Doctoral Thesis

Regulation of the mitotic spindle during asymmetric cleavage in the nematode Caenorhabditis elegans

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Regulation of the mitotic spindle during asymmetric cleavage in the nematode Caenorhabditis elegans

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Summary

Asymmetric cell division is of fundamental importance for the generation of cell diversity during the development of organisms. Establishment of polarity and proper alignment of the mitotic spindle are both essential prerequisites for an asymmetric cell division.

Here we investigated the molecular mechanisms underlying spindle positioning using the Caenorhabditis elegans 1-cell embryo as a model system. This embryo is highly polarized displaying two cortical protein domains, created by the PAR (partitioning defective) proteins, which play an important role in spindle regulation. PAR-3, PAR-6 and PKC-3 are found on the anterior cortex, while PAR-1 and PAR-2 are localized on the posterior cortex. Downstream of the PAR proteins, heterotrimeric G protein (HGP) signaling is essential for regulation of spindle positioning. It has previously been shown, that the HGP controlling spindle positioning is activated in a receptor independent manner. However, the precise mechanisms that regulate HGP signaling and the targets of the HGP in spindle positioning are still unknown.

In this thesis, I have investigated the role of RIC-8 in these processes. RIC-8 is a protein that is also important for spindle regulation, as loss of RIC-8 results in a symmetric cleavage. RIC-8 interacts physically and genetically with Ga proteins. In mammalian cells, RIC-8 is a guanine exchange factor (GEF) for Ga. We also suggest a GEF role for RIC-8 in the early C. elegans embryo. In our model, RIC-8 participates in activating HGP signaling.

To identify more genes required in spindle orientation and regulation, we performed a screen for genes that can enhance the lethality of a temperature-sensitive allele of gpr-1 and lin-5. In this screen, we identified dyrb-1 as a putative candidate genetically interacting with gpr-1 and lin-5. dyrb-1 is the C. elegans homologue of Drosophila Roadblock (robl), a member of the dynein light chain family of proteins. Interestingly, we find that DYRB-1 can also co-immunoprecipitate with LIN-5 and GPR-1/2. Our data suggests that heterotrimeric G protein signalling may control mitotic spindle positioning by regulating dynein activity.
Zusammenfassung


Wir untersuchten die molekularen Mechanismen der Spindel-Positionierung im 1-Zell Embryo des Model Organismus Caenorhabditis elegans. Im Embryo sind zwei Membrandomänen zu finden, welche von den PAR Proteinen gebildet werden, die eine wichtige Rolle in der Regelung der Spindel ausüben. Dies führt zu einem hochgradig polarisierten Embryo. Auf der anterioren Seite befinden sich PAR-3, PAR-6 und PKC-3, während PAR-1 und PAR-2 am posterioren Ende lokalisiert sind. Abgesehen von den PAR-Proteinen nehmen die heterotrimeren G Proteine (HGP) eine wichtige Rolle in der Spindel-Regulierung ein. Es wurde gezeigt, dass die HGP unabhängig von membrangebundenen Rezeptoren aktiviert werden können. Die genaue Kontrolle der HGP ist bisher immer noch unklar, was auch die Moleküle einschliesst, die von dem HGP kontrolliert werden.


CHAPTER I

1. Introduction

1.1 Asymmetric cell division - an overview

In order to create higher organisms, a variety of cells are needed. Therefore, cell diversity is very important during development and for the functional specification of many different cell types. One way to generate unequal cells is by induction. This type of division is a symmetric cell division, where one mother cell initially gives rise to two identical daughter cells. Subsequently one of the daughters is induced by extrinsic signals and becomes different from its sister. Through this method, two identical daughter cells become different because they encounter different environments (Figure 1A).

Another way to ensure cell diversity is by asymmetric cell division. Several steps are required to guarantee an asymmetric cell division. The first step is the establishment of an axis of polarity within the cell. This is followed by the asymmetric segregation of cell-fate determinants along this axis of polarity (Figure 1B). The third important step is the proper regulation of mitotic spindle orientation, which must be parallel to the axis of polarity. Cell division results in the unequal distribution of the polarized factors, leading to the generation of two daughter cells with different fate. Interestingly, many asymmetric cell divisions also have an effect on cell size (Salmon et al. 1989; Doe and Bowerman, 2001).

Since spindle positioning along the axis of polarity plays a crucial role in asymmetric cell division, it is important to understand how spindle positioning is regulated and how it is coordinated with cellular polarity. Most of the mechanistic insight into this process comes from invertebrate model systems, for instance Drosophila melanogaster and Caenorhabidits elegans.
Figure 1. Cell differentiation

A) Cell induction
One cell gives rise to two equal cells in cell fate and size. Cell inductions change the fate of a cell leading to differentiation. The blue circles represent equal cells. The red cell is inducing one of the cells, changing its fate (green).

B) Asymmetric cell division
The circles are a schematic representation of cells, the orange dots represent cell components in the cytoplasm, while the blue domain represents cortically localized components.
1. establishment of axial polarity (double arrow)
2. separation of cell components along the axis of polarity (blue cortical components, orange cytoplasmic components)
3. spindle alignment along the axis of polarity
4. generation of two different daughter cells in cell size and cell fate
1.2 *C. elegans* as a model system

*Caenorhabditis elegans* (*C. elegans*) is a free-living nematode, about 1 mm in length, transparent, which lives in temperate soil environments. Research into the molecular and developmental biology of this nematode was started in 1974 by Sydney Brenner. *C. elegans* has since been used extensively because it offers several advantages as a model system:

- The genome is entirely sequenced (Sequencing Consortium, 1998).
- The developmental fate of every single cell is mapped (959 in the adult hermaphrodite; 1031 in the adult male).
- Development is invariant
- The worm is transparent which makes it possible to follow developmental defects directly by microscopy.
- The use of RNA interference (RNAi, Fire et al. 1998) by feeding, injection or soaking of double stranded DNA, makes it possible to directly explore the effects resulting in protein depletion.
- It is easy to maintain populations in the laboratory.

The *C. elegans* embryo is especially suited to the study of asymmetric cell division because the embryo is rather big (around 50 μm at its longitudinal axis) and transparent, which makes it easy to directly observe changes during development and, more importantly, the first divisions are asymmetric.

With these advantages and the completely sequenced genome it is possible to undertake genome-wide screens that could potentially identify most genes required for specific biological processes. Such screen work was pioneered by the Ahringer group, who constructed a feeding library covering about 86 % of the predicted *C. elegans* genes (Fraser et al. 2000; Kamath et al. 2003).
1.3 Asymmetric cell division in the model system C. elegans

1.3.1 An overview of early processes in C. elegans

The body plan of the C. elegans embryo is established during the first few cleavages. The newly fertilized embryo undergoes a series of four unequal divisions to produce five somatic founder cells (AB, MS, E, C and D) and the primordial germ line (P4) by the 28-cell stage (Figure 2). The reproducible orientations of these cleavages coupled with asymmetric localization of cytoplasmic components initiate processes that establish the axes of polarity and set cell fates.

---

**Figure 2. The first divisions of C. elegans embryos are asymmetric**

Cell divisions are indicated by horizontal lines; the anterior daughter of each division is placed on the left. A series of unequal divisions of the germ line, or P cells, results in the formation of five somatic founder cells (AB, MS, E, C and D) and the primordial germ cell (P4). The tissues generated by each founder cell are indicated. Adapted from Sulston et al. (1983) and Schierenberg et al. (1987).
The first division of the *C. elegans* embryo in particular has been the focus of intense studies. The zygote, also called P0, divides asymmetrically along the anterior-posterior axis and produces a larger anterior AB cell and a smaller posterior P1 cell. The two daughter cells are different in size and cell fate (Figure 3). The AB cell will form mainly ectoderm, whereas P1 will differentiate into germline cells, as well as endo- and mesoderm.

**Figure 3. Asymmetric cell division in the *C. elegans* zygote**

The *C. elegans* zygote (schematically represented above) divides along its anterior-posterior axis and gives rise to a bigger anterior AB (green) and a smaller posterior P1 daughter cell (red), which are committed to different cell lineages. The AB cell gives rise to the ectoderm while P1 is the germline precursor cell and also forms meso- and ectoderm.

Observations of the first cleavage show that a subset of actions have to occur to guarantee an asymmetric cleavage: Firstly, the oocyte becomes fertilized by the sperm. This triggers the completion of meiosis I and II (Iwasaki et al. 1996),leads to the secretion of the strong and impermeable chitinous eggshell that gives the embryo its distinctive ovoid shape, and initiates the process of polarity establishment. By approximately 20 minutes after fertilization meiosis is completed and the haploid maternal and paternal pronuclei form, with the paternal pronucleus (sperm) position defining the posterior pole of the embryo. The maternal pronucleus is localized in the anterior part of the embryo, opposite to the sperm pronucleus. In the next step, the maternal pronucleus migrates towards the paternal pronucleus in the embryo posterior
while the paternal pronucleus moves slightly anterior. The two pronuclei meet in the more posterior half of the embryo (Albertson et al. 1984; Hyman et al. 1987). During this time the embryo has become highly polarized. Thereafter, the nuclei-centrosome-complex (NCC) undergoes a 90° rotation while moving towards the center with the centrosomes aligned along the anterior-posterior axis of the cell (Albertson et al. 1984; Hyman and White 1987). The nuclear envelope breaks down (Figure 4C), the spindle starts to elongate along to the longitudinal axis of the embryo (Albertson et al. 1984; Hyman and White 1987) and gets displaced to the posterior of the embryo. During anaphase spindle elongation the posterior centrosome oscillates, a so-called “rocking” movement, along the short axis of the embryo (Figure 4D). Different forces act on the poles during anaphase resulting in a more round anterior aster and a flattened posterior aster (Severson et al. 2003). The spindle is asymmetrically positioned during cytokinesis, leading to the formation of a bigger anterior AB cell and a smaller posterior P1 cell (Figure 4E).
Figure 4. Early embryo stages of *C. elegans*

(A) Sperm entry defines the posterior pole of the zygote (PO, right), after fertilization the maternal and the paternal pronucleus migrate toward each other (B) and meet in the posterior third of PO. The nuclear complex is centered (C), the nuclear envelope breaks down and the mitotic spindle elongates parallel to the longitudinal axis of the embryo (D). PO gives rise to two unequal cells (AB and P1) different in size and fate. The posterior aster has a disk-like shape (E) while the anterior is more rounded. In the next cell cycle AB divides before P1 into ABa and ABp, while P1 gives rise to EMS and P2 (F). The scale bar in (F) represents 10 μm.
1.3.2 Polarity in the one-cell embryo

1.3.2.1 The PAR proteins

As previously mentioned, the sperm entry defines the anterior-posterior axis of the embryo. Goldstein and Hird showed that there is no pre-fixed axis of polarity in the oocyte. The side of sperm entry will become the posterior pole (Goldstein et al. 1996). Sperm entry results in the asymmetric partitioning of the PAR proteins along the anterior-posterior axis of the 1-cell embryo. The par genes were identified by a pioneering genetic screen searching for mutants affecting the first asymmetric cell division in *C. elegans*, and were called *par* for partitioning-defective (Kemphues et al. 1988). Mutations in these genes lead to disruption of several aspects of A-P polarity in the zygote, including asymmetric placement of the first mitotic spindle and P-granule localization (Kirby et al. 1990). There are six PAR proteins, PAR-1 to PAR-6, which, together with PKC-3, are required for polarization of the embryo. These proteins are key regulators of cell polarity and are conserved across species and cell types (Ohno et al. 2001; Macara et al. 2004).

The PAR proteins form two distinct cortical domains. The anterior domain is formed by PAR-3 and PAR-6, both PDZ (Postsynaptic Density 95/Discs Large/Zonula Occulens-1) containing proteins, together with atypical Protein Kinase C, PKC-3 (Kurzchalia et al. 1996; Hung et al. 1999; Tabuse et al. 1998; Etemad-Moghadam et al. 1995). In meiotic embryos, PAR-3, PAR-6 and PKC-3 associate with the entire actomyosin cortex, but during polarity establishment become anteriorly localized (Munro et al. 2004). At the 2-cell stage the anterior PAR domain covers the entire AB cortex and the anteriormost part of the P1 cortex.

Localized to the opposite pole is PAR-2, a RING-finger protein (Boyd et al. 1996). Its RING-finger domain plays a crucial role in maintaining its cortical localization, since mutants lacking this domain are displaced from the cortex (Hao et al. 2006). Furthermore, PAR-2 plays a role in restricting PAR-3, PAR-6 and PKC-3 to the anterior half of the embryo (Boyd et al. 1996; Cuenca et al. 2003; Hao et al. 2006).

PAR-1, a serine/threonine kinase, colocalizes with PAR-2 at the posterior cortex. PAR-1 localization is dependent on functional PAR-2 and the anterior PAR domain (Guo et
al. 1995). The kinase activity of PAR-1 appears crucial since mutations in the kinase domain are equivalent to null mutations (Guo et al. 1995). It has been proposed that PAR-1 may also play a role in excluding the PAR proteins from the posterior domain (Cuenca et al. 2003; Hao et al. 2006). PAR-1 is also essential for correct segregation of cell fate determinants such as MEX-5 and MEX-6 in the 1-cell embryo, as discussed below. Two PAR proteins are uniformly distributed throughout the cortex and in the cytoplasm of the early embryo: PAR-4, a serine/threonine kinase, and PAR5, a 14-3-3 protein (Morton et al. 1992, 2002; Watts et al. 2000; Table 1).

1.3.2.2 Establishment of polarity

Which upstream events act to localize the PAR proteins to the anterior or posterior domains? After fertilization and completion of meiosis the embryo cortex is highly contractile. Recently, it has been shown that two molecules, the small GTPase RHO-1 and the RhoGEF ECT-2, together with myosin, are important for this cortical contractility (Jenkins et al. 2006; Motegi and Sugimoto, 2006; Schoenegg and Hyman, 2006). Studies from several laboratories show that the paternally provided centrosomes play a crucial role in establishing polarity in the zygote (Sadler et al. 2000; O’Connell et al. 2000; Cowan et al. 2004). It has been shown that ablation of the sperm centrosome prior to polarity establishment prevents the polarization of the embryo (Cowan et al. 2004). In contrast to this, later centrosome ablation no longer has an effect on polarity (Cowan et al. 2004). More recent data has shown that the sperm provides an additional molecule, the RhoGAP CYK-4 (Jenkins et al. 2006). One possible model is that CYK-4 inactivates RHO-1 at the posterior pole resulting in punctual relaxation of the actin cytoskeleton (Figure 5), which retracts towards the anterior pole.

This asymmetric contraction of the cortical actomyosin meshwork drives the cortical flows, which result in the relocalization of the uniformly distributed PAR-3 and PAR-6 towards the anterior pole (Munro et al. 2004; Figure 5). This movement creates a posterior gap, free of PAR-3, PAR-6 and PKC-3, allowing PAR-2 to become cortically localized to the posterior pole (Figure 5). PAR-2 is not required to establish polarity but it accumulates on the posterior cortex and prevents the anterior PAR complex from
becoming re-localized to the posterior cortex (Munro et al. 2004). The same cortical movements also lead to the redistribution of cytoplasmic factors to the posterior pole (e.g. the P-granules, Hird et al. 1996). Mutations or chemical disruption of the actomyosin activity destroys asymmetric PAR-distribution (Guo and Kemphues, 1995; Severson and Bowerman, 2003; Shelton et al. 1999, Munro et al. 2004).

**Figure 5. Establishment of cortical polarity in the *C. elegans* zygote**

The cloud-shaped structures represent the early *C. elegans* embryo in different stages, from sperm entry (left side) to the completely polarized embryo (right side). The first row represents the establishment of the cortical PAR-domains (green), with PAR-3, PAR-6 and PKC-3 on the anterior cortex and PAR-2 (red) on the posterior cortex. Below that, the acto-myosin network is illustrated. It is getting reduced on the posterior pole of the embryo. The reason for this is the loss of uniform distribution of RHO-1 and ECT-2, getting excluded from the anterior part of the embryo. The embryo is depicted from a surface view. Centrosomes normally lie in the embryo mid-plane. Posterior is right. (Figure adapted and modified from Cowan et al. 2007)
To summarize, during polarity establishment the localization of the PARs changes from an uniform distribution of PAR-3, PAR-6 and PKC-3 across the entire cortex to a fully polarized situation where the PAR-complex is localized to the anterior while PAR-1 and PAR-2 are localized on the posterior cortex (Guo and Kemphues, 1995; Boyd et al. 1996; Cuenca et al. 2003). Interestingly, loss of *par-3, par-6* and *pkc-3* leads to uniform distribution of cortical PAR-1 and PAR-2 (Etemad-Moghadam et al. 1995; Watts et al. 1996; Tabuse et al. 1998; Hung and Kemphues, 1999; Cuenca et al. 2003). On the other hand depletion of PAR-2 leads to posterior directed cortical expansion of the anterior PAR domain. This indicates that the anterior PAR domain works antagonistically to the posterior PARs. For further understanding the distribution of the described molecules is depicted in Figure 5. How CYK-4 and the sperm signal contribute to polarization is still unclear. The question is if the centrosome associated microtubules act in parallel to CYK-4 or as a part of the CYK-4 in the polarization process of the zygote.

### 1.3.2.3 *par* mutants

Observations in *par* mutants showed that *par-1, par-2, par-3, par-5* and *par-6* share a common phenotype. Depletion of any of these PARs results in a central spindle leading to daughter cells of equal size and cell cycle timing. But there are more differences between the anterior and the posterior PAR proteins. Mutant embryos of *par-3, par-6* and *pkc-3* display flat asters at both spindle poles in P0 and rotation of both AB and P1 spindles along the anterior-posterior axis (Etemad-Moghadam et al. 1995; Watts et al. 1996; Tabuse et al. 1998; Gotta et al. 2001). In contrast, *par-2* has two round P0 asters and neither AB nor P1 spindles rotate (Levitan et al. 1994; Boyd et al. 1996). PAR-2 is known to recruit PAR-1 to the posterior cortex. In *par-1* mutant embryos, the PAR-2 domain expands more rapidly and advances further towards the anterior pole, with a corresponding reduction of the anterior PAR domain (Cuenca et al. 2003) and a more anterior position of the pseudocleavage furrow (Cuenca et al. 2003; Kirby et al. 1990). The *par-4* phenotype is distinct from the other *pars*. The first division is still asymmetric, but AB and P1 display synchronous cell-cycle timing as in the other *pars*. PAR-5 is known to separate the anterior domain from the posterior domain, since *par-5*
embryos show invasion of the PAR-3 complex to the posterior. The phenotypes are summarized in Table 1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Structure</th>
<th>Function</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>Serine-Threonine kinase</td>
<td>AB and P1 asymmetry</td>
<td>Zygote, posterior cortex</td>
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<tr>
<td></td>
<td></td>
<td>P-granule localization</td>
<td></td>
</tr>
<tr>
<td>PAR-2</td>
<td>Ring-finger protein</td>
<td>AB and P1 asymmetry</td>
<td>Zygote, posterior cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spindle orientation in P1</td>
<td></td>
</tr>
<tr>
<td>PAR-3</td>
<td>PDZ-domain protein</td>
<td>AB and P1 asymmetry inhibition of</td>
<td>Zygote, anterior cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spindle orientation in AB.</td>
<td></td>
</tr>
<tr>
<td>PAR-6</td>
<td>PDZ-domain protein</td>
<td>AB and P1 asymmetry inhibition of</td>
<td>Zygote, anterior cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spindle orientation in AB.</td>
<td></td>
</tr>
<tr>
<td>PKC-3</td>
<td>Atypical protein kinase C</td>
<td>AB and P1 asymmetry inhibition</td>
<td>Zygote, anterior cortex</td>
</tr>
<tr>
<td></td>
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<td>of spindle orientation in AB.</td>
<td></td>
</tr>
<tr>
<td>PAR-4</td>
<td>Serine-Threonine kinase</td>
<td>AB and P1 cell cycle timing</td>
<td>entire cortex and cytoplasm</td>
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<td></td>
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<td>maintaining exclusivity of PAR</td>
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<td></td>
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<td>domains</td>
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<tr>
<td>PAR-5</td>
<td>14-3-3 protein</td>
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<td>entire cortex and cytoplasm</td>
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Table 1: Overview of the partitioning in the early *C. elegans* embryo

1.3.3 Segregation of cytoplasmic factors along the anterior-posterior axis in the one-cell embryo

The first mitotic division segregates different cytoplasmic proteins that control cell-fate potential to each daughter cell. This segregation depends on the cortical PAR proteins. Downstream of PAR-2 and PAR-3, PAR-1 also has an important role in the segregation of two redundant cytoplasmic CCCH finger proteins, MEX-5 and MEX-6, which are 70% identical (Schubert et al. 2000), by restricting them from the posterior part of the cell. During division MEX-5/6 are segregated into the AB cell. The localization of MEX-5/6 is uniformly cytoplasmic in *par-1* mutant embryos. Therefore, MEX-5/6 play
a role downstream of PAR polarity in restricting P-granules (cytoplasmic ribonucleoprotein complexes which are important for germline development) and germline proteins such as PIE-1 to the posterior cytoplasm (Kemphues et al. 2000; Cuenca et al. 2003; Schubert et al. 2000). However, recent data suggests that MEX-5/6 collaborate with the PAR proteins to generate intracellular motility (Cheeks et al. 2004). Depletion of MEX-5/6 results in similar phenotypes as observed for par mutants, including equal first cleavage and reduction of flows (Cheeks et al. 2004). To summarize this, MEX-5/6 are important for intracellular motility, which is needed for the correct localization of cortical PAR proteins and segregation of cell fate determinants. After recruitment of PAR-1 to the posterior cortex, PAR-1 excludes MEX-5/6 from the posterior cytoplasm and stabilizes P-granule localization at the posterior pole. MEX-5/6 subsequently excludes PIE-1 from the anterior cytoplasm. This hierarchy of regulation converts the cortical polarity of the PAR proteins into a complementary pattern of cytoplasmic proteins. Elimination of any of these proteins results in abnormal cell fate specification.

