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The Role of NHL-2 in Regulating *C. elegans* P granule Function

par

Rana Amini

Département de Pathologie et Biologie Cellulaire

Faculté de Médecine

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Présenté par :

Rana Amini

a été évalué par un jury composé des personnes suivantes :

Dr. Marc Therrien,

président-rapporteur

Dr. Jean-Claude Labbé

directeur de recherche

Dr. Katherine Borden

membre du jury

Abstract

Asymmetric cell division is a process that enables stem cells to simultaneously self-renew and generate progeny committed to differentiation. For instance the totipotent and immortal germ lineage in *C. elegans* is a stem cell lineage that contains ribonucleoprotein organelles called P granules. P granules are present specifically in germ cells and have been proposed to function as germ line determinants. Interestingly, various RNP granules, such as P bodies that regulate post-transcriptional control have been also observed in all organisms. Nevertheless, the precise function of *C. elegans* P granules remains elusive. Recently our lab showed that NHL-2, a *C. elegans* homologue of *Drosophila* Mei-P26, localizes to P granules in early embryos and plays a role in asymmetric cell division and cell polarity. All P granules contain NHL-2, raising the possibility that NHL-2 plays a role in regulating P granule biogenesis and function. We investigated this possibility by imaging and quantifying the intensity of PGL-1, a core component of P granules, in fixed embryos. This analysis revealed that there is a reduction in: 1) the number of P granules and 2) the mean fluorescence intensity (MFI) and total fluorescence intensity (TFI) of PGL-1 staining in *nhl-2* null mutant embryos compared to wild type. Our further analysis of *nhl-2* loss of function demonstrates that NHL-2, similar to P granule core components such as DEPS-1, GLH-1 and PGL-1, is required for proper germ cell proliferation and fertility at elevated temperature. One aspect of the *nhl-2* loss of function phenotype is that *nhl-2* mutants are distributed in different groups based on both their P granules number and PGL-1 intensity: one population has wild type PGL-1 intensity while the second group has severely reduced amounts of PGL-1. Strikingly, such variability is observed even in the sterility phenotype of *nhl-2* mutants at elevated temperatures. Taken together, our results suggests that NHL-2 loss of function disrupts germ cell proliferation as well as P granule formation or stability during early stages of germ cell precursor development and that NHL-2 function may be partially redundant with other regulators of P granule stability.

Keywords : P granules, NHL-2, Germline.

Résumé

La divison cellulaire asymétrique est un processus essentiel qui permet aux cellules souches de s'auto-renouveller et de produire une cellule fille destinée à la différenciation. La lignée germinale de *C. elegans*, totipotente et immortelle, est une lignée de cellules souches qui contient des organites ribonucléoprotéiques appelés granules P. Au cours du développement ces derniers sont toujours localisés spécifiquement dans les cellules précurseurs de la lignée germinale, suggérant qu'ils sont des déterminants de la lignée germinale. De façon intéressante, des granules ribonucléoprotéiques, comme les *P bodies* impliqués dans le contrôle post-transcriptionnel, ont été observés chez tous les organismes. Néanmoins, la fonction précise des granules P de *C. elegans* est inconnue. Récemment, notre laboratoire a montré que NHL-2, un homologue de Mei-P26 de *Drosophile,* colocalise avec les granules P dans des embryons précoces et joue un rôle dans la division cellulaire asymétrique et dans la polarité cellulaire. Tous les granules P contiennent NHL-2, ce qui nous a mené à poser l'hypothèse que NHL-2 régule la biogenèse et la fonction des granules P. Nous avons testé cette hypothèse par imagerie et quantification de l'intensité de PGL-1, un composant essentiel des granules P, dans des embryons fixés. Nos résultats montrent que dans des embryons mutants pour *nhl-2* il y a une réduction du nombre de granules P, de l'intensité de fluorescence moyenne (IFM) et de l'intensité de fluorescence total (IFT) de PGL-1. Une analyse plus poussée a montré qu'il existe deux populations distinctes d'embryons mutants pour *nhl-2* : l'une présente une intensité de PGL-1 comparable à celle d'une population sauvage alors que le second groupe présente une forte réduction des quantités de PGL-1 et est comparable à des mutants pour *pgl-1*. Cette variabilité est aussi observée dans le phénotype de stérilité de *nhl-2* mutant à des températures élevées. Globalement, nos résultats suggèrent que la perte de fonction de NHL-2 perturbe la prolifération des cellules germinales ainsi que la formation et/ou la stabilité des granules P au cours des étapes précoces du développement des précurseurs de la lignée germinals. D'autre part, ils suggèrent que la fonction de NHL-2 pourrait être partiellement redondants avec les autres régulateurs de la stabilité des granules P.

Mots-clés : Granules P, NHL-2, Cellules germinals.

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To my mother for her endless love and

support

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1. Introduction

1.1. Asymmetric cell division:

In contrast to normal cell division, which gives rise to equivalent daughter cells, **asymmetric cell division** (ACD) is a process by which a mother cell divides to generate two daughter cells that adopt distinct cell fates. In principle, asymmetric cell division occurs through two different mechanisms: extrinsic (induced) or intrinsic (Horvitz and Herskowitz, 1992). In the case of extrinsic or induced ACD, the daughter cells are initially equivalent but signaling between the cells induces a difference, whereas in the intrinsic ACD the difference in two daughter cells is generated by the asymmetrical segregation of intracellular proteins before division. These proteins are subsequently partitioned differentially into the two daughter cells so that they are inherited to only one of the daughter cells, causing that cell to be different from its sibling. Because these proteins determine what becomes of a cell they are called **cell fate determinants**.

Asymmetric cell division is a conserved mechanism used by different organisms to generate cell diversity during animal development (Horvitz and Herskowitz, 1992). For instance, intrinsic asymmetric cell division is a fascinating property of stem cells that enables them to undergo self-renewal and differentiation at the same time (Morrison and Kimble, 2006). In addition, during *Drosophila* neurogenesis, neuroblasts divide asymmetrically giving rise to two daughter cells, with different sizes, different cell cycle times and distinct fates. Later the larger cell keeps dividing asymmetrically, whereas the smaller cell called GMC (Ganglion Mother Cell) undergoes differentiation to generate two neurons or glial cells. Another classic example of intrinsic asymmetric cell division is the *C. elegans* zygote, which divides along its anterior–posterior (a–p) axis to produce a large anterior blastomere (AB) and a small posterior blastomere (P_1).

1.2. Polarity is required for asymmetric cell division:

Cell Polarity is a fundamental property of many cells and refers to the asymmetry either in shape or in the content of a cell created through unequal distribution of its molecular components. Cell polarity is essential for various cellular processes such as cell migration. Strikingly, cell polarity plays a critical role in governing asymmetric cell division and therefore for the development of multicellular organisms.

In *C. elegans*, cell polarity is established by the asymmetric distribution of a group of proteins called PAR proteins (for 'partitioning defective'). The seven *par* genes (*par-1* to *par-6* and *pkc-3*) in *C. elegans* encode proteins that are highly conserved across species. PAR-1, PAR-4 and PKC-3 are serine-threonine protein kinases; PAR-3 and PAR-6 are PDZ domain proteins, PAR-2 contains a RING finger domain and PAR-5 is a 14-3-3 protein (Kemphues et al., 1988). In *C. elegans*, polarization is initiated at fertilization with sperm entry into the oocyte. The sperm defines the posterior end of the zygote and also brings the microtubule-organizing center (MTOC) to the zygote. After a couple of maternal meiotic cycles, the uniformly distributed PAR proteins start to rearrange so that the PAR-1 and PAR-2 are restricted to the posterior cell cortex of the embryo whereas PAR-3, PAR-6 and PKC-3 are enriched at the anterior cell cortex. Polarity along the anterior-posterior axis then influences spindle positioning, leading to the first asymmetric segregation and division of cell fate determinants, including P granules and MEX-5 protein into two different daughter cells (Figure 1.1). The AB cell will mainly form ectoderm, whereas P_1 will give rise to the germline, as well as endoderm and some mesoderm. Removing any of the PAR proteins disrupts the establishment of polarity, leading to an abnormal cell division and ultimately to embryonic lethality.
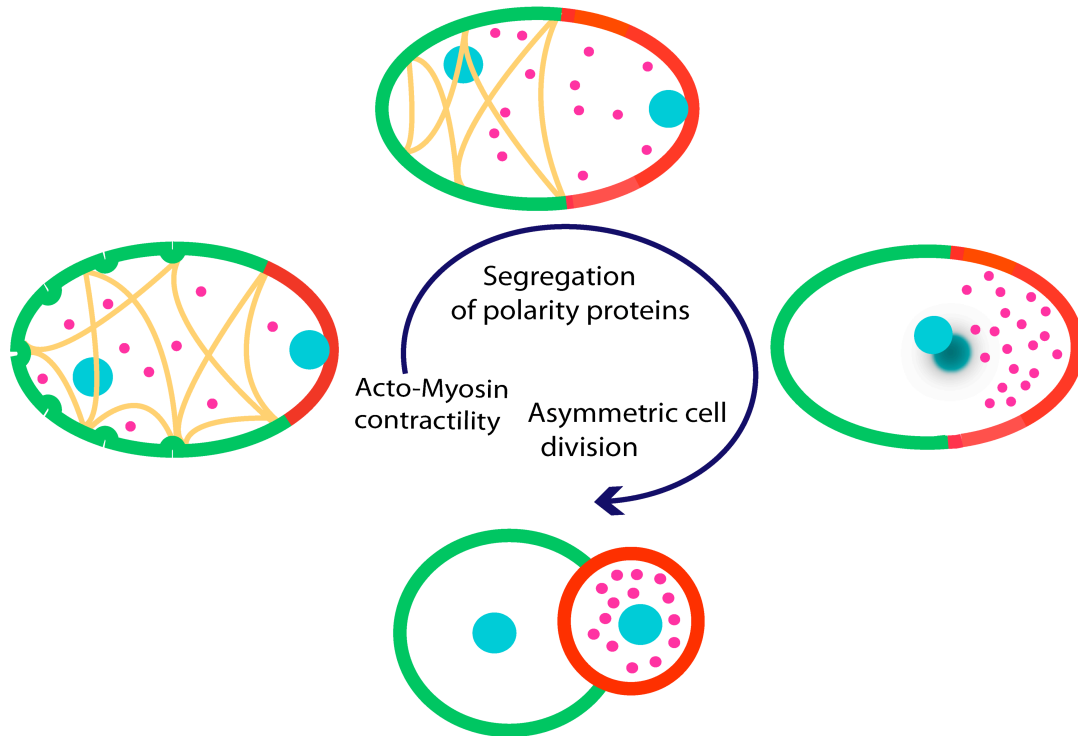
Figure 1.1: Polarity establishment and asymmetric cell division in *C. elegans*. Acto-myosin network: yellow lines; pronuclei and nuclei: blue, cortical PAR-3/PAR-6/PKC-3: green; cortical PAR-2 and PAR-1: red, P granules: pink. In *C. elegans*, the cortex contractions that occurs shortly after fertilization, determines polarity by the unequal distribution of a group of conserved polarity proteins or PARs. This anterior-posterior polarity in turn directs the differential segregation of cell fate determinants to opposite ends of the embryo, leading to an asymmetric cell division, after which two unequal cells are formed, each possessing a particular set of molecules and thus a distinct identity.

