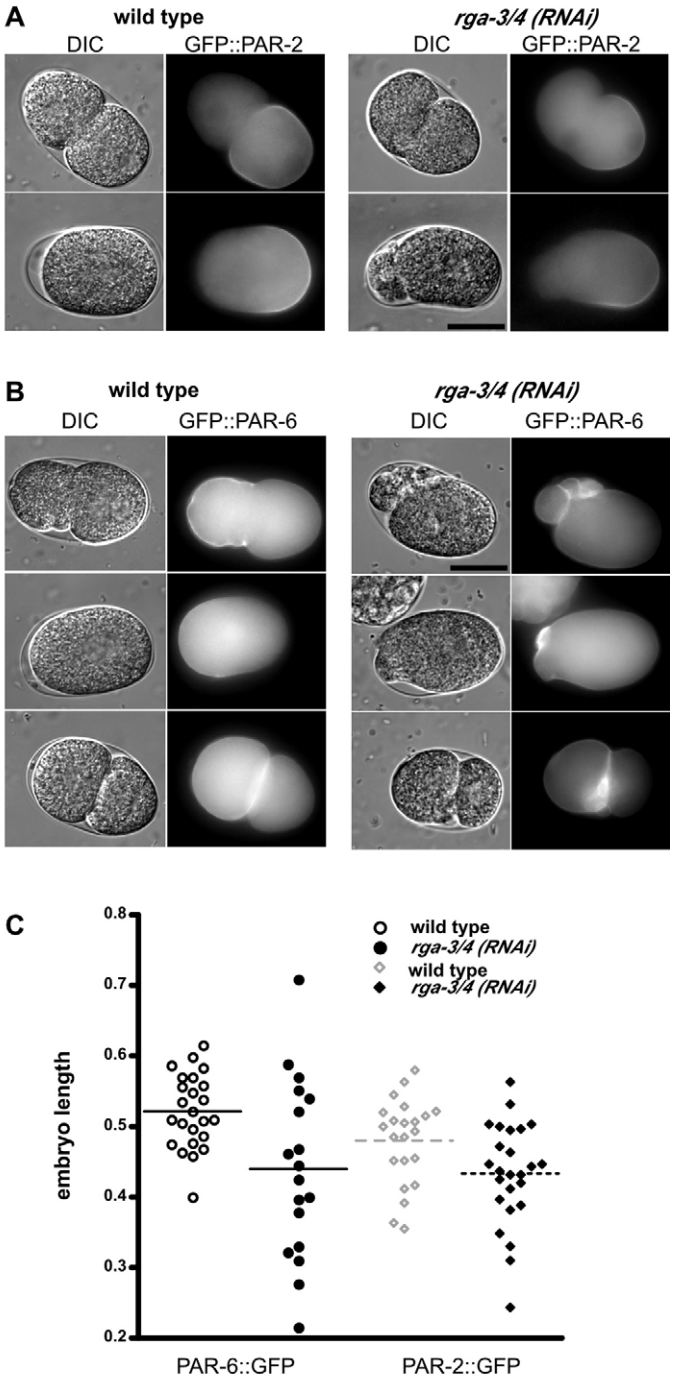

Fig. 6. Polarity establishment is not affected in *rga-3/4(RNAi)* embryos but the anterior PAR-6-domain size fluctuates more in *rga-3/4(RNAi)* embryos compared with wild type. (**A**) GFP::PAR-2 and (**B**) GFP::PAR-6 localization is determined in vivo in wild-type and in *rga-3/4(RNAi)* embryos. GFP::PAR-2 as well as GFP::PAR-6 is correctly localized in *rga-3/4(RNAi)* embryos at the posterior and anterior cortex, respectively. (**C**) The relative size of the GFP::PAR-2 (wild type: n=24 embryos; RNAi: n=18) and GFP::PAR-6 (wild type: n=22; RNAi: n=25) domains was determined by drawing a line from the anterior to the posterior end of the embryo and then measuring the size of the individual domain. The size of the GFP::PAR-6 domain fluctuated more in *rga-3/4(RNAi)* embryos, compared with wild type. The variations in the domain sizes are statistically significant ($P<0.01$). The size of the GFP::PAR-2 domain was less affected. Scale bars: 20 μm.

502(sb106) showed barely any gonadal defects at the restrictive temperature of 23°C (Fig. 8A,B). Knockdown of RGA-3/4 aggravated the *let-502(sb106)* phenotype such that the gonadal defects were very similar to those observed in *let-502*$^{-/-}$ animals; 'holes' and strong defects in distal and proximal rachis formation were observed. Conversely, *rga-3/4(RNAi)* by itself had no effect on gonad migration or brood size (Fig. 8A,C). The only noticeable phenotype was a problem in the formation of the distal rachis. A similar, mild gonad defect was observed upon *cyk-4(RNAi)*. However, no enhancement of the gonad phenotype was observed in *let-502(sb106)* mutants. Taken together, our data suggest a role for RGA-3/4 as GAPs for RHO-1 in the germ line and in the early embryo, and that these functions might be, at least in part, temporally and spatially distinct from the functions of CYK-4.

DISCUSSION

We have identified two novel RhoGAPs, RGA-3/4, that are essential to turn off RHO-1 signalling to the acto-myosin network at the anterior cortex in the *C. elegans* zygote. Furthermore, RGA-3/4 are required for proper germline formation. The two novel RhoGAPs belong to an uncharacterized subfamily. They carry the GAP domain at the N-terminus and the remainder of the proteins do not contain any known interaction or signalling motifs, which is unusual for RhoGAP proteins (Jaffe and Hall, 2005; Moon and Zheng, 2003). The two vertebrate orthologues, the mammalian ARHGAP11A and the amphibian MGC83907, have the same domain structure and also lack known motifs, and might therefore fulfil similar functions. How do these proteins interact with effector molecules or provide specificity for Rho GTPases? It seems rather likely that these GAPs contain yet unidentified interaction domains. Further analysis of this novel class of regulators is likely to reveal interesting and new roles for Rho during development.

In *C. elegans*, RGA-3/4 are expressed in the germ line and in the early embryos, probably up to the 100- to 200-cell stage [Nematode Expression Pattern DataBase (NEXTDB), Ver. 4.0]. Another RhoGAP, CYK-4, is expressed in a similar pattern. Although these GAPs are expressed in the same cells, and target the same GTPase, their biological functions are largely non-overlapping. RGA-3/4 seem to control the contractility of the acto-myosin network, whereas CYK-4 is involved in cytokinesis, and in polarity establishment and maintenance (Jenkins et al., 2006) (Fig. 7 and Table 4). By contrast, RGA-3/4 do not seem to play any primary role in meiosis, mitosis, polarity establishment or cytokinesis. The occasional failure in these processes upon *rga-3/4(RNAi)* might be due to the destruction or compromised assembly of cellular structures by the extensive furrowing and the hyper-contractility. Polarity setup and maintenance were also mostly normal. We observed, however, a significant fluctuation in the size of the GFP::PAR-6 domain, indicating that the boundaries separating the anterior and posterior domains might not be stable. RGA-3/4 clearly control contractility-related functions of RHO-1, and seem to be specific for this process. However, the bulk of CYK-4 is located at the mitotic spindle and only some paternal CYK-4 is found at cortex at the sperm entry site (Jantsch-Plunger et al., 2000; Jenkins et al., 2006).

Three RhoGAPs expressed in the germ line have been characterized so far: CYK-4, and now RGA-3/4; all of which act on RHO-1. No GAP for CDC-42 in the early *C. elegans* embryo has been identified yet. However, CYK-4 and RGA-3/4 act on RHO-1, which is upstream of CDC-42 in the zygote. Therefore, despite the result of the epistatic analysis, we cannot rule out that one or more of the three GAPs also turns off CDC-42. Another

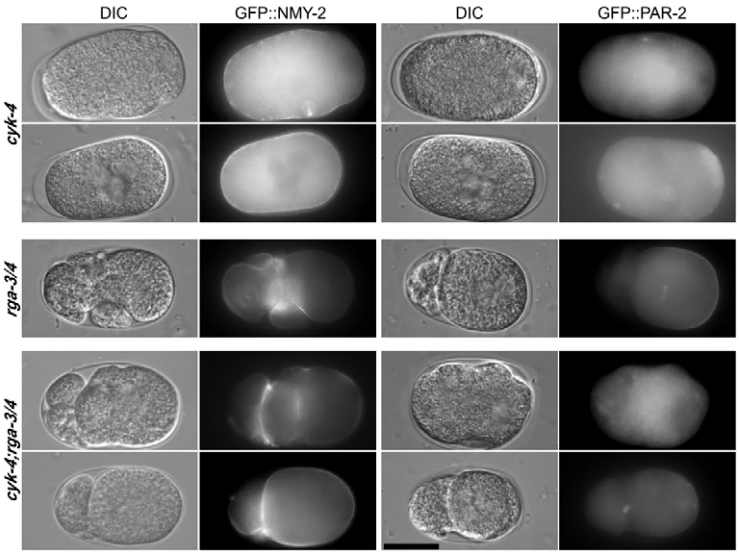

Fig. 7. CYK-4 and RGA-3/4 fulfil different functions in the early *C. elegans* embryo. To compare the functions of the three RhoGAPs – RGA-3/4 and CYK-4 – present in the early embryo, RNAi epistasis experiments were performed by injecting dsRNA into the embryos. Whereas CYK-4 was essential for the first division and to recruit GFP::PAR-2 to the posterior cortex (upper panels), RGA-3/4 played a role in downregulating contraction (middle panels). Simultaneous knockdown of all three RhoGAPs resulted in a combination of both phenotypes; embryos did not undergo cytokinesis, the cortex remained contractile and NMY-2 spread over the entire cortex (lower panels). Scale bar: 20 μm.

possibility is the presence of at least one other GAP, which is likely to be the case (NEXTDB, Ver. 4.0). But why would we need so many different RhoGAPs? Perhaps the GAPs provide the specificity for Rho activity (Moon and Zheng, 2003) and hence provide the basis for temporal and spatial control of RHO-1 activity. This concerted action by the GAPs might be extremely important because there is only one RhoGEF expressed in the *C. elegans* zygote.

The RhoGEF ECT-2 might be the first target of the polarity cue provided by the sperm (Jenkins et al., 2006; Motegi and Sugimoto, 2006). Then, ECT-2 activates RHO-1, which in turn leads to the rearrangement of the acto-myosin network. To control the contractions performed by the acto-myosin network, the RhoGAPs RGA-3/4 inactivate RHO-1. If RHO-1 cannot be inactivated at the anterior cortex, cytoplasmic and cortical streaming still occurs, and therefore polarity establishment and maintenance are normal.