1.3.4 Regulation of spindle positioning - physical mechanisms

To ensure the correct segregation of polarity determinants, like PAR proteins, the mitotic spindle must align along the axis of cell polarity. Spindle positioning in *C. elegans* can be separated into two different events: First, the alignment of the spindle along the axis of polarity, followed by a second process, the asymmetric displacement of the spindle towards the posterior pole. Both processes are linked to cortical polarity and, as described above, the PAR proteins influence these events.

How is the mitotic spindle displaced to the posterior? One possible explanation could be given by microtubule-based force generation. Laser or genetic ablation of the mitotic spindle gave indications for pulling forces acting on the astral microtubules. Evidence for these pulling forces comes from observations on the two centrosomes. If the spindle midzone is destroyed the anterior and the posterior centrosome move apart from each other. Interestingly, the posterior one moves faster to the posterior pole than the anterior centrosome to the anterior pole (Grill et al. 2001). The forces acting on the spindle poles
cannot directly be measured, therefore the peak velocities of the moving spindle poles are suggesting that at the posterior higher pulling forces act from the cortex on the posterior spindle pole. This experiment demonstrated an asymmetry of forces exerted on the anterior and posterior spindle poles in wild-type embryos, which translated into an approx. 40% faster movement of the posterior spindle pole. This result is due to a greater net force pulling on the posterior aster (Grill et al. 2001). This force difference is under the control of cortical polarity. In par-2 and par-3 mutants, the measured peak velocities for both poles are equal (Grill et al. 2001). Interestingly, in par-2 mutants, where PAR-3 is uniformly distributed and both asters are round like the wild-type anterior aster, the peak velocities are lower on both spindle poles, similar to the forces acting on the anterior wild-type pole. In contrast, in par-3 mutants, where PAR-2 covers the entire cortex and both asters are flat like the wild-type posterior aster, forces acting on both spindle poles are high and comparable to the forces acting on the posterior wild-type pole. There is also a correlation between the so-called “rocking” events prior to aster flattening and the higher forces acting on the posterior wild-type pole. These results indicate that PAR proteins regulate posterior spindle displacement by controlling forces acting on astral microtubules that contact their cortical domains (Grill et al. 2001).

An increase in net force on the posterior pole relative to the one acting on the anterior aster could be explained by differences in the number, strength, or distribution of force generators (Sharp et al. 2000; Goenczy et al. 2002). More recent work suggests that the imbalance in cortical pulling forces is due to more force generators being active at the posterior than at the anterior pole (Grill et al. 2003; Pecreaux et al. 2006). The molecular nature of the force generator is not yet known. One possibility is that the minus-end directed motor protein dynein generates the forces necessary to displace the spindle (Schmidt et al. 2005; Dujardin et al. 2002). Full depletion of dynein activity results in spindle formation defects, preventing observations of spindle positioning. Studies performed using temperature sensitive (ts) mutants of dynein heavy chain-1 (DHC-1) indicate that dynein inactivation at the time of spindle positioning results in a delay of spindle positioning (Schmidt et al. 2005). Another non-exclusive explanation is that microtubule dynamics contribute to posterior spindle displacement. The time period
that an individual microtubule remains near the cortex is shorter in the posterior than in
the anterior of the embryo (Labbe et al. 2003), suggesting that microtubules are more
dynamic at the posterior cortex. This difference also depends on PAR protein polarity
(Labbe et al. 2003). Furthermore, if shortening of astral microtubules is induced during
metaphase/anaphase transition, either by microtubule depolymerizing drugs or the zyg-8
mutation, the spindle moves from the cell center to the posterior pole (Hyman et al. 1987; Bellanger et al. 2001; Grill et al. 2001; Bellanger and Gonczy, 2003). To
conclude, both motor proteins such as dynein and/or microtubule depolymerization
could contribute to generate the force required for spindle displacement.

1.3.5 Asymmetric spindle positioning - molecular mechanisms

1.3.5.1 Heterotrimeric G Proteins

Although the force generators at the cortex have not been unambiguously identified,
heterotrimeric G protein signaling is known to control pulling forces downstream of the
PAR-proteins. The heterotrimeric G protein consists of three subunits, Go, Gβ and Gγ.
In C. elegans there are two Go proteins of redundant function, GOA-1 and GPA-16. In
1-cell embryos lacking Go, pronuclear meeting is central, the spindle is shorter and
displays two round asters, rocking movements and its displacement to the posterior is
absent, resulting in a symmetric cleavage similar to the par-2 phenotype (Gotta and
Ahringer, 2001). This is a consequence of lower forces acting on both spindle poles
(Colombo et al. 2003).

The single Gβ subunit, GPB-1, has been shown to oppose Go function. Embryos
depleted of gpb-1 display abnormal rocking of the pronuclear/centrosomal complex and
failure in centration, possibly from too much force being applied to astral MTs (Tsou et
al. 2003). Both spindle poles oscillate during spindle elongation. Measurements of the
forces acting on the spindle poles showed equal and high peak velocities on both poles
in gpb-1 embryos comparable to posterior wild-type intensity. However, cleavage is
still asymmetric, because this phenotype of Gβ depletion appears to be due to the
centration defect, since the spindle sets up in the posterior part of the cell. Interestingly,
PAR distribution is not affected by depletion of the HGP subunits, leading to the conclusion that the PARs act either upstream or on the same level of HGP (Ahringer et al. 1996; Emerson et al. 2000; Gotta and Ahringer 2001; Figure 6).

Figure 6. Schematic representation of a polarized metaphase C. elegans embryo
Anterior domain consisting of PAR-6, PAR-3 and PKC-3 (green) and posterior domain, where PAR-1 and PAR-2 are localized (red). The mitotic spindle is posteriorly displaced, the posterior aster flattened, resulting in an asymmetric cleavage in cell size. Heterotrimeric G protein signaling is regulated by PAR genes and involved in spindle positioning. Mutations in the PAR proteins, as well as mutations in the heterotrimeric G Proteins lead to a more symmetric cleavage. 1. eggshell, 2. spindle pole, 3. mitotic spindle, chromosomes (blue) are aligned at the metaphase plate, 4. microtubules.

GOA-1 and GPA-16 are distributed all over the cortex (Ahringer et al. 1996; Gotta and Ahringer 2001). Taken together these data indicate that GOA-1 and GPA-16 are required to generate the forces that displace the mitotic spindle. Therefore, something
has to regulate G proteins asymmetrically to guarantee the asymmetric forces present in the wild-type embryo.

1.3.5.2 Regulation of heterotrimeric G protein complex in C. elegans

Heterotrimeric G-Proteins are well known for their role in transducing extracellular signals via seven transmembrane receptors (Hamm et al. 1998). Upon ligand binding to the receptor, the G-Protein coupled receptor (GPCR) promotes exchange of GDP for GTP in the Gα subunit which then leads to dissociation of the Gβγ from the Gα-subunit and of all three subunits from the receptor. Signaling is terminated by the GTPase activity of the Gα-subunit. Both Gα and Gβγ have the potential to signal to downstream effectors (Figure 7A).

Interestingly, the C. elegans one-cell embryo is surrounded by an impermeable eggshell and is unlikely to be polarized by extracellular signals. GoLoco domain containing proteins are known to mediate the activation of G-proteins in several organisms. This class of proteins are able to bind Gα subunits and appear to provide a receptor independent activation of Gα proteins to influence mitotic spindle positioning (Cai et al. 2003; Schaefer et al. 2001; Schaefer et al. 2001; Yu et al. 2000). In C. elegans these GoLoco motif containing proteins are represented by two nearly identical proteins, GPR-1 and GPR-2, that are widely conserved (Figure 7B). GPR-1/2 is the functional homologue of AGS-3 in mammals and PINS in Drosophila. The generation of asymmetric GOA-1/GPA-16 dependent cortical forces may be regulated by GPR-1/2 in C. elegans, since its localization is concentrated on the posterior cortex in an asymmetric manner during metaphase and anaphase (Colombo et al. 2003; Gotta et al. 2003). One theory for regulation is that instead of a GPCR, GPR-1/2 binds to Gα and triggers a signal, promoting the dissociation of Gα from Gβγ. GPR-1/2 stabilizes Gα in its GDP bound form free of Gβγ. Genetic data indicates that both Gα and GPR-1/2 play a positive role in spindle positioning. Therefore, one possible model is that GPR-1/2-GαGDP is the signaling molecule. Another possibility is that GPR-1/2-GαGDP is activated by another protein- this model is discussed below.
Figure 7. Regulation of the heterotrimeric G protein in C. elegans

(A) General mechanism for regulation: The heterotrimeric G Protein (HGP) consists of three subunits: Ga and Gßy. (A) ligand bound G-protein coupled receptor (GPCR) acts as guanine exchange factor (GFF) forming GTP-bound Ga, thereby dissociating Ga from Gßy.

(B) Possible model of regulation: GPR-1/2 GoLoco domain containing protein dissociates the HTG by binding to GDP-bound state of Ga. The GPR-1/2-Ga complex can be converted by GEF proteins into GTP-bound Ga. Signaling is either terminated by the release of GPR-1/2 or by the GTPase activity of Ga.

Further indication for GPR-1/2's role in regulating Gα function is derived from phenotypical observations. Loss of GPR-1/2 results in a similar phenotype to that
described for goa-1; gpa-16 embryos and in reduced forces acting on the spindle poles, leading to a shorter, mispositioned spindle and a more symmetric cleavage (Gotta and Ahringer, 2001; Colombo et al. 2003). GPR-1/2 is a good candidate for the asymmetric regulation of HGP signaling downstream of PAR polarity. Localization of GPR-1/2 is dependent on PAR proteins. Loss of PAR-2 leads to loss of cortically localized GPR-1/2, while depletion of PAR-6 results in high levels of GPR-1/2 all over the cortex. Certainly, forces in wild-type embryos are higher on the posterior pole where GPR-1/2 is enriched, while the forces are reduced on both spindle poles in par-2 mutants, where GPR-1/2 levels are lost (Grill et al. 2001, Gotta et al. 2003), but in par-6 forces are high on both spindle poles (Grill et al. 2001). Therefore, by a yet unknown mechanism, still to be determined, the cortical polarity of the embryo acts through the PAR proteins (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003) to up-regulate G protein signaling in the posterior, thereby increasing the total force (Colombo et al. 2003), which then leads to asymmetric spindle positioning.

Another large coiled-coil protein called LIN-5 has previously been shown to physically interact with GPR-1/2. Interestingly, mutations in lin-5 also lead to a symmetric spindle, lacking any displacement during metaphase to anaphase transition and resulting in an equal cleavage. This phenotype is reminiscent of the phenotypes of gpr-1/2 or Ga, as described above.

GPR-1/2 are counteracted by another protein called LET-99, a non-conserved DEP (Dishevelled/EGL-10/Pleckstrin) domain protein, localized on the cortex and also required for spindle alignment along the anterior-posterior axis (Rose and Kemphues 1998; Tsou, Hayashi et al. 2002). LET-99 is required for nuclear rotation and asymmetric anaphase spindle movements in P0 and P1. It is enriched in an asymmetrically positioned band in P lineage cells in response to PAR-polarity cues (Rose and Kemphues 1998; Tsou et al. 2002). The mislocalization of LET-99 leads to nuclear centrosome complex (NCC) rotation defects and strong oscillations of the NCC and the mitotic spindle during anaphase reminiscent of observations in par-3 mutant embryos (Tsou et al. 2002; Tsou et al. 2003). These observations raised the hypothesis that the cortical LET-99 band is an intermediate, translating PAR cues into the asymmetric forces needed for nuclear rotation and anaphase spindle movement (Tsou et
al. 2002; Tsou et al. 2003). Interestingly, it was shown that the migration of the NCC complex, that is accompanied with a smooth 90° rotation to orient the centrosomes along the longitudinal axis of the embryo, is under control of the dynein/dynactin complex. The strong reduction of dynein activity inhibits this rotation event in wild-type, while weaker reduction suppresses the hyperactive oscillations in let-99 mutants. To summarize, Gα, GPR-1/2 and LIN-5 play a positive role in spindle positioning while LET-99 counteracts Gα and GPR-1/2 function. The mode of activation of the HGP is still unclear and further discussed below.

1.3.6 RIC-8 is involved in spindle positioning

1.3.6.1 RIC-8 in C. elegans

To better understand HGP signaling and in order to find physical interactors with the mammalian Gα subunits, Gαq or Gαo, several mutant screens were performed and suppression analysis was carried out (Miller et al. 2000; Hajdu-Cronin et al. 1999). The outcome of these screens was one class of mutations termed Ric (resistant to inhibitors of cholinesterase), selected for their ability to survive the neurotoxin effects of cholinesterase inhibitors (Miller et al. 2000). One of these, ric-8, functions with the G-protein subunit goa-1 (Miller et al. 2000). Interestingly, RIC-8 is a maternally expressed gene important for embryo development. Mutations in ric-8 result in weak or absent centrosomal rocking. In the background of goa-1 mutants this phenotype increases, showing complete absence of rocking, while goa-1 mutants alone display only less pronounced movements. Furthermore, goa-1;ric-8 mutants show posterior displacement of the spindle to a lesser extent, resulting in an AB cell only slightly bigger than P1 (Miller and Rand, 2000). Both proteins are also important for proper centrosome migration in C. elegans. Absence of one of them results in weakly misplaced nuclei, while depletion of both results in strong misplacement (Miller and Rand, 2000). In general the time required for wild-type to progress through the first two cycles of cell
division was significantly longer in the single mutants and even longer in goa-1;ric-8 embryos (Miller and Rand, 2000). At later stages, ric-8 embryos display spindle misalignments, particularly in AB, P1, ABa and ABp. In these embryos spindle orientation appears random. However, polarity is normal.

In conclusion, RIC-8 is needed for several aspects during early embryogenesis in C. elegans.

### 1.3.6.2 Mammalian RIC-8 is a GEF for Gα

Two RIC-8 proteins exist in mammals, Ric-8A (or Ric-8/synembryn) and Ric-8B, both are homologous to C. elegans RIC-8. In a former Yeast-2-Hybrid screen to identify novel G protein signaling factors, with Gα0 and Gαs subunits as baits, it was found that both, Ric-8A and Ric-8B, are able to physically interact with Gα (Tall et al. 2003). Further biochemical characterization showed that Ric-8A can be a potent guanine exchange factor (GEF) for Gαq, Gα11 and Gαs. RIC-8 interacts with GDP bound Gα subunits in the absence of Gβγ, causing the release of GDP to form a stable, nucleotide free Gα-Ric-8A complex. In the next step, GTP binds to Gα and disrupts the binding with Ric-8A, resulting in an activated GoGTP protein (Tall et al. 2003). Ric-8A is not able to bind to GαGTP. To conclude, mammalian RIC-8 in vitro behaves as a GEF for free Gα subunits. This data suggests that RIC-8 may play a similar role in C. elegans and contribute to spindle positioning by activating Gα.
1.4. Asymmetric cell division in the model system *Drosophila*

1.4.1 Asymmetric division in *Drosophila* neuroblasts

The *Drosophila* central nervous system progenitor cells, the neuroblasts (NB), are specified within a monolayered epithelium, the ventral neuroectoderm. They delaminate from the epithelium, come to lie beneath the epithelial cell layer and undergo repeated rounds of asymmetric cell divisions in a stem cell like fashion (Figure 8). Each division gives rise to a small basal daughter cell called ganglion mother cell (GMC), and a larger apical daughter cell, the NB. The GMC divides only once more to form neuron and glia cells, whereas the apical daughter cell continues to divide asymmetrically.

![Figure 8: Asymmetric cell division in *Drosophila* neuroblast cells](image)

*Drosophila* neuroblasts (NB) are located underneath the neuroectoderm. They divide along their apical-basal axis to generate a bigger apical daughter cell that retains neuroblast characteristics. The smaller basal ganglion mother cell (GMC; green) divides once more into neuronal and glia cells.

The neuroblast asymmetric cell division is achieved by asymmetric localization and segregation of cell fate determinants to the basal cortex, followed by the positioning of the spindle along the axis of polarity. Asymmetry of daughter cell size is the result of an highly asymmetric spindle (Kaltschmidt et al. 2000; 2002; see also Figure 9). In
addition, the spindle is also displaced towards the basal cortex during ana- to telophase transition (Kaltschmidt et al. 2002).

Neuroblast spindle orientation and asymmetry is controlled, in part, by the localized activity of apical cortical proteins. This includes the evolutionary conserved Par-domain consisting of Bazooka, the *Drosophila* orthologue of PAR-3 (Kuchinke et al. 1998), together with *Drosophila* PAR-6 (DmPAR-6; Pedronczki et al. 2001) and *Drosophila* atypical PKC (DaPKC; Wodarz et al. 2000). Each of these three proteins binds to each other. The absence of one of these proteins results automatically in re-localization of the others to the cytoplasm. Also found to co-localize together with the PAR-domain in NB cells is Inscutable (Insc), which also forms a crescent at the apical cortex (depicted in Figure 9). This crescent is maintained during the first divisions until it disappears in telophase. Lack of Inscutable leads to rotation failure of the mitotic spindle and to an apical-basal orientation (Kaltschmidt et al. 2000). Interestingly, Insc interacts with Pins (Partner of Inscutable), the functional homologue of *C. elegans* GPR-1/2. As in *C. elegans*, HGP is also required in *Drosophila* to control spindle orientation and positioning. The heterotrimeric G protein subunit Go1 is found in a complex with Baz, DmPAR-6, DaPKC, Insc and Pins.

**Figure 9. Apical-basal polarity of a Drosophia NB cell**

Baz (Par-3), Par-6 and aPKC (blue) are found in one complex, localized to the apical cortex of NB cells. Go1 (yellow) and its two GDIs (Pins and Loco) colocalize with the apical PAR-domain (blue). Cell fate determinants like Prospero and Numb (green) are relegated to the basal side of the NB cell and later found in the GMC descendant. The mitotic spindle is asymmetric in shape with longer MTs at the apical side and additionally positions more towards the basal cortex, resulting in a bigger NB cell and a smaller GMC. (figure adapted from Matsuzaki, 2005)
As with GPR-1/2, Pins functions as a guanine nucleotide dissociation inhibitor (GDI) for Gαi (Parmentier et al. 2000; Schaefer et al. 2000; Yu et al. 2000). In terms of asymmetric spindle formation, Baz, DmPAR-6 and aPKC act redundantly to Pins/Gαi in NB cells. The simultaneous disruption of the two pathways prevents the formation of an asymmetric spindle, which results in a symmetric cleavage (Cai et al. 2003).

Interestingly, the localization of the cell fate determinants Numb, Prospero and Miranda remains asymmetric if only one protein of the Par-domain is lacking, but their crescents form at random positions around the cell cortex and not at the basal position, like in wild-type NB (Kraut et al. 1996; Knoblich et al. 1995). In contrast, Numb and Miranda crescents do not form at all in absence of both Bazooka and DmPAR-6 (Schober et al. 1999; Wodraz et al. 1999; Petronczki et al. 2001).

Gβ13F, the β subunit of HGP, affects the segregation of cell fate determinants and determines the difference in daughter cell size during NB division. Loss of Gβ13F results in a large, symmetric spindle with emerged MTs on both spindle poles and division into two cells similar in size (Fuse et al. 2003). Interestingly, overexpression of Gα (Gαi/Gαo) leads to a similar phenotype as described for Gβ13F mutants (Schaefer et al. 2001; Yu et al. 2003; Fuse et al. 2003; Izumi et al. 2004). As overexpressed Gα should be largely in the GDP bound state, it can sequester free Gβγ. Therefore, the symmetry of division in Gα overexpressed cells could be due to Gαi mediated repression of Gβ activity. However, it is not clear whether Gβγ mediates spindle geometry independently of the Gαs, or alternatively, by controlling the localization of Gα together with Pins, by promoting dissociation of Gβγ from the heterotrimer, leading to a stabilized Gα,GDP state (Schaefer et al. 2001). Intriguingly, loss of Pins function alone does not result in severe spindle defects as seen in Gβ13F or Gγ1 mutant NB, suggesting that the absence of Pins GDI activity does not prevent generation of free Gβ13F.

Another GoLoco-motif-containing protein identified in Drosophila is Loco (Locomotion defects), which is also a GDI for Gαi. The removal of both GoLoco proteins, Pins and Loco, results in defects similar to those observed for Gβ13F and Gγ1 mutants (Yu et al. 2005). This suggests that Loco and Pins act synergistically to release Gβγ in NBs.
Recently, another candidate termed Mud (Mushroom body defect) was found to be important for asymmetric cell division in NB cells (Izumi et al. 2006). Mud (homologue to *C. elegans* LIN-5) physically interacts with Pins, which recruits it to the apical cortex. Furthermore, Mud localizes to centrosomes during mitosis, binds to microtubules and enhances MT polymerization (Izumi et al. 2006; Bowman et al. 2006; Siller et al. 2006). In the absence of Mud, the spindle is not able to align within the axis of polarity although cortical polarity is normal (Siller et al. 2006) resulting in missegregation of cell fate components. These findings lead to the suggestion that Mud plays an important role in linking spindle orientation to cortical polarity (Bowman et al. 2006; Siller et al. 2006).

### 1.4.2 Asymmetric cell division in SOP cells

In *Drosophila*, sensory organ precursor (SOP) cells of the peripheral nervous system also divide asymmetrically (Jan and Jan 2001; Bardin et al. 2004). The SOP cells, also known as pI cells, give rise to four founder cells later creating the external sensory organs. pI cells divide asymmetrically along the anterior-posterior axis within the plane of the epithelium, generating an anterior pIIb and a posterior pIIa cell (Figure 10 and 11). These daughter cells are different in morphology and developmental potential. The pIIb descendants are important for the formation of internal structures and give rise to glia cells, which undergo apoptosis, while pIIa daughters create external structures.