1.3. Asymmetric Cell Division and Cell Polarity in Development and Disease:

Asymmetric cell division enables stem cells to self-renew and differentiate with a single division; however symmetric stem cell divisions have been also observed during both vertebrate and invertebrate development. For instance, germline stem cells divide

symmetrically in the distal tip of the gonad in *C. elegans* larvae, whereas they divide asymmetrically during embryogenesis. Indeed symmetric cell division is the strategy that stem cells use in order to increase their population (expansion). However, this unique feature of stem cells, which is essential for the development of organisms, can be deleterious if the balance between asymmetric and symmetric cell divisions becomes defective. While an excess of stem cell self-renewal may lead to tumorogenesis, an excess of differentiation may lead to tissue degeneration and / or tissue aging (Morrison and Kimble, 2006). But what causes the disruption of this balance and deregulation of asymmetric cell divisions?

Cell polarity mechanisms are responsible for regulating the asymmetric cell divisions of stem cells (Wodarz, 2005; Wodarz and Nathke, 2007). Several lines of evidence indicate that disruption of cell polarity is a hallmark of cancer: several cell polarity proteins function either as proto-oncogenes or tumor suppressors (Lee and Vasioukhin, 2008). For instance, the *Drosophila* polarity proteins Lgl, Dlg and Scrib are potent tumor suppressors. It has been shown that removing any of these proteins results in the growth of tumor-like structures in larval imaginal discs and brains (Bilder, 2004). Moreover, there is clear evidence that aPKC is also a potent proto-oncogene in mammalian cells and is involved in the regulation of cell proliferation as well as tumor development (Regala et al., 2005; Stallings-Mann et al., 2006). In addition, it has been shown that aPKC is involved in the squamous-cell carcinoma of the head and neck (Cohen et al., 2006) as well as the proliferation of human glioblastoma cell lines (Donson et al., 2000). It was also shown that overexpression of PKCι, a human homologue of aPKC results in ovarian, lung and colon cancers. Interestingly, tumors with elevated levels of PKCι had lost their polarity demonstrating that PKC could contribute to the development, establishment and maintenance of cell polarity (Eder et al., 2005, Wodarz and Nathke, 2007). Moreover, mutations in LKB1, a human homologue of PAR-4 cause the Peutz-Jeghers syndrome (PJS) by impairing LKB1's ability to induce cell polarity (Wodarz and Nathke, 2007).

Taken together, these studies directly connect cell polarity pathways to the development of cancer in vertebrates and invertebrates.

1.4. Germline development in *C. elegans*

In sexually reproducing animals, germ lineage is responsible for the development of an organism by transmitting genetic information from one generation to the next.

1.4.1. Germ line specification occurs during embryogenesis

In *C. elegans,* the germ line is set apart from the soma early in embryogenesis through a series of asymmetric divisions. The zygote (P_0) undergoes an asymmetric cleavage in order to give rise to the AB and P_1 blastomeres. AB and P_1 are unequal in size and fate and are also different in their cell cycle lengths with AB dividing prior to P_1. AB then divides to produce somatic descendents while P_1 divides asymmetrically to give rise to germ line blastomeres (P_2, P_3 and P_4). P_4 is a germline founder cell that divides only once (at the 100-cell stage) during embryogenesis in order to generate the two primordial daughter cells Z_2 and Z_3. During larval development, Z_2 and Z_3 begin to divide symmetrically to give rise to the entire germ cells of the adult gonad (Figure 1.2).
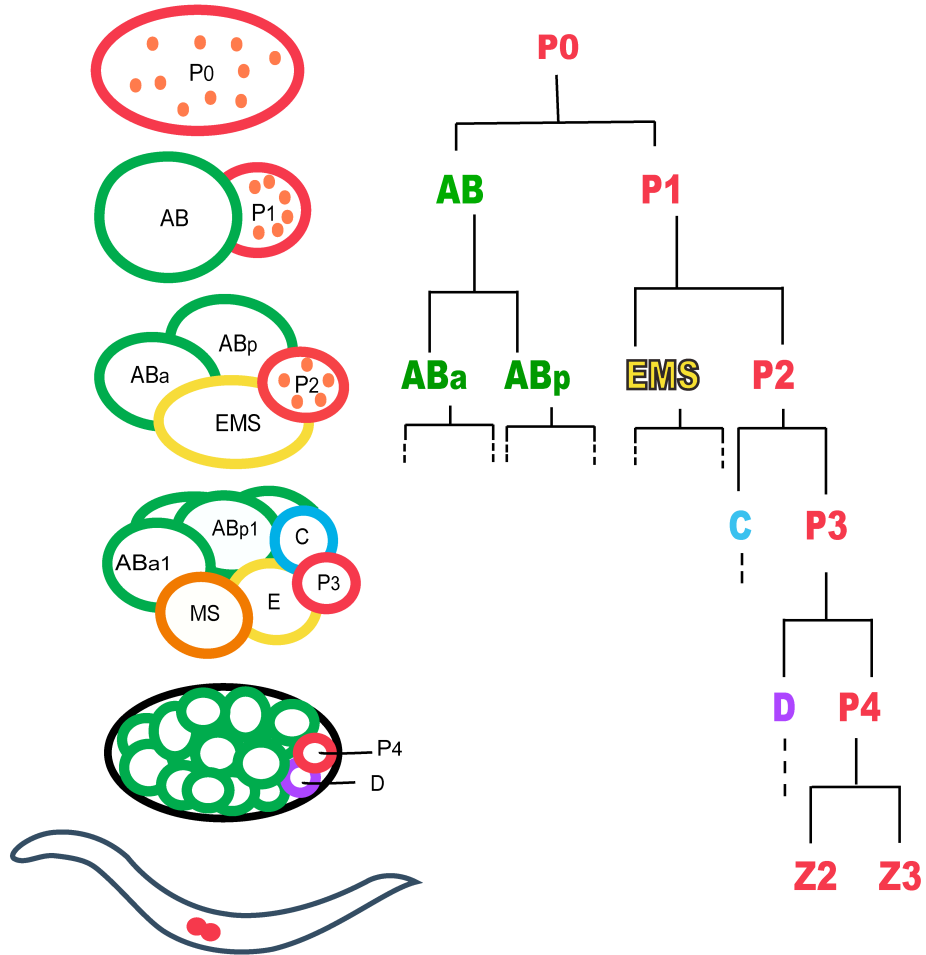
Figure 1.2: Germ Cells are set apart from somatic cells during early embryogenesis in _C. elegans._ The germline precursor cells or P cells (shown in red). Embryos divide asymmetrically to give rise to AB and P_1. The AB lineage then loses the germline determinants, P granules (small dots shown in orange) and therefore all her daughters are restricted to form the somatic tisuues of the animal such as hypodermis and neurons: EMS produces the somatic gonad, muscle, the majority of the pharynx, neurons, gland cells and intestine; C produces muscle, hypodermis and neurons; D produces muscle. On the contrary, the germline determinants are transferred to the second daughter cell, P_1 that gives rise to all germline blastomeres (P_2, P_3, and P_4). The asymmetric cell division continues so that at L1 two primordial germ cells, Z_2 and Z_3 are present (shown in red).