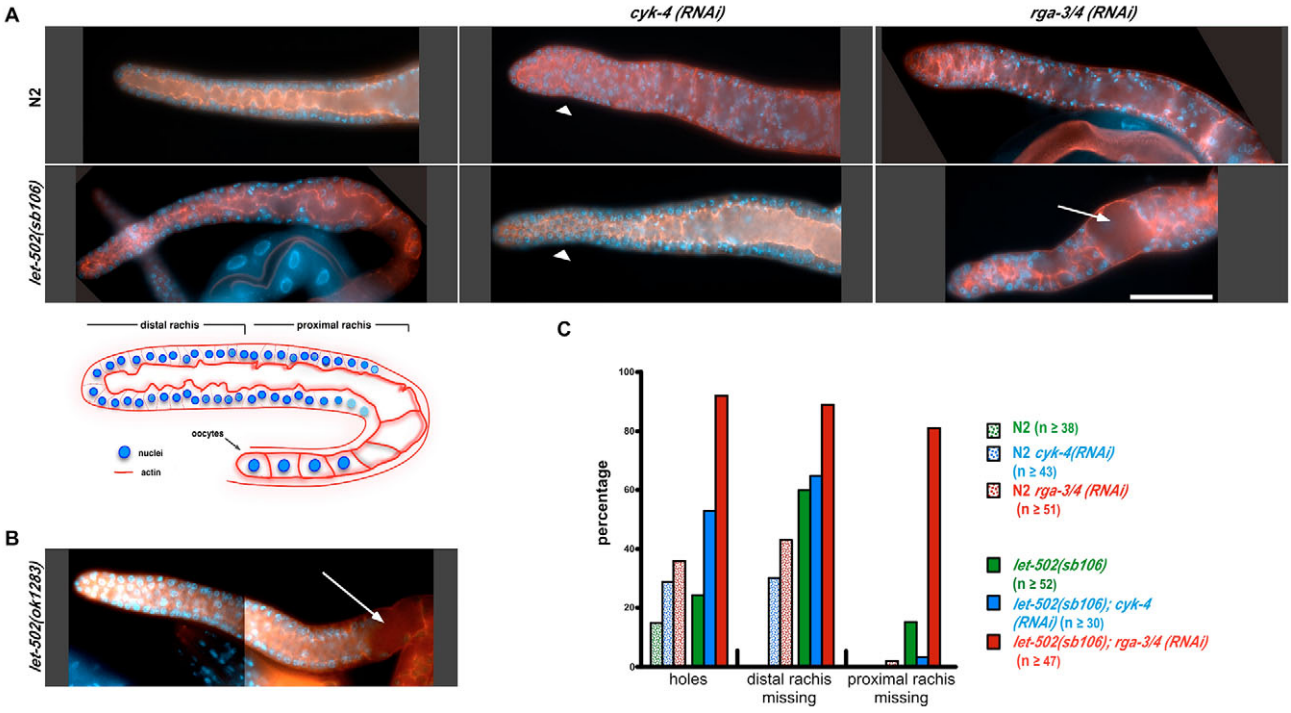

Fig. 8. *rga-3/4(RNAi)* causes strong gonad defects in *let-502(sb106)* mutants. (**A**) Gonad arms were extracted from worms and stained with rhodamin-phalloidin and DAPI. The gonads are oriented with the distal tip pointing towards the left. A schematic drawing of the gonad is included. The arrowheads point to the rachis formation defects. The arrow points to the 'hole' in the *let-502(sb106) rga-3/4(RNAi)* gonad. (**B**) A gonad from the *let-502(ok1283)* mutant, which represents a knock-out allele, was stained as in A. The arrow points to the 'hole'. (**C**) Quantification of gonad defects of N2 and *let-502(sb106)* animals treated with *rga-3/4* or *cyk-4* dsRNA. The number of gonads used for quantification of a particular phenotype is given in parenthesis. Depending on how the gonads adhered to the glass surface, not all phenotypes could be quantified in the same gonad. Ambiguous-looking gonads were not included in the analysis. Scale bar: 50 μm.

Motegi and Sugimoto suggested that CDC-42 maintains polarity by controlling the acto-myosin network in a second phase in the one-cell embryo (Motegi and Sugimoto, 2006). Our data are in agreement with this interpretation, because after centration, the entire cortex becomes smooth even upon *rga-3/4(RNAi)*, before the anterior part starts to contract again during cytokinesis. Therefore, CDC-42 still functions normally in the absence of RGA-3/4. CDC-42 might be involved in turning off RHO-1 signalling at the cortex during centration, perhaps by interacting with a RhoGAP. This GAP would be different from RGA-3 or RGA-4, because cortical ruffling stopped at centration upon loss of RGA-3/4 function. Interestingly, the original *cyk-4* mutant displayed less-pronounced furrowing, which is the opposite phenotype than that observed after *rga-3/4(RNAi)* (Jantsch-Plunger et al., 2000).

A possible scenario for the control of RHO-1 function is that first, upon fertilization, CYK-4 initiates cytoplasmic and cortical streaming, allowing the establishment of polarity. In a second step, RGA-3/4 control membrane contractility and the pseudo-cleavage at the anterior cortex by keeping RHO-1 activity at a certain threshold. Finally, CYK-4 takes over again; however, this time at the mitotic spindle, where CYK-4 controls cytokinesis. This rather simple model provides an explanation concerning the control of RHO-1 action by spatially and temporally separated RhoGAP activities.

In addition to the function of RGA-3/4 at the anterior cortex in the zygote, we found a requirement for RGA-3/4 in gonad development. Expression of GFP::RGA-3 led to over-proliferation of the gonad and to gonad migration defects. More importantly, whereas neither *rga-3/4(RNAi)* nor *let-502(sb106)* alone, or *cyk-4(RNAi) let-502(sb106)* showed severe gonad defects, *rga-3/4(RNAi) let-502(sb106)* worms contained non-functional and abnormal gonads, similar to those observed in *let-502*$^{-/-}$ animals. RGA-3/4 could either act in the same pathway or in a parallel pathway as LET-502 in the gonad. The manner in which RGA-3/4 and CYK-4 control RHO-1 activity in the early embryo and in the gonad might be different. Further components of these signalling pathways must be identified to shed light on the precise function of the RhoGAPs RGA-3/4 and CYK-4.

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Note added in proof

Stephanie Schonegg et al. have obtained similar results to those reported here (Schonegg et al., 2007).

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/19/3495/DC1

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Figure S1

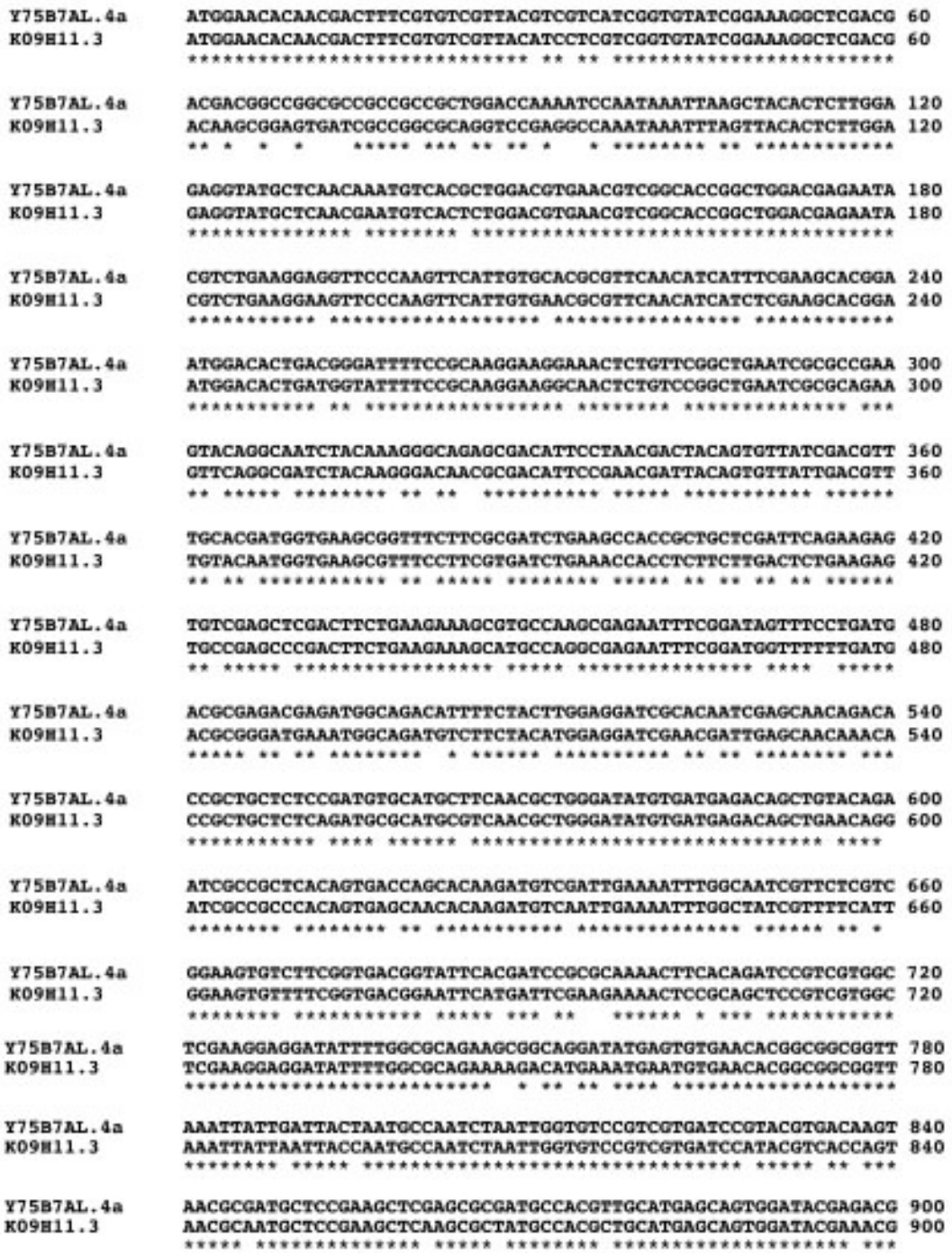

Fig. S1: Nucleotide alignment covering the first thousand bases of the spliced K09H11.3 and Y75B7AL.4 gene.
This region covers in both cases the predicted RhoGAP domain (approximately bp 141-831 in K09H11.3 sequence). Clearly visible is the high similarity between both sequences. The alignment was done by using ClustalW.