![Figure 10. Drosophila SOP cell division](image)

*Drosophila* SOP cells divide in an epithelial cell layer along their anterior-posterior axis and generate two nearly equal sized morphologically different daughter cells that form the internal (pIIb, green) and external (pIIa, red) cells of the sense organ.
Similar to NB cells, the Par-domain consists of Baz, DmPar6 and DaPKC. In contrast to NB cells, this complex is found anteriorly in the SOP cells (Jan and Jan, 2001; Figure 11), regulated by epithelial planar polarity cues ignoring apical-basal polarity (described above). Another major difference to NB cells is that InsC is not expressed in SOP cells, resulting in a Par-domain that is localized in a cortical crescent opposed by Pins/Ga. This establishment occurs in two steps: First the planar polarity induces polarization of Pins, followed by a second phase where Pins/Ga and the PAR complex are maintained separately from each other, independent of planar polarity cues (Bellaiche et al. 2001). Another important difference to NB division was observed in spindle positioning. In SOP cells the spindle itself is not asymmetric (as in NB cells, described above), the posterior centrosome is instead pushed off the cell cortex (Roegiers et al. 2001) and this asymmetrically positioned spindle results in unequal descendants.

In summary, this shows that although the molecules important for asymmetric cell division are similar in *C. elegans* and *Drosophila*, the mechanisms that lead to asymmetry can vary from cell to cell.

Figure 11. Asymmetric cell division in *Drosophila* SOP cells

In SOP cells the Par domain, consisting of Baz, DmPar6 and aPKC (blue), is localized to the posterior cell cortex. On the opposite side, Ga and its GDIs (Pins and Loco) are found (yellow). The serpentine receptor Frizzled is also asymmetrically localized and found on the posterior cortex. The mitotic spindle is symmetric in shape but asymmetrically localized during division. The posterior centrosome (red) is pushed off the cell cortex, which results in an asymmetric cleavage in cell size and fate (Figure adapted from Matsuzaki, 2005).
1.5 Asymmetric cell division in mammals

1.5.1 Asymmetric division in mammalian cells

The basal epidermal cells derived from mouse use their polarity to divide asymmetrically, orienting the mitotic spindle perpendicularly to the basement membrane, generating a committed suprabasal cell and a proliferative basal cell. Since the two daughter cells of the apical-basal division adopt different developmental fates, it has been proposed that asymmetric cell division is essential for the initiation of stratification during epidermal morphogenesis.

Similar to the molecular core in Drosophila and C. elegans, described above, the mammalian Par-domain, containing Par-3, Par-6 and PKCζ, is found as cortically localized complex. This exclusively anterior localized complex recruits the adaptor protein LGN. There are two proteins present in mammals, LGN and AGS3, which are functional homologues of Drosophila Pins and C. elegans GPR-1/2. They have been shown to bind specifically to Go1 and Go2 subunits of the HGP (Willard et al. 2004). It was recently shown that LGN behaves as a conformational switch molecule: In its closed state its N- and C-termini interact, leading to a closed conformation (Figure 12). The N-terminus of LGN can bind to NuMA (homologue of Drosophila Mud and orthologue of C.elegans LIN-5), a large coiled-coil protein that is nuclear in interphase and organizes the spindle poles during mitosis in cultured cells (Gaglio et al. 1995; Du et al. 2001; Du and Macara 2004; Kisurina-Evgenieva et al. 2004). With its C-terminus it can bind to Go1. Therefore, the closed conformation of LGN can be disrupted either by NuMA or by Go1, allowing LGN to interact simultaneously with both proteins, resulting in their cortical localization (Figure 12). Interestingly, overexpression of LGN or Go1 results in pronounced oscillations and rotations of the mitotic spindle in epithelia cells. This indicates stronger forces acting on astral microtubules of the spindle apparatus (Du and Macara, 2004). The complex consisting of Go1GDP, LGN and NuMA seems to promote spindle oscillations by increasing the pulling forces exerted on the mitotic spindle during mitosis. This raises the question, how does the complex interact with the mitotic spindle.
Recently, another adaptor molecule, the mammalian homologue of *Drosophila* Inscutable, mInsc (mouse Inscutable), has been found in perpendicularly dividing basal cells (Lechler and Fuchs, 2005). Intriguingly, mInsc was also found in a complex with LGN and Par-3 (Lechler and Fuchs, 2005). Additionally, NuMA was shown to participate in the proper alignment of the spindle poles with the LGN-Insc-Par-3 complex to govern asymmetric cell divisions (Lechler and Fuchs, 2005). At the molecular level NuMA contributes to the formation of microtubule bundles that are organized in a focused array at each spindle pole, thereby enhancing microtubule polymerization (Du et al. 2002) and interacting with the minus end-directed motor protein dynein (Merdes et al. 1996). Both effects could provide a mechanistic explanation for a potential role in spindle orientation. These findings indicate that NuMA and dynein-dynactin dependent pulling forces at the apical cortex might function together to generate asymmetric division of cells. The spindle poles could be formed and stabilized by NuMA, dynein and dynactin, bound in a large complex that moves toward microtubule minus ends, thereby also regulating spindle length (Gaetz and Kappor 2004).

![Diagram](image)

**Figure 12. Molecular switch mechanism to regulate microtubule attachment to the cell cortex in mammalian cells**

In interphase LGN (blue) is in its inactive closed conformation and NuMA (green) localization is nuclear (1.). Goi (yellow) is localized at the cortex (2.). After release of NuMA, it can bind to the N-terminus of LGN, allowing the C-terminus of LGN to interact with cortical Goi. This leads to the formation of a cortical localized trimeric complex. Cortical NuMA might induce pulling forces acting on astral microtubules (black lines), by interacting with dynein/dynactin complex (orange), which moves in a minus end direction along MTs (Figure adapted from Hampoe/L, 2004 and modified).
1.5.2 Mammalian neuronal asymmetric cell division

Other insights into asymmetric cell division derive from mouse cerebral cortical progenitor cells. The neuronal progenitor cells generate neurons over multiple divisions at different times during mammalian development. Prior to the onset of neurogenesis, most progenitor cells undergo symmetric cell division to generate two daughter cells that both adopt the progenitor fate. As cortical development proceeds, the progenitor cells start to divide asymmetrically giving rise to one neuron and one progenitor cell (Chenn and McConnell 1995; Takahashi et al. 1996; Miyata et al. 2001; Noctor et al. 2001). Later on, the progenitor cells return to symmetric divisions, producing two neuronal cells (Kornack and Rakic, 1995; Takahashi et al. 1996; Miyata et al. 2001; Cai et al. 2002). During these processes, the progenitors change their cleavage plane from perpendicular to the ventricular surface to parallel and back to the vertical axis (Chenn and McConnell, 1995; Haydar et al. 2003). The orientation of the mitotic spindle correlates with the cell fate decision of the two daughter cells in each step. As known from C. elegans and Drosophila, G proteins are very important for spindle orientation in these cells. Moreover, they are regulated by nonreceptor activators GPR-1/2 in C. elegans and Pins in Drosophila, as mentioned above. The mammalian ortholog AGS3 (activator of G protein signaling 3) plays the same role in spindle regulation. Silencing of AGS3 results in similar defects due to impairment of Gβγ (Sanada and Tsai, 2005). It was previously shown that inhibition of Gaαi results in the reduction of proliferating cells in the neurocortex, showing that Gaαi/Gβγ are important for proliferation of neuronal progenitor cells (Shinohara et al. 2004). Gaαi normally sequesters the free Gβγ, thereby inhibiting Gβγ signaling, resulting in misoriented spindles (Federman et al. 1992; Crespo et al. 1994). Overexpression of Gaαi results in higher percentage of misaligned spindles, leading to more neuronal cells (from symmetrically dividing mothers) and less progenitor cells (Sanada and Tsai, 2005). These observations indicate that impaired Gβγ signaling in progenitor cells leads to overproduction of neurons as a consequence of both daughter cells adopting the neuronal fate. Mammalian AGS3 physically interacts with Gaαi, raising the possibility that these molecules are likely to function together in progenitor cells to regulate the division plane (Sanada and Tsai, 2005). Moreover, Gβ is associated with Gaαi, and the localization of Gβ within the
progenitor is important for the cleavage plane, apical-basal Gβ resulting in a horizontal cleavage plane, whereas lateral or apical-lateral Gβ results in a vertical one. Deduced from this data it is likely that Gβγ subunits establish the axis of division by localizing to the interface between astral microtubules and the plasma membrane, thereby regulating their interactions (Sanada and Tsai, 2005) and AGS3 is needed for the activation of Gβγ (see above and Sanada and Tsai, 2005).

In short, AGS3-Gβγ signaling regulates apical-basal division and asymmetric cell fate decisions, which are linked to the mitotic spindle orientation in neuronal progenitor cells.

1.6. Similarities and differences

The study of asymmetric cell division in different model systems has shown that several core mechanisms involved in establishing cell polarity and in signaling are conserved. In particular, the PAR-3, PAR-6, aPKC complex and the GαGPR-1/2 complex have functions in regulating asymmetric cell divisions. However, in different cell types the role of these complexes differ, suggesting that the regulation of asymmetric division can be a plastic process, where cells use the activities of these complexes to achieve different modes of asymmetric cell divisions (e.g. apical-basal versus planar division or the positioning of the spindle).

To summarize, in the C. elegans embryo the posterior spindle pole is pulled to the posterior, whereas it is pushed away in Drosophila SOP cells, both resulting in an asymmetrically localized spindle. In Drosophila neuroblast cells, the asymmetry itself is ensured by generation of an asymmetric spindle in addition to a slight displacement of minor importance.

Another major difference between NB cells and C. elegans embryos is found at the molecular level. As described above for C. elegans, GPR-1/2 is localized opposite to the PAR-domain. Its depletion results in similar phenotypes to the depletion of Gα (GOA-1 and GPA-16). In Drosophila, the functional homologue Pins is co-localized with the Par-complex and depletion has only minor effects, due to their roles in two redundant pathways.
Another very important difference between the discussed organisms is the role of the G\(\beta\gamma\) subunits in spindle regulation. In *Drosophila* NB and mammalian neuronal progenitor cells, disrupted G\(\beta\gamma\) function results in misoriented spindles, showing that G\(\beta\gamma\) signaling is important for spindle orientation. In contrast, the *C. elegans* G\(\beta\) subunit has no influence on spindle orientation and positioning.

Another candidate of interest in all species is *C. elegans* LIN-5 (*Drosophila* Mud/mammalian NuMA). This protein could build a bridge between cortical polarity and mitotic spindle regulation. It physically interacts with GPR-1/2 (Pins/LGN), localizing to centrosomes and microtubules (Izumi et al. 2006; Yu et al. 2006; Bowman et al. 2006). Therefore, it could provide a new idea for signaling, connecting MT dynamics with cortical polarity (Figure 13). NuMA and Mud are also reported to play a role in MT polymerization and interaction with the dynein/dynactin complex. From the conformation model, described above for LGN and its binding partners G\(\alpha\) and NuMA, dynein is a good candidate for a target of the HGP signaling pathway to directly regulate the mitotic spindle. It would be interesting if this were also true for *C. elegans* LIN-5.
Components of the known spindle regulation pathway are conserved throughout evolution. In vertebrates LGN is interacts with NuMA and Ga. NuMA is proposed to bind to LGN first, later Ga is able to interact with LGN. NuMA is released from the membrane and transported to the spindle pole via the MTs. A similar ternary complex is proposed for C. elegans and Drosophila. Here, LIN-5 or Insc interact with GPR-1/2/Pins which bind to GOA-1/Gα.

**Figure 13. Schematic representation of interaction models in different organisms**

1.7 Open questions for spindle orientation and positioning in C. elegans

Today, the significance of asymmetric cell division for the development of multicellular organisms is widely recognized. Most of the mechanistic insight into this process comes, however, from invertebrate model systems, especially Drosophila and C. elegans. In recent years, a conserved general mechanism for asymmetric cell division has been discovered: The asymmetric localization of PAR proteins polarizes the cell cortex, orients the mitotic spindle through heterotrimeric G-proteins and directs the segregation of determinants into only one of the two daughter cells. Despite this progress, there are still many open questions. How are cell fate determinants
segregated? How are the asymmetrically localized proteins regulated and their asymmetric distribution achieved? How are G-proteins regulated and how do they act on microtubules? Could it be possible that HGP signaling works via dynein to generate forces acting on the mitotic spindle?

1.8 Aim of the thesis

The aim of my thesis was to better understand the molecular mechanism of spindle regulation in the early *C. elegans* embryo. The problems of regulating the mitotic spindle can be formulated in three main questions that were addressed in the different parts of my work:

1. How are the G protein Gα subunits regulated in the early *C. elegans* embryo?
2. Are GOA-1 and GPA-16 acting on one substrate in terms of spindle regulation?
3. What are the targets of the HGP?
CHAPTER II

Control of Embryonic Spindle Positioning and Gα Activity by

*C. elegans* RIC-8

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Annina C. Spilker's contribution to this work:

Construction and cloning of the constitutively active form of GOA-1 (GOA-1 Q205L) into pDONR201.

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2.1 Summary

Asymmetric spindle positioning is of fundamental importance for generating cell diversity during development. In the *C. elegans* 1-cell embryo, spindle positioning has been shown to depend on heterotrimeric G protein signaling. Two Gα subunits, GOA-1 and GPA-16 (hereafter Gα), and receptor independent activator of G protein signaling GPR-1 and GPR-2 (GPR-1/2) are required for proper regulation of spindle positioning (Schneider and Bowerman, 2003). However, it remains unclear whether Gα regulates spindle positioning in its GDP or GTP bound form. Here, we investigate the role of RIC-8 in this pathway. RIC-8 was genetically shown to act in concert with *goa-1* to regulate centrosome movements in *C. elegans* (Miller et al. 2000; Miller and Rand, 2000). Interestingly, mammalian RIC-8 was recently found to behave as a GEF for Gα subunits in vitro (Tall et al. 2003). We show that reduction of function of *ric-8* results in a 1-cell embryo very similar to the phenotype of embryos depleted of Gα. RIC-8 is able to bind to GOA-1, preferentially to GOA-1-GDP, consistent with a GEF role. RIC-8 is localized at the embryo cortex, and its activity is essential for the asymmetric localization of GPR-1/2. We suggest that RIC-8 directly modulates Gα activity and that Gα-GTP is the signaling molecule regulating spindle positioning in the early embryo.
2.2 Introduction

During the first division of *C. elegans* embryos, the mitotic spindle is asymmetrically positioned along the anterior-posterior axis (reviewed in (Schneider and Bowerman, 2003)). This asymmetry is the result of differential pulling forces acting on the spindle poles, with a stronger net force acting on the posterior pole (Grill et al., 2001). The regulation of pulling forces is under the control of partitioning-defective (PAR) proteins (Grill et al. 2001). Ga and GPR-1/2 are required for regulating spindle positioning downstream of the PAR proteins (Colombo et al. 2003; Gotta and Ahringer, 2001; Gotta et al. 2003; Srinivasan et al., 2003). Like the mammalian AGS3 and *Drosophila* PINS (Bernard et al. 2001; De Vries et al. 2000; Natochin et al. 2001; Natochin et al. 2000; Peterson et al. 2000; Schaefer et al. 2001), GPR-1/2 has been shown to be a GDP dissociation inhibitor for GOA-1 (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003). Because loss of GPR-1/2 and loss of Ga result in identical phenotypes, a model was proposed in which Ga-GDP, or a GPR-1/2-Ga-GDP complex, is the active molecule in spindle positioning, as has also been proposed in the *Drosophila* neuroblast (Colombo et al. 2003; Gotta et al. 2003; Izumi et al. 1998; Schaefer et al. 2001; Srinivasan et al. 2003; Yu et al. 2003). This model was challenged by the recent finding that Synembryn, the mammalian RIC-8 homolog, acts as a guanine exchange factor (GEF) for Ga subunits (Tall et al. 2003). Because *C. elegans* ric-8 has been shown to be required for early aspects of embryogenesis (Miller and Rand, 2000), an alternative model in which Ga-GTP is the active molecule promoting spindle positioning was proposed (Gotta et al. 2003; Srinivasan et al. 2003).

In *C. elegans*, ric-8 reduction-of-function mutants were shown to exhibit embryonic lethality and to be required for mitotic spindle rocking and spindle orientation (Miller and Rand, 2000). The phenotype of ric-8 mutants was shown to be greatly enhanced by a 50% reduction in maternal goa-1 dosage (Miller and Rand, 2000). This indicates that goa-1 and ric-8 are likely to function in the same processes, but the phenotype of ric-8 mutants has not been directly compared to the phenotype of embryos depleted of GOA-1 and GPA-16 by RNA interference (RNAi, Fire et al. 1998), goa-1(RNAi);gpa-16(RNAi), hereafter referred to as Ga(RNAi) and embryos depleted of GPR1/2.
Chapter II

2.3 Results and Discussion

To explore the relationship between ric-8 and the Gα pathway, we reexamined the ric-8 phenotype and focused on the early events in the 1-cell embryo. In ric-8(mdl1909) mutant embryos, early events such as meiosis, pronuclear migration, meeting, and centration are normal. However, nuclear rotation is delayed in 30% of the embryos (n=24), the mitotic spindle fails to rock as it elongates, and it is only weakly displaced to the posterior, resulting in a cleavage that is more symmetric than in the wild-type (see Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Spindle length(^a)</th>
<th>Position of First Cleavage(^b)</th>
<th>Percentage of Embryonic Lethality(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n=27)</td>
<td>42.5 ± 3.7</td>
<td>58.3 ± 1.9</td>
<td>0</td>
</tr>
<tr>
<td>goa-1(RNAi) (n=13)</td>
<td>42.0 ± 2.5</td>
<td>56.6 ± 2.7</td>
<td>11</td>
</tr>
<tr>
<td>goa-1(n1134) (n=21)</td>
<td>41.2 ± 1.8</td>
<td>57.5 ± 2.4</td>
<td>16</td>
</tr>
<tr>
<td>gpa-16(RNAi) (n=14)</td>
<td>40.8 ± 1.7</td>
<td>55.6 ± 2.5</td>
<td>10</td>
</tr>
<tr>
<td>ric-8(mdl1909) (n=32)</td>
<td>39.7 ± 2.6</td>
<td>54.6 ± 2.2</td>
<td>30</td>
</tr>
<tr>
<td>ric-8(mdl1909);ric-8(RNAi) (n=25)</td>
<td>37.5 ± 2.0</td>
<td>52.8 ± 1.9</td>
<td>72</td>
</tr>
<tr>
<td>ric-8(mdl1909);goa-1(RNAi) (n=18)</td>
<td>37.0 ± 3.6</td>
<td>52.3 ± 2.4</td>
<td>91</td>
</tr>
<tr>
<td>goa-1(n1134);ric-8(RNAi) (n=18)</td>
<td>37.4 ± 2.4</td>
<td>51.6 ± 2.3</td>
<td>100</td>
</tr>
<tr>
<td>ric-8(mdl1909);gpa-16(RNAi) (n=25)</td>
<td>38.1 ± 1.9</td>
<td>52.9 ± 1.8</td>
<td>54</td>
</tr>
<tr>
<td>Gα(RNAi)(^d) (n=10)</td>
<td>36.7 ± 2.0</td>
<td>50.9 ± 1.3</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Spindle length and position of first cleavage

Both the spindle length and the position of first cleavage were calculated as percentage of egg length. n is the number of embryos analyzed.

\(^a\)Spindle pole separation was calculated at anaphase (by dividing spindle length by embryo length).

\(^b\)Anterior is 0%.

\(^c\)For the embryonic lethality, more than 100 progeny were counted in each case.

\(^d\)Gα(RNAi) is goa-1(RNAi);gpa-16(RNAi).

The difference in the position of the first cleavage of ric-8(mdl1909) embryos compared to ric-8(mdl1909);ric-8(RNAi) embryos, ric-8(mdl1909);goa-1(RNAi), or ric-8(mdl1909);gpa-16(RNAi) embryos is significant (p < 0.0025, p < 0.006; p < 0.001 respectively, t test).

In addition, spindle morphology is symmetric with both anterior and posterior asters having a round morphology at late anaphase in contrast to wild-type embryos, in which
the anterior aster is round but the posterior one has a flat appearance (Figure 14B, Miller and Rand, 2000).

**Figure 14.** The phenotype of *ric-8(md1909); ric-8(RNAi)* embryos is similar to that of *Ga(RNAi)* embryos

(A) Wild-type embryo. The posterior aster is disc shaped, and the first cleavage is asymmetric, with the posterior cell smaller than the anterior.

(B) *ric-8(md1909); ric-8(RNAi)* The posterior aster is round, like the anterior one, and the first cleavage is symmetric as in (C) *Ga(RNAi)* embryos. The arrowheads mark the aster. Posterior is right. The scale bar in (C) represents 10 μm.
None of the ric-8 mutants are null mutants, and depletion of RIC-8 by RNAi results in 30 % embryonic lethality (see below and Miller and Rand, 2000). We therefore tested whether depletion of RIC-8 by RNAi in a ric-8 mutant would increase the observed phenotype. Lethality increased from 30 % in ric-8(md1909) to 72 % in ric-8(md1909);ric-8(RNAi). It also resulted in a significantly more symmetric first cleavage (see Table 2). The percentage of embryos with a more symmetric cleavage (cleavage furrow position at less than 54 % egg length) increased from 31 % in ric-8(md1909) to 72 % in ric-8(md1909);ric-8(RNAi) (Table 3). The 1-cell embryo phenotype of symmetric embryos is very similar to that of Ga(RNAi) and gpr-1/2(RNAi) embryos (Figures 14B and 14C, Table 2), suggesting that ric-8 functions in the heterotrimeric G protein pathway during spindle positioning in the 1-cell embryo.

<table>
<thead>
<tr>
<th>Percentage egg length</th>
<th>49% - 54%</th>
<th>&gt; 54%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ric-8(md1909)</td>
<td>31%</td>
<td>69%</td>
</tr>
<tr>
<td>ric-8(md1909); ric-8(RNAi)</td>
<td>72%</td>
<td>28%</td>
</tr>
</tbody>
</table>

Table 2. Percentage of ric-8 embryos with a symmetric first cleavage
The method of clustering the embryos is described in the experimental part.

We therefore asked whether ric-8 genetically interacts with the two Ga subunits to position the spindle asymmetrically. It was previously shown that goa-1/+;ric-8 mothers produce only inviable progeny (Miller and Rand, 2000). We found that, compared to any single mutant, each mutant/RNAi combination of ric-8 and the Ga subunits goa-1 and gpa-16 results in a higher percentage of embryonic lethality (see Table 2). In addition, analysis of these embryos by differential interference contrast (DIC) microscopy revealed that the position of the first cleavage in each mutant/RNAi combination is significantly more symmetric than in any single mutant (see Table 2). These results suggest that RIC-8, GOA-1, and GPA-16 function together or in parallel pathways to promote posterior spindle displacement in the 1-cell embryo.