1.4.2. Postembryonic development of the germline

At hatching, the gonad contains four cells: the two germline precursor cells, Z_2 and Z_3, and the somatic gonad founder cells, Z_1 and Z_4, which are derived from the MSt lineage. In the middle of L1 larval stage, Z_1 and Z_4 descendants form the Distal Tip Cells (DTCs) while Z_2 and Z_3 undergo extensive proliferation to give rise to all the germ cells of the developing gonad. DTCs are located at the distal tips of the two U-shaped gonad arms throughout development in hermaphrodites and at the distal tip of the single J-shaped gonad in males (Kimble and Hirsh, 1979). The adult gonad of hermaphrodite *C. elegans* has two symmetrical U-shaped tubes consisting of an ovary in a distal arm and an oviduct in the proximal arm, connected through a spermetheca to the common uterus (Figure1.3). The gonad in *C. elegans* exhibits distal-proximal polarity that corresponds to the development of the germ line from mitosis through meiosis and gametogenesis. The distal tip (mitotic region) of the adult gonad contains a population of stem cells that are mitotically dividing to form a syncitium in the distal gonad arm (Hirsh et al., 1976). The DTC functions as a niche providing the necessary signals that regulate the balance between self-renewal and differentiation that is needed for proper progression through gametogenesis.

Later, during the third larval stage, as germ cells move away from the mitotic region, they enter a transition zone where they progress into different stages of meiotic prophase from prophase I to diakinesis. As germ cells move in the transition zone they exit pachytene in the loop region of the gonad to form oocytes then undergo cellularization. Oocyte maturation occurs in the most proximal end of the gonad arm upon receiving a signal from sperm. Oocytes are then fertilized by stored sperm as they move through the spermatheca into the uterus. After fertilization embryogenesis starts in the uterus and the embryos are then eventually extruded from the uterus. In hermaphrodites when sperm is abundant, oocytes undergo maturation through diakinesis; however, in the absence of sperm, oocytes are arrested in diakinesis (Dernburg et al., 1998; Francis et al., 1995).
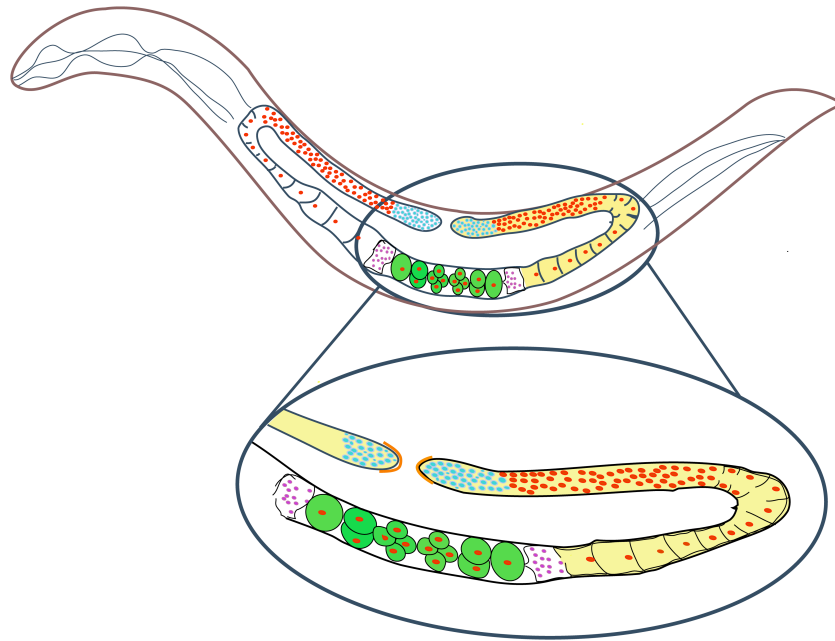
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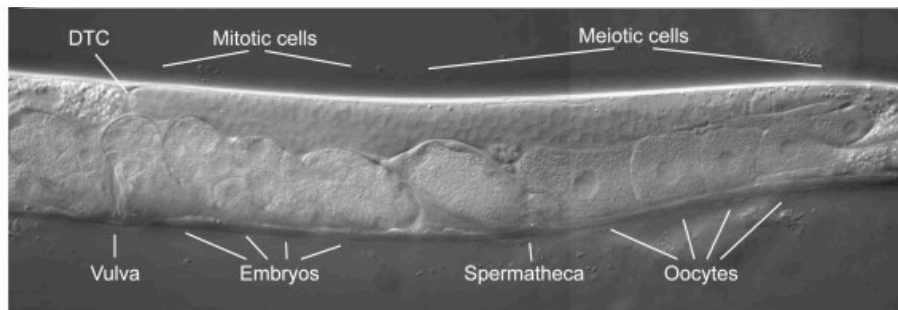
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Figure 1.3: The *C. elegans* Gonad. (A) Schematic and (B) DIC image of the gonad in wild type hermaphrodite. The gonad has two identical arms, each of which produces first sperm, and later oocytes. The oocytes are fertilized as they move towards the uterus, which is common to both gonad arms. The most distal region of the gonad contains a pool of germline stem cells that continuously undergo mitosis. These cells are capped by the somatic distal tip cell (DTC). As the cells move away from DTC, they enter into meiotic phase; they go into meiosis (transition zone) where they undergo an extended period of pachytene. Germ cells specified to become oocytes exit pachytene in the loop region of the gonad, undergo cellularization, and begin swelling from increased cytoplasmic volume.

1.5. Characteristics of the Germline

Germ cells are the founder cells of all sexually reproducing organisms and must be established in early embryogenesis and then maintained throughout development. Due to their unique role, germ cells possess special properties that are essential for reproduction and species survival. Several defining features that distinguish germ cells from somatic cells are conserved among several species including *Drosophila melanogaster*, *Caenorhabditis elegans* and *Xenopus laevis*.

1.5.1. Germ cells behave like stem cells:

Because the germline is the only lineage to contribute its genetic information to the next generation, it is often referred to as an immortal and totipotent lineage. Totipotency is defined as the ability of a single cell or a cell lineage to generate all the cell types of an organism, including the germline. One unique feature of germ cells in most animals is their ability to divide and give rise to all the differentiated tissues of future generations of multi-cellular organisms. Unlike somatic cells, germ cells undergo meiosis to reduce their ploidy in order to generate gametes. The germ lineage is often referred to as an immortal and totipotent lineage, since at fertilization the haploid gametes unite and give rise to a fully differentiated organism, more germ cells and thus the endless germ line cycle continues from one generation to the next. In contrast to somatic cells that undergo only mitosis and ultimately die with each generation, the germline escapes mortality (Cinalli et al., 2008, Kawasaki et al., 2004).

1.5.2. Germ Cells are in a Transcriptionally Repressed State:

Germ cells are considered to function as totipotent stem cells that give rise to highly differentiated cells: sperms and oocytes. However, during development, the totipotency of germ cells is suppressed, and it is not until fertilization that this totipotency is unleashed (reactivated) and transferred to the zygote to form a new organism. In other

words, although germ cells contain somatic factors, including those required for the patterning of the early embryo, germ cells are prevented from responding to these factors that determine (promote) somatic differentiation in their somatic neighboring cells.

But what protects germline cells from following a somatic fate? In *C. elegans*, after the initial determination of the germ line, germ cells are kept separated from somatic cells throughout development. During asymmetric cell divisions, proteins important for germline development are segregated preferentially to the germline blastomeres while being excluded from somatic daughter cells. One of these proteins is a maternally encoded CCCH zinc finger proteins named PIE-1. PIE-1 is a global transcriptional repressor that accumulates in the developing germline of *C. elegans*. PIE-1 interferes with phosphorylation of the carboxy terminal domain (CTD) repeats of RNA polymerase II to repress the RNA polymerase II-dependant gene expression through blocking transcriptional elongation. Due to PIE-1 function, germline blastomeres lack many embryonically transcribed RNAs that somatic cells contain (Seydoux et al., 1996). It has been shown that loss of *pie-1* function affects the patterns of transcription; embryonic transcribed RNAs are observed in both somatic and germline cells resulting in transforming of the germ cells into somatic cells. In addition, ectopic expression of PIE-1 in somatic blastomeres inhibits transcription in these cells, suggesting that PIE-1 has a key role in protecting early germ cells from expressing somatic differentiation and therefore promoting germline fate (Seydoux et al., 1996).

1.5.3. Germ Granules:

One fascinating feature of germline cells is their continuity from one generation to the next. This property is due to the segregation of a special cytoplasm called germ plasm, passed on from parent germ cells to the germ cells of the succeeding individual (Seydoux and Braun, 2006). In most, if not all animals germ plasm contains large, non-

membrane bound, ribonucleoprotein (RNP) organelles referred to as germ granules, on which germ lineage differentiation depends. Germ granules are inherited maternally with the germ plasm and segregated preferentially to the primordial germ cells (PGCs) during germ line development and they have been called by various names in different organisms; polar granules in flies, P granules in worms, mitochondrial clouds in frogs and chromatid body in mammalian spermatocytes (Seydoux and Braun, 2006).

It has been shown that mRNAs coding for important germline factors are present in germ granules. For instance, Vasa a conserved germ-granule component is required for germ plasm assembly in the posterior pole of *Drosophila*; *vasa* mutants lack localized polar granules, fail to form the germ cell lineage and develop into sterile adults (Lasko and Ashburner, 1988; Hay et al., 1990; Lehmann, 1992; Lang et al., 1994). The presence of germ granules in germline cytoplasm across species and the germline defects after depleting germ granule components, suggests that germ granules are essential for germ cell formation and development. However, one question remains: How do germ granules play their role?