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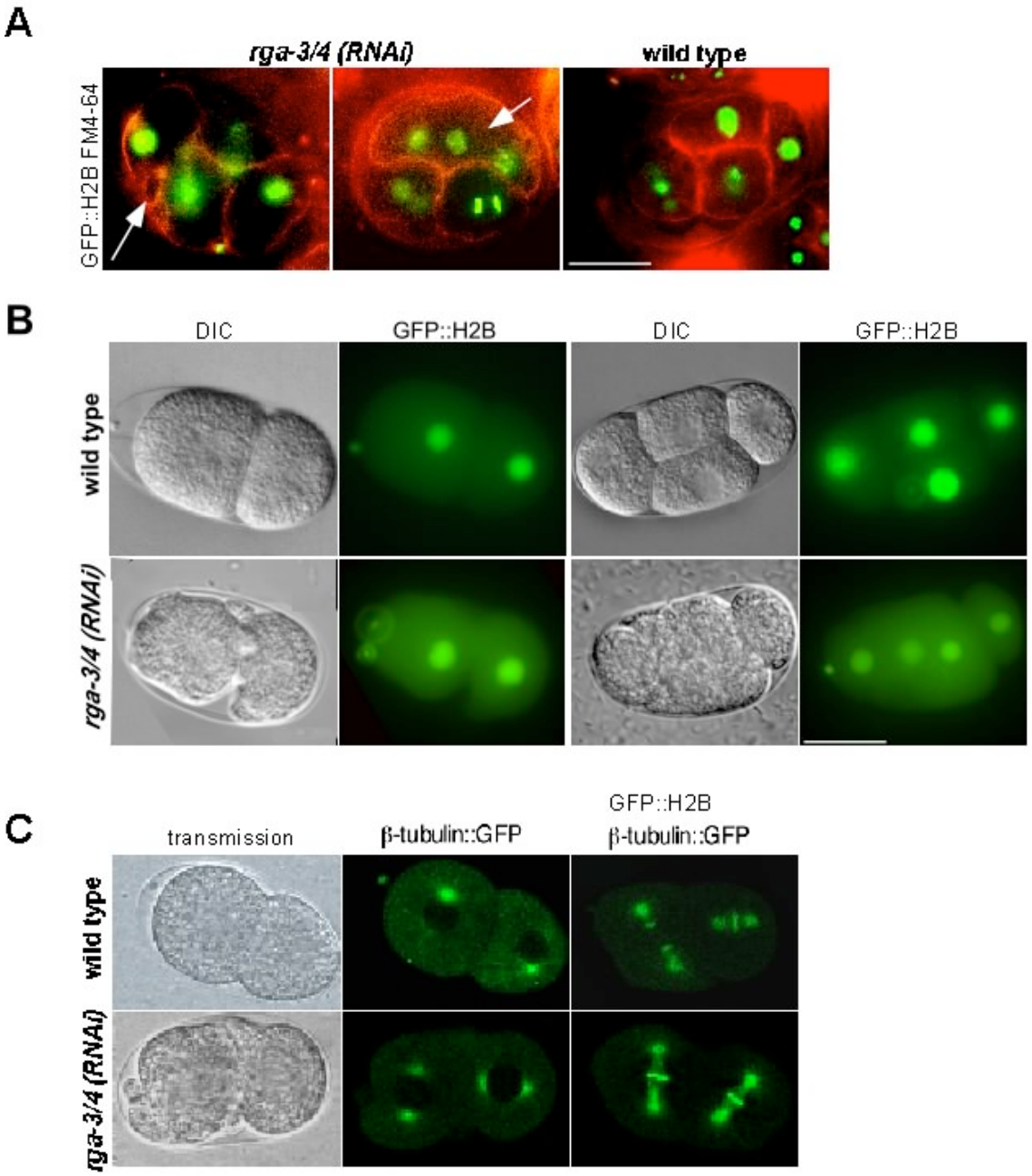
S2



Mitosis and nuclear division is fine in *rga-3/4(RNAi)* and cytokinesis is sometimes affected. (A) Wild type and *rga-3/4 (RNAi)* expressing H2B::GFP embryos were stained with FM4-64 to visualize membranes and nuclei at the same time. *rga-3/4(RNAi)* embryos showed cytokinesis defects and formation of cytoplasts (white arrows). (B) Nuclear division is not affected in *rga-3/4 (RNAi)* embryos. Chromatin is visualized by H2B::GFP. (C) The formation of the mitotic spindle is not affected in *rga-3/4 (RNAi)* embryos. The mitotic spindle was visualized using a strain expressing ≤-tubulin::GFP, although spindle rotation is sometimes affected (≤-tubulin::GFP and H2B::GFP).

Schmutz et al., Figure S3

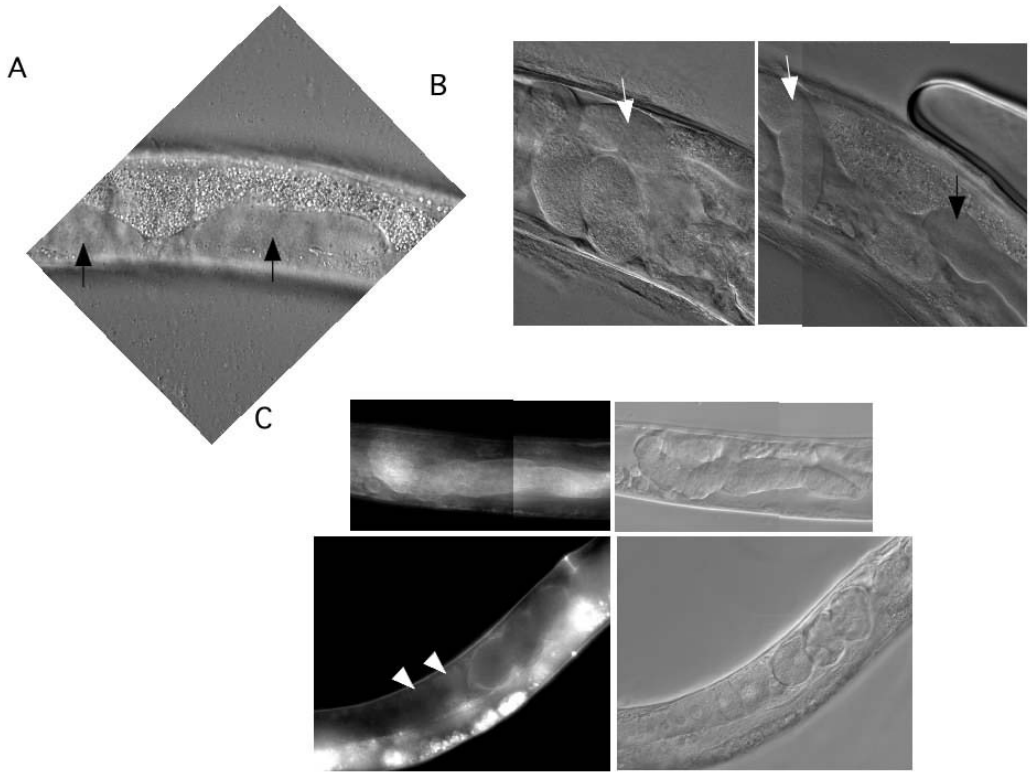


Figure S3. Analysis of GFP::RGA-3 expressing worms. **(A)** Gonad migration defect in L4 larvae. The black arrows point to the 2 different gonad arms. **(B)** Gonad migration in adults. One gonad arm overshoots and bents into the uterus. The other gonad arm has an unusual swelling in the tip area. The white arrows point to the same gonad arm in two different frames. The black arrow points to the swelling at the tip of the second gonad arm. **(C)** GFP::RGA-3 expression in the gonad and oocytes. The upper panel shows the gonad of a larva. The gonad seems crippled. The lower panel displays part of an adult worm. The strong fluorescence is derived from auto fluorescing gut granules. The arrowheads point to 2 oocytes positive for GFP::RGA-3.

II.2. Knockdown of the centrosomal component SAS-5 results in defects in nuclear morphology in *Caenorhabditis elegans*

Schmutz, C. and Spang, A. (2005). Knock-down of the centrosomal component SAS-5 results in defects in nuclear morphology in *Caenorhabditis elegans*. Eur J Cell Biology **84**, 75-82.

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SHORT COMMUNICATION

Knockdown of the centrosomal component SAS-5 results in defects in nuclear morphology in *Caenorhabditis elegans*

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Abstract

Several different processes must be completed in order to proceed through cell division. First, the centrosomes have to be duplicated and the genomic material is replicated. The separation of the chromatin is achieved by a bipolar spindle, which in turn is organized by the two centrosomes. The last step of cell division involves the separation of cellular content and the cleavage of the cell by cytokinesis. We used RNAi to study the centrosomal component SAS-5 in the early *Caenorhabditis elegans* embryo. While the first cell division and the establishment of polarity of *sas-5* dsRNA-treated embryos was indistinguishable from wild type, subsequent cleavages were abnormal. Time-lapse microscopy studies of worms expressing β-tubulin::GFP revealed that the absence of SAS-5 results in a failure of mitotic spindle assembly starting at the two-cell stage embryo. Furthermore, the chromatin in at least one of the two cells in the early embryo was dispersed. Yet, this dispersion did neither trigger apoptosis nor affect nuclear envelope assembly. No intrinsic size control for the nucleus seems to exist in the early embryo.

Keywords: *C. elegans*; Centrosome; Mitosis; RNAi; Nucleus; Cell cycle

Introduction

The proliferation of cells is tightly regulated and controlled. The events that lead to the duplication of one cell are concerted and a number of checkpoints ensure the correct order of events. First, the cell needs to replicate the DNA, so that each daughter cell inherits a full complement of the genetic information. The DNA is distributed towards two poles during mitosis via the spindle apparatus. The organelles that organize the microtubules and setup the mitotic spindle, the centrosomes, have to be duplicated exactly once per cell cycle (Palazzo et al., 2000; Sluder and Hinchcliffe, 1999). In higher eukaryotes, this happens concomitant with DNA replication during S-phase. Finally, after the genetic material is equally distributed, the cell divides also the cellular content by inserting membrane exactly perpendicular to the middle of the meanwhile mostly disassembled spindle apparatus. Thus, the spindle plays a major role in determining the division plane. The cleavage furrow ingresses and separates the two daughter cells, which then can go on to undergo another division cycle. While a DNA replication checkpoint exists in the *Caenorhabditis elegans* zygote, other checkpoint control is only observed later in development (Brauchle et al., 2003).