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To distinguish between these two possibilities, and because mammalian RIC-8 has been shown to behave as a GEF for Gα subunits (Tall et al. 2003), we decided to test whether RIC-8 is able to bind to Gα. GEFs bind to the inactive GDP bound conformation of G proteins, stimulate release of GDP, and stabilize a Gα subunit nucleotide-free transition state that is disrupted by GTP binding (Coleman et al. 1994). We therefore tested whether RIC-8 was able to interact with GOA-1 in the two-hybrid system and whether this interaction was specific for the GDP bound form, as would be expected for a GEF. A full length RIC-8 bait was transformed with wild-type GOA-1 prey and GTPase-deficient GOA-1 prey, which is locked in the GTP bound form (Mendel et al. 1995) or alternative an GOA-1 prey locked in the GDP bound form. This analysis demonstrated that RIC-8 is able to interact with GOA-1 and that it has a clear preference for wild-type GOA-1 or GOA-1-GDP when compared to the GTPase-defective counterpart (Figure 15).

**Figure 15. RIC-8 preferentially interacts with Gα-GDP.**
The interaction of full-length RIC-8 with full length wild-type GOA-1 or full length GOA-1(G204A) but not with GOA-1(Q205L). β-galactosidase activity indicating interaction was measured on a colony lift filter assay.
We then investigated whether RIC-8 can also bind GOA-1 in vitro and whether it preferentially binds GOA-1-GDP as compared to GOA-1-GTP. Consistent with the two-hybrid results, RIC-8 preferentially bound to GOA-1-GDP (Figure 16). We could not detect binding of RIC-8 to GPA-16 by two-hybrid analysis (see the experimental procedures), and we were unable to purify GPA-16 in native conditions, preventing an equivalent analysis of the binding of RIC-8 to GPA-16.

These results show that, like mammalian RIC-8, *C. elegans* RIC-8 preferentially binds to the GDP form of GOA-1. This is consistent with a role as a GEF.

**Figure 16. RIC-8 preferentially interacts with Gα-GDP.**

SDS-PAGE gel stained with Coomassie blue. Lane 1 shows RIC-8, lane 2 HIS-GOA-1-GDP binding to RIC-8, and lane 3 HIS-GOA-1-GTP binding to RIC-8. The protein complexes were isolated by a HIS-GOA-1 pull down assay with Talon resin (see experimental procedures).
It has recently been shown that LET-99, a DEP domain-containing protein required for spindle positioning in the early embryo (Rose and Kemphues, 1998; Tsou et al. 2002), functions antagonistically to the Gα/GPR1/2 signaling pathway (Tsou et al. 2003).

Figure 17. Hyperactive nuclear movement of let-99 mutant embryos is suppressed in let-99(or204ts);ric-8(RNAi) embryos

(A-D) DIC images of the wild-type. (E-H) let-99(or204ts) and (I-L) let-99(or204ts);ric-8(RNAi) 1 cell embryos recorded by time-lapse videomicroscopy. Black arrowheads mark the position of centrosomes. Note the lack of hyperactive nuclear movement in let-99(or204ts);ric-8(RNAi) embryos (see movies 1-3). At least 15 embryos were analyzed in each case. Relative time points are indicated. Posterior is right. The scale bar in (L) represents 10 μm.

let-99 mutants exhibit hyperactive nuclear and spindle movements (Figure 17). These phenotypes appear to be produced by excess Gα activity because they are suppressed in let-99;Gα(RNAi) embryos (Tsou et al. 2003). If RIC-8 is required for activation of Gα signaling, depletion of ric-8 in let-99(or204ts) should also suppress the nuclear and spindle hyperactive movement. Indeed, in let-99(or204ts);ric-8(RNAi) embryos, the nuclear and spindle rocking phenotypes are completely suppressed (Figure 17), consistent with a model in which RIC-8 functions to activate Gα.

Gα and GPR-1/2 have been shown to modulate the cortical pulling forces responsible for posterior spindle displacement. In Gα(RNAi) and gpr-1/2(RNAi) embryos, the
spindle pulling forces are reduced compared to those in the wild-type (Colombo et al. 2003; Gotta et al. 2003). Our data suggests that RIC-8 plays a positive role in promoting spindle positioning. We therefore investigated whether spindle pulling forces are reduced in *ric-8* mutants with two measures: elongation of the mitotic spindle during anaphase B and a spindle breakage assay performed with *mcak(RNAi)* (Grill et al. 2001).

The length of the spindle at anaphase correlates with the intensity of the pulling forces (Aist et al. 1993; Waters et al. 1993; Gotta et al. 2003). We measured the length of the
mitotic spindle at anaphase and found that in ric-8 mutants and in all the different mutant-RNAi combinations the spindle is significantly shorter than in wild-type (Table 2). In addition, depletion of C. elegans MCAK by RNAi provokes spindle rupture in wild-type embryos, whereas it was not sufficient to provoke spindle rupture in ric-8(md1909) embryos (Figure 18), indicating that the pulling forces are reduced in this mutant.

Taken together, the preferential binding of RIC-8 to GOA-1-GDP and the reduction in pulling forces in ric-8(md1909) suggest that RIC-8 participates in spindle positioning by modulating the activity of Goα.

The cortical localization of GOA-1 and GPR-1/2 and the posterior enrichment of GPR-1/2 suggested a model in which asymmetric activation of Goα by GPR-1/2 would result in an asymmetry of pulling forces, with stronger forces at the posterior cortex, where GPR-1/2 levels are highest (Colombo et al. 2003; Gotta and Ahringer, 2001; Gotta et al. 2003; Miller and Rand, 2000). RIC-8 plays a positive role in spindle positioning, and our data show that it binds to GOA-1. To investigate whether the localization of RIC-8 is similar to that of GOA-1, we raised antibodies against RIC-8 and we constructed a green fluorescent protein (GFP) fusion expressing the full-length RIC-8 protein. We found that the cellular localization of RIC-8 is very similar to the localization of GOA-1, for example at the cortex and on the asters of the mitotic spindle (Figure 19). In addition, we found RIC-8 to be localized on the central spindle, as GPR-1/2, at the nuclear envelope and around the chromatin (Figure 19). With the exception of the staining at the nuclear envelope and around the chromatin, the RIC-8 staining is reduced in ric-8(md1909);ric-8(RNAi) but not completely abolished, consistent with a partial RNAi effect (Figure 19A6). All staining patterns are absent in a preadsorbed antibody preparation (Figure 19A4 and 6A4), confirming the specificity of our antibodies. Two additional lines of evidence suggest that the nuclear envelope staining is specific. This localization was also observed with a different antibody generated by Miller (2000), and GFP::RIC-8 embryos show the same pattern (Figure 19A6). However, because none of the other players of the heterotrimeric G protein pathway has been shown to localize at the nuclear envelope and around the chromatin, the significance of this localization and whether it plays any role in spindle positioning remains unclear. One possibility is that
in early embryo, RIC-8 has functions other than spindle positioning that do not depend on this heterotrimeric G protein pathway.
The cortical localization of GPR-1/2 depends on Gα and LIN-5 (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003; Tsou et al. 2003). We therefore investigated whether the cortical localization of RIC-8 also depends on any of the other known components of the pathway. Depletion of Gα, GPR-1/2, and LIN-5 does not result in any significant difference in the localization pattern of RIC-8 (Figures 19A_j and 19A_k, data not shown). These results suggest that the cortical localization of RIC-8 is either intrinsic to the protein or depends on another protein that has yet to be identified.

Figure 19B. RIC-8 and GPR-1/2 localization
GPR-1/2 asymmetry depends on ric-8. (l, n, and p) show wild-type embryos. (m, o and q) show ric-8(md1909) embryos. (l and m) show anaphase, (n and o) telophase, and (p and q) 2-cell embryos. Posterior enrichment of GPR-1/2 (in red) is lost in ric-8(md1909) embryos, consistent with reduced pulling forces in this mutant. We measured the posterior to the anterior relative intensity of GPR-1/2 staining in wild-type and ric-8(md1909) mutants, 1-cell embryos from metaphase to telophase, and 2-cell embryos. 1-cell embryos: wild-type = 1.46±0.4 (n=21), ric-8(md1909) = 0.91±0.20 (n=18, t test p < 0.000008). 2-cell embryos: wild-type = 1.37±0.23 (n=17), ric-8(md1909) = 0.92±0.20 (n=11, t test p < 0.00003). DNA is stained with DAPI (blue). Posterior is right. The scale bar in (q) represents 10 μm.
We and others have previously shown that asymmetric localization of GPR-1/2 correlates with an asymmetry of pulling forces (Colombo et al., 2003; Gotta et al., 2003). Because pulling forces appear weaker in ric-8(md1909), we investigated whether the localization of GPR-1/2 was affected. Indeed, asymmetric localization of GPR-1/2 is abolished in ric-8(md1909) embryos (Figure 19Bm, 19Bo, and 19Bq; see Figure 19B legend for a quantification of the localization in the wild-type and ric-8(md1909)). Moreover, the GPR-1/2 levels are low on both the anterior and posterior cortices, consistent with the observed weaker pulling forces. This result suggests that active Gα is required for the asymmetry of GPR-1/2. However, asymmetry of GPR-1/2 has been proposed to be required for asymmetric activation of Gα. One possible explanation is that a feedback loop could exist so that a weak initial asymmetry of GPR-1/2 would be reinforced by active Gα. A second hypothesis is that asymmetric modulation of GPR-1/2 activity would promote spindle positioning and posterior spindle displacement. What could be the role of GPR-1/2 in this process? Whereas mammalian RIC-8 is able to act as a GEF only on monomeric Gα subunits (Tall et al. 2003), mammalian AGS3 and Drosophila PINS have been shown to bind Gα and lead to the dissociation of Gβγ, and make Gα accessible to RIC-8.
2.4 Conclusions

Here, we have shown that RIC-8 plays an essential role in spindle positioning in the 1-cell \textit{C. elegans} embryo. Our phenotypic and binding data suggest that RIC-8 controls spindle position by modulating the activity of Go and, therefore, modulating the pulling forces exerted on the spindle. Our work and the recent finding that mutation in \textit{C. elegans rgs-7}, a regulator of G protein signaling that stimulates GTP hydrolysis by Go, results in spindle positioning defects (Hess et al. 2004) support a model in which Go-GTP is the active signaling molecule during spindle positioning in \textit{C. elegans}. PINS and a heterotrimeric G protein have been shown to play a role in the asymmetric cell division of the \textit{Drosophila} neuroblast and sensory organ precursor (SOP) cells (Bellaiche et al. 2001; Schaefer et al. 2001; Yu et al. 2000). However, although all the molecular players, including RIC-8, are conserved, it is still unclear which molecule is responsible for giving the signal (Fuse et al. 2003; Izumi et al. 1998; Schaefer et al. 2001; Yu et al. 2003). Future genetic, biochemical, and \textit{in vitro} studies should help to uncover the interplay between these regulators in both systems.
Chapter II

2.5 Experimental procedures

2.5.1 Strains

*Caenorhabditis elegans* Bristol strain N2 was used as the standard wild-type strain. Strains used in this study are *ric-8*(md1099) (Miller et al., 2000), *let-99*(or204ts) (Tsou et al., 2003), and *goa-1*(n1134) (Segalat et al., 1995). We used the *ric-8*(md1099) rather than the *ric-8*(md303) strain because we could not observe a significant difference in terms of embryonic lethality and spindle-positioning defects (data not shown). However, the *ric-8*(md303) has a stronger egg-laying defect, making it difficult to analyze early embryogenesis. The *let-99*(or204ts) was grown at 15°C and shifted to 20°C 24 hrs before the embryos were examined.

For the GFP::Ric-8 strain, full length RIC-8 cDNA was cloned into pID3.01 (Pellettieri et al., 2003) with Gateway technology (Invitrogen). GFP lines were created by the microparticle bombardment technique (Praitis et al., 2001).

RNA synthesis was performed as described (Zipperlen et al., 2001). Embryos from injected mothers were analyzed at least 24 hrs after injection.

In Table 3, two categories of position of first cleavage were used: percentage of egg length 49-54 % and percentage of egg length > 54 %. These cathegories have been chosen based on measurements of spindle position in the par mutants, which all fall into the more symmetric first category (percentage of egg length: 51 % ± 2 % in *par-2*(it5); 52 % ± 1 % in *par-3*(e2074), and 53 % ± 2 % in *par-1*(b274) (Kemphues et al., 1988)).

2.5.2 Antibodies and Immunostaining

Antibody staining was carried out as described (Gotta et al., 2003). For the competition experiment, RIC-8 purified antibodies were incubated overnight at 4°C with 170 μg/ml MBP-RIC-8 or only maltose binding protein (MBP) as control. Embryos were then stained with the preadsorbed solution as described. In this study, the following primary antibodies were used: anti-RIC-8, anti-GOA-1, and anti-GPR-2.

Antibodies were raised against glutathione S-transferase (GST) fusions of GOA-1, RIC-8, and GPR-2. The proteins were expressed, purified, and injected into rabbits as described (Pintard et al., 2003). Antibodies were affinity purified on immobilized protein.
2.5.3 In vitro binding assays

The vector pMalC2 (NEB) was used to express MBP fusions of full length RIC-8 in *E. coli*. After purification of the protein with standard methods, the MBP moiety was cut out with factor Xa (Amersham). His-GOA-1 expression and purification and the binding of RIC-8 to GOA-1 were performed as described (S8). His-GOA-1 was pulled down with Talon resin (Clonetech). Our bacterially purified RIC-8 was not functional in the *in vitro* GEF assay. Guanosin diphosphate (GDP) and guanosine 5'-O-3-thiotriphosphate (GTP\(_\gamma\)S) were purchased from Sigma.

2.5.4 Yeast Two-Hybrid experiments

The yeast strain Y190 (Clonetech) was used for two-hybrid experiment. β-galactosidase activity was measured by a colony filter assay, carried out according to published protocols (Golemis et al., 1996). Full-length wild-type RIC-8 fused to the DNA binding domain of GAL4, full length wild-type GOA-1, and full length Q205L mutant GOA-1 fused to the activation domain of GAL4 were used in this experiment. Later on full-length wild-type RIC-8 fused with the DNA binding domain of GAL4, full length G204A mutant GOA-1 fused either with the DNA binding domain or with the activation domain of GAL4, and full length GPR-1 fused with the activation domain of GAL4 were used. GPA-16 interaction with RIC-8 was tested with GPA-16 as bait as well as prey and with two yeast strains (Y190 as before and MAV203, Invitrogen).

2.5.5 Microscopy and analysis of living embryos

For DIC analysis of living embryos, embryos were mounted as described (Gotta et al., 2003). The first cell cycle of embryos was recorded (1 image every 10s) with a Zeiss 200M microscope. For immunofluorescence experiments, the DeltaVision system was used for capturing images of embryos. Quantification of GPR-1/2 signal was performed with the ImageJ software. Average pixel intensities of the posterior and anterior cortices were determined after background subtraction, and the posterior-to-anterior ratio was calculated. Posterior and anterior cortices were defined as the region in the cell cortex that is found within the most posterior and most anterior 15% of embryo length, respectively.
2.6 References


DYRB-1, a light chain of dynein, regulates spindle positioning with LIN-5 and GPR-1/2

3.1 Abstract

During asymmetric cell division proper orientation of the mitotic spindle is crucial to ensure correct segregation of cell fate determinants. In *C. elegans*, heterotrimeric G proteins are required to promote spindle positioning in the 1-cell embryo. Three other proteins, LIN-5, GPR-1 and GPR-2 (collectively called GPR-1/2), the NuMA/Mud and Pins/LGN homologues respectively, have been shown to be essential for spindle positioning. Although heterotrimeric G proteins have been postulated to promote spindle positioning by regulating dynein activity, no link could be established between heterotrimeric G proteins and dynein. Here we show that *dyrb-1*, a light chain of dynein, genetically interacts with the heterotrimeric G protein pathway. Using spindle severing as an assay to measure pulling forces, we show that DYRB-1 is required to generate asymmetric pulling forces at spindle poles. Our work demonstrates that dynein activity is required with LIN-5 and GPR-1/2 to generate pulling forces and promote spindle positioning in the *C. elegans* 1-cell embryo. We propose that G proteins act through dynein in all systems.

3.2 Introduction

Asymmetric cell division plays a crucial role in generating cell diversity. Proper positioning of the mitotic spindle is an essential step of an asymmetric division. In *C. elegans*, asymmetric positioning of the mitotic spindle has been shown to depend on unbalanced cortical force generators that act on astral microtubules and pull on spindle poles. Although the molecular nature of the force generators is not known, spatial and
temporal activation is controlled by heterotrimeric G protein signaling. Inactivation of two Gα subunits, GOA-1 and GPA-16, and receptor independent activators of G protein signaling, GPR-1 and GPR-2 (collectively called GPR-1/2, two proteins containing a GoLoco domain), results in strongly reduced and symmetric pulling forces. The coiled-coil protein LIN-5 also plays a crucial role in spindle positioning. LIN-5 physically interacts with GPR-1/2, and its inactivation results in a phenotype very similar to the phenotype of embryos lacking Gαs or GPR-1/2.

The role of heterotrimeric G proteins in spindle positioning is conserved in other organisms. In the fly neuroblast, Gα and Pins, the functional homologue of GPR-1/2, are required for apical-basal orientation of the mitotic spindle. G proteins are also required for spindle orientation in vertebrates. In mammalian cells, Gα, the GoLoco containing protein LGN and the microtubule binding protein NuMA were shown to form a complex that has been suggested to regulate the interaction of astral MTs with the cell cortex. Interestingly, NuMA can bind to the dynein/dynactin complex and recent work has shown that Drosophila Mud and C. elegans LIN-5 are homologues of NuMA. Therefore, one possible model is that cortically localized NuMA/LIN-5/Mud interacts with dynein on the mitotic spindle and that the forces exerted by this minus end-directed motor pull the mitotic spindle to the cortex (Hampoelz and Knoblich, 2004).

While there is no evidence for dynein requirement in spindle positioning in Drosophila, recent work in C. elegans using temperature sensitive mutants has shown that inactivation of dynein does not abolish spindle displacement to the posterior. However, the spindle moves to the posterior with slower dynamics suggesting that dynein could participate in spindle positioning.

Here we show that inactivation of dyrb-1, a light chain of dynein, also plays a role in spindle regulation. dyrb-1 belongs to the highly conserved Roadblock/LC7 family. One member of this family, robl, was identified in flies. In Drosophila, mutations of the robl gene result in defects in mitosis, accumulation of vesicles in axons, and larval or pupal lethality (Bowman et al. 1999). It was shown that Robl/LC7-like proteins are components of cytoplasmic dynein in Drosophila and are involved in modulating specific dynein functions (Bowman, 1999). This incorporation of Robl/LC7 into the
The dynein complex is mediated by protein-protein interactions (Sulsalka et al. 2002; DiBella et al. 2004). The exact function of Roadblock/LC7 is unknown. We have identified dyrb-1 in two screens aimed at identifying genes that when knocked down enhance the phenotype of gpr-1 and lin-5 temperature-sensitive mutants. We show that the forces pulling on the astral microtubules are reduced following inactivation of dyrb-1. Notably, we find that dyrb-1 co-immunoprecipitates with gpr-1 and lin-5. We suggest a model in which GPR-1 and LIN-5 control spindle positioning, especially events leading to the posterior spindle displacement at least in part by regulating the interaction with dynein motor proteins.

### 3.3 Results

#### 3.3.1 Identification of dyrb-1 as an enhancer of gpr-1 and lin-5 lethality

In order to identify additional genes that act with heterotrimeric G proteins in spindle positioning, we performed enhancer screens by RNAi in liquid using lin-5 and gpr-1 temperature sensitive alleles (Jean-Claude Labbe, Thomas Marti and Monica Gotta, unpublished). This has led to the identification of one RNAi clone that showed enhanced lethality in both lin-5 and gpr-1 mutant strain backgrounds. The clone corresponds to the gene T24H10.6, which codes for a homologue of the Roadbock/LC7 dynein light chain family and in C. elegans has been recently named dyrb-1 (dynein light chain roadblock type-1). While depleting dyrb-1 by feeding (see Material and Methods) in wild-type animals resulted in less than 1% embryonic lethality, the lethality of both lin-5 and gpr-1 mutants was significantly increased (Table 4). Increased lethality was also observed when dyrb-1 was fed to goa-1 and gpa-16 mutants. Although by feeding dyrb-1 animals display less than 1% embryonic lethality, injection in wild-type animals results in 50% embryonic lethality due to stronger RNAi efficiency (Table 4). Injection of dyrb-1 dsRNA in lin-5 and gpr-1 also resulted in a significantly increased embryonic lethality. To conclude, dyrb-1 was isolated in a RNAi screen as an enhancer of lin-5 and gpr-1 lethality. Our data indicates that dyrb-1 genetically interacts with the heterotrimeric G protein pathway.
Table 4. Increased lethality of dyrb-1(RNAi) in different mutant backgrounds by feeding at 17°C or injection at 15°C

L4440 and L4440 DYRB-1 corresponds to the feeding experiment, non-injected and dyrb-1(RNAi) to the dsRNA injections. L4440 is the feeding vector into which the gene thought to be depleted must be cloned. The L4440 vector is transformed in HT115 E. coli cells and used as a negative feeding control. L4440 dyrb-1 is fed under the same conditions in parallel. In the non-injected sample the worm strains were cultured on OP50 E. coli plates, while in parallel dyrb-1 dsRNA was injected in the same strains, cultured under the same conditions.