Germ granules in all species contain various RNAs and different RNA-binding proteins, many of which are associated with RNA metabolism. It has been revealed that during most of the germline development, germ granules are perinuclear and are associated with nuclear pores (Pitt et al., 2000) (Figure 1.4). Furthermore, germ granules in *Drosophila* associate with ribosomes (Mahowald, 1968). Altogether this suggests that germ granules among species might be involved in mRNA processing and/or mRNA trafficking from the nucleus to the cytoplasm (Seydoux and Braun, 2006).
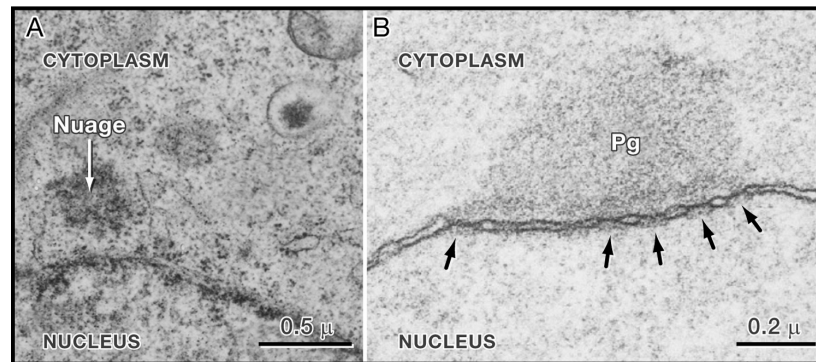
Figure 1.4: Germ Granules are Perinuclear in most organisms (Seydoux and Braun, 2006). Electron micrographs of P granules on germ nuclei. Anti-nuclear pore antibody (mAb414) and anti-P granule antibody (K76) were used. Germ granules in (A) the primordial germ cell of a rat at embryonic day 10 (nuage) and (B) in an adult *C. elegans* germ cell (Pg). P granules (Pg) appear as electron-dense zones around each nucleus. Arrows show nuclear pores. Five pores are visible beneath the P granules.

1.6. *C. elegans* P granules

In *C. elegans*, the germ granules are called P granules and are thought to have determinative roles in germline development. P granules are cytoplasmic particles present throughout life in the germline of *C. elegans* (with the exception of mature sperm), are exclusively localized to germ cells and can be visualized by immunofluorescence (Strome and Wood, 1982) or by electron microscopy (Wolf et al., 1983).

1.6.1. P granule Localization and Physical Nature

In 1996, Hird et al., injected fluorescently labeled OIC1D4 monoclonal antibody, which presumably recognize the P granule specific protein PGL-1, into the germline syncitium of adult hermaphrodites in order to monitor the germline-specific P granule movements in the early *C. elegans* embryo. Their studies showed that P granules are maternally contributed to the embryo and are initially distributed equally throughout the cytoplasm of the unpolarized 1-cell embryo. During pseudo-cleavage and before the oocyte pronucleus migration toward the sperm pronucleus, P granules start to move posteriorly so that by the end of pseudo-cleavage, almost all P granules are located at the posterior end of the embryo. At the first asymmetric division, all the P granules are segregated preferentially to the posterior germline blastomere (P_1), while the anterior P granules disappear progressively during mitosis, so that none are detectable in the AB cell. The asymmetric segregation of P granules continues at each asymmetric cleavage resulting in the inheritance of P granules by the germline blastomeres (P_2, P_3 and P_4). P_4 then divides and passes the P granules to the two primordial germ cells, Z_2 and Z_3 (Figure 1.5).
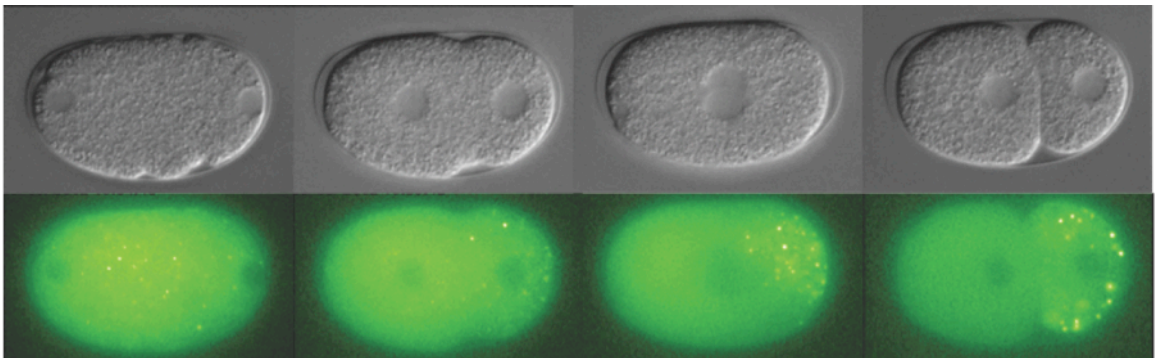
Figure 1.5: P granule segregation in *C. elegans.* The images are taken with an Axio-Imager microscope, using a PGL-1:GFP strain. The P granules are initially distributed throughout the cytoplasm, but begin to move posteriorly before the oocyte pronucleus starts to migrate toward the sperm pronucleus. After the two pronuclei meeting, most P granules are localized to the posterior cell so that after the first division, all the P granules are inherited by P_1; no P granules are seen in the anterior cell AB.

How are P granules targeted specifically to germline blastomeres during *C. elegans* divisions? For a long time it was thought that P granule posterior partitioning is either controlled by their migration with the cytoplasmic flow toward the posterior pole (Hird and White, 1993, Cheeks et al., 2004 and Strome and Lehmann, 2007), or by the degradation or disassembly of the P granules remaining in the anterior (Hird et al., 1996

and Spike and Strome, 2003). However, it has recently been shown that a dissolution/condensation process controls P granule localization. Using a 3D particle tracking approach, Brangwynne et al. showed that before symmetry breakage, there is an overall P granule dissolution throughout the cytoplasm. Breaking of the symmetry however, results in the condensation of the P granules at the posterior while dissolution continues in the anterior pole of the zygote. Thus the rapid transition between P granules dissolution and condensation causes the migration of P granule components to the posterior, which eventually leads to the disappearance of the anterior components (Figure

1.6). Interestingly, they also reported that P granules behave like liquid droplets. During the condensation phase, P granules are spherical but since their transition from a soluble phase to a condensed structure is required for their proper segregation, they become nonspherical as they associate with the nucleus at the four-cell stage (Brangwynne et al., 2009). In addition, there is also evidence that some polarity proteins such as PAR-1 and MEX-5 function in P granule localization mainly by regulating dissolution/condensation of P granules (Brangwynne et al., 2009).

P granules are known to have a dynamic morphology. During the early embryogenesis, P granules get progressively larger; as they increase in size, their number decreases, suggesting that P granules aggregate in order to form larger granules. In addition, during most of the germline development, P granules have a perinuclear positioning; however during oogenesis; they dissociate from the nuclear surface and become localized to the cytoplasm. It has been shown that P granules reattach to the nucleus during embryogenesis, in 4-cell stage germline blastomeres (Strome and Wood, 1982, Pitt et al., 2000).

Before Symmetry Breakage

After Symmetry Breakage
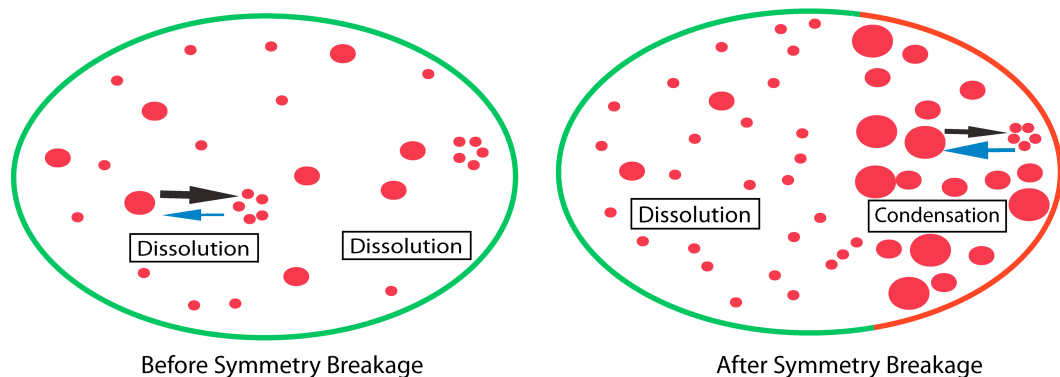
Figure 1.6: P granules are localized by dissolution/condensation mechanisms. (A) Before symmetry breakage the high concentration of P granule components leads to the dissolution of P granules into diffusing components (small red circles) in both anterior and posterior cells. (B) However, symmetry breaking causes the P granule condensation in the posterior while P granules at the anterior pole undergo dissolution.

<u>1.6.2. P granule composition</u>

P granules are large non-membrane bound ribonucleoprotein (RNP) organelles and their function is essential for germline development but they have not been characterized biochemically. However it has been shown that P granules are composed of some RNAs and proteins important for proper germline specification and development.