Centrosomes are essential to establish a bipolar spindle during mitosis. Failure in centrosome duplication leads to a monopolar spindle, while more than two centrosomes form multipolar spindles. Therefore,

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centrosome duplication has to occur once and only once during the cell cycle. Components of the centrioles and the pericentriolar matrix as well as several kinases are required for centrosome duplication. In *C. elegans*, centriolar components that are essential for this regulated process comprise SPD-2, SAS-4 and ZYG-1 (Hamill et al., 2002; Hannak et al., 2001, 2002; Kemp et al., 2004; Kirkham et al., 2003; Leidel and Gonczy, 2003; O'Connell et al., 2001; Pelletier et al., 2004; Strome et al., 2001). SPD-2 and SAS-4 are coiled-coil proteins, which localize to the centriole throughout the cell cycle, whereas the Ser/Thr kinase ZYG-1 is present at the centrioles only during mitosis. Targets of this kinase are not known.

We are interested in the mitotic cycle in the *C. elegans* embryo. We have characterized a new protein, F35B12.5, involved in centrosome duplication using RNAi and in vivo imaging techniques. During the process of our studies, Delattre et al. (2004) reported mutants in F35B12.5 and named the gene *sas-5* (*s*pindle *as*sembly defective-*5*). We will therefore refer to F35B12.5 as *sas-5*. Delattre et al. (2004) and we identified a failure of the two-cell stage embryo to develop normally due to a mitotic spindle assembly defect. SAS-5 is an essential component of the centrosome and localizes to both the centrioles as well as the pericentriolar matrix. Depletion of SAS-5 led to the inability to duplicate centrosomes and therefore to establish a bipolar spindle during mitosis (Delattre et al., 2004) (our unpublished results). Here, we investigated the nuclear morphology upon knockdown of *sas-5*. While the equal mitotic DNA distribution failed and cytokinesis could not be completed, nuclear division continued. Non-separated DNA was often dispersed, but this dispersion of DNA in the attempt to complete mitotic cycles did not trigger apoptosis and did not affect nuclear envelope formation. Nuclear division was aberrant because giant nuclei were observed in at least one part of the embryo, which seemed to collapse into smaller nuclei.

Materials and methods

General methods and strains

C. elegans was cultured and maintained as described before on NGM medium at 20 °C unless otherwise noted (Brenner, 1974). The *C. elegans* Bristol strain N2 was used for immunofluorescence and Nomarski phenotype time-lapse studies. Strain AZ212, which is homozygous for a H2B::GFP histone fusion in the germ line (Praitis et al., 2001), and WH204, a strain expressing *β*-tubulin::GFP (Strome et al., 2001), were obtained from the *Caenorhabditis* Genetics Center. XA3507 expressing a MAN-1::GFP fusion was obtained from Iain Mattaj (Galy et al., 2003).

Cloning of F35B12.5

Standard molecular biology protocols were used. Primers used throughout this study were based on sequences determined by The *C. elegans* Sequencing Consortium (1998). RNA from mixed developmental stages was prepared for RT-polymerase chain reaction (PCR) reactions using oligo dT_{16-18} primers (Invitrogen). The coding sequence of F35B12.5 was generated by amplification from cDNA using the PCR and primers: F35B12.5 forward: 5′-ATGAATAATTACGACGACTTACCC-3′ and F35B12.5 reverse 5′-TTGATATCATTTCCTGCGAGC-3′. The PCR product was cloned into the TOPO PCR 2.1 plasmid (Invitrogen) according to the manufacturer's recommendations. The sequence was confirmed by sequencing. The plasmid for expression of dsRNA of F35B12.5 in *E. coli* was constructed by subcloning the corresponding cDNA sequence from the TOPO PCR 2.1 plasmid into the SpeI and XhoI sites of vector L4440 (Timmons et al., 2001).

RNAi experiments

For RNAi experiments using feeding as the method of delivering dsRNA, plasmid L4440 containing the full-length coding sequence of F35B12.5 was transformed into *E. coli* strain HT115 (Timmons et al., 2001). NGM plates containing 1 mM IPTG and 25 μg/ml carbenicillin were inoculated with transformed HT115 bacteria. The expression of dsRNA was induced for 3.5 h at room temperature. The plates were subsequently inoculated with L1 or L2 larvae. Animals were cultured at 15 or 20 °C and their progeny were analyzed. For RNAi experiments using injection as the method of delivering dsRNA, the TOPO PCR 2.1 plasmid containing the F35B12.5 coding sequence was used for PCR amplification with the following primers: F35B12.5-RNAi-f 5′-TAATACGACTCACTATAGGGGCATTAAGCGGGAATGAATAATTACGAC-3′, F35B12.5-RNAi-r 5′-TAATACGACTCACTATAGGGTTGCAGTTCTGTTTTCATAGCGTCCCGC-3′, F35B12.5-RNAi-(long)-f 5′-TAATACGACTCACTATAGGGCAGTTAACGTTTCCCAAACTGT-3′, and F35B12.5-RNAi-(long)-r 5′-TAATACGACTCACTATAGGGCTGATGAATCTTCCACGTAGTATCC-3′ were used. PCR products were used as templates for in vitro transcription using the T7 polymerase (Promega). DsRNA was produced according to the manufacturer's recommendation (Promega). The RNA was purified by phenol/chloroform extraction and resuspended in 20 μl DEPC-H_2O. Double-stranded RNA was injected into the gonad of young

adult worms, which were subsequently incubated at 20 °C. The progeny of injected animals was analyzed.

Live embryo imaging

Embryos were mounted in a drop of M9 buffer using the hanging drop method. Observation and recording of epifluorescence and transmitted light was done either with a Leica confocal microscope TCS SP2 with an HCX PL APO 63 × /1.32–0.6 oil objective. Images were captured using integrated Leica software (Leica Microsystems, Wetzlar, Germany) and processed with Adobe Photoshop 7.0. Alternatively, the recording was performed with a Zeiss Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany), with a Plan Apochromat 63 × / 1.4 oil objective equipped with a Zeiss AxioCam MRm camera. Zeiss Axiovision 3.1 software was used to control hardware and acquire and process images. With both systems, laser intensities and UV light were adjusted so that no effect on development was observed during the recording. Depending on the system and on the GFP strain being analyzed, images were collected at 60 or 30-s intervals for a total time period of 15–35 min.

DAPI staining

Worms from OP50 seeded plates or RNAi plates were dissected directly on poly L-lysine-coated glass slides. The eggshell was opened by freeze cracking and the embryos were fixed in methanol for 20 min at −20 °C and 7 min in 100% acetone (−20 °C). The embryos were incubated with 1 μg/ml DAPI for 20 min in a humid chamber in the dark. After staining of the DNA, a drop of Citifluor (Agar Scientific) was added, covered with a coverslip and sealed. Images were acquired on a Zeiss Axioplan 2 microscope as described above.

FM 4-64 staining

Eggs from RNAi-treated or untreated AZ212 worms were used to visualize nuclei and membranes of the endocytic pathway at the same time. To visualize the membranes of an embryo with the lipophilic dye FM 4–64 (Molecular Probes), the embryos were mounted onto a slide in 10 μl M9 buffer containing FM 4–64 (1:200 dilution). The eggshell was cracked by carefully squeezing a coverslip on top of the eggs in order to allow the penetration of FM4–64. The staining was recorded with a Zeiss Axioplan 2 microscope as mentioned above.

Results

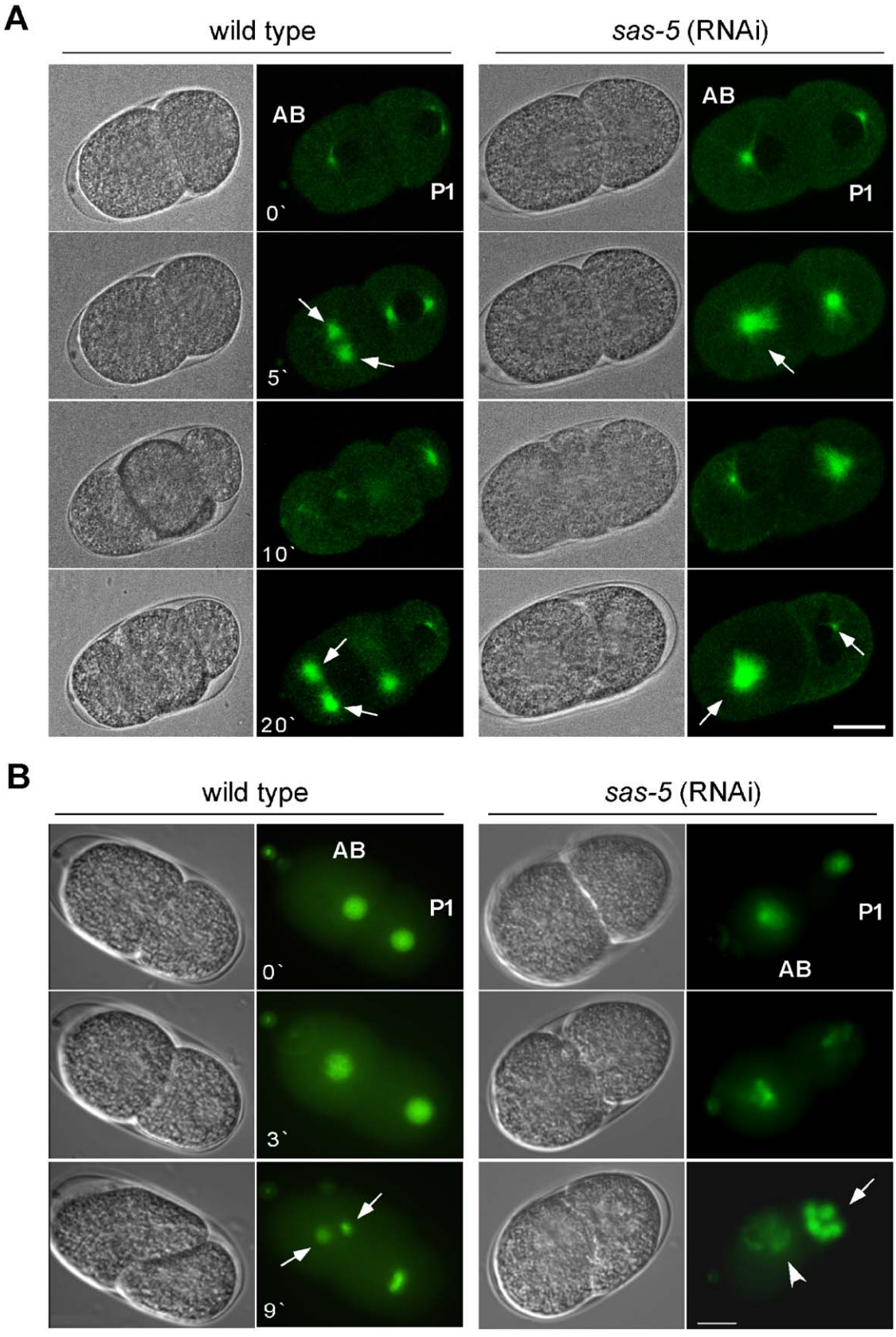
sas-5 (RNAi) embryos show defects in bipolar spindle formation