3.3.2 Inactivation of dyrb-1 affects dynein dependent processes

To assess which events in early embryogenesis are impaired by depletion of dyrb-1, we compared wild-type to dyrb-1 depleted embryos by time-lapse DIC microscopy. The progression of events in wild-type is illustrated in Figure 20 (A-D). In wild-type the oocyte pronucleus migrates to the posterior to meet the sperm pronucleus. This migration is divided into two phases, the slow phase that is followed by a shorter fast phase (Table 5, Figure 21). The sperm pronucleus also moves slightly to the center of the embryo. As a result the two pronuclei meet at about 68% embryo length (0% most anterior, 100% most posterior). The two pronuclei and associated centrosomes then migrate towards the cell center whilst undergoing a 90% rotation that aligns the centrosomes along the anterior-posterior axis of the embryo (Table 6).
Figure 20. Early development of wild-type and dyrb-1(RNAi) embryos

(A-D) Wild-type embryo. (E-H) DYRB-1 depleted embryo (22°C). (A and E) show pronuclear meeting. (B and F) show the embryo at nuclear envelope break down (NEBD). In the wild-type embryo centration of the nuclear-centrosome complex has already taken place (B). In dyrb-1(RNAi) embryos centration is incomplete (F) and rotation of the nuclear-centrosome complex also delayed. (C and G) display the mitotic spindle at anaphase. Rocking of the mitotic spindle is observed in wild-type (C) but is absent in dyrb-1(RNAi) embryos (G). (D and H) embryos after cytokinesis. The posterior aster has a disc shape morphology in wild-type (D). This is not observed in dyrb-1(RNAi) embryos (H). Posterior is to the right. The scale bar in (H) represents 10 μm. Arrowheads mark centrosomes.
The spindle sets up in the center of the cell along the anterior-posterior axis and at metaphase/anaphase is displaced towards the posterior by an imbalance of cortical pulling forces. During this displacement the posterior spindle pole undergoes transverse oscillations called “rocking”. At telophase, the posterior spindle pole flattens while the anterior remains round. This asymmetric displacement of the spindle results in an asymmetric cleavage with a larger anterior cell, AB, and a smaller posterior cell, P1 (Table 7 and Figure 20). *dyrb-1(RNAi)* embryos (24 hours post-injection at 15°C and 22°C) display delayed migration of the oocyte pronucleus, where the relative movement of the maternal pronucleus over time is significantly reduced during the fast phase compared to wild-type (Table 5, Figure 21).

**Figure 21.** DYRB-1 depleted embryos display a less pronounced fast phase compared to wild-type

Pronuclear migration is divided into two phases. The slow phase (blue) where pronuclear movement is flow driven, followed by the fast phase (red) which is due to MT capturing. This graph displays the reduction of relative movement per time in the “fast phase” of pronuclear migration upon depletion of DYRB-1, while the “slow phase” is not significantly changed.
Maternal genotype | Rate of slow phase of oocyte pronuclear migration (μm/sec) | Rate of fast phase of oocyte pronuclear migration (μm/sec)
--- | --- | ---
wild-type (n=10) | 0.046 ± 0.017 | 0.334 ± 0.094
*dyrb-1(RNAi) (n=11)* | 0.033 ± 0.014 | 0.212 ± 0.025

**Table 5. Pronuclear migration in wild-type and dyrb-1(RNAi) embryos**
Measurements ± error were determined in 1-cell embryos obtained from mothers growing at 22°C. All events were monitored by DIC microscopy; pronuclei are visible as granule-free spheres. The rate of slow phase is not significantly different between wild-type and *dyrb-1(RNAi)* slow phase (p values of less than 0.05 are considered statistically significant). The fast phase is significantly different p=0.0007; student t-test.

This data is an indication for DYRB-1 being involved in MT driven processes, since the fast phase of pronuclear migration is dependant on MTs growing out from the centrosomes capturing the maternal pronucleus. Furthermore, these embryos display strongly reduced pronuclear centration (Table 7) and delay in rotation of the pronucleus-centrosome complex (Figure 20B and F). In addition, the P0 spindle is significantly shorter than in wild-type animals (Table 7), spindle rocking is absent and the posterior aster does not flatten like in wild-type (22/22 embryos at 22°C, see Figure 20). These phenotypes are highly penetrant and can therefore not be the cause of lethality, since lethality in these conditions is only 50%.

<table>
<thead>
<tr>
<th>strain</th>
<th>wt-like 0°-10°</th>
<th>oblique 11°-90°</th>
</tr>
</thead>
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<tr>
<td>wildtype (n=27)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td><em>dyrb-1(RNAi) (n=22)</em></td>
<td>13%</td>
<td>87%</td>
</tr>
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</table>

**Table 6. Centrosome orientation at the timepoint of NEBD in the 1-cell C. elegans embryo**
By measuring the positioning of the centrosomes at the timepoint of nuclear envelope break down (NEBD) we could observe that in 87% of the *dyrb-1* depleted embryos rotation of the nuclear centrosome complex was not properly finished, due to a delay in rotation. The embryos were put into two classes, either wild-type like or oblique. The embryos represented in the second class display a misalignment of the nuclear centrosomes compared with the longitudinal axis of the embryo that is more than 10 degree.
Table 7. Position of pronuclear meeting, nuclear centrosome complex, first cleavage and spindle length

<table>
<thead>
<tr>
<th>Condition</th>
<th>PNM a</th>
<th>position of NCC b</th>
<th>spindle length c</th>
<th>position of first cleavage d</th>
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<td><strong>15°C</strong></td>
<td></td>
<td></td>
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<tr>
<td>wild-type (n=18)</td>
<td>69.62 ± 5.26</td>
<td>50.53 ± 1.21</td>
<td>43.44 ± 1.92</td>
<td>56.69 ± 1.17</td>
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<td>gpr-1(or574ts) (n=15)</td>
<td>73.04 ± 9.02</td>
<td>50.75 ± 1.54</td>
<td>41.41 ± 3.24</td>
<td>55.92 ± 2.51</td>
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<td>lin-5(ev571ts) (n=22)</td>
<td>71.39 ± 6.88</td>
<td>52.94 ± 2.30</td>
<td>44.04 ± 2.56</td>
<td>54.93 ± 2.03</td>
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<td>dyrb-1(RNAi) (n=23)</td>
<td>70.58 ± 6.46</td>
<td>62.87 ± 4.66</td>
<td>40.29 ± 2.29</td>
<td>56.51 ± 1.62</td>
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<td>gpr-1(or574ts);dyrb-1(RNAi)</td>
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<td>(n=19)</td>
<td>67.42 ± 5.81</td>
<td>64.53 ± 6.17</td>
<td>35.29 ± 3.18</td>
<td>59.58 ± 4.02</td>
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<td>(n=17)</td>
<td>74.78 ± 5.11</td>
<td>69.67 ± 5.47</td>
<td>34.10 ± 5.74</td>
<td>59.97 ± 5.86</td>
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<td><strong>22°C</strong></td>
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<tr>
<td>wild-type (n=27)</td>
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<td>51.08 ± 2.25</td>
<td>44.32 ± 3.01</td>
<td>57.29 ± 1.41</td>
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<td>gpr-1(or574ts) (n=17)</td>
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<td>50.41 ± 1.85</td>
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<td>50.08 ± 3.81</td>
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<td>53.16 ± 2.11</td>
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<td>57.56 ± 2.19</td>
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<td>gpr-1(or574ts);dyrb-1(RNAi)</td>
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<td>(n=16)</td>
<td>67.33 ± 3.74</td>
<td>68.33 ± 5.77</td>
<td>37.73 ± 4.55</td>
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<td>(n=17)</td>
<td>72.47 ± 7.46</td>
<td>76.81 ± 12.16</td>
<td>36.08 ± 4.03</td>
<td>59.82 ± 7.42</td>
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Table 7. Position of pronuclear meeting, nuclear centrosome complex, first cleavage and spindle length

All events were calculated as a percentage of egg length. n is the number of embryos analyzed.

aPosition of NCC was calculated at the timepoint when pronuclei met.

bPosition of NCC was calculated just before nuclear envelope breakdown (by dividing distance of midpoint to anterior cortex by embryo length).

cSpindle pole separation was calculated at anaphase (by dividing spindle length by embryo length).

dAnterior is 0%.
The difference in spindle length of dyrb-1(RNAi) embryos compared to gpr-1(or574ts);dyrb-1(RNAi), lin-5(ev571ts);dyrb-1(RNAi) and wild-type embryos is significant (p < 0.00001, student t test).
Further the difference of NCC position and spindle length was significant for all dyrb-1(RNAi) combinations compared to the background strain (p < 0.00001 for all, student t test) at 15°C and 22°C.

The phenotypes observed in DYRB-1 depleted embryos are very similar to the phenotypes observed upon weak depletion of dynein heavy chain 1 (DHC-1) or when dhc-1 is inactivated using temperature-sensitive alleles. Analysis of such embryos showed that the two pronuclei meet, but migration to the center and rotation of
centrosomes fail to occur, the spindle sets up perpendicular to the longitudinal axis at about 70% egg length due to a rotation delay of the NCC. The spindle is later rescued to wild-type orientation by ingestion of the cleavage furrow. These phenotypes are highly reminiscent of the phenotypes observed in dyrb-1 depleted embryos. Strong RNAi depletion of DHC-1 results in more severe phenotypes, including defects in centrosome separation, bipolar spindle formation and cytokinesis. Furthermore, migration of anterior and posterior pronuclei is abolished leading to a spindle formed only by the male pronucleus.

Depletion of dyrb-1 does not result in a phenotype comparable to that seen after strong loss of dhc-1 even when RNAi is performed at 25°C and the embryos are analyzed 48 to 72 hours post injection (data not shown). Under our RNAi conditions, GFP-DYRB-1 is strongly depleted (see below). However, we cannot exclude the possibility that we are unable to fully deplete endogenous DYRB-1. Unfortunately, two attempts to raise anti-DYRB-1 antibodies and every attempt to isolate a mutant have failed. In conclusion, DYRB-1 is required for several dynein dependent processes in the 1-cell embryo including proper spindle orientation.

3.3.3 Distribution of DYRB-1 in early C. elegans embryos

To study the localization of DYRB-1 in early C. elegans embryos we constructed a transgene expressing GFP::DYRB-1 under the control of the PIE-1 promoter. We find that, similar to the distribution of dynein heavy chain (Gonczy et al. 1999), DYRB-1 localizes in a punctate manner in the cytoplasm and is found at the periphery of pronuclei and nuclei. In cells progressing through mitosis, GFP::DYRB-1 localizes to the spindle poles, to the spindle and around the chromosomes at metaphase. We also observe a weak localization to the cortex that is more apparent in 2- and 4-cell embryos or older embryos (Figure 22). Therefore, consistent with a role in regulating dynein activity, DYRB-1 localization reflects DHC-1 localization.
Figure 22. Localization pattern of GFP::DYRB-1 in early embryos

Panel A. (A) through (G) show the localization pattern of GFP::DYRB-1 at different cell cycle stages. At all stages embryos display a diffuse and punctate cytoplasmic GFP::DYRB-1 distribution. (A) One-cell embryo during pronuclear migration. GFP::DYRB-1 is enriched at the periphery of the female and male pronuclei. (B) Metaphase embryo. (C) One-cell embryo at anaphase. GFP::DYRB-1 is enriched on the spindle and at the spindle poles. (D) Two-cell embryo. GFP::DYRB-1 is found at the periphery of AB and P1 nuclei and throughout the cortex between AB and P1. (E,F) Three-cell embryo. P1 is dividing and GFP::DYRB-1 is enriched on the spindle on both sides of the metaphase plate. (G) Four-cell embryo. GFP::DYRB-1 is enriched at the cortex, at the periphery of nuclei and in a punctate cytoplasmic manner. This localization pattern is very similar to the one observed with antibodies to DHC-1 (Gonczy and Schmidt). The scale bar in (I) represents 10 μm. Posterior is to the right.

Panel B. Western Blot of C. elegans embryos probed with anti-GFP antibodies and αtubulin antibodies. αGFP antibodies recognize a band of 40 kDa (lane 2), which is absent in the extract from wild-type embryos (lane 1). GFP::DYRB-1 is fully depleted in dyrb-1(RNAi) embryos (lane 3). αtubulin is used as a loading control.
Depletion of Go, GPR-1/2, LIN-5 or DHC-1 did not affect this localization pattern (data not shown). Conversely, depletion of DYRB-1 did not affect the localization pattern of GPR-1/2, GOA-1, LIN-5 and DHC-1 (see below or data not shown).

3.3.4 dyrb-1 suppresses hyperactive nuclear movements in let-99

LET-99 is a DEP domain protein, which was suggested to be a link between polarity cues and the downstream machinery that determines spindle positioning in C. elegans embryos. In let-99 1-cell embryos, the nuclear-centrosome complex exhibits a hyperactive oscillation, instead of the normal wild-type like anterior directed migration and rotation of the nuclear-centrosome complex (Rose and Kemphues, 1998; Tsou et al. 2002). Thus, it appears that let-99 mutant embryos exhibit an increase in the net forces acting on the nuclear-centrosome complex, and that those forces require dynein. This was suggested after weak depletion of DHC-1 in the background of let-99(or204) led to suppression of the hyperactive oscillations, indicating that dynein is essential for generation of the net forces acting on the centrosomal complex and the spindle during elongation (Tsou et al. 2002).

Next, we wanted to investigate if dyrb-1 plays a role in regulating the forces acting on the nuclear-centrosome complex during migration and rotation. From our previous investigations, we have concluded that dyrb-1 depletion leads to incomplete migration and delayed rotation of the nuclear-centrosome complex (see above). Thus, we tested whether dyrb-1 is required for the excessive nuclear movements observed in let-99 embryos. Depletion of dyrb-1 in the let-99 mutant background results in normal nuclear migration and formation of a robust bipolar spindle by suppression of the hyperactive movements, but failure of centration and rotation (Figure 23). These observations are similar to data presented about DHC-1 (Goenczy et al. 1999).

Thus it appears that let-99 mutant embryos exhibit an increase of the net forces acting on the nuclear-centrosome complex, and this force increase requires dynein or dyrb-1, which positively regulates dynein, suggesting that let-99 acts upstream of dynein.
We also investigated whether *dyrb-1* influences the localization pattern of LET-99 in the early embryo. Interestingly, LET-99 is localized in an asymmetrically enriched cortical band, present on the border where the anterior PAR complex meets the posterior PAR proteins and the spindle midzone is localized. This LET-99 localization is PAR protein dependent (Tsou et al. 2002) but unchanged in *let-99; dyrb-1(RNAi)* embryos (n= 27).

Therefore, *dyrb-1* is not needed to target *let-99* to the membrane but is needed for force generation acting on the NCC or the mitotic spindle.

**Figure 23. Suppression of the hyperactive nuclear movement of let-99 mutant embryos by DYRB-1 depletion.**

(A-D) DIC images of the wild-type embryos. (E-H) *dyrb-1(RNAi)* and (I-L) *let-99(or204ts)* embryos. (M-P) *let-99(or204ts); dyrb-1(RNAi)* 1-cell embryos recorded with time-lapse videomicroscopy. Arrowheads mark the position of centrosomes. Notice the lack of hyperactive nuclear movement in *let-99(or204ts); dyrb-1(RNAi)* embryos. At least 15 embryos were analyzed in each case. Relative time points are indicated (M-P). Posterior is to the right. The scale bar in (P) represents 10 μm.
3.3.5 *dyrb-1* is required to generate pulling forces on spindle poles

Recent work has shown that dynein is not strictly required for spindle positioning since inactivation of dynein using several temperature sensitive alleles does not result in a major spindle positioning defect. However, spindle positioning to the posterior occurs with lower dynamics suggesting that dynein plays a role in this process. In *dyrb-1* depleted embryos the mitotic spindle forms at the posterior, preventing the observation of spindle displacement. However, a spindle forms and aligns along the axis of polarity making it possible to measure peak velocities of the two spindle poles. Indeed several observations indicate that pulling forces are weaker in *dyrb-1* depleted embryos: the spindle is significantly shorter than in wild-type, spindle rocking does not occur and the posterior aster does not flatten (see above and Table 7). We therefore investigated whether astral pulling forces are compromised in *dyrb-1(RNAi)* embryos by performing laser microbeam-severing experiments and analyzed the resulting spindle pole movement using timelapse DIC microscopy. In wild-type embryos the peak velocities of the posterior spindle pole are about 1.6 times higher than that of the anterior pole. As shown in Figure 24, the peak velocities of both anterior and posterior centrosomes in *dyrb-1(RNAi)* embryos after spindle severing are significantly reduced compared to wild-type and the asymmetry is lost.

![Figure 24](image)

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**Figure 24. dyrb-1(RNAi) lowers the forces acting on the spindle poles.**

Average peak velocities ± standard deviation of anterior (orange) and posterior (green) spindle poles measured after spindle severing in one-cell stage embryos of the indicated genotypes. Values and statistical analyses are given in Table 9, below.
Interestingly, we found, that *dyrb-1* depletion in DHC-1 mutants does not significantly differ from *dyrb-1(RNAi)* in wild-type (Figure 25). For this approach we used a weaker *dhc-1* mutant, which displays pronuclear migration and formation of a mitotic spindle (Schmidt et al. 2005).

**Figure 25.** *dyrb-1(RNAi)* does not further reduce forces acting on the mitotic spindle of *dhc-1* mutant embryos.

Average peak velocities ± standard deviation of anterior (orange) and posterior (green) spindle poles measured after spindle severing in one-cell stage embryos of the indicated genotypes.

The reduced forces are not a consequence of polarity defects as PAR proteins are properly localized in *dyrb-1(RNAi)* embryos (Figure 26: anti-PAR6: wild-type (n=13), *dyrb-1(RNAi)* (n=9), anti-PAR-2: wild-type (n=8), *dyrb-1(RNAi)* (n=16)). The distribution of GPR-1/2 is also unaffected (Figure 26, n=42). This data indicates that *dyrb-1* plays an important role in generating the forces that pull on astral microtubules and that it works downstream or in parallel to polarity cues and GPR-1/2.
Figure 26. DYRB-1 depleted embryos have normal polarity

Panel A. (A) PAR-6 is localized at the cortex of the anterior AB cell in wild-type embryos while PAR-2 (B) is enriched at the cortex of PI. In *dyrb-1(RNAi)* embryos localization of PAR-6 (C) and PAR-2 (D) are as in wild-type. PAR-6 and PAR-2 arc in red (Alexa 568). DNA is stained with DAPI (in blue). The scale bar in (D) represents 10 μm.

Panel B. GPR-1/2 (Alexa488) is enriched at the posterior cortex of one-cell embryos (A). This cortical localization is unchanged in *dyrb-1(RNAi)* embryos (B). The embryo is co-stained with actin antibodies (Alexa568) and DAPI (blue). The scale bar in (B) represents 10 μm. For both panels posterior is to the right.
3.3.6 Depletion of *dyrb-1* in *gpr-1* and *lin-5* mutants results in a strong synthetic phenotype.

To further investigate the genetic interaction between *dyrb-1* and the heterotrimeric G protein pathway, we analyzed the phenotype resulting from *dyrb-1* depletion in *lin-5* and *gpr-1* mutants. Depletion of *dyrb-1* in *gpr-1* and *lin-5* results in a strong synthetic phenotype. While pronuclear migration is not affected in *lin-5* and *gpr-1* single mutants (data not shown), it is slowed down in *dyrb-1* single mutants (see above). In the double mutants, pronuclear migration is further delayed resulting in NEBD of the male pronucleus before the pronuclei meet. This is the case in 13/37 *dyrb-1*(RNAi) embryos at 22°C with increasing numbers in the mutant backgrounds (9/17 *gpr-1*(or574ts);*dyrb-1*(RNAi), 9/16 *lin-5*(ev571ts) *dyrb-1*(RNAi)). While we cannot observe a clear defect in centrosome separation in P0 of *gpr-1* and *lin-5* single mutants, or in *dyrb-1*(RNAi) embryos, *lin-5* *dyrb-1* and *gpr-1;dyrb-1* embryos show a defect in centrosome separation in 7/17 and 6/10 1-cell embryos, respectively.

Observations of the mitotic spindle just before cytokinesis revealed that 100% of *dyrb-1*(RNAi), *gpr-1* and *lin-5* embryos showed a wild-type like spindle orientation along the longitudinal axis. In contrast, in 76% of *lin-5;dyrb-1*(RNAi) embryos (n=17) and 19% of *gpr-1;dyrb-1* embryos (n=16) display a spindle that is mis-aligned (Figure 27 and Table 8). Therefore, *dyrb-1* *lin-5* and *dyrb-1;gpr-1* double mutant embryos have a stronger phenotype than any single mutant.

This genetic interaction suggests that GPR-1, LIN-5 and DYRB-1 have a common function in mitosis in the one-cell *C. elegans* embryo.
Figure 27A. Inactivation of dyrb-1 in lin-5 and gpr-1 mutants results in aberrant spindle orientation in the one-cell embryo prior to cytokinesis.

(A-F) 1-cell embryos at anaphase. The circles mark the spindle poles. In (A), (B), (C) and (D) the spindle is aligned along the anterior-posterior axis at cytokinesis. In (E) and (F) the mitotic spindle is not properly aligned along the A-P axis. (see also Table 8). Posterior is to the right. The scale bar in (F) represents 10 μm.
Figure 27B. Inactivation of *dyrb-1* in *lin-5* and *gpr-1* mutants results in aberrant spindle orientation in the one-cell embryo prior to cytokinesis.

Panel B. Spindle orientation at cytokinesis in wild-type and mutant embryos of the indicated genotype. Alignment along the anterior-posterior axis corresponds to 0°C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>WT-like 0°-10°</th>
<th>Oblique 11°-90°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype (n=27)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td><em>gpr-1</em>(or574ts) (n=17)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td><em>lin-5</em>(ev571ts) (n=26)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td><em>dyrb-1</em>(RNAi) (n=22)</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td><em>gpr-1</em>(or74ts);<em>dyrb-1</em>(RNAi) (n=16)</td>
<td>81%</td>
<td>19%</td>
</tr>
<tr>
<td><em>lin-5</em>(ev571ts);<em>dyrb-1</em>(RNAi) (n=17)</td>
<td>24%</td>
<td>76%</td>
</tr>
</tbody>
</table>

Table 8. Spindle orientation at the time point of furrow ingestion in the 1-cell *C. elegans* embryo

The embryos were put in two classes, either wild-type like or oblique. The embryos represented in the second class display a misalignment of the nuclear centrosomes compared with the longitudinal axis of the embryo that is more than 10 degrees. The timepoint of measuring was at furrow ingestion.
3.3.7 Peak velocities are strongly affected in double mutants

In order to investigate whether LIN-5, DYRB-1 and GPR-1/2 have a common function in spindle positioning in the one-cell embryo, we measured the peak velocities of spindle poles in the single mutants and in combinations of double and triple mutants.