- ***RNAs***

P granules like their mammalian counterparts contain RNA molecules. After the onset of asymmetric cell divisions, one group of maternal RNAs known as the class II maternal RNAs, show unequal distribution between somatic and germline blastomeres. Although these RNAs are distributed to both daughter cells after each cleavage, they are maintained in germline cells while being selectively degraded in somatic blastomeres. In addition, several clusters of poly(A)+ RNAs co-localize with the germline-specific P granules (Seydoux and Fire, 1994). *nos-2* (Subramaniam and Seydoux, 1999) and *pos-1* (Tabara et al., 1999, Schisa et al., 2001), are two mRNAs which associate with P granules during early embryogenesis. For instance, the *nos-2* mRNA is present throughout the cytoplasm of early embryonic blastomeres, but appears to be concentrated and enriched in P granules of germ cells (Subramaniam and Seydoux, 1999). *nos-2* encodes a protein similar to the *Drosophila* Nanos and functions redundantly with *nos-1* to regulate germ-cell proliferation and viability (Subramaniam and Seydoux, 1999). *pos-1* encodes a CCCH-type zinc-finger protein which is also essential for the proper germline development. In addition to *nos-2* and *pos-1*, some other class II maternal mRNAs shown to localize to P granules in adult gonads are also degraded rapidly in somatic cells while they are protected in germline blastomeres (Schisa et al., 2001).

- ***Proteins***

P granules contain different proteins most of which serve an essential role in *C. elegans* germline development. One group of proteins is transiently associated with P granules; they are detected in P granules only during early embryogenesis while they disappear in late-stage embryos or in larval or adult germlines. These include PIE-1 (Mello et al., 1996, Tenenhouse et al., 1998), MEX-1 (Guedes and Pries, 1997), MEX-3 (Draper et al., 1996), MEX-5 (Schubert et al., 2000), POS-1 (Tabara et al., 1999b) and IFE-1, an isoform of eIF4E (Amiri et al., 2001). Almost all proteins in this group are known to function during early embryogenesis by preventing the germline blastomeres from adopting somatic fate or the opposite.

Some of the protein components of P granules, like PGL-1/3, GLH-1/4 and DEPS-1, are structurally associated with P granules throughout the life cycle of the worm and regulate assembly and function of these particles, as removing any of them affects proper germline proliferation and development in *C. elegans* (Kawasaki et al., 1998, 2004; Spike et al., 2007). It was also shown that removing GLH and PGL family proteins alters the proper P granule morphology (Schisa et al., 2001). DEPS-1 and PGL proteins are nematode specific components while the GLH family is homologous to Vasa; a highly conserved Dead-box RNA helicase with a role in germline development and fertility in several species, including *Xenopus* and mouse (Hay et al., 1988; Lasko and Ashburner, 1988). Several other proteins are also found to be localized to P granules. For instance, our lab showed that NHL-2, a homologue of Brat in *Drosophila*, also localizes to P granules in *C. elegans* early embryo (Hyenne et al., 2008).

Interestingly, all known protein components of P granules contain putative RNA-binding motifs, suggesting that they might regulate either the structure or function of the P granules through their RNA-related function.

1.6.3. P granule assembly pathway:

Several molecular epistasis analyses have revealed the mechanism of P granule assembly, stability and function. Studies have shown that loss of some components of P granules affects the structure of P granules in germlines, suggesting their role in P granule assembly pathway. In addition, removing of these components results in sterility and germ cell underproliferation (Kawasaki et al., 1998, Kawasaki et al., 2004, Spike et al., 2008b). These properties provide clues that the role of P granule components is perhaps to regulate the postembryonic germline development. For instance, DEPS-1, a P granule-associated protein is required for the proper localization of PGL-1. Moreover, RT-PCR and western blot analysis revealed that *deps-1* mutant germlines display a reduction in the levels of *glh-1* mRNA and protein, suggesting that DEPS-1 functions upstream of GLH and PGL family of proteins in the P granule assembly pathway (Spike et al., 2008b). Another P granule component is a DEAD-box RNA helicase, GLH-1, loss of which causes a partial dispersal of PGL proteins (PGL-1, PGL-2 and PGL-3), suggesting that in addition to DEPS-1, GLH-1 participates in the recruitment of PGL proteins to P granules (Spike et al., 2008 a, Spike et al., 2008b). In contrast, it has been shown that PGL-1 depletion is not needed for the assembly of GLH-1, GLH-4 and DEPS-1 to P granules. However, IFE-1, an isoform of eIF4E that binds mRNA caps is a component of P granules that requires PGL-1 associate with P granules (Amiri et al., 2001, Kawasaki et al., 2004, Spike et al., 2008a, Spike et al., 2008b). Together these findings place DEPS-1 at the top of the P granule assembly pathway, while GLH-1 is upstream and IFE-1 is downstream of PGL-1 (Kawasaki et al., 2004, Updike and Strome, 2009).

Moreover, studies showed that mutations in other P granule components such as MEX-1 (Mello et al., 1992; Guedes and Priess, 1997), MEG-1 (Leacock and Reinke, 2008) and OMA-1 (Shimada et al., 2006) also disrupt PGL-1 in the germline. Recently, a genome-wide RNAi screen for PGL-1 accumulation and localization defects was performed and it has been shown that 173 genes are required for the normal P granule stability, localization and function in *C. elegans*. Interestingly many of these genes are involved in

important cellular processes such as protein degradation, translation, splicing and nuclear export (Updike and Strome, 2009). For instance, some Sm components of the splicesome have been shown to colocalize with P granules within the germ cells and it has been revealed that RNAi depletion of some Sm proteins affects the proper localization of all three PGL proteins to P granules (Barbee et al., 2002). The finding that RNAi depletion of other splicing components did not affect P granule distribution pattern supported the idea that the role of Sm proteins in P granule localization is not through their function in splicing (Barbee et al., 2002). Strikingly however, the RNAi screen performed by Updike and Strome revealed that some pre-mRNA splicing factors are also required for the proper localization of PGL-1 in germline blastomeres. As the germline cells are transcriptionally silenced (Seydoux et al., 1996), this data suggests that proper mRNA splicing might affect P granule localization, assembly and/or stability (Kawasaki et al., 2004, Updike and Strome, 2009). In addition, the proper localization of P granules to the nuclear periphery was also shown to depend on nuclear pores and nuclear transport components, suggesting that mRNA export from the nucleus is also required for the stability and proper localization of P granules to the nuclear periphery (Updike and Strome, 2009).

1.6.4. What are the functional consequences of loss of P granule components?

Phenotypic analysis of mutants for P granule components provide clues that the role of P granule components is perhaps to regulate postembryonic germline development, as *pgl-l*, *glh-1* and *deps-1* mutants display temperature sensitive sterility and germ cell under-proliferation (Kawasaki et al., 1998, Kawasaki et al., 2004, Spike et al., 2008b). It is now known that absence of any of these genes does not severely compromise fertility at low temperature, while mutations in any of them lead to 100% sterility at high temperatures (26°C for *pgl-1* and *glh-1* mutants, 24.5°C for *deps-1* mutants). *pgl-1*, *glh-1* and *deps-1* mutants have under-proliferated germlines that generally lack gametes suggesting that the primary role of these components is in regulating germ cell proliferation and gametogenesis

to ensure fertility. Furthermore, there is evidence that removing of PGL-1 affects proper oogenesis. Interestingly, this sterility phenotype has both a maternal and a non-maternal (zygotic) component indicating that both maternal and zygotic PGL-1, GLH-1 and DEPS-1 are required to ensure normal germline development in *C. elegans* (Kawasaki et al., 1998, Spike et al., 2008a,b).

Another P granule component is IFE-1, one of five eIF4E (Eukaryotic translation initiation factor 4E) isoforms identified in *C. elegans*. IFE-1 is expressed primarily in the germline and associates with P granules. Complete loss of *ife-1* function affects the translation of several maternal mRNAs (*pos-1*, *pal-1*, *mex-1* and *oma-1*) as well as male germ cell mRNAs (Henderson et al., 2009). IFE-1 is involved in spermatogenesis since loss of IFE-1 affects the proper cytokinesis of spermatocytes (Amiri et al., 2001, Henderson et al., 2009). In addition, *ife-1* mutants also show oogenesis defects and therefore *ife-1* mutants have abnormal gametogenesis resulting in 100% sterility at 25°C (Henderson et al., 2009).

One fascinating feature of the sterility observed in *deps-1*, *glh-1*, *pgl-1* and *ife-1* mutants is its temperature sensitivity. One explanation is that there is a functional redundancy with other members of their respective gene family at lower temperatures. For instance, in *glh-1* mutants, at lower temperature other GLH proteins compensate for the absence of GLH-1, whereas GLH-1 depletion leads to sterility at 26°C (Kawasaki et al., s2004). Another explanation is that the processes that are regulated by P granule components, such as germline proliferation and/or mRNA export from germ nuclei are temperature sensitive (Kawasaki et al., 2004).

<u>1.7. P granules and Translational Control</u>

Germline development and embryogenesis rely on gene expression and cytoplasmic mRNA control. Transcription is active during oogenesis allowing the cell to produce maternal messenger RNAs required for the development. Messenger RNAs are transcribed in the nucleus and exported to the cytoplasm for translation. The maternal mRNAs then are packaged into oocytes until fertilization. Upon fertilization the maternal mRNAs will be actively translated in a specific spatial and temporal manner.