To study the defect upon knockdown of SAS-5 by RNAi, we used transgenic GFP lines to visualize different cellular processes. To this end, we knocked down SAS-5 in worms expressing β-tubulin::GFP. As observed before, the first cell division occurred normally and the mitotic spindle formed correctly in the RNAi-treated embryos (Delattre et al., 2004) (Fig. 1A and data not shown). However, at the onset of mitosis in the AB cell, no bipolar spindle was formed (Fig. 1A 5′). This led to a failure in DNA separation. The AB cell tried to divide its cellular content, but here again cytokinesis was aborted (Fig. 1A 10′). Similarly, when P1 entered mitosis, the centrosome failed to duplicate (Fig. 1A 10′). Consequently, we observed nuclear division but no cytokinesis (Fig. 1A 20′). The centrosomes in the AB and the P1 cell formed mono-polar spindles. Both centrosomes retained the ability to nucleate and stabilize microtubules. If there were differences at all, they appeared to nucleate more microtubules (Fig. 1A 20′, compare wild type to *sas-5* (RNAi)). These results confirmed a defect in mitosis after SAS-5 depletion and the inability to duplicate the centrosome properly after the first cell division (Delattre et al., 2004) (our unpublished results).

Failure in centrosome duplication leads to a defect in nuclear separation

In order to investigate the defects following the mitosis-failure further, we performed time-lapse epifluorescence microscopy in *C. elegans* embryos expressing histone::GFP (H2B::GFP). Upon knockdown of SAS-5 by RNAi, the distribution of histone in the first mitotic cell cycle of the embryo occurred like in wild type, which is consistent with the centrosome duplication defect (Delattre et al., 2004) (our unpublished results). However, the subsequent mitosis and the separation of nuclei did not proceed properly. In both the AB cell and the P1 cell, the histone distribution was abnormal (Fig. 1B). The DNA seemed to be dispersed (Fig. 1B, arrowhead). Alternatively, the DNA replication might have continued after the first round of replication. The nuclei in *sas-5* (RNAi)-treated embryos appeared to be much bigger compared to wild-type embryos (Fig. 1B 9′, arrows). However, these bigger nuclei seemed to collapse into smaller nuclei later.

To establish a link between mitosis and cytokinesis, we investigated this phenotype further by performing FM4–64 staining in early *C. elegans* embryos expressing histone::GFP, which allows us to follow chromatin.


A
wild type
sas-5 (RNAi)
AB
P1
0`
5`
10`
20`
B
wild type
sas-5 (RNAi)
AB
P1
0`
3`
9`

FM4–64 is a lipophilic dye that marks the plasma membrane and the endocytic pathway to the lysosomes. Because in the early embryo, the endosomes and lysosomes are very small compared to the size of the cell, the plasma membrane is predominantly stained while the endocytic pathway appears as a haze. In wild-type embryos, all cells were of similar size, and each cell contained one single nucleus as monitored by H2B::GFP (Fig. 2A). In contrast, in RNAi-treated embryos the cells had various sizes (Fig. 2A). These data confirmed a partial defect in cytokinesis. Interestingly, we also noted the presence of both multinucleated and anucleated cells (Fig. 2A). Thus in some cases cytokinesis must occur in the absence of a successful mitosis. Therefore, RNAi of *sas-5* affects both cytokinesis and mitosis. However, because mitosis precedes cytokinesis, the failure in cellularization is a secondary effect of depleting SAS-5.

The integrity of the nuclear envelope is not affected by the nuclear defects in *sas-5* (RNAi)

We were wondering whether the nuclear envelope could form around this dispersed DNA or whether the nuclear envelope assembles during the formation of the smaller nuclei. To distinguish between these two scenarios, we depleted SAS-5 in worms expressing MAN1::GFP, which is a marker for the nuclear envelope (Galy et al., 2003). The nuclear envelope surrounded the DNA in wild-type as well as in *sas-5* (RNAi) embryos (Fig. 2B). Thus, nuclear envelope assembly is not affected upon RNAi treatment of SAS-5. The larger nuclei that formed in the RNAi-treated embryos were not round but had indentations that grew larger and finally gave rise to multiple smaller nuclei (Fig. 2B). The resulting nuclei were of various size, indicating that they might form around different numbers of chromosomes. In subsequent division cycles, the nuclear envelope dispersed before entry into the next attempt of mitosis and reformed after the failure to segregate DNA properly. Thus, the giant nuclei appear to be a transition state and an intrinsic size restriction or size control does not seem to exist for nuclei in *C. elegans*.

In order to rule out that knockdown of SAS-5 might interfere with nucleosome assembly and this would be the reason for the dispersed histone::GFP signal, we stained embryos expressing histone::GFP with DAPI after treatment with *sas-5* (RNAi) (Fig. 2B). Both signals overlapped perfectly. Therefore, it was indeed the DNA, which was dispersed during *sas-5* RNAi treatment.

Given the dispersed DNA and the abnormal nuclear morphology in the embryo treated with *sas-5* dsRNA, we were curious, whether this phenotype involved apoptosis. This was also indicated because we observed membrane blebbing in RNAi-treated embryos, which is a hallmark of apoptosis. Although apoptosis is well defined in the germ line, in late embryonic as well as in larval development, no apoptosis has been reported in the early *C. elegans* embryo (Sulston et al., 1983). We treated different cell-death mutants (*ced-4* (*n1416*) *egl-1* (*n986*); *egl-1* (*n1084*, *n3082*); *ced-3* (*n1286*); *ced-4* (*n1894*); *ced-3* (*n1165*) *egl-1* (*n487*)) with *sas-5* dsRNA. CED-3 is an aspartate-specific cysteine protease involved in cell death and can be activated by CED-4 and EGL-1 (del Peso et al., 1998; Wu et al., 1997). However, no alleviation of the dispersion of the DNA in either the AB or the P1 cell was detected. Furthermore, we stained wild-type embryos and *sas-5* (RNAi) embryos with acridine orange, a marker for apoptosis. In early embryos no staining was observed, while in further developed wild-type embryos the dying cells were clearly detectable (data not shown). Thus, the failure to properly organize the DNA upon depletion of SAS-5 does not induce apoptosis. This result is consistent with the ability of the embryos to correctly assemble the nuclear envelope.

Discussion

Using a systematic approach to elucidate the function of SAS-5 in cell division, we found that inactivation of SAS-5 by RNAi results in a failure of centrosome duplication at the two-cell stage. This phenotype is indistinguishable from that observed in embryos deprived of ZYG-1 or SAS-4 (Kirkham et al., 2003; Leidel and Gonczy, 2003; O'Connell et al., 2001). While this work was in progress, Delattre et al. (2004) reported that SAS-5 is essential for centrosome duplication. Therefore, we focused in this report on the strange nuclear

Fig. 1. Nuclear morphology is disturbed in *sas-5* dsRNA-treated embryos. (A) Time-lapse confocal microscopy of embryos expressing β-tubulin::GFP. Untreated and SAS-5-depleted embryos are shown. No bipolar spindle was formed after RNAi treatment. The centrosome maintained the capacity to nucleate and stabilize microtubules. The white arrows point to individual centrosomes/MTOCs. (B) Epifluorescence time-lapse series of embryos expressing histone::GFP. The embryos are oriented that the anterior is to the left and the posterior to the right. The white arrows at 9′ point to the DNA in the AB cell during mitosis while at the same time point in the RNAi-treated embryo multiple nuclei are observed in P1 (white arrow) and dispersed DNA in the AB cell (white arrowhead). The time points in the panels are referenced to the first picture of the time lapse shown (0′). Bars: 10 μm.