Peak velocities in *lin-5* mutants were not previously measured. We therefore measured peak velocities in *lin-5(ev571) lin-5(RNAi)* at 22°C and 25°C and found that the peak velocities of both anterior and posterior aster are strongly reduced and symmetric (Figure 28), as in GPR-1/2 depleted embryos, consistent with the fact that LIN-5 and GPR-1/2 bind to each other. In *dyrb-1(RNAi)* embryos, as mentioned previously, peak velocities of both spindle poles are reduced but are still higher than the peak velocities measured in *gpr-1/2* and *lin-5* depleted embryos. However, if we deplete *dyrb-1* in *gpr-1/2(574ts)* at semi-permissive temperature, the peak velocities are now lower than in the single mutants under the same conditions, and comparable to those seen in *gpr-1/2(RNAi)* embryos. Similarly, the peak velocities of both asters of *lin-5 dyrb-1(RNAi)* embryos are also lower than those in single mutants (Figure 29, Table 9). These data show that *dyrb-1*, *gpr-1* and *lin-5* work together in generating the forces that pull on the mitotic spindle.

![Figure 28. *lin-5(ev574)* display reduced peak velocities](image)

**Figure 28. *lin-5(ev574)* display reduced peak velocities**

Peak velocities in *lin-5* mutant embryos are reduced on both spindle poles at 22°C compared to wild-type. At higher temperature the peak velocities are symmetric and further reduced.
**Figure 29. dyrb-1(RNAi) lowers the forces acting on the spindle poles.**

Average peak velocities ± standard deviation of anterior (orange) and posterior (green) spindle poles measured after spindle severing in one-cell stage embryos of the indicated genotypes. Values and statistical analysis are given in Table 9.

### Table 9. Spindle severing experiments

Average peak velocities ± standard deviation were calculated as described in the "material and methods" for anterior and posterior spindle poles. p values are calculated by student t-test and the multiplication factor of forces acting on the posterior, compared to the anterior spindle, to get an idea of the asymmetry of forces.
3.3.8 GOA-1 and GPA-16 act redundantly on spindle regulation

As mentioned previously, one question still to be answered is whether the heterotrimeric G protein subunits GOA-1 and GPA-16 (Gα's) act in two parallel pathways in terms of spindle regulation and orientation, or whether they are completely redundant and able to act on one common substrate to control mitotic spindle positioning. Therefore, we present two models for regulation via Ga in the early C. elegans embryo (Figure 30). In these models we also place GPR-1/2, which is known to physically interact with Gα and LIN-5 (see introduction).

To distinguish between these two modes of regulation, different approaches are possible. One is the quantification of synthetic lethality, an other experiment is by spindle severing.

We reasoned that if GPA-16 and GOA-1 are essential for distinct pathways, GPA-16 should not compensate for the loss of GOA-1 and vice versa. Therefore, the lethalities of GOA-1 and GPA-16 should differ in a mutant background. Furthermore, spindle severing experiments should result in different peak velocities measured for dyrb-1(RNAi) in both backgrounds.

A first indication for the mode of regulation, came from observations where it was shown that GOA-1 and GPA-16 become completely redundant for GPR-1/2 localization and symmetric spindle positioning, when Gβγ is inactivated (Afshar et al. 2005). Parallel to this, the peak velocities measured on anterior and posterior spindle poles are diminished in goa-1 or gpa-16 mutant embryos compared to wild-type, whilst remaining asymmetric. In contrast, Gβ (GPB-1) mutant embryos display high and symmetric peak velocities on both spindle poles. Investigations of double mutant embryos showed no distinguishable difference between peak velocities measured in gpb-1, goa-1;gpb-1 or gpa-16;gpb-1 mutant embryos. This suggested that excess GPA-16 liberated from Gβγ can compensate for a loss of GOA-1 and vice versa. Therefore, instead of being essential for distinct pathways, GPA-16 and GOA-1 appear to each contribute to partial activation of the same pathway.

This data did not confirm observations made at the same time using lethality assays.
Initially it was investigated, whether DYRB-1 also displays enhancement of lethality, phenotype or forces in the background of *goa-1* and *gpa-16*, since it is known that Gαs are important for spindle regulation, as is the case for *LIN-5* and GPR-1/2.

Depletion of DYRB-1 by feeding, results in almost no lethality in wild-type background. Whereas, the depletion of DYRB-1 by injection in two different Gα mutants, *goa-1(sa734)*, a deletion allele (Robatzek and Thomas, 2000) or *gpa-16(it143)* a strong reduction of function allele (Bergmann et al. 2003), leads to increased lethality.

**Figure 30. Spindle regulation by Gα**

GPR-1/2 physically binds to *LIN-5*, GPR-1/2 binds to Gα and triggers its dissociation from Gβγ, keeping Gα in the GDP bound state. All four players are involved in spindle regulation. One method of regulation might be that GOA-1 and GPA-16 are regulated independently and act in two different pathways on effectors that are involved in spindle regulation (A). Another possibility is that GOA-1 and GPA-16 act in concert on one substrate and therefore regulate simultaneously (B).

By feeding there was no significant increase of lethality. However, the results from injection could still be due to an additive effect. We expect that if dynein were
controlled by GOA-1, then a higher lethality would be seen in the combination depleting *dyrb-1* in the background of *gpa-16*, or *vice versa*. In fact, the lethality data may suggest this, since the lethality of *goa-1;dyrb-1* is higher than the combination *gpa-16;dyrb-1*. Still, we have to take into consideration that we are not using null mutants for our approaches since we were not able to obtain a *dyrb-1* mutant.

As the lethality leaves the open question, "which is the correct mode of regulation", we next tested whether GOA-1 and GPA-16 are in distinct pathways with a more sensitive assay. We investigated the extent of pulling forces using laser microbeam-mediated spindle severing experiments, analyzing the resulting spindle pole movements with time-lapse differential interference contrast (DIC) microscopy.

We investigate whether *dyrb-1*, and therefore dynein, is regulated by either *goa-1* or *gpa-16* independently. After spindle severing in wild-type one-cell stage embryos, the peak velocity of the anterior pole is \( \approx 0.74 \mu\text{m/sec} \), whereas that of the posterior is \( \approx 1.14 \mu\text{m/sec} \), reflecting the imbalance of net pulling forces acting on the two spindle poles (see Figure 31 and Table 10). If *goa-1* and *gpa-16* act fully redundantly, we would expect to see a similar result of peak velocities in these mutants. Inactivation of either *goa-1* or *gpa-16* in a *dyrb-1*(RNAi) background, results in diminished peak velocities of both spindle poles, but retained asymmetry of forces between the anterior and posterior poles (see Table 10). From the genetic data, we expect to see no difference between the combination *goa-1;dyrb-1* or *gpa-16;dyrb-1*. In fact, in double mutant embryos the forces are reduced on both spindle poles and the asymmetry of these forces is lost. We could not observe a significant difference between *goa-1*(n1134);*dyrb-1*(RNAi), *gpa-16*(it143ts);*dyrb-1* peak velocities and *dyrb-1*(RNAi) alone. Furthermore, we also expect similar forces for the triple combination, depleting both G\(\alpha\)’s together with DYRB-1. The result however was unexpected, as the triple inactivation of *goa-1* *gpa-16* and *dyrb-1*, resulted in further reduced peak velocities (anterior \( \approx 0.05 \mu\text{m/sec} \); posterior \( \approx 0.06 \mu\text{m/sec} \)) compared to those observed in *goa-1/gpa-16*(RNAi) embryos (anterior/posterior both \( \approx 0.07 \mu\text{m/sec} \)). These peak velocities were even lower than forces described for G\(\alpha\) or GPR-1/2. Interestingly, the peak velocities are similar to those obtained in *lin-5 dyrb-1* depleted embryos (see above). As mentioned previously, we were not able to obtain a mutant for *dyrb-1*. Furthermore, it is not possible to work
with 

\textit{goa-1;gpa-16} mutants, as this combination leads to embryonic lethality. Due to these technical problems we are not able to draw strong conclusions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure31.png}
\caption{Depletion of \textit{dyrb-1} lowers the forces acting on the spindle poles in \textit{goa-1} and \textit{gpa-16} mutants to the same level.}
\end{figure}

Average peak velocities ± standard deviation of anterior (orange) and posterior (green) spindle poles measured after spindle severing in one-cell stage embryos of the indicated phenotype. Values and standard deviation are given in Table 10.
3.3.9 LIN-5, GPR-1/2 and DYRB-1 are in the same complex

The data presented above is consistent with two models: dyrb-1 could act in a pathway parallel to the lin-5/gpr-1/2 pathway, or it could act in the same pathway. It has recently been shown that LIN-5 is the C. elegans homologue of mammalian NuMA and fly Mud (Du and Macara, 2004; Kisurina-Evgenieva et al. 2004; Izumi et al. 2006; Bowman et al. 2006). NuMA is a microtubule binding protein essential for proper organization of the mitotic spindle, and binds to LGN. Interestingly, NuMA has also been shown to interact with the motor protein dynein. However, there is no evidence of an interaction between dynein motor proteins and C. elegans lin-5 or Drosophila Mud. To assess whether dyrb-1 works in the heterotrimeric G protein pathway or in a parallel pathway, we investigated whether DYRB-1 can be recovered in a complex with LIN-5 and GPR-1/2.

By using embryonic extracts, we found that LIN-5 and GPR-1/2 can co-immunoprecipitate with GFP::DYRB-1 (Figure 32A). Conversely, LIN-5 antibodies can precipitate GPR-1/2 and GFP::DYRB-1 (Figure 32B). This interaction is specific, as no LIN-5 or GPR-1/2 is detected in control immunoprecipitations using both wild-type,
which does not express GFP::DYRB-1, or GFP::DYRB-1 lines upon depletion of DYRB-1 by RNAi (Figure 32C and D). This interaction is specific since LIN-5 is not interacting with GFP. Therefore, we performed immunoprecipitation experiments with other GFP-fusion proteins (data not shown).

**Figure 32. LIN-5 and GPR-1/2 co-immunoprecipitate together with DYRB-1.**

(A) LIN-5 and GPR-1/2 co-immunoprecipitate with DYRB-1, whilst upon depletion of DYRB-1 in GFP::DYRB-1 embryonic extracts neither LIN-5 nor GPR-1/2 was detectable in the immunoprecipitate (B). The IP is also possible vice versa. With LIN-5 antibodies, GPR-1/2 and DYRB-1 are detectable in the input and the IP (C). To test the antibody affinity we performed an αGFP pulldown in wild-type embryonic lysate (D). We could not detect any LIN-5 or GPR-1/2 in the IP lane. All IPs were performed the same way (see material and methods). The membrane was cut at 75 kDa and 50 kDa to allow incubation with different antibodies in parallel.
Data from the mammalian system suggests an interaction of NuMA with microtubules, as is the case for the dynein/dynactin complex. Our localization studies, showed that DYRB-1 also localizes to the spindle. Therefore, DYRB-1 could interact with LIN-5 via microtubules. Alternatively, the interaction could be microtubule independent. To distinguish between these two possibilities, we tested whether LIN-5 still interacts with DYRB-1 when microtubules are disrupted upon Nocodazole treatment. To that end, the embryonic extract was pre-incubated with 50µM Nocodazole. We found that LIN-5 still co-immunoprecipitates with DYRB-1, suggesting that the LIN-5/DYRB-1 interaction is microtubule independent (Figure 33).

Figure 33. Interaction between LIN-5, GPR-1/2 and DYRB-1 is microtubule independent.
(A) GFP::DYRB-1 embryonic extract, untreated, LIN-5 and GRP-1/2 co-immunoprecipitate with DYRB-1. (B) Upon nocodazole treatment (50µM) LIN-5 and GPR-1/2 still co-immunoprecipitate with DYRB-1. Immunoprecipitation conditions described in Material and Methods, Nocodazole treatment parallel to αGFP binding, 3 hours at 4°C rotating.
Consistent with the biochemical interaction, we observed co-localization of LIN-5 and GFP::DYRB-1 at the cortex and on the central spindle (see Figure 34). However, this localization is not interdependent, since depletion of LIN-5 does not effect DYRB-1 localization and *vice versa*, suggesting that it could be important to regulate the activity of the complex (data not shown).

In conclusion, we can recover LIN-5, GPR-1/2 and DYRB-1 in the same complex. DYRB-1 interacts with LIN-5 in a microtubule independent manner. However the question whether or not DYRB-1, LIN-5 and GPR-1/2 act together in a parallel pathway to regulate dynein still remains unclear.
Figure 34. Co-localization of LIN-5 and GFP::DYRB-1 in the early embryo.

(A), (C), (E) and (G) one-cell embryo, (B), (D), (F) and (H) two-cell embryo expressing GFP::DYRB-1. Embryos are fixed and stained with anti-GFP antibodies in (A) and (B) and with anti-LIN-5 antibodies in (C) and (D). (F) and (H) show the DNA and (G) and (H) the merge of all three channels. LIN-5 (Alexa568) and GFP::DYRB-1 (Alexa488) colocalize at the cortex and on the central spindle (yellow overlay). Posterior is to the right. The scale bar in (H) represents 10 μm.
CHAPTER IV

4. Discussion

4.1 PART I: RIC-8

In *C. elegans* a nontraditional G-protein signaling pathway is thought to direct centrosome movements during cell division. This works via a GPCR-independent activation of Go. Two almost identical GoLoco-domain containing proteins, GPR-1 and GPR-2, activate HGP signaling. GPR-1/2 acts as a GDI for Go, thereby stabilizing it in its GDP bound state. Since loss of Go and loss of GPR-1/2 display the same phenotype, GoGDP could be the signaling molecule in this pathway. In the mammalian system a molecule, RIC-8, was found to act as a GEF for monomeric Go subunits (Tall et al. 2003). Therefore, one possibility is that RIC-8 exchanges GDP with GTP in Go, thereby activating it. Indeed here we show that RIC-8 plays an important role for regulating the position of the mitotic spindle in *C. elegans*. We found a genetic interaction of ric-8 with two Go subunits goa-1 and gpa-16. We find that RIC-8 physically interacts with GOA-1 but we are unable to detect a physical interaction with GPA-16. We observed in our experiments that RIC-8 binds preferentially to GOA-1 in its GDP bound form, consistent with RIC-8 being a GEF for Go. Furthermore, we show that RIC-8 localizes to the cortex, to the cytoplasm and to the mitotic spindle in the early embryo. Depletion of RIC-8 leads to a similar phenotype as loss of both Go’s together and results in lower forces acting on the mitotic spindle. The asymmetric localization of GPR-1/2 that correlates with an asymmetry of pulling forces (Gotta et al. 2003, Colombo et al. 2003) is abolished in ric-8 mutant embryos. This data suggests RIC-8 also plays a major role in the regulation of HGP signaling. Our phenotypic and binding data indicates that RIC-8 controls spindle positioning by modulating the activity of Go and therefore modulating the pulling forces exerted on the spindle. Our results provided indication for a putative GEF-role of RIC-8.
4.1.1 RIC-8 is a GEF for the Ga subunit GOA-1

RIC-8 has recently been shown to be a regulator of HGP function in C. elegans and Drosophila by different groups independently (Manning et al. 2003; Hampoezlz et al 2004; Siderovski et al. 2005; Hess et al. 2004; Couwenbergs et al. 2004; Srinivasan et al. 2003).

From our data (presented in Chapter II), we deduced a model in which RIC-8 has a putative GEF role for the HGP Ga subunit GOA-1. Our data indicates that RIC-8, like Ga and GPR-1/2, plays a positive role in spindle positioning.

We suggest that GPR-1/2 initially binds to the heterotrimer GaGDPβγ, dissociates βγ and stabilizes GOA-1 in its GDP bound state. RIC-8 then promotes the exchange of GDP for GTP (Manning et al. 2003; Hampoezlz et al 2004; Siderovski et al. 2005; Hess et al. 2004; Couwenbergs et al. 2004; Srinivasan et al. 2003; Figure 35). In our model GOA-1GTP is the active signaling molecule and can act downstream on different target molecules to regulate the mitotic spindle.

Figure 35. Our working model of HGP regulation in the early C. elegans embryo
GPR-1/2 binds to the HGP complex thereby promoting the dissociation of Gβγ from Ga, keeping Ga stabilized in its GDP bound state. RIC-8 binds to free GaGDP leading to exchange of GDP for GTP. In our model we favoured GaGTP as the signaling molecule for regulating the mitotic spindle.

However, our model has been challenged by data from Afshar et al. 2004, showing that the regulatory order in which GPR-1/2 and RIC-8 interact with HGP is different. RIC-8...
binds to Goα prior to interaction with GPR-1/2 in the sequence of events that lead to Goα activation. This was concluded from data, showing that RIC-8 is able to interact with GOA-1 in the absence of GPR-1/2. The depletion of Gβγ also results in free Goα that is available for RIC-8 interaction. In conclusion, the association of GPR-1/2 to Goα is not essential for RIC-8 binding. But indeed, RIC-8 is essential for the asymmetrically higher levels of gpr-1/2 at the posterior cortex. These are significantly diminished in ric-8 mutant embryos (Couwenbergs et al. 2004, Afshar et al. 2004). Other data showed that RIC-8 co-immunoprecipitates with GPR-1/2 (Afshar et al. 2004). Which in turn, would indicate that GPR-1/2 and RIC-8 form a complex together with GOA-1. This contradicts the finding, that RIC-8’s GEF activity is inhibited by the GoLoco domain of GPR-1/2 (Afshar et al. 2004). The mode of regulation is not completely clear from this data.

The discovery that RIC-8 acts prior to GPR-1/2, raised the possibility that RIC-8 activates Goα dependent force generation by supporting stable levels of free GOA-1. In this case, inactivation of Gβγ should enable GOA-1 to interact together with GPR-1/2 even in the absence of RIC-8. Observations of the forces acting on spindle poles in gpb-1 mutants (Gβ') supported this data (Afshar et al. 2004). Depletion of Gβ results in significantly higher forces acting on the posterior spindle pole, than observed in wild-type embryos. As mentioned previously, reduction of RIC-8 results in lower forces on both spindle poles, resulting in a more symmetric cleavage, similar to Goα depleted embryos where forces on both poles are equalized. Embryos lacking Gβ and RIC-8 together show high forces on both spindle poles, equal to Gβ' embryos, leading to asymmetric division. This data shows that loss of Gβ alleviates the need for RIC-8 in the one-cell embryo. This finding was supported by data showing that depletion of Gβ restores GPR-1/2 localization in ric-8 mutant embryos (Afshar et al. 2004), similar to wild-type GPR-1/2 distribution.

In light of this more recent discovery, a different model is proposed: RIC-8 acts before GPR-1/2 in the activation cycle (depicted below in Figure 36A). As RIC-8 is a GEF for Goα, RIC-8 may act on an intermediate GoαGDP, dissociated from Gβγ, thereby exchanging GDP for GTP. Thereafter, GoαGTP can be converted to GoαGDP by the
intrinsic GTPase activity or by an RGS (Regulator of G protein Signaling) protein. GaGDP is then capable of binding GPR-1/2, which represents the signaling molecule in spindle positioning. In this model, RIC-8 mutants should show a defect in generating GaGDP-GPR-1/2. Indeed, depletion of RIC-8 results in lower amounts of GaGTP, leading to lower levels of free GaGDP capable for GPR-1/2 binding and therefore less signaling molecules (Figure 36B). Inactivation of Gßy alleviates the need for RIC-8 in one-cell stage embryos (Figure 36C). The dissociation and re-association of the heterotrimer is not explained in this model.

![Figure 2. Working Model of RIC-8 and GPR-1/2-mediated Ga activation during asymmetric cell division](image)

(A) RIC-8 (GEF) promotes exchange of GDP for GTP. Thereafter, intrinsic GTPase activity of Ga converts Ga to a GDP bound state, ready for interaction with GPR-1/2.

(B) Inactivation of RIC-8 results in reduced levels of GaGTP, which also decreases levels of GaGDP available for GPR-1/2 (GDI).

(C) Additional inactivation of Gßy diminishes the importance of RIC-8, levels of GaGDP are increased. Also increased levels of GaGTP may occur without requirement for GEF activity under these circumstances, either due to spontaneous exchange or to a RIC-8 independent GEF activity (Figure adapted from Afshar et al. 2004).

The release of Gßy subunits plays a major role in the GDP/GTP cycle. RIC-8 cannot act on the heterotrimer Gaßy and the Afshar model does not clarify how dissociation may occur. Furthermore, there remains an open question as to why the intermediate GaGDP binds to RIC-8 and not GPR-1/2. This model is consistent with some data, but does not completely explain the mechanism of regulation. E.g. it remains unclear how the disassembly of the GaGDP-GPR-1/2 complex occurs, to close the regulation cycle.
4.1.2 RGS-7 is a GAP for Gα

Another protein, RGS-7, a GAP for GOA-1 and GPA-16 is required to negatively regulate posterior centrosome movements in the early *C. elegans* embryo (Hess et al. 2004). *rgs* mutants display exaggerated transverse oscillations of the posterior centrosome, stronger displacement of the spindle towards the posterior pole and formation of a smaller posterior cell. These phenotypes could be provoked either by enhanced pulling forces on the posterior aster and/or reduced anterior pulling forces. In *rgs-7* embryos the posterior centrosome movement was unchanged compared to wild-type embryos. But interestingly, the anterior centrosome moved significantly slower to the anterior pole, indicating lower forces acting on the anterior pole but not to such an extent as observed in GPR-1/2 or Gα depleted embryos (Colombo et al. 2003; Hess et al. 2004; Afshar et al. 2004). These observations lead to the conclusion that the RGS protein described in the Afshar model cannot be RGS-7. The role of RGS-7 in spindle regulation is still unclear. If the model postulated by Afshar is right, another RGS protein would be involved in the cycling process.