In the germline, mRNAs programmed for the delayed translation are accumulated and stored in P granules (Seydoux and Braun, 2006). Thus P granules are thought to play an essential role in regulating the accurate temporal and spatial mRNA translation or degradation. In addition, the finding that IFE-1 associates with PGL-1 in P granules, raises the possibility that P granules might are involved in activation or inhibition of translation (Henderson et al., 2009). However the biochemical function of P granules remains elusive. Nevertheless, several lines of evidence support the idea that P granules are involved in the posttranscriptional regulation of gene expression:

<u>1.7.1. P granule localization and composition:</u>

Mahowald in 1968 observed that *Drosophila* germ granules associate with ribosomes. This observation led him to propose that mRNAs required for germ cell development might store into *Drosophila* polar granules (Mahowald, 1968). Today it is clear that P granules contain mRNAs encoding germline-specific proteins and poly(A)+ RNAs (Seydoux and Fire 1994). In 2001, Schisa et al showed that mRNAs that function during development are enriched in P granules whereas the ubiquitously expressed actin and tubulin mRNAs are distributed uniformly throughout the cytoplasm (Schisa et al., 2001). Moreover, there is evidence that SL1 sequences, which are trans-spliced onto the 5' end of most *C. elegans* mRNAs (Zorio *et al.* 1994), also colocalize with P granules (Pitt et al., 2000; Schisa et al., 2001; Updike and Strome, 2009). In addition, P granules are composed of multiple proteins that all contain RNA-binding motifs, which might enable

them to interact with RNAs exiting from the nucleus. The question is whether this association is important?

GLH-1, a core component of P granules, contains two different regions, a DEAD-box helicase domain and a Gly-rich region containing 3 CCHC zinc fingers, which are both able to bind to RNA (Sengoku et al., 2006). GLH-1 proteins lacking any of these two regions are not able to localize properly to P granules. Together these observations suggest that the interaction between GLH-1 and RNA contributes to P granule function, perhaps by regulating the assembly of GLH-1 to P granules (Spike et al., 2008b). It has also been reported that the Phe-Gly (FG) domain of GLHs mediates their association with the FG-rich nuclear pore protein (Frey et al., 2006). Furthermore, it was shown that 75% of nuclear pores in the germ cells are associated with the P granules (Pitt et al., 2000). According to a genome-wide RNAi screen that was performed recently, removal of nuclear pore proteins (NPP) leads to the detachment of P granules from the nuclear envelope and disrupts the proper localization and assembly of PGL-1 to P granules. Thus the association between NPPs and P granule components is required for proper P granule location and PGL-1 intensity levels (Updike and Strome, 2009).

Studies revealed that some P granule components such as DEPS-1 are required for proper RNAi response (Spike et al, 2008b). Moreover, *csr-1*, one of the *C. elegans* Argonauts, is also involved in a germline endo-RNAi pathway. *csr-1* worms have larger P granules in germline blastomeres (Updike and Strome, 2009). Thus one possibility is that P granules are involved in the regulation of the posttranscriptional gene expression.

P granule composition together with their distribution over nuclear pores suggests that they are most likely to encounter mRNAs during their transport from nucleus to cytoplasm and therefore could potentially contribute to the majority of post-transcriptional gene regulation procedures such as mRNA trafficking, translation and stability that occur in germ cells (Schisa et al., 2001; Seydoux and Braun, 2006 and Spike et al., 2008b).

1.7.2. Links between different RNP granules in *C. elegans*:

Messenger RNAs that are not immediately translated after being exported from the nucleus accumulate in special discrete cytoplasmic domains referred to as RNA granules. These granules contain various ribosomal subunits, ribosomal RNAs, translation factors, decay enzymes, helicases, scaffold proteins and RNA-binding proteins in order to control the localization, stability, and translation of their RNAs. Strikingly P granules share certain components with these RNA granules of somatic cells. They have various names; P bodies, processing bodies and stress granules and are widely conserved among species.

Untranslated mRNAs localize to translationally repressed mRNP granules called Processing bodies or P bodies (also referred to as GW182 or Dcp bodies) of somatic cells in order to be degraded or reactivated (Brengues et al., 2005; Anderson and Kedersha, 2006 and Seydoux and Braun, 2006). P bodies contain factors essential for translational repression (eIF4e, Dhh1/RCK/p54, Scd6/RAP55) and components of mRNA decapping (DCP1 and DCP2), activators of decapping (LSM-1) and deadenylation (CCR4-NOT deadenylase complex) machinery and are involved in mRNA repression and degradation (Seydoux and Braun, 2006).

Non-translating mRNAs can also accumulate in other cytoplasmic structures termed stress granules. Certain P body components (e.g. Dhh1/RCK/p54, Scd6/RAP55 and XRN1) also localize to stress granules (Kedersha and Anderson, 2002). Stress granules are formed when translation initiation is compromised in response to environmental stress such as heat or UV irradiation. Under these conditions, translation of mRNAs encoding housekeeping proteins is arrested, and mRNAs accumulate in stress granules. The T-cell intracellular antigen-1 (TIA-1), an mRNA binding protein, is essential for the accumulation of stalled translational pre-initiation complexes to stress granules (Kedersha et al., 1999, Kedersha et al., 2005). Although they have some similarities, some features distinguish stress granules from P bodies. Unlike P bodies, stress granules contain ribosomal subunits. In addition, the initiation factors such as, eIF3, G3BP, eIF4G, TIA-1 and PAB-1 are found

exclusively in stress granules but not in P bodies, whereas DCP1and DCP2 are only restricted to P bodies. Another difference is that mRNA degradation does not occur in stress granules, which makes them available for rapid reinitiation after recovering from stress (Sheth and Parker, 2003; Cougot et al., 2004a). Thus mRNA degradation machinery must be somehow disabled during the stress response, suggesting that stress granules function in mRNA sorting rather than decay (Kedersha et al., 2005) whereas P bodies are sites of mRNA degradation (Sheth and Parker, 2003).

Several lines of evidence indicate that P bodies and stress granules are distinct granules that interact with each other, suggesting that stress granules and P bodies may function together to regulate the balance of translated, repressed and degraded mRNAs in somatic cells (Kedersha et al., 2005; Gallo et al., 2008). Interestingly, P bodies and stress granules contain the same species of mRNAs. One explanation could be that some mRNAs exiting translation assemble into stress granules. Later, mRNAs destined for degradation are transported from stress granules to P bodies through the physical association between these two RNA granules (Kedersha et al., 2005).

Several lines of evidence suggest parallels between P granules, P bodies and stress granules. P granules share components with both processing bodies and stress granules of somatic cells in *C. elegans*. P granules contain protein involved in translation initiation, translation control and mRNA decay:

- Decapping proteins including PATR-1, DCAP-1, DCAP-2, CGH-1 and CAR-1 (Audhya et al., 2005, Boag et al., 2005, Gallo et al., 2008, Lall et al., 2005, Navarro et al., 2001, Squirrell et al., 2006)

- RNA dependent silencing machinery components (Argonauts and microRNAs) (Updike and Strome, 2009)

- Translational initiation factors and markers of stress granules: IFE-1 (Amiri et al., 2001), TIA-1 and PAB-1 (Gallo et al., 2008)

P granules and stress granules have been shown to have similarities. P granules and stress granules, in addition to their dynamic structures, both share some components of P bodies while they have some factors such as TIA-1 and PAB-1 that are not found in P bodies. Furthermore, it has been shown that *nos-2* mRNA accumulates in both P granules and stress granules whereas its association to P bodies is not very stable, suggesting that P granules might associate with stress granules (Gallo et al., 2009).

An interesting issue consists of identifying how different RNP granules regulate mRNA translation. CGH-1, a highly conserved DEAD-box family protein localizes to both P granules and P bodies. *cgh-1* mutants show a reduction in their mRNA level. In addition the *nos-2* mRNA that should be translationally silenced during oogenesis, is prematurely translated in the oocytes of the *cgh-1* worms (Boag et al., 2008, Gallo et al., 2008), suggesting that CGH-1 functions in both mRNA translational repression and protection from degradation. In addition, it has been shown that while PATR-1 has a key role in P body formation, P granules are formed independently of PATR-1 (Boag et al., 2008). Based on these observations, Rajyaguru and Parker proposed that CGH-1 has different roles in posttranscriptional gene expression, depending on its assembly to P granules or P bodies. In germline blastomeres, CGH-1 colocalization to P granules leads to the recruitment of CAR-1 and PAB-1 to P granules. This complex protects the germline mRNA from being degraded by blocking the association of decapping and degrading enzymes to P granules. In contrast, CGH-1 associates with PATR-1 and other decapping and degrading factors of the somatic P bodies in order to degrade the mRNA in somatic cells (Rajyagur and Parker, 2009). Altogether, these observations support the idea that different RNA granules use distinct mechanisms to control maternal mRNA translation and stability.

1.8. NHL-2 and Translational Control

NHL-2, a homologue of the *Drosophila* Brain Tumor (Brat), is enriched in P granules during early embryogenesis in *C. elegans* (Hyenne et al., 2008). Brat is a member of the NHL family of proteins found in *Drosophila* (Arama et al., 2000; Sonoda, 2001). The NHL family name derives from three of the founding members: NCL-1, HT2A, and LIN-41, which have been shown to be involved in RNA metabolism: NCL-1 is required for ribosomal RNA synthesis (Frank et al., 1998) and LIN-41 is involved in the temporal regulation of the expression of the LIN-29 transcription factor (Slack, 2000). The Brat protein contains conserved motifs: N-terminal B-box zinc-finger domains and several NHL repeats in their C-terminus. All these motifs are reported to be involved in protein-protein interactions. In addition to its role as a cell growth inhibitor in *Drosophila*, Brat is also known as a tumor suppressor functioning in the posttranscriptional gene regulation. Brat forms a complex with Pumilio (homolog of *C. elegans* FBF-1/2) and Nanos (homolog of *C. elegans* NOS-1/2 and 3) and interacts with the 3'-UTR of the Hunchback (HB) mRNA through its NHL domains in order to inhibit the translation of HB in the posterior pole of the *Drosophila* embryo, suggesting a role for other NHL family of proteins in controlling the posttranscriptional gene expression (Sonoda et al., 2001).