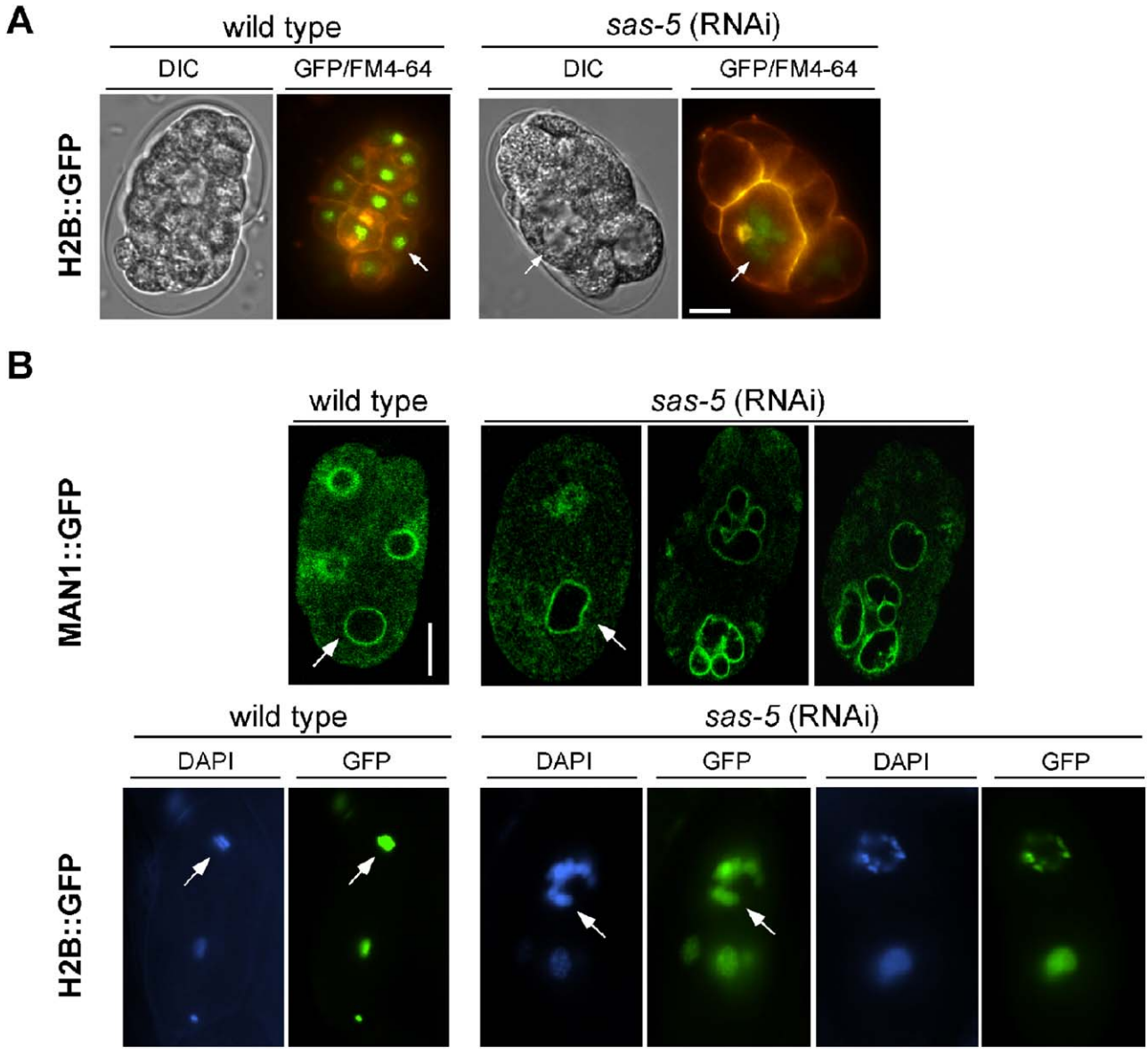


Fig. 2. Large nuclei collapse into smaller nuclei in *sas-5* RNAi-treated embryos. (A) FM4–64 staining of embryos expressing H2B::GFP. The plasma membrane is stained in red by FM4–64. The green fluorescence shows the localization of H2B::GFP. The white arrows point to nuclei in wild-type or *sas-5* (RNAi) embryos. (B) Nuclear envelope membranes were visualized in wild-type and *sas-5* RNAi-treated embryos with MAN1::GFP. The nuclei in the RNAi-treated embryos are bigger at first but then the nuclear envelope ingresses giving rise to a number of small nuclei. The dispersed or strong H2B::GFP signal in SAS-5 knockdown embryos co-localizes with DNA. H2B::GFP images were taken from wild-type and *sas-5* RNAi-treated embryos that had been counterstained for DNA with DAPI. The arrows point to individual nuclei. Bars: 10 μm.

morphology and dynamics observed upon depletion of SAS-5 by RNAi.

After depletion of SAS-5, we observed occasionally cytokinesis in the absence of DNA segregation leading to anucleated and multinucleated cells. This indicates that the checkpoint(s) controlling the correct order of mitosis and cytokinesis might not be activated at each cell division. Alternatively, this checkpoint is not fully developed in the early *C. elegans* embryo. Yet, some control must occur, because at least the first attempts of furrow ingression after unsuccessful chromosome segregation in the AB and the P1 cells failed. In addition, the DNA did not only seem to be dispersed, but also the amount of DNA present in the cells appeared to be elevated. A possible explanation is that the DNA replication did not stop in the absence of centrosome duplication. Because the centrosomes in higher eukaryotes duplicate during S-phase, a sensor for the time

frame of replication could be linked to centrosome duplication. In the absence of the signal indicating the successful duplication, the DNA replication continued. This feedback mechanism would be different from the recently reported DNA checkpoint in the early *C. elegans* embryo, because the latter one is connected to asymmetry (Brauchle et al., 2003). However, we did not detect any defects in polarity in embryos depleted of SAS-5 (data not shown).

We observed the formation of an abnormal nucleus in the early embryo and the DNA seemed to be dispersed. The dispersal of the DNA was transient, because we did not observe giant nuclei in later stages. Alternatively, the DNA could have been degraded in the big nucleus. However, we did not detect any apoptosis or acidification of the *sas-5* (RNAi) embryos. Moreover, we found that the nuclear envelope around the large nuclei became indented and smaller nuclei were formed. Therefore, it is conceivable that upon establishment of the nuclear architecture, the nuclear skeleton assembles around the chromatin and pulls in the nuclear envelope, which subsequently results in the formation of smaller nuclei. Thus, the formation of a nucleus should occur in multiple steps. The nuclear breakdown in the attempt of proceeding through the next cell division cycle still took place. This indicates that unlike cytokinesis, the nuclear envelope assembly and disassembly cycles are not disturbed when interfering with centrosome duplication.

While the number of proteins required for centrosome duplication increases, the precise role of different components is still very much a mystery. And certainly, more experiments are required to elucidate the function of SAS-5.

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III Discussion and Outlook

The aim of this work was to discover new genes required for the first cell division cycle in *C. elegans*. By undertaking an RNAi candidate approach, RGA-3/4 and SAS-5 were discovered and their functions in the embryo were analyzed. The phenotypic characterization of these genes highlights the multi-layered regulation of zygotic polarity and the cell cycle and raises interesting questions about the regulation of the different further components of the different pathways.

III.1. The novel RhoGAPs RGA-3/4 regulate RHO-1 GTPase activity during cortical polarization in the embryo and function in the germ line

Much progress has been made in recent years towards understanding of the role of RHO-1 signaling during polarization of the *C. elegans* embryo. Tight regulation of RHO-1 signaling is necessary to ensure proper polarization of the embryo. RHO-1 signaling is needed to establish contractile polarity, which in turn is essential for PAR mediated polarization of the embryo (Cowan and Hyman, 2007). Subsequently, the second GTPase in the embryo, CDC-42, is required to maintain the established polarity (Aceto et al., 2006).

III.1.1. Deciphering the different roles of RHO-1 and CDC-42 in the embryo

Triggered by a signal provided by the sperm and its centrosome, the RHO-1 pathway is activated in the embryo and orchestrates the rearrangement of the cortical acto-myosin network (Munro et al., 2004). This rearrangement of the zygotic cortex is at least partially mediated by the RHO-1 effector NMY-2, which localizes into contractile foci. Dependant on RHO-1 signaling is the PAR protein mediated polarization. CDC-42 function instead is needed for the maintenance of PAR-6 and the remainder of the complex at the anterior cortex.

In this thesis, the RhoGAP proteins RGA-3/4 were identified as negative regulators of RHO-1. Furthermore, I found that RHO-1 effector proteins were enriched at sites of contractility and furrowing in *rga-3/4 (RNAi)* embryos. Surprisingly, I observed, although RHO-1 signaling was not down-regulated properly in *rga-3/4 (RNAi)* embryos, that PAR

protein mediated polarization of *rga-3/4 (RNAi)* embryos was not heavily affected. How can this be explained?

Motegi and Sugimoto proposed a model in 2006 trying to enlighten the different roles of RHO-1 and CDC-42 during polarization. In this model, active RHO-1 is needed to establish polarity. CDC-42 would then be activated afterwards during a second (maintenance) phase of polarization and be responsible for proper acto-myosin network regulation during this phase. Therefore, one could hypothesize that excessive RHO-1 activity did not result in strong up-regulation of CDC-42 activity, and hence, that control of the second phase of polarization was more or less normal (in *rga-3/4 (RNAi)* embryos). Therefore, a feedback loop could signal from CDC-42 back to RHO-1 verifying that polarization has been established and that active RHO-1 is not needed anymore (and can be switched off). One could test this hypothesis easily by monitoring the CDC-42 levels in *rga-3/4 (RNAi)* embryos, since YFP::CDC-42 animals are available (Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006). CDC-42 levels could be monitored in a similar way like it was done for the investigation of RHO-1::GFP enrichment in *rga-3/4 (RNAi)* embryos (see II.1). Another tool, which could be used to address this question would be to look at the localization of a GFP-tagged version of wild type and especially dominant active CDC-42 (Q61L), which is localized more asymmetrically at the anterior cortex than the wild-type CDC-42::GFP (Aceto et al., 2006).

Apart from the role of RGA-3/4 in regulating RHO-1 and CDC-42, open questions are the discrimination between the molecular function of RHO-1 and CDC-42 during polarization and the elucidation of the CDC-42 activation. In particular, one important question would be if there is a signaling cascade connecting RHO-1 and CDC-42. Are the two GTPases acting in the same pathway or in different ones? Many insights should come from the elucidation of effector proteins. Consistent with RHO-1`s role in contractile polarity, several effector proteins like NMY-2 and MLC-4 or PFN-1 were shown to result in a decreased contractility phenotype if mutated or knocked-down by RNAi (Severson et al., 2002; Shelton et al., 1999; Swan et al., 1998). But what is the connection between contractility mediated by RHO-1 and maintenance of the PAR-3/6/aPKC complex at the anterior cortex? Is CDC-42 activated via a RHO-1 effector? One way to address these questions and to get a better molecular insight into the signaling cascade would be to express recombinant proteins like RHO-1 and CDC-42 and to prepare affinity purification columns with the purified proteins. Applying embryo extract to these columns and washing the non-binding proteins away might allow the identification of specific interactors and would allow a direct comparison between the RHO-1 and CDC-42 effectors, as well as putative regulatory proteins.

Importantly, all published studies on RHO-1 and CDC-42 function have used RNAi, since neither *rho-1* nor *cdc-42* mutant alleles specifically affecting the early embryo are available. In some studies mentioned, the penetrance of CDC-42 knock-down was tried to enhance by combining different modes of dsRNA delivery, i.e. feeding and injection (Schonegg and Hyman, 2006). Still, it cannot be excluded that the described phenotypes do not represent full the loss-of-function phenotypes but are the consequence of a partial knock-down. Low residual amounts of CDC-42 could therefore have masked an earlier essential function after RNAi treatment. Hence, a potential function of RGA-3/4 in regulating CDC-42 could have been missed.