4.1.3 RIC-8 is required for the cortical localization of GPA-16

The regulation of the mitotic spindle via the HGP complex is even more complicated in *C. elegans*. RIC-8 plays different roles for the Gα subunits GOA-1 and GPA-16. Most recent data has also shown the association of RIC-8 with GPA-16. The localization of GOA-1 was shown to be independent of RIC-8 in the early embryo (Afshar et al. 2004; Couwenbergs et al. 2004), while GPA-16’s cortical localization is dependant on RIC-8. Furthermore, RIC-8 does not exhibit any GEF activity for GPA-16 (Afshar et al. 2005) but it does for GOA-1 (Afshar et al. 2004; Couwenbergs et al. 2004; Hess et al. 2004). Future work will establish how these different roles contribute to spindle positioning.
4.1.4 What is the signaling molecule in HGP signaling in *C. elegans*?

An interesting open question, is whether GoGDP or GoGTP represents the active signaling molecule. In our model, we favoured GoGTP as an active molecule signaling downstream on specific targets for spindle regulation and GoGDP as being only an intermediate state capable for the binding of RIC-8. The discrepancy in our model is the fact that loss of Gβ diminishes the need for RIC-8 in the one-cell embryo (Afshar et al. 2004). This data leads to the conclusion that RIC-8 functions before GPR-1/2 to make GOA-1 available for its interaction with GPR-1/2. In this case, the signaling molecule would be the GOA-1/GPR-1/2 complex.

It is still unclear whether GoGDP or GoGTP is the signaling molecule in *C. elegans*. But if GoGTP were the signaling molecule, then *C. elegans* would be different form other organisms, e.g. *Drosophila* (Schaefer et al. 2001). Another possibility might be that the important step for regulation is the cycling between GoGDP and GoGTP and not the activity of one signaling molecule.

4.1.5 The role of RIC-8 in *Drosophila melanogaster*

Ric-8 is a highly conserved molecule. Recent studies in *Drosophila* NB and SOP cells have shown that spindle positioning is regulated by HGP signaling (David et al. 2005). In these cells, the pathway is activated by two types of Ga-binding proteins, first GDP-dissociation inhibitors (Pins and Loco) and second Ric-8. Ric-8 was recently shown to also be essential for spindle regulation and differences in daughter cell size in *Drosophila*. RIC-8 is thought to act as a GEF, promoting exchange of GDP by GTP in NB and SOP cells (Wang et al. 2005; Hampoezl et al. 2005). This was shown by co-immunoprecipitations of Ric-8, which found it to be in a complex with Pins and GoGDP and not GoGTP. As in *C. elegans*, Ric-8 is required for proper membrane targeting of HGP in *Drosophila* (Wang et al. 2005; Hampoezl et al. 2005; David et al. 2005). In the absence of Ric-8, however, all G-protein subunits (Gαi, Gαo, Gβ- and by extension, presumably Gγ) fail to reach the cell cortex.
RIC-8 has different functions for the HGP subunits. It regulates the cortical localization of all three subunits. Additional, RIC-8, as a GEF, is regulating Gα’s activity. Gβγ inhibits the activity of Gα, since the signaling molecule has to be released from Gβγ. The PAR proteins together with Gα regulate spindle positioning. This data is summarized in Figure 37, depicted below.

![Diagram of asymmetric spindle regulation](image)

**Figure 37**: A model on the regulation of heterorimeric G proteins in the model organism *Drosophila melanogaster*

Ric-8 localizes multiple Gα subunits to the cortex of the NBs and/or acts as a putative GEF towards Gα, whereas Gβγ act to restrict Gα localization to the apical cortex only. Ric-8 also indirectly regulates the cortical localization of Gβγ. Asymmetrically localized Gα subunits, in conjunction with Par proteins, mediate asymmetric spindle geometry and differences in daughter-cell size (based on genetic analysis by Wang 2005).

### 4.1.6 What is the signaling molecule in HGP signaling in *Drosophila*?

In *Drosophila*, the question “which is the signaling molecule” in HGP signaling seems to be partially solved. The signaling molecule could be GαGDP, GαGTP or Gβγ. To distinguish between these possibilities wild-type Gαi and Gαi,Q205L (GTPase deficient) were over-expressed. The wild-type Gαi should bind and deplete Gβγ thereby inhibiting downstream interactions, while Gαi,Q205L in contrast should be unable to bind Gβγ. The Gβγ signaling would be unaffected while Gα signaling should be enhanced.
Interestingly, over-expression of wild-type Gαi resulted in embryos with changed protein localizations and an equalized daughter cell size (Schaefer et al. 2001; Yu et al. 2003). E.g., Gαi and Pins were found all over the cortex, in contrast to wild-type cells where they are localized on the apical cortex. Furthermore, Pins was recruited to the cortex in higher levels (Schaefer et al. 2001). If these effects are due to depletion of Gβγ, over-expression of GαiQ205L should not display any defects. This was the case, indicating that over-expression of Gαi results in depletion of Gβγ. This cannot be the sole reason, as the phenotypes observed in cells over-expressing Gαi differ from Gβ13F mutant cells (Schaefer et al. 2001). These results show that GαGTP cannot be the signaling molecule in flies. Therefore, it was suggested that other mechanisms, such as depletion of another Gβ subunit or signaling via the GDP-bound form of Gαi may contribute to these phenotypes. More recent work on Gαo suggested that the GDP-bound form of Gαo might be responsible for the defects in size asymmetry observed in the over-expression experiments (Yu et al. 2003). This was shown by over-expression of a putative constitutively active GαoQ205L, which does not show any defects in spindle geometry in NB cells.

This data shows that in flies GαGTP cannot be the signaling molecule in this process. The signaling molecule is represented by GαGDP, Gβγ or both together. In contrast to C. elegans, in flies, Gβ also plays an important role in the signaling pathway.

4.1.7 RIC-8 in mammalian cells

In mammals RIC-8 was first shown to behave as a GEF for HGP signaling, however there is still no evidence for a role in spindle positioning. In mammals, a receptor-unbound activation of heterotrimeric G protein signaling also exists (Tall et al. 2003). Recent work in mammals should answer the question of whether or not the GoLoco bound Gαi serves as a substrate for RIC-8A-mediated guanine nucleotide exchange. As mentioned previously, two GoLoco domain containing proteins exist in mammals, LGN and AGS3, both of which are important for HGP signaling. LGN, the microtubule-binding nuclear mitotic apparatus protein NuMA and Gαi regulate a similar process.
Tall and Gillman (2005) performed several experiments demonstrating that mammalian RIC-8 recognizes LGN- and AGS3-Gαi,GDP complexes in vitro. Furthermore, they could show that RIC-8 dissociates GoLoco protein/Gαi,GDP complexes while activating Gαi catalytically in the presence of GTPγS. In another experimental set it was shown, that activation of the Gαi/LGN/NuMA complex by RIC-8A also stimulates the release of NuMA from LGN.

### 4.1.8 Conclusion

In all three model organisms RIC-8 was shown to behave as a GEF for Gα. In *C. elegans* the importance of RIC-8 was shown to be diminished in the absence of Gβγ, which contradicts the findings in *Drosophila* that ric-8 mutants are genetically epistatic to Gβ mutants, both with respect to Gαi-Pins localization and to spindle geometry. Interestingly, the mammalian RIC-8 shows no influence on spindle positioning at all. An important open question pertains to the form of Gα that generates force. It is possible that GαGDP, perhaps in a complex with GPR-1/2, is the active species, since GPR-1/2 is a GDI that acts as a positive regulator of Gα activity in the embryo and because GPR-1/2 acts after RIC-8 in the activation cycle. The working models do not exclude, however, GαGTP as the active species. For instance, GPR-1/2 may serve as a cortical reservoir for GαGDP, facilitating local transit through the entire Gα nucleotide cycle and ultimately promoting local generation of GαGTP. Other data from mammals, suggests that Gα is only the cortical anchor for the other proteins. Gα is localized to the cortex and interacts with LGN (*C. elegans* GPR-1/2, *Drosophila* Pins/Loco), which in turn can bind to NuMA (LIN-5, Mud). NuMA interacts with the dynein/dynactin complex, linking the cortical signal to the mitotic spindle. These recent findings show one thing in common, a core set of proteins that can be regulated in a similar fashion in all discussed animal systems and that perhaps an “active molecule” is not required. Only the cycling between GDP and GTP may be important for the HGP activity in spindle positioning.
4.2 PART II: DYRB-1

In the second part of the project, we were interested in new molecular players important for the regulation of the mitotic spindle. One candidate confirmed in our enhancer screens is showing genetic interaction with both gpr-1 and lin-5, two genes well known to regulate spindle positions. This gene, T24H10.6, recently named dyrb-1, with so far unknown function in C. elegans, was shown to be homologous to members of the Roadblock family of dynein light chains in Drosophila melanogaster, with a hypothesized regulatory function for dynein. Our data suggests that DYRB-1 also plays an important role in spindle positioning. The localization pattern of GFP::DYRB-1 together with the phenotypical data gave indications for a possible regulation of dynein activity by dyrb-1. We further investigated if dyrb-1 is needed for force generation on the mitotic spindle and dissected the forces and the spindle orientation in the early C. elegans embryo. We found that the forces acting on the mitotic spindle in embryos depleted of DYRB-1 are strongly diminished on both spindle poles, leading to loss of asymmetric forces. We further investigated if the genetic interaction of dyrb-1 is also a physical one. We found DYRB-1 to co-immunoprecipitate with LIN-5 and GPR-1/2 in embryonic extracts. Our data allowed us to draw further conclusions about the signaling pathway leading to correct spindle regulation in the 1-cell C. elegans embryo.

4.2.1 Cytoplasmic dynein- an overview

Biological motor molecules in vivo possess many of the characteristics required to power nanomachines. They can generate force and torque, transport specific cargoes over appropriate substrates, and the character and rate of their action can be controlled. In the background of this work, the motor protein dynein is of special interest. Dynein is a large, multimeric, minus-end directed microtubule motor that has been implicated in numerous cellular functions. Cytoplasmic dynein contains two approx. 520 kDa heavy chains (HC) that form the stems and globular heads of the complex (see Figure 38). The heavy chain contains a large fundamental motor domain in the C-terminal fragment, incorporating four ATP-binding sites, including the ATP-hydrolytic site that provides
the energy for its movement along microtubules and a microtubule binding site (Gee et al. 1997; Koonce et al. 2000; Nishiura et al. 2004). Proteins associated with the stem domains of the HCs include a dimer of 74 kDa intermediate chains (IC74), which mediates dynein-dynactin interactions, several light intermediate chains (LIC) and different classes of light chains (LC).

![Diagram of a dynein molecule](image)

**Figure 38: Schematic representation of a dynein molecule**

Cytoplasmic dynein consists of a homodimer of heavy chains (HC: green), a dimer of intermediate chains (IC74: light blue), four light intermediate chains (LIC: blue) and several light chains (LC: yellow, magenta and red).

### 4.2.2 Dynein function

In cytoplasm, the dynein motor transports various vesicles and other cargos towards the minus end of microtubules. To date, these enzymes are known to be involved in vesicular transport, maintenance of the Golgi apparatus, nuclear envelope breakdown and the transport of a variety of protein complexes (Vallee et al. 1996; Karki et al. 1999). Cytoplasmic dynein is also clearly involved in mitosis, during which it participates in spindle orientation, aspects of chromosome movement and spindle pole organization. In our work we showed special interest in understanding how spindle orientation, positioning and the asymmetry in forces acting on the spindle poles is regulated. We wonder what the role of dynein could be in this process and how it is regulated.
4.2.3 Dynein light chains

While multiple motors have been identified in mammals, *Drosophia* or *Chlamydomonas* and in some cases their cargoes could be determined, little is known about the regulation of the movement they drive. Recent work suggests that dynein light chains (DLCs) may play crucial roles in dynein function and regulation. Dynein heavy chains (DHCs) are tightly associated with one or more light chains that may directly regulate motor function. To date, in this context three classes of cytoplasmic DLCs have been identified. The highly conserved LC8 (King et al. 1995; Jaffrey and Snyder, 1996) and Tctex1/Tctex2 (Patel-King et al. 1997; King et al. 1996) are two classes which have been shown to bind to distinct regions of IC74, thereby modulating dynein-cargo attachment and motor functions. Recently, the third class has been identified in a mutant screen, based upon larval phenotypes, searching for posterior sluggishness and axonal cargo accumulation in *Drosophila*: The Roadblock/LC7 family of DLC. This family of DLC was shown to directly bind to the intermediate chains of cytoplasmic dynein (Song et al. 2005). Investigations of the binding sites showed that a single intermediate chain could bind to each of the three DLC families in parallel (Susalka et al. 2002).

4.2.4 The Roadblock/LC7 family of dynein light chains

The roadblock light chains seem to be crucial for the proper functioning of the cytoplasmic dynein complex.

The sequence of the Roadblock/LC7 family appears to be highly conserved through evolution. To date 14 representatives of this family are known: 6 in *Drosophia*, 6 in mammals, 1 in *Chlamydomonas* and 1 in *C. elegans*. The exact function of these proteins is unknown, but mutations of the roadblock gene in *Drosophila* result in defects in mitosis, accumulation of vesicles in axons and larval or pupal lethality (Martin et al. 1999; Hurd et al. 1996; Bowman et al. 1999). In *Drosophila*, roadblock was suggested to control some functions of dynein (Bowman et al. 1999). Recent data from mammals showed that Roadblock/LC7 rob1 (mouse) forms a homodimer, binding
to the IC74 at a site close to the DHCs (Figure 38, mouse rob1 is represented in red, Song et al. 2005) and thereby modulating the dynein-cargo attachment and motor functions. We want to discuss now if the C. elegans homologue to roadblock/LC7, dyrb-1, plays also a role in dynein regulation, as it is suggested in other organisms.

### 4.2.5 Possible roles for dyrb-1 in the early C. elegans embryo

Our data (see chapter III) suggests that DYRB-1 is positively regulating dynein dependent processes in the early C. elegans embryo. This will be discussed now in more detail in the background of dynein function. Our first indications came from the characterization of dyrb-1 (RNAi) embryos, displaying a significant delay in pronuclear migration, a delay in rotation and centration of the nuclear centrosomal complex (NCC) and the suppression of hyperactive oscillations of the NCC in the background of let-99ts mutant embryos. All this processes are connected to microtubules and have been shown to be dynein dependent (Gonczy et al. 1999, Schmidt et al. 2005). From this we could deduce that dyrb-1 is positively regulating dynein functions. But what is regulated? As mentioned before, the two processes discussed here are MT dependent. During the fast phase the maternal pronucleus is pulled towards the paternal pronucleus via MT interactions. Furthermore, the NCC is rotated and centrated with help of MTs. In dyrb-1(RNAi) embryos it seems that the relative net forces acting on the centrosomes are reduced, resulting in the observed phenotypes. From this data we deduce a role for dyrb-1 in the positive regulation of dynein dependent force generation in the early C. elegans embryo.

This data is in concert with observations which show that GFP::DYRB-1 exhibits a similar localization pattern to DHC-1. If we compare the localization pattern in C. elegans with data from mouse, we find that the rob11 and the rob12 localization pattern (Nikulina et al. 2004) overlaps. This would harmonize with the finding that the roadblock LCs are incorporated as homodimers into the dynein molecule, thereby regulating DHC functions (see above, Song at al. 2005). DYRB-1 plays not only an important role in pronuclear migration, centration and orientation of the NCC, it is as
well involved in events concerning the mitotic spindle, as reported before for \( dhc-1 \) (Gonczy et al. 1999; Schmidt et al. 2005). This will be discussed later on.

Interestingly, during our observations we never found a full \( dhc-1 \) phenotype or lethality upon depletion of \( dyrb-1 \). For this we have different explanations. First, we cannot exclude that we fully deplete \( DYRB-1 \) by RNAi. Second, the lethality is maybe not related to the phenotype. The phenotype is highly penetrant in the 1-cell embryo. Maybe the resulting lethality (by RNAi injection) is due to an effect in the later embryo. Third, the dynein molecule is created of different components (described above, and Figure 38). It is possible that different classes of light chains can bind parallel to one intermediate chain. Maybe the depletion of one class (here the roadblock/LC7 family) leads only to a partial dynein phenotype. It would be interesting if the depletion of two classes of DLC in one organism would already result in a strong \( dhc-1 \) phenotype. Therefore, \( dyrb-1 \) is regulating only some functions of dynein and for the maximum force generation another protein may be also involved, which acts parallel to the \( dyrb-1 \) regulation. This could be a light chain or another protein. Possible candidates will be discussed in the following paragraphs.

### 4.2.6 \( dyrb-1 \) genetically interacts with \( lin-5 \) and \( gpr-1 \)

As previously presented, \( dyrb-1 \) depletion in the background of \( gpr-1 \) and \( lin-5 \) leads to enhanced lethality. This lethality is a result of the strong synthetic interaction between \( dyrb-1 \) and these genes. The phenotype described for \( dyrb-1 \) is stronger in these backgrounds, leading to more severe defects. In these embryos the spindle is not rescued anymore to an anterior-posterior orientation. This could be due to the incomplete centration and rotation of the NCC. As mentioned before, these are MT dependent processes, which are dynein dependent. Therefore, a stronger reduction of relative net forces acting on the centrosomes in \( dyrb-1(\text{RNAi}); gpr-1ts \) or \( dyrb-1(\text{RNAi}); lin-5ts \) embryos could be the reason for an oblique posterior elongated spindle. This phenotype is highly penetrant and overcomes the symmetric phenotype of \( gpr-1/2 \) or \( lin-5 \) embryos. To summarize, the phenotypes (characterized in Chapter III) are stronger
Chapter IV

than the one of *dyrb-1* alone and partially reminiscent of the phenotype reported for *dhc-1* (Goenczy et al. 1999; Schmidt et al. 2005). From this data we conclude that *dyrb-1* interacts genetically with *lin-5* and *gpr-1/2* and that these molecules are important for the regulation of microtubule dependant movements within the early embryo. We will discuss this in more detail in the background of the forces acting on the mitotic spindle.

4.2.7 How is the mitotic spindle asymmetrically positioned?

The astral MTs extending from the centrosomes to the cortex are required to transmit cortical information to the spindle. Mutations that result in short astral MTs fail dramatically in spindle positioning (Albertson et al. 1984; Bellanger et al. 2003; Hyman et al. 1987; LeBot et al. 2003; Sryko et al. 2003). The most likely mechanistic basis for both, the rotation (see above) and the spindle displacement is the interaction of astral MTs with cortical force generators, such as dynein, coupling the dynamics of MTs to movement. Direct evidence for pulling forces came from laser ablation experiments in which the midzone of the early anaphase spindle was physically destroyed (Grill et al. 2001, see chapter I). The force imbalance results in one pole being selectively pulled toward the cortical region. For this different models of cortical inhomogeneity exist (see also chapter I). How is the imbalance in the forces acting on the two poles of the spindle generated? The measured relative force difference is under the control of cortical polarity, since mutations in the PAR proteins equalize the forces acting on the poles. Recent work suggests that the preferential posterior displacement of the spindle probably results from a combination of greater number of force generators at the posterior cortex (Grill et al. 2003) together with a greater frequency of shrinking MTs in the posterior (Labbe et al. 2003). Microtubule dynamics where also shown to be under the control of the PAR proteins and HGP signaling (Labbe et al. 2003).

The force generators at the cortex have not been unambiguously identified, but pulling forces are under the control of a receptor-independent G protein pathway. Mutants that reduce the activity of Gα-dependent signaling reduce the forces acting on the spindle (Gotta and Ahringer, 2001; Grill et al. 2003), and at least one of the regulators of G protein signaling, GPR-1/2, is asymmetrically localized when force on the posterior
spindle pole is at its maximum (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003). Therefore, it is in some way still to be determined, if the cortical polarity of the embryo acts through the PAR proteins (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003) to up-regulate G protein signaling in the posterior, increasing the total force (Colombo et al. 2003) thereby generating asymmetric spindle position. It remains still unclear which molecules are linking the HGP signaling pathway to the mitotic spindle. We will try to give a more defined picture of the possibilities.

4.2.8 dyrb-1 is needed for force generation in the early embryo

Data from different laboratories concerning the mitotic spindle led to the conclusion that components of the HGP pathway are important for regulation of forces acting on the spindle poles during asymmetric cell division. Depletion of LIN-5, GPR-1/2 or Gα leads to symmetric and reduced peak velocities measured at the anterior and posterior spindle poles (presented in Chapter III). We know that also dynein is important for force generation on the mitotic spindle poles (Schmidt et al. 2005). As already mentioned before, the phenotypes resultant from DYRB-1 depletion (e.g. delayed pronuclear migration, incomplete NCC rotation and centration, absence of posterior spindle pole oscillations and flattening of the posterior aster) give indications that dyrb-1 is necessary for force generation in the early embryo. Indeed, spindle severing experiments in dhc-1(ct77)i+ and dyrb-1(RNAi) embryos showed a reduction of peak velocities on both spindle poles when compared to wild-type measurements.

The depletion of dyrb-1 results in lower and symmetric peak velocities measured on both spindle poles, but forces are still higher than in lin-5, gpr-1/2 or Gα mutant embryos. What could be the reason for this? The explanation is simple. First, we maybe cannot fully deplete DYRB-1, and this results in still higher forces. Second, dyrb-1 is not exclusively regulating dynein function, other molecules could be also involved in this regulation. Third, dynein is not exclusively responsible for the forces acting on the spindle poles. If dyrb-1 is depleted in the background of the mentioned mutants (above and chapter III), the peak velocities acting on the spindle poles are significantly reduced and similar to those forces observed in Gα, or GPR-1/2 depleted embryos. To
remember, Gα was shown to be important for higher MT dynamics in the posterior of the embryo (Labbe et al. 2003). But why are the forces lower in Gα than in dyrb-1 depleted embryos? One possibility might be that the HGP signaling pathway is not only regulating the MT dynamics, but also the force generation of dynein. All together leads to the conclusion that DYRB-1 functions together with LIN-5, GPR-1/2 and therefore HGP to generate forces in the early embryo.