Drosophila Brat has five homologues in *C. elegans*: NCL-1, NHL-1, NHL-2, NHL-3 and LIN-41, which are collectively referred to as CeBrats (for *C. elegans* Brats) (Hyenne et al., 2008). In addition to the conserved domains present in Brat, all CeBrats except for NCL-1 also contain an N-terminal RING domain (Figure 1.7). In *C. elegans*, the ortholog of Brat, NCL-1, was shown to function as a cell growth inhibitor, since worms lacking NCL-1 have enlarged nucleoli, increased amounts of ribosomal RNA and a higher rate of protein synthesis (Frank and Roth, 1998), whereas NHL-1, NHL-2 and NHL-3 are not likely to play the same role (Hyenne et al., 2008). Interestingly, it has been reported that while LIN-41 cannot affect *par-2* lethality, 4 of the CeBrats; NCL-1, NHL-1, NHL-2 and NHL-3 are able to suppress *par-2* lethality and therefore are involved in regulating embryonic polarity (Hyenne et al., 2008).

Recently it has been shown in *Drosophila* that Brat and another member of the TRIM-NHL family of proteins, Mei-P26, bind to Argonaute-1, a core miRISC (miRNA-induced silencing complex) component. Mei-P26 inhibits the microRNA biogenesis in *Drosophila* ovaries, since its removal leads to a general upregulation of most microRNAs. In addition, both Mei-P26 and Brat, bind to AGO-1 (Argonaute-1), a core component of RISC machinery, through their NHL domains. Mei-P26 lacking its NHL domains is not able to bind to AGO-1, leading to the possibility that NHL domains in other proteins might be also involved in regulating microRNAs (Neumuller et al. 2008). In support of this idea, it was recently shown that NHL-2 controls microRNA activity in *C. elegans* larvae. It has been also reported that NHL-2 similar to its ortholog Mei-P26, has a physical interaction with key factors of miRISC pathway: ALG-1 and AIN-1 (Hammel et al., 2009). Similarly, TRIM32, a mouse homolog of *Drosophila* Brat and Mei-P26 also binds Argonaute-1 and is involved in the regulation of microRNAs (Schwamborn et al., 2009).

It was shown that NHL-2 localizes to somatic P bodies together with CGH-1, another key component of P bodies that binds to ALG-1 and AIN-1. NHL-2 and CGH-1 have been shown to interact physically and both are involved in regulating the posttranscriptional repression of several microRNA targets in somatic cells of developing worm larvae (Hammell et al., 2009). In addition, there is evidence that NHL-2 functions in posttranscriptional gene regulation mainly via modulating the efficacy of the interactions between miRNAs and their targets in *C. elegans* larvae (Hammell et al., 2009), however it is not known whether NHL-2 has the same role during embryogenesis.
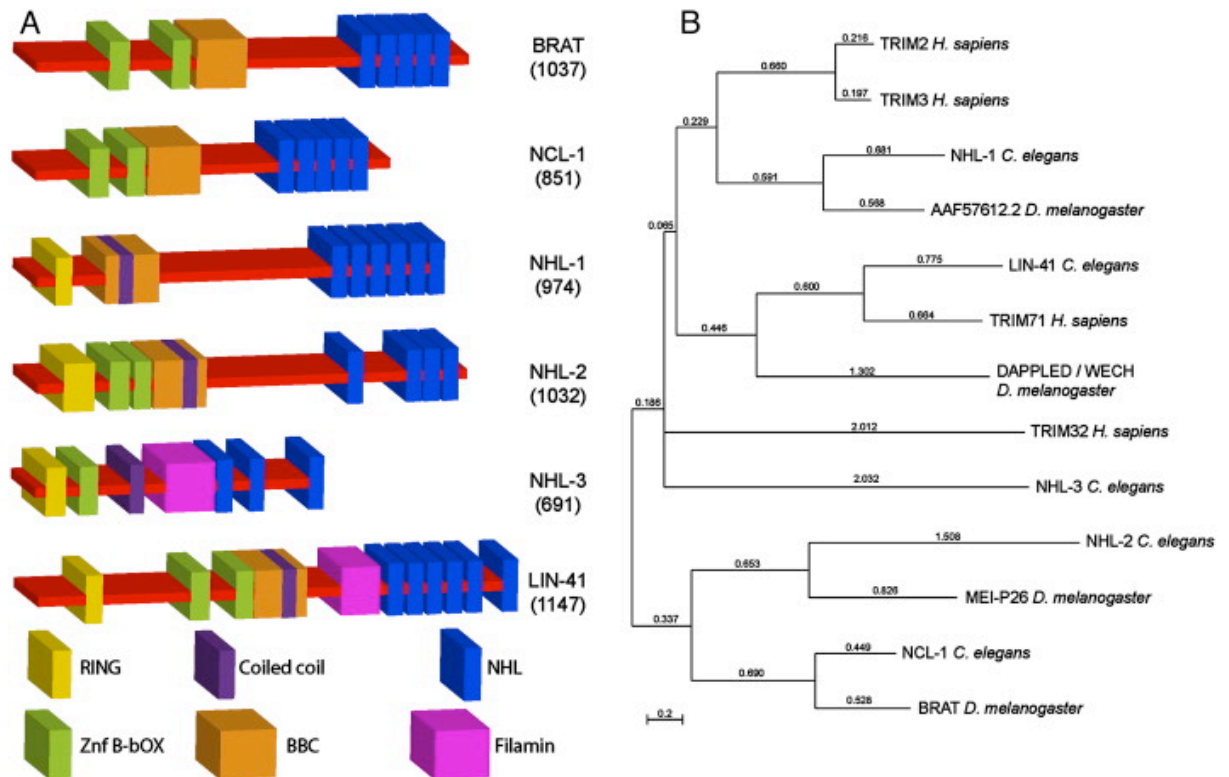
Figure 1.7: NHL-2 is a member of the conserved TRIM-NHL- family of proteins (Hyenne et al., 2008). (A) Schematic representation of *D. melanogaster* Brat and its homologs in *C. elegans*. All of them contain N-terminal B-box zinc-finger domains, a BBC or coiled-coil domain and several NHL repeats in their C-terminus. All of these but NCL-1 and Brat contain an N-terminal RING domain. Protein amino acid length is given in brackets. (B) Phylogenetic reconstruction of Brat homologues in *C. elegans*, *D. melanogaster* and *H. sapiens*. *C. elegans* NCL-1 and NHL-2 together with *D. melanogaster* Brat and Mei-P26, are separated from their other homologs. The length of the tree branches are proportional to the relative evolutionary distance between Brat homologs which is numbered above.

1.9. Objectives of the Master's Project

Recently, our lab has shown that NHL-2 localizes to P granules in the embryo (Figure 1.8.A). In addition, studies in our lab revealed that NHL-2 functions in embryonic polarity in *C. elegans* (Hyenne et al., 2008). NHL-2 is also known to be involved in the posttranscriptional repression in *C. elegans* larvae (Hammell et al., 2009). Nevertheless the precise function of NHL-2 at the embryonic level is not known. Interestingly, it was shown that two markers of stress granules; PAB-1 and TIA-1 have the same distribution pattern as NHL-2 as they both localize to P granules (1.8.B) (Gallo et al., 2008). In addition, little is known about the regulation and exact function of P granules in *C. elegans*. In order to investigate the underlying mechanisms of P granule function and stability, we postulated that NHL-2 plays its role in early embryogenesis by regulating P granule function, perhaps through association with PAB-1 and/or TIA-1. Furthermore, the presence of NHL-2 on P granules makes it also possible that, similar to P granule core components, NHL-2 also functions in germline development and proliferation. Thus we decided to explore the molecular function of NHL-2 in P granule biogenesis, in order to gain a better understanding of P granules function.