III.1.2. RGA-3/4 and CYK-4 together regulate the temporal and spatial down-regulation of RHO-1 function in the early embryo

All three RhoGAPs with an essential function in the early embryo, RGA-3, RGA-4 and CYK-4, act as GAPs for RHO-1 (Jantsch-Plunger et al., 2000; Jenkins et al., 2006; Schmutz et al., 2007; Schonegg et al., 2007). Epistasis analysis of RGA-3/4 and RHO-1 *in vivo* as well as GAP assays *in vitro* have demonstrated that RGA-3/4 most likely act as GAPs for RHO-1 (Schonegg et al., 2007). CYK-4 does exhibit activity towards RHO-1 as well as RAC-2 and CDC-42 (Jantsch-Plunger et al., 2000) *in vitro*, but *in vivo* data points towards a specific role as a RhoGAP for RHO-1 during polarization and cytokinesis. If all three GAPs, RGA-3/4 and CYK-4, inactivate the same signaling molecule, why are their knock-down phenotypes so different from each other? One possible explanation is that these regulators may affect temporally and spatially distinct functions of RHO-1 in the embryo. RGA-3/4 function during the establishment phase of polarization and their inactivation affects the anterior part of the embryo, whereas CYK-4 action is needed to initiate posterior polarization before the action of RGA-3/4 and central spindle assembly during cytokinesis when polarization is already established and maintained (Jantsch-Plunger et al., 2000; Jenkins et al., 2006). Triple RNAi experiments with *rga-3/4* and *cyk-4* have shown that the different phenotypes are additive (Schmutz et al., 2007), consistent with them regulating different RHO-1 functions in the early embryo.

I would therefore like to propose the following model of RhoGAP function (Figure 5): shortly after fertilization, the paternal CYK-4 pool is needed to inactivate RHO-1 at the posterior cortex to initiate polarization. Later, RGA-3/4 come into play by down-regulating RHO-1 at the anterior cortex. Finally, RHO-1 is negatively regulated once more by CYK-4 during cytokinesis.

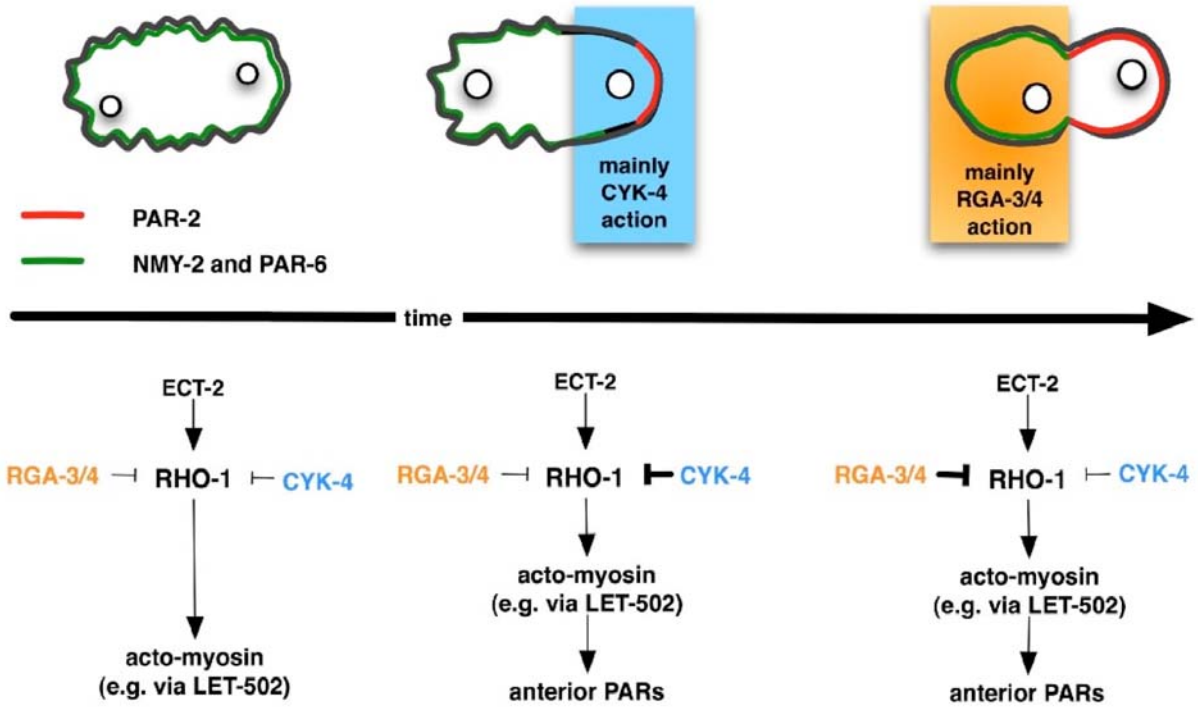

Figure 5: Model on RHO-1 regulation during cortical polarization on the *C. elegans* embryo.

Immediately after fertilization, NMY-2 and PAR-6/3/aPKC are spread across the entire embryonic cortex (first picture) due to RHO-1 mediated signaling. RHO-1 in turn is activated by ECT-2, which responds to a signal provided by the sperm. With the beginning of polarization and posterior cortex smoothening (second picture), CYK-4 down-regulates RHO-1's spreading from the point of sperm entry and leads to the establishment of a smooth posterior cortex. During pseudo-cleavage, RGA-3/4 are needed to reduce anterior contractility and down-regulate RHO-1 function. Red: PAR-2; green: NMY-2 and PAR-6/3/aPKC; orange: RGA-3/4; light blue: CYK-4.

Alongside the phenotypic discrimination by RNAi, it would be nice to distinguish the different functions of RGA-3/4 and CYK-4 in more detail and try to exclude overlapping functions. To assess that, one could use GFP-lines to follow the localization of RGA-3/4 and CYK-4 *in vivo.*

Schonegg and Hyman (2006) have shown that the localization of RGA-3::YFP is similar to that of NMY-2::GFP, except for the clear asymmetric localization during polarity establishment. To date, no CYK-4::GFP line has been established, but CYK-4 has been detected at the posterior cortex and in the male pronucleus as well as at the central spindle by immuno-histochemistry (Jantsch-Plunger et al., 2000; Jenkins et al., 2006). This localization is consistent with the proposed action of CYK-4 at the posterior cortex.

The distinct functions of RGA-3/4 and CYK-4 might therefore be a consequence of the different sub-cellular localizations of these proteins, thereby allowing RHO-1 inactivation at different sites of the cell.

To tackle the question whether the RGA-3/4 and CYK-4 functions are really exclusively additive, one could simply monitor the RGA-3::YFP behavior in a *cyk-4 (RNAi)* embryo. Is the RGA-3::YFP pattern in *cyk-4 (RNAi)* different from that in an untreated embryo? In-line with that one could also test if the penetrance of *cyk-4 (RNAi)* is weaker in a RGA-3::YFP expressing strain compared to wild-type background. If so, the over-expression of RGA-3::YFP can really compensate to some extent the loss of CYK-4 suggesting overlapping functions of the RhoGAPs.

Alternatively one could address the question by looking at RHO-1 function. *rho-1* loss-of-function mutants are lethal, but it would be helpful to generate some dominant-active as well as dominant-negative *rho-1* mutants. A dominant active *rho-1* mutant is, according to my model (Figure 5), expected to mimic the triple RNAi knockdown of its negative regulators and display a failure in polarization initiation, hyper-contractility and problems during cytokinesis.

One important question concerning CYK-4 and RGA-3/4 is how they are regulated themselves. How is CYK-4 targeted to the posterior cortex and the central spindle and how is cortical localization of RGA-3 achieved? One way to address the regulation of RGA-3 would be to screen for mis-localization of RGA-3::YFP in the embryo. This could be done in a candidate approach by choosing genes, which are expressed during this early stage of development. Again, combining a biochemical purification approach with recombinant protein expression to identify interacting proteins could shed light on the regulatory network.

III.1.3. Regulation of CDC-42

CDC-42 does function during polarity maintenance and its interaction with PAR-6 is required to maintain the latter at the cortex. Yet, neither RGA-3/4 nor CYK-4 seem to be required for its regulation. Schonegg and Hyman (2006) proposed CDC-42 to act in a parallel pathway in polarity establishment, whereas Motegi and Sugimoto (2006) proposed that it functions also downstream of RHO-1.

To date, no GEF nor GAP acting on CDC-42 *in vivo* has been identified in the early embryo and the mechanism of its activation is unknown. Immediately after fertilization, CDC-42 covers the entire cortex and becomes restricted to the anterior cortex after initiation of polarization similar to ECT-2, RHO-1 and NMY-2 (Aceto et al., 2006). As mentioned above, all the phenotypic studies performed to date rely on RNAi knock-down approaches. As this

might not lead to a complete depletion of the protein, large-scale RNAi screens may have missed possible regulators of CDC-42 due to incomplete knock-down. Unfortunately, it is difficult to assess the knock-down efficiency in early *C. elegans* embryos in a similar way as it is commonly done in mammalian cells, due to the difficulties of obtaining enough eggs for Western blot analysis and the lack of specific antibodies. But potentially, one could monitor the knock-down by RT-PCR.

Alternatively, CDC-42 GEFs or GAPs might act redundantly, as is the case for RGA-3/4, and therefore single-gene RNAi might not yield a visible phenotype. A more detailed analysis of RhoGAPs or GEFs by targeting more than one protein by RNAi simultaneously might uncover the sought-after CDC-42 regulators. Alternatively, one could also address this question by undertaking a screen/candidate approach assaying the mis-localization of CDC-42::YFP or CDC-42::GFP. Also here, another opportunity would be a biochemical approach (see III.1.1). One could also modify the suggested procedure by fishing with dominant active or inactive version of CDC-42 to identify potential activators or inactivators (Trautwein et al., 2004).

III.1.4. ECT-2 and putative other RhoGEFs in the early embryo

ECT-2 is the only RhoGEF that has been identified to function in the early embryo, based on its RNAi phenotype in the zygote (Lundquist, 2006). No *in vitro* data is available to indicate the target GTPase of ECT-2, yet, but the protein localizes in a similar pattern as RHO-1 and CDC-42 in the early embryo (Schonegg and Hyman, 2006; Motegi and Sugimoto, 2006). The asymmetric localization upon fertilization combined with the failure of pseudo-cleavage and cytokinesis in ECT-2 RNAi knock-down experiments suggest that it might be a GEF for RHO-1 rather than CDC-42. But these RNAi data do not exclude the possibility that besides regulating RHO-1, ECT-2 may also act on CDC-42, since the *cdc-42 (RNAi)* phenotype can only be observed later than that of *rho-1 (RNAi*).