### 4.2.9 Polarity is not affected in dyrb-1 depleted embryos

As the asymmetry of forces in the early embryo is mostly dependent on asymmetric distribution of certain proteins, the PARs or GPR-1/2, we investigated whether depletion of DYRB-1 leads to disrupted protein distribution. But what would explain the symmetric forces in these embryos? We found PAR polarity unchanged in dyrb-1 (RNAi) embryos. Moreover, we further observed unchanged asymmetric localization for GPR-1/2. We therefore concluded, that polarity is not affected in these embryos and that DYRB-1 acts either at the level of PAR polarity or downstream. We will discuss the role of DYRB-1 in the background of asymmetric spindle regulation and PAR regulation in more detail (see below).

### 4.2.10 A possible role for dyrb-1 in the HGP signaling pathway

We suggest a role for dyrb-1 in spindle regulation, possibly acting together with LIN-5 and GPR-1/2, since we could show genetic and physical interaction. We learned from other model systems that the mammalian NuMA (C. elegans LIN-5, Drosophila Mud) and LGN (GPR-1/2, Pins) are found in a complex together with Gα, all of them important for spindle regulation (Du and Macara, 2004; Lechler and Fuchs, 2005; Siller et al. 2006; Izumi et al. 2006; Bowman et al. 2006; Du et al. 2006).

Like the mammalian NuMA, Drosophila Mud can directly bind to microtubules (Merdes et al. 2000; Bowman et al. 2006) and enhance microtubule polymerization. In the absence of Mud, mitotic spindles in Drosophila neuroblasts fail to align with the
polarity axis (Siller et al. 2006; Izumi et al. 2006; Bowman et al. 2006) which can lead to symmetric segregation of cell fate determinants. The C. elegans LIN-5 could play a similar role and interact with MTs, as deduced from its localization pattern (Chapter III). On the other hand, we know that LIN-5 binds to GPR-1/2, which in turn interacts with Gαi and is thereby asymmetrically targeted to the cortex.

Data from Bowman (2006) suggests a model in which asymmetrically localized Pins-Gαi complexes regulate spindle orientation by directly binding to Mud. The idea is that Gαi is anchored to the cell cortex and bound to Pins, which is itself directly bound to Mud, which in turn sits on MT and therefore coordinates the orientation of the mitotic spindle with cortical polarity (Bowman et al. 2006; Siller et al. 2006). Gαi and Pins are also needed for cell size asymmetry. Interestingly, LGN and GPR-1/2 cortical association depends on NuMA and LIN-5, respectively (Gotta et al. 2003; Srinivasan et al. 2003; Du et al. 2004), whereas Mud can localize to the cortex in the absence of cortical Gαi and Pins (Siller et al. 2006; Izumi et al. 2006; Bowman et al. 2006).

From our co-immunoprecipitation data, we conclude that DYRB-1 is in a complex with LIN-5 and GPR-1/2 even in the absence of MTs. Therefore, LIN-5 could be the key to help close the gap between HGP signaling and spindle regulation via dynein, regulated by DYRB-1. In this case, the regulation of the spindle in C. elegans would be similar to the processes occurring in Drosophila. From our data in the background of the known data, we draw a model in which the asymmetric positioning of the mitotic spindle is regulated in one pathway. To summarize shortly:

1. Which molecules are needed to generate asymmetric forces on the spindle poles? We have on one side the PAR proteins, the HGP subunits Gα: GOA-1 and GPA-16, GPR-1/2 and LIN-5. Gα directly targets GPR-1/2 to the posterior cortex, which then in terms interacts with LIN-5. LIN-5 would have a similar role to the one of NuMA, as a molecular link between MTs and dynein/dynactin. And on the other side we have dyrb-1, found in a complex with LIN-5 and GPR-1/2 and regulating some, but not all, aspects of dynein, connecting the HGP pathway to dynein.

2. Why don’t we loose all forces acting on the spindle poles in dyrb-1(RNAi) or dhc-1ts embryos? As mentioned before, the theory exists, that we need force generators on the cortex combined with MT dynamics to guaranty the asymmetry of forces. It was shown
that MT dynamics are under control of PAR-Polarity and Gaαs (Labbe et al. 2003). On the other hand is dynein a candidate acting as "the" force generator. Now we have a new link between dynein and HGP signaling, because of the genetic and physical interaction of DYRB-1 with GPR-1/2 and LIN-5.

3. How could the mitotic spindle be regulated in terms of posterior displacement? As mentioned before GPR-1/2, downstream of the PARs, is asymmetrically enriched at the posterior cortex when the forces on the posterior pole are highest (Gotta et al. 2003; Colombo et al. 2003; Srinivasan et al. 2003). If GPR-1/2 is depleted, we have the lowest
measured forces acting on the spindle poles. This is also the case for Gα, which is regulated by GPR-1/2. Therefore we suggest that the HGP signaling pathway regulates on one hand the microtubule dynamics (directly via the cortical PARs via Gα) and on the other hand the motor protein dynein. Dynein is partially regulated via dyrb-1, which interacts with GPR-1/2 and LIN-5. Thereby, we have the connection between HGP and dynein. It still has to be investigated how dyrb-1 is regulated through the HGP pathway, if it is binding to GPR-1/2 and LIN-5 or only to one of these two. For the asymmetric regulation of dynein, GPR-1/2 is the perfect candidate. But maybe it is only the molecular switch to target LIN-5 for its interaction with the cortical dynein complex (as supposed in other organisms, see above). Further it would be interesting if Gα is only the anchoring protein in this process, targeting GPR-1/2 and LIN-5 to the cortex, where LIN-5 can bind to MTs and gets transported towards the asters. This could be investigated by a mutation of the Gα cortical binding site. Clearly there remain many open questions that have to be answered in the future.

4.2.11 GOA-1 and GPA-16 act redundantly to regulate spindle positioning

Former work has shown that the two Gα subunits, GOA-1 and GPA-16, act redundantly to control spindle positioning downstream of the PAR proteins in the early *C. elegans* embryo (Gotta et al. 2003). In par mutant embryos, the pulling forces exerted on the spindle poles are equal, resulting in a symmetric cleavage compared to the asymmetric wild-type cleavage, where the forces acting on the posterior spindle pole are higher than on the anterior pole (Grill et al. 2001). Depletion of both Gα subunits also results in reduced forces and therefore leads to a symmetrically positioned spindle (Colombo et al. 2003, Chapter III), the same is true for loss of GPR-1/2 (Colombo et al. 2003, Gotta et al. 2003, Srinivasan et al. 2003). We hypothesized, that if GOA-1 and GPA-16 would act on two different substrates to regulate the mitotic spindle, animals completely depleted for both GOA-1 and GPA-16 would have the same phenotype as the single mutants. Alternatively, if GOA-1 and GPA-16 acted in parallel on one common
substrate the double mutant would have a stronger phenotype than the individual single mutants. We observed the phenotypes of both \textit{goa-1}(RNAi) and \textit{gpa-16}(RNAi) mutants depleted of DYRB-1. We could not find significant epistatic differences, neither in increased lethality nor phenotypically.

\textbf{Figure 40. Both G\textalpha}s act together on one common substrate to regulate spindle positioning}

Downstream of the PAR polarity, heterotrimeric G protein subunits GOA-1 and GPA-16 are important for spindle regulation. The molecular link between the PARs and HGP still has to be uncovered. LIN-5 is known to physically interact with GPR-1/2. Both are localized on the cortex, as is Ga. GPR-1/2 interacts with Ga, leading to further signaling downstream on one common substrate for spindle regulation. This player of the heterotrimeric G protein pathway still needs to be identified.

Furthermore, we found that \textit{goa-1} and \textit{gpa-16} together with \textit{dyrb-1}(RNAi) had similar peak velocities (Chapter III), which suggests that the two G\textalpha}s are acting together on one common substrate in the regulatory pathway.
From these data, we conclude that GOA-1 and GPA-16 act in concert on one substrate for spindle regulation.

4.2.12 Conclusions

In both organisms, Drosophila and C. elegans, the HGP signaling is clearly involved in regulation of cell size during asymmetric cell division. Recent work suggests that this function is conserved in higher organisms as well (Sanada et al. 2005). Asymmetric spindle displacement in C. elegans is regulated via modulation of the forces acting on astral microtubules. A similar mechanism might also exist for Drosophila. One possibility how spindle positioning is controlled, could be explained by a cortically anchored motor protein such as dynein, combined with MT dynamics under the control of PAR polarity. Our data shows a genetic and physical interaction between dyrb-1 and lin-5 and gpr-1/2, suggesting a connection between the heterotrimeric G protein signaling pathway and the motor protein dynein, where on one hand MT dynamics are regulated by the PARs and DYRB-1 on the other hand is regulating dynein force generation, maybe directly via LIN-5/GPR-1/2 interaction. We suggest that dyrb-1’s role in dynein regulation is similar to the one in other organisms.

The huge number of spindle assembly factors with partially redundant functions makes it difficult to test and understand the contribution of each individual protein. Moreover, several spindle assembly factors may play more than one role in cell division. It is clear, however, that spindles are highly dynamic structures that are organized and maintained by a complicated balance of forces. Further investigations are needed to answer all the questions arising with each new observation.
Materials and Methods

Strains and alleles

*C. elegans* Bristol strain N2 was used as the standard wild type strain. Nematode culturing was performed as described (Brenner et al. 1974). The mutation stains used for this thesis are *ric-8(mdl909)* (Miller et al. 2000), *gpr-1(or574ts)* (kindly provided by Bruce Bowerman and Morgan Golding), *gpr-2(tm0964)*, *lin-5(ev571ts)*, *let-99(or204ts)* (Tsou et al. 2003), *goa-1(n1134)* (Segalat et al., 1995), *goa-1(sa734)* (Robatzek and Thomas, 2000), *gpa-16(it143)* (Bergmann et al. 2003), *par-2(lw32), par-6(zu222)*. Temperature sensitive mutant strains were grown at 15 °C and shifted to restrictive temperature 24 h before the embryos were examined. The worms were usually been observed at 15°C, 20°C, 22°C and 25°C.

For the GFP::RIC-8 strain and the GFP::DYRB-1 strain, full length RIC-8 cDNA or DYRB-1 cDNA was cloned into pID3.01 (Pellettieri et al. 2003) using Gateway technology (Invitrogen). GFP lines were created using the microparticle bombardment technique (Praitis et al. 2001).

RNA synthesis was performed as described (Zipperlen et al. 2001). Embryos from injected mothers were analyzed at least 24 hrs after injection.

In Table 3 (Chapter II), two categories of position of first cleavage were used: percentage of egg length 49-54% and percentage of egg length >54%. These categories have been chosen based on measurements of spindle position in the par mutants, which all fall in the more symmetric first category (percentage of egg length: 51% ± 2% in *par-2(it5); 52% ± 1% in par-3(e2074), and 53% ± 2% in par-1(b274), Kemphues et al. 1988).
Materials and Methods

*In vitro* binding assays

The vector pMalC2 (NEB) was used to express MBP fusions of RIC-8 in *E. coli* C41 and later the fusion was cut via factor Xa (Amersham). pDEST17 (Gateway) was used to express HIS fusions of GOA-1 in *E. coli* C41. The *in vitro* binding experiments were performed in buffer A (20 mM TRIS, 10 mM MgSO₄, 130 mM NaCl) and with HIS-affinity beads (Talon resin, Clonetech). Binding of RIC-8 was performed as described (Natochin et al. 2004) with the following modifications: 0.05 % NP40 was added and 0.5 mM of each protein was used in each assay. Guanosine diphosphate (GDP) and guanosine 5'-O-(3-thiotriphosphate (GTPgS) were purchased from SIGMA. During the GDP binding to Galpha 10mM EDTA was added.

Yeast Two-Hybrid Experiments

The yeast strain Y190 (Clonetech) was used for the two-hybrid experiment. β-galactosidase activity was measured by a colony filter assay carried out according to published protocols (Golemis et al. 1996). Full-length wild-type RIC-8 fused to the DNA binding domain of GAL4, ful-length wild-type GOA-1, and full length Q205L mutant GOA-1 fused to the activation domain of GAL4 were used in this experiment. Later on, full-length wild-type RIC-8 fused with the DNA binding domain of GAL4, full-length G204A mutant GOA-1 fused with either the DNA binding domain or the activation domain of GAL4, and full-length GPR-1 fused with the activation domain of GAL4 were used.

GPA-16 interaction with RIC-8 was tested with GPA-16 as bait and prey and with two yeast strains (Y190 as before and MAV203, Invitrogen).

Microscopy and analysis of living embryos

For DIC analysis of living embryos, embryos were mounted as described (Gotta et al. 2003). The first cell cycle of embryos was recorded (1 image every 10s) with a Zeiss 200M microscope. For immunofluorescence experiments, the DeltaVision system was used for capturing images of embryos. Quantification of GPR-1/2 signal was performed with the ImageJ software. Average pixel intensities of the posterior and anterior cortices
were determined after background subtraction, and the posterior-to-anterior ratio was calculated. Posterior and anterior cortices were defined as the region in the cell cortex that is found within the most posterior and most anterior 15% of embryo length, respectively.

RNA-mediated interference
Double-stranded RNA produced by PCR with T7 oligos from full length cDNAs as described (Zipperlen et al. 2001) was introduced by either injection or feeding. Animals were harvested after 24 hours for live imaging, protein characterization via embryo immuno-histochemistry or used for protein extracts.

Live Microscopy and GFP imaging
Microscopy experiments were carried out on a Zeiss 200M microscope, and photographed with an Orca camera, a DeltaVision (Olympus 3000) or a Zeiss LSM 510 confocal system was also used. Live imaging was performed as described (Gotta and Ahringer, 2001).

Spindle severing and microscopy
Experiments were performed using a Zeiss 220 Axiovision microscope equipped with a PALM laser system (Mikrolaser Technologie GmbH). We focused the pulsed laser to a approx. 1 μm thick spot in the focal plane. C. elegans embryos were mounted on the inverted microscope, and cut along the spindle midzone when the beginning of anaphase B was indicated. For monitoring spindle severing, 1 DIC image was captured every second. Measurements of peak velocities were performed by manual tracking with ImageJ (tracking the center of the aster), conditions used: laser output 45%, cut speed 6%, focus: 61%.
Indirect Immunofluorescence

Indirect immunofluorescence of embryos was carried out by cutting adult worms on a specimen stage previously coated with 1% poly-L-lysine (Sigma, P1524) in 1x M9. The embryos were fixed by the “freeze-crack” procedure, followed by our standard methods for antibody stainings (Guo et al. 1995) with the following modifications: the slides were blocked in PBS, 0.2 % Tween-20, 1% BSA (SIGMA) and all primary and secondary antibodies were diluted in PBS-Tween before using. We used anti-GPR-1/2 (1:80), anti-α-tubulin (1:1000, SIGMA), anti-LIN-5 (1:30), anti-GOA-1(1:25), anti-DHC-1 (1:50), anti-GFP (1:150, Abcam) as primary antibodies and anti-rabbit-Alexa488, anti-mouse-Alexa568 as secondary antibodies, as well as DNA observations with DAPI. The Antibody to GPR-1/2 and GOA-1 was raised against the full length protein fused to GST. Images were recorded using a Delta Vision microscope (Olympus 3000) and deconvolved.

For the competition experiment, RIC-8 purified antibodies were incubated overnight at 4°C with 170 µg/ml MBP-RIC-8 or only maltose binding protein (MBP) as control. Embryos were then stained with the preadsorbed solution as described. In this study, the following primary antibodies were used: anti-RIC-8, anti-GOA-1, and anti-GPR-2. Antibodies were raised against glutathione S-transferase (GST) fusions of GOA-1, RIC-8, and GPR-2. The proteins were expressed, purified, and injected into rabbits as described (Pintard et al., 2003). Antibodies were affinity purified on immobilized protein.

Protein extracts, antibodies and immunoblotting

Embryo extracts were prepared by hypochlorite treatment. Adult worms were bleached, washed 3 times in 1x M9 buffer. The embryos for extracts were resuspended in 1 volume of Laemmli buffer 4X and boiled for 10 min at 95 °C. For immunoprecipitation, the embryos were washed in IP-Lysis buffer (20mM Tris-HCl pH 7.5, 100mM NaCl, 5mM MgCl₂, 1mM EGTA, 1mM DTT, 1% Triton X-100, protease inhibitor cocktail) and broken with glass beads in a bead beater (3x30sec, 1min pause, 4°C). Lysate was spun down at 14,000 rpm, 15min, 4°C. Antibody binding was performed at 4°C for 3
hours, bead binding over night at 4°C. For SDS-PAGE and Western blotting the standard procedures were used.

In this study the following antibodies were used:

<table>
<thead>
<tr>
<th>antibody</th>
<th>species</th>
<th>concentration</th>
<th>concentration</th>
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<tr>
<td>α-RIC-8</td>
<td>rabbit</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>α-GOA-1</td>
<td>rabbit</td>
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</tr>
<tr>
<td>α-GFP (Roche)</td>
<td>mouse</td>
<td>1:1000</td>
<td>3μg</td>
</tr>
<tr>
<td>α-GFP (Abcam)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>2μg</td>
</tr>
<tr>
<td>α-GFP (Torrypines)</td>
<td>rabbit</td>
<td>1:2000</td>
<td>2μg</td>
</tr>
<tr>
<td>α-αtubulin (Sigma)</td>
<td>mouse</td>
<td>1:3000</td>
<td></td>
</tr>
<tr>
<td>α-LIN-5</td>
<td>mouse</td>
<td>1:1000</td>
<td>2μg</td>
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<tr>
<td>α-GPR-1/2</td>
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<td>4μg</td>
</tr>
<tr>
<td>α-GPA-16</td>
<td>rabbit</td>
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<td>3μg</td>
</tr>
</tbody>
</table>

The vector pGEX-4T (Pharmacia) was used to generate GST-fusions of RIC-8 in *E. coli* BL21. Antibodies were affinity-purified via strips. Secondary antibodies conjugated to peroxidase against rabbit or mouse antibodies were purchased from BioRad (1:3000). For IPs we used “trueblot” horse-radish-peroxidase antibodies against rabbit (1:5000), which recognizes only native folded IgG, because the heavy chain of IgG is similar in size to GPR-1/2.

**Microscopy, immunocytochemistry and GFP imaging**

Microscopy experiments were carried out on a Zeiss 200M microscope, and photographed with an Orca camera, a DeltaVision system was also used. Live imaging was performed as described (Gotta and Ahringer, 2001). Antibody staining was carried out as in (Gou et al. 1995) with the following modifications: the slides were blocked in PBS, 0.2 % Tween-20, 1% BSA (SIGMA) and all primary and secondary antibodies were diluted in PBS-Tween before using. We used anti-RIC-8 (1:50), anti-GOA-1
(1:25), anti-GPB (1:100, Roche), anti-GPR-1 (1:25) as primary antibodies and anti-
rabbit-Cy3, anti-mouse-FITC, anti-goat-FITC and DAPI. The Antibody to RIC-8 was
raised against the full length protein fused to GST.
References


References


References


agalpha_1\) and \(G_{beta3F}\) subunits of the heterotrimeric G protein complex in the mediation of Drosophila neuroblast asymmetric divisions. J Cell Biol 162, 623-633.


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>Activator of G protein Signaling</td>
</tr>
<tr>
<td>CYK</td>
<td>CYtoKinesis defect</td>
</tr>
<tr>
<td>DEP</td>
<td>Dishevelled/Egl-10/Pleckstrin</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>Dm</td>
<td><em>Drosophila melanogaster</em></td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine exchange Dissociation Inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine Exchange Factor</td>
</tr>
<tr>
<td>GDP</td>
<td>GuanineDiPhosphate</td>
</tr>
<tr>
<td>GMC</td>
<td>Ganglion Mother Cell</td>
</tr>
<tr>
<td>GOA</td>
<td>G protein, O, Alpha subunit</td>
</tr>
<tr>
<td>GPA</td>
<td>G Protein Alpha subunit</td>
</tr>
<tr>
<td>GPCR</td>
<td>G Protein Coupled Receptor</td>
</tr>
<tr>
<td>GPR</td>
<td>G Protein Regulator</td>
</tr>
<tr>
<td>GPMS</td>
<td>G Protein Signaling Modulator</td>
</tr>
<tr>
<td>GTP</td>
<td>GuaninTriPhosphate</td>
</tr>
<tr>
<td>HGP</td>
<td>Heterotrimeric G Protein</td>
</tr>
<tr>
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<td>Inscutable</td>
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<tr>
<td>IP</td>
<td>ImmunoPrecipitation</td>
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<tr>
<td>LET</td>
<td>LEThal</td>
</tr>
<tr>
<td>LGN</td>
<td>GPSM-2</td>
</tr>
<tr>
<td>LIN</td>
<td>abnormal cell LINEage</td>
</tr>
<tr>
<td>Loco</td>
<td>Locomotion defects</td>
</tr>
<tr>
<td>MEX</td>
<td>Muscle EXcess</td>
</tr>
<tr>
<td>MT</td>
<td>MicroTubule</td>
</tr>
<tr>
<td>Mud</td>
<td>Mushroom body defect</td>
</tr>
<tr>
<td>NB</td>
<td>NeuroBlast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NCC</td>
<td>Nuclear Centrosome Complex</td>
</tr>
<tr>
<td>NEBD</td>
<td>Nuclear Envelope Break Down</td>
</tr>
<tr>
<td>NuMA</td>
<td>Nuclear Mitotic Apparatus</td>
</tr>
<tr>
<td>PAR</td>
<td>PARtitioning-defective</td>
</tr>
<tr>
<td>PDZ</td>
<td>Postsynaptic Density 95/ Zonula occludens</td>
</tr>
<tr>
<td>PINS</td>
<td>Partner of INScutable</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>RIC</td>
<td>Resistant to Inhibitors of Cholinesterase</td>
</tr>
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<td>RGS</td>
<td>Regulator of G protein Signaling</td>
</tr>
<tr>
<td>robl</td>
<td>roadblock gene</td>
</tr>
<tr>
<td>SOP</td>
<td>Sensory Organ Percursor</td>
</tr>
<tr>
<td>ts</td>
<td>temperature sensitive</td>
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</table>
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Thanks to my supervisor Monica Gotta giving me the opportunity to work on exciting projects and to take part in setting up our laser ablation system. And special thanks for always being there for discussions.

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