If ECT-2 is truly the only GEF active during early embryogenesis, specific regulation of GTPase activity is mainly provided by RhoGAPs. Here again, due to redundancy, other RhoGEFs of the early embryo might have been missed in RNAi screens (see III.1.3.).

ECT-2 is excluded from the posterior cortex in response to a cue from the sperm. But the nature of this cue remains elusive so far. As single reverse-genetic approaches have failed to identify potential candidates, the future identification of possible interactors of ECT-2 using biochemical approaches might shed light on these early events of embryogenesis.

III.1.5. Are RGA-3/4 required at different stages or in different tissues than in the early embryo ?

The most obvious phenotype of RGA-3/4 knock-down is the extensive ruffling in the one-cell stage embryo. However, in a background sensitized for LET-502 (*let-502 (sb106))* function, a requirement for RGA-3/4 in gonadal development was revealed.

LET-502 positively regulates contractions in the embryo and has also been implicated, due to its localization, to play a role in gonadal development. Strong *let-502* (*ok1283*) alleles show severe gonadal defects and defects in spermatogenesis. In contrast, weak alleles only interfering with the kinase activity of LET-502 (e.g. *let-502 (sb106)*) do not display these defects.

When *rga-3/*4 *(*RNAi) knock-down experiments were performed in the background of a weak *let-502 (sb106)* mutant allele, strong gonadal defects were observed, similar to those seen in the strong alleles. In other words, RNAi of the negative RHO1- regulator *rga-3/4* enhances the phenotype of the weak *let-502 (sb106)* allele. This demonstrates that RGA-3/4 do have a function in the germ line. It remains to be seen if this function is similar to that the one in the early embryo. One could test here, if the same pathway is needed in the germ line as in the embryo again with epistasis experiments and ask whether *rho-1* and *rga-3/4* also genetically interact in the germ line. Another way to study this would be to investigate the regulation of NMY-2 in the germ line. Does the GFP::NMY-2 pattern differ between a wild-type and a *rga-3/4 (RNAi)* gonad? If so, the same RHO-1 pathway as in the early embryo would be needed also in the germ line.

LET-502 was initially identified as a factor required for embryonic elongation, during which the spherical embryo develops into a thin long worm. Hypodermal cells in the outermost layer of the embryonic constrict the embryo circumferentially, a process controlled by Rho GTPase signaling. The Rho kinase LET-502 positively regulates these contractions, whereas its counterpart, the phosphatase MEL-11, negatively regulates elongation (Piekny et al., 2003; Piekny and Mains, 2002; Piekny et al., 2000; Wissmann et al., 1997). Given the genetic interactions of LET-502 and RGA-3/4, it will be interesting to explore whether the RhoGAPs are also involved in regulating morphogenesis.

III.2. SAS-5 is needed for centrosome duplication in *C. elegans*

When I started the work presented in this thesis, forward genetic as well as functional genomics by RNAi had led to the identification of 3 proteins essential for centriole formation in the *C. elegans* embryo. ZYG-1, SPD-2 and SAS-4 were believed to represent the proteins required for the formation of daughter centrioles. In this study, I have identified SAS-5 as an additional central component.

III.2.1. The role of SAS-5 during centrosome duplication

The discovery of the core components for centriole duplication and their sequential recruitment to centrioles in *C. elegans* has provided an initial overview of daughter centriole formation, but there is still a need to get a deeper insight into the molecular mechanism and regulation of daughter centriole formation (see I.3.2.1.).

SAS-5 localizes to centrosomes (Schmutz and Spang, 2005; data not shown), more specifically to the centrosome`s centriole (Delattre et al., 2004). In 2004 Delattre et al. also showed that SAS-5 shuttles freely between the cytoplasm and the centriole throughout the cell cycle, a behavior, which has not been reported for any other centrosomal protein. The localization of SAS-5 and its recruitment to the daughter centriole depends on two additional factors, SDP-2 and the kinase ZYG-1 (Delattre et al., 2004) (Leidel and Gonczy, 2005). SAS-5 might be posttranslationally modified *in vivo*, as several bands are specifically recognized in Western blot experiments. Future experiments could elucidate whether SAS-5 really is phosphorylated and whether is may be directly targeted by ZYG-1.

SAS-5 and SAS-6 can interact with each other in a yeast-two-hybrid assay (Leidel et al., 2005). As opposed to SAS-5, though, SAS-6 does not shuttle between the centriole and the cytoplasm. This interaction might therefore be regulated depending on the stage of the centrosomal duplication cycle. While SAS-6 is conserved in mammalian genomes, so far no SAS-5 sequence ortholog has been identified outside the worm.

III.2.2. Are there functional homologs of SAS-5 in other species?

Whereas molecules distantly related to SAS-4 as well as SAS-6 exist, no clear orthologs of ZYG-1 and SAS-5 have been identified.

Are there functional homologs of the coiled-coil protein SAS-5 and the kinase ZYG-1 present in vertebrates? Several vertebrate kinases have been implicated in the regulation of centrosome duplication (Sluder 2004). It is likely, that cyclin-dependent kinases play a role in regulating centrosome duplication, but the question remains if their primary function is mainly centrosome duplication or rather regulation of the cell cycle (Matsumoto et al., 1999; Meraldi et al., 1999). The most promising candidate for a functional homolog of ZYG-1 in vertebrates is the polo like kinase 4 (Plk4), since it has been identified as a positive regulator of centriole duplication in human cells and *Drosophila* (Habedanck et al., 2005; Bettencourt-Dias et al., 2005). The kinase activity of Plk4 is required at the centrosome, similar to the function of ZYG-1 in *C. elegans* (Leidel and Gonczy, 2005). It is tempting to speculate that these kinases might carry out analogous functions.

Identifying functional homologs for SAS-5 is more difficult, as there is no other function predicted for it other than mediating protein-protein interactions via its coiled-coil domains. So far, no protein with similar function could be identified in other systems than *C. elegans*, although the same semi-conservative mechanism of centrosome duplication seems to be conserved in other species.

It is possible that the core mechanism of MTOC duplication may rely on proteins with limited primary sequence homology but analogous functions. In this case, SAS-5 function might be conserved, but has been taken over by a protein unrelated in amino acid sequence.

III.2.3. Consequences and causes of the altered nuclear morphology in SAS-5 knock-down embryos

SAS-5 knockdown resulted in a clear defect in nuclear morphology at the two-cell stage (see II.2.). In the AB and P1 of *sas-5 (RNAi)* embryos, only one centriole remained, which could not be duplicated, and assembly of a bipolar spindle in AB or P1 is not possible. This failure in spindle assembly influences the nuclear morphology as embryos enter mitosis and try to separate their DNA, but fail. Subsequently, the nucleus enlarges, possible because large amounts of chromatin are generated in additional cell cycles lacking successful mitosis. Although the nuclear envelope can still form, pronounced indentations in

the nuclear envelope can be observed (see II.2). Epistasis experiments with *ced*-mutants clearly demonstrated that this change in nuclear morphology is not a consequence of apoptosis induction (see II.2). Also, neither the morphology of other cellular organelles, like the ER, nor endocytotic uptake of yolk protein was significantly affected (data not shown).

IV References

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V Abbreviations

a-p	anterior-posterior
ATP	adenosin triphosphate
aPKC	a typical protein kinase C
BrDU	bromodeoxyuridin
cDNA	coding DNA
cdk	cyclin-dependent kinase
C. elegans	*Caenorhabditis elegans*
CGC	*C. elegans* Gene Consortium
d	day
DIC	differential interference contrast
DNA	deoxyribonucleotid acid
D. melanogaster	*Drosophila melanogaster*
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
ER	endoplasmic reticulum
F-actin	filamentous actin
FM 4-64	N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)-hexatrienyl) pyridiniumdibromid
GAP	GTPase activating protein
GDI	guanine-dinulceotide-dissociation inhibitor
GEF	guanine-nucleotide-exchange factor
GFP	green fluorescent protein
GTP	Guanosin-5`-triphophosphate
GDP	Guanosin-5`-diphophosphate
GTPase	GTP hydrolase
h	hour
IVTR	*in vitro* transcription
kD	kilo Dalton
M	Molar
min	minutes
mRNA	messenger RNA
MT	microtubules
MTOC	microtubule organizing center
µm	micro meter
nt	nucleotide
NGM	normal growth media
nm	nano meter
ORF	open reading frame
PC	pseudo cleavage
PH	pleckstrin homology
PM	pronuclear meeting
ROCK	Rho associated kinase
RNA	ribonucleotide acid
RNAi	RNA mediated interference
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
qRT-PCR	quantitative RT-PCR
S. cerevisiae	*Saccharomyces cerevisiae*

SPB	spindle pole body
toca	transducer of Cdc42 dependant actin assembly
WASP	wiskott-aldrich-syndrome-protein
WT	wild type

Origin of gene names

arp	actin related protein
ani	ANIllin (actin binding protein)
ced	cell death abnormal
cyk	cytokinesis defective
cdc	cell division cycle
ect	mammalian Rho GEF homolog
emb	embryonic lethal
gld	germline differentiation abnormal
gpr	G protein regulator
let	lethal
mel	maternal effect lethal
mex	muscle in excess
mig	abnormal cell migration
mlc	myosin light chain
nmy	non-muscle-myosin
par	partitioning defective
pgl	P granules abnormal
pfn	profilin
pie	pharynx and intestine in excess
pal	posterior alae
rac	rac related
rga	rho GTPase activating
rol	roller
sas	spindle assembly defective
skn	skin in excess
syd	synapse defective
tbb	tubulin, beta
unc	uncoordinated
zyg	zygotic defective

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European Worm Meeting, May, 22nd-25th ,2004,
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ELSO Meeting 2004, September, 4th-8th, 2004,
Nice, France

Dmitry Poteryaev, Cornelia Schmutz, Hanah Fares, Bruce Bowerman and Anne Spang
***„Caenorhabditis elegans* SAND-1 is an essential effector of early to late endosomal traffic.“**
International Worm Meeting, 25th-29th June 2005,
Los Angeles, USA

Cornelia Schmutz, Julia Stevens and Anne Spang
„Functions of the novel RhoGAP proteins RGA-3 and RGA-4 in the germ line and in the early embryo of *C. elegans.“*
ELSO Meeting 2007, 1st-4th, September, 2007,
Dresden, Germany

Hiermit erkläre ich, dass ich die Dissertation “Characterization of the RhoGAP proteins RGA-3 and RGA-4 and the centrosomal protein SAS-5 in the early *Caenorhabditis elegans* embryo” nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Basel, den

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