The aim of this project is to understand the possible role of NHL-2 in controlling P granule function and germline development. Thus, we developed an image analysis approach to determine whether NHL-2 functions in P granule stability. Furthermore, we are also interested in investigating how P granules are regulated in *C. elegans* during development and to identify proteins that mediate this control. Studying how NHL-2 and P granules function in *C. elegans* could elucidate and provide key insights into the roles and relationships of various RNA granules in different species.
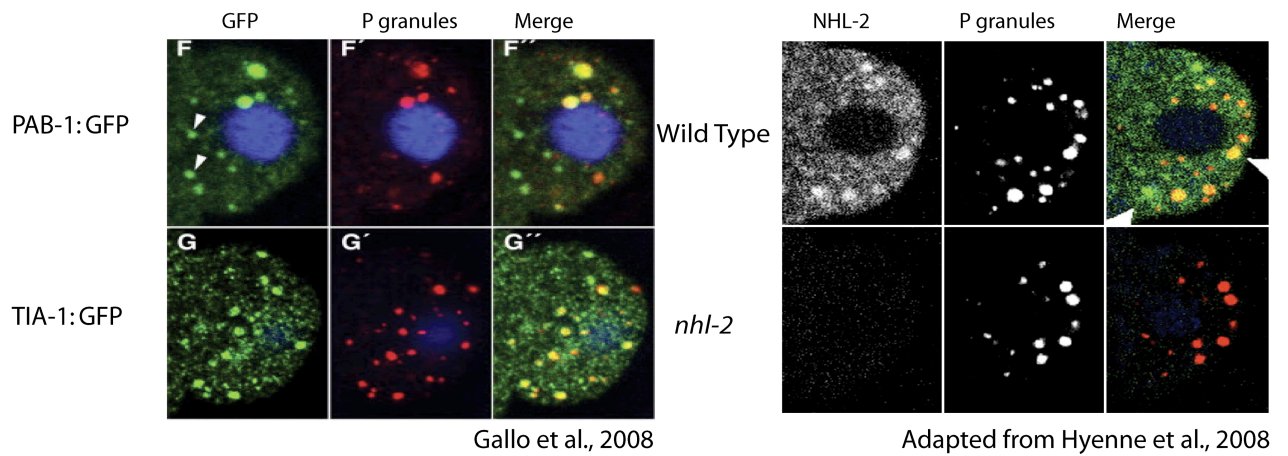
Figure 1.8: NHL-2, PAB-1 and TIA-1 granules have similar distribution patterns. They all colocalize partially to P granules. (A) Confocal images of embryos expressing the indicated GFP fusion and double-stained with anti-GFP and anti-P granules antibodies (B) Immunofluorescence of 4-cell embryos stained with anti-P granules and anti-NHL-2 antibodies. Note that some NHL-2, PAB-1 and TIA-1 positive granules colocalize with P granules, while others are devoid of P granule components.

2. Materials and Methods

<u>2.1. Strains and Alleles</u>

All strains were maintained as described by Brenner (1974) and were grown at 15°C unless otherwise stated. The wild-type strain was the N2 (Bristol) strain. The following mutants have been used:

1- *nhl-2(ok818)* III

Deletion allele considered as a null allele (Hyenne et al., 2008).

2- *pgl-1(bn101)* IV

Point mutation considered as a null allele (Kawasaki et al., 2004).

3- *nhl-3(tm2516)* II; *nhl-2(ok818) ncl-1(e1942) nhl-1(gk15)* III

nhl-1(gk15), *nhl-3(tm2516)* are deletion alleles and *ncl-1(e1942)* is a point mutation allele and it can be considered as a null allele since it induces a strong decrease in its respective protein expression levels (Frank et al., 2002).

4- *nhl-2(ok818)* III*; pgl-1(bn101)* IV

same as above

Mutant strains were generated by genetic crosses. *nhl-3(tm2516)*; *nhl-2 (ok818) ncl-1(e1942) nhl-1(gk15)* strain was generated by Vincent Hyenne (Hyenne et al., 2008).

I generated *nhl-2(ok818); pgl-1(bn101)* double mutants. The presence of *nhl-2(ok818)* was confirmed by PCR. The presence of *pgl-1(bn101)* in this mutant was confirmed by examining the sterility of mutants at the elevated temperature of 26°C under the dissecting microscope.

2.2. *C. elegans* techniques- Dissection, Immunofluorescence and Microscopy

2.2.1. Antibodies and Markers

Table I: List of the markers and antibodies used for embryo staining.

Antibodies		Source	Dilution
Primary Antibodies	Mouse OIC1D4 anti-P granules antibody (anti-PGL-1)	Developmental Studies Hybridoma Bank	1/300
	Rabbit anti-PAR-6 antibody	Hyenne et al., 2008	1/150
	Rabbit anti-CENPA antibody	Maddox et al., 2007	1/1000
Secondary Antibodies	Alexa Fluor 555-coupled goat-anti mouse	Invitrogen	1/500
	Alexa Fluor 488-coupled goat-anti-rabbit	Invitrogen	1/500

2.2.2. Immunofluorescence of *C. elegans* embryos

L4s were kept for 24h at 25°C and were then fixed and stained using the following protocol:

1. 20-30 worms were placed in 6μl water on a polylysine-coated slide and were sliced twice, once near each spermatheca, using two 25-gauge needles. This releases embryos (Fig 2.1).
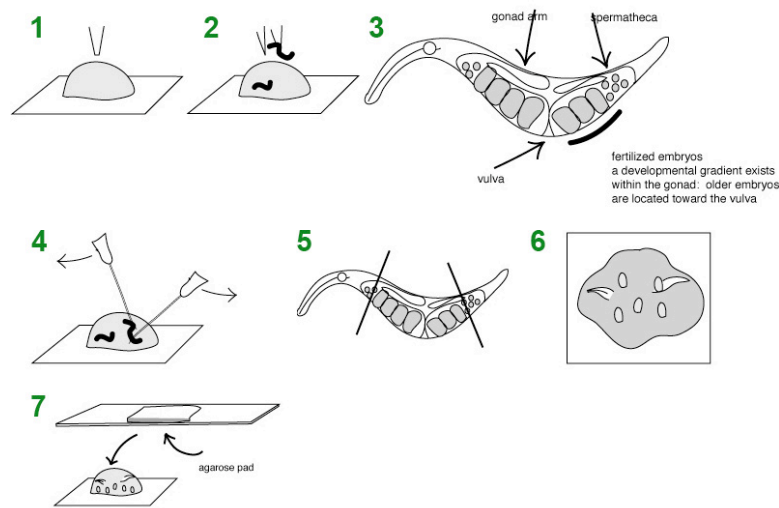

Figure 2.1: *C.elegans* **dissection for immunofluorescence (Prasad, Jang et al. 2007)**.

2. The slides were immediately frozen on dry ice. The coverslips were then flicked off the slides with a razor blade (to crack the egg shell) and the slides were put into cold methanol for fixation for 20 minutes at -20°C.

3. The slides were taken out of methanol and were dried using a Kim wipe (to remove all the liquid surrounding the embryos). To remove the methanol, the slides were washed (3-4 times) with PBS.

4. The PBS was aspirated off and then 100μl of PBT (PBS + 0.05% Tween 20) was added on top of the slides.

5. The PBT was aspirated off and 25 μl of goat serum was then added on embryos. The slides were then left in a humid chamber at room temperature for 30 minutes.

6. The goat serum was aspirated and 20 μl of OICD4 (1:300 dilutions in PBT) was added on each slide.

7. The slides were incubated at 4° C overnight in a sealed humidity chamber.

8. The next day the slides were washed with PBT 3 times and then 25 μl of secondary antibody was put on them. The slides were incubated at room temperature for 1 hour and they were washed with PBT 3 times.

9. Six μl of mounting medium containing DAPI was added on each slide, the coverslips were then put gently on each slide and later the slides were sealed with nail polish.

2.3.3. Imaging

2.3.3.1. P granule Quantification:

- Image Acquisition

   For P granule numbers quantification, images were acquired with a Zeiss LSM 510 Meta laser scanning confocal microscope using a Plan Apochromat 63x/1.4 NA objective. Images from wild type and mutant embryos were acquired using identical parameters. The principle of confocal microscopy is based on the fact that only a portion of the emitted light passes through a "pinhole" for the high-resolution observation of very thin optical sections of cells. This allows for a higher resolution image by preventing out of focus light from reaching the detector. Then a 3D reconstruction can be performed by superposition of these sections.

- Image Analysis

   The images acquired in confocal fluorescence were analyzed with the LSM program, and then processed using Adobe Photoshop. Since P granules are only present in the posterior pole of the embryo, a threshold was applied in order to eliminate the background puncta in the anterior and then we counted the total number of P granules in the posterior of each embryo (Figure 2.2). The number of P granules in each embryo is the average of three times quantification.
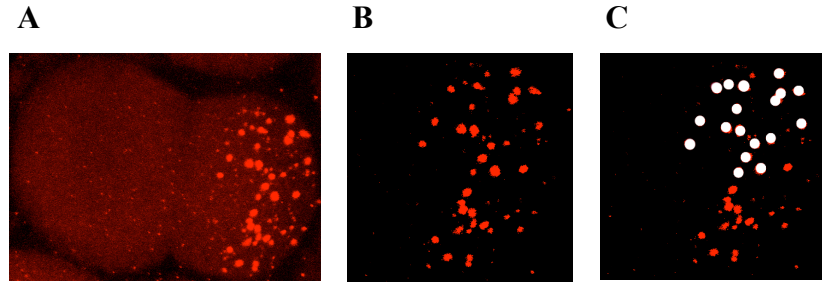
Figure 2.2: P granules quantification. (A) Embryos were stained with a P granule marker (OIC1D4). (B) A threshold was applied to subtract the background staining and then (C) P granules were quantified.

2.3.3.2. PGL-1 Fluorescence Intensity Measurements

- Image Acquisition

The images for PGL-1 intensity measurements have been made using a Delta Vision microscope, using the 60x objective. The total exposure time of each sequence is 0.1 msec, and the optical section spacing is 0.5 microns. The Delta Vision uses wide-field epifluorescence and a high precision robotic stage to take Z-sections through the specimen.

- Image Analysis

The images were analyzed with the NIS-Elements AR 3.0 program. For PGL-1 intensity measurements, one line was drawn around the anterior cell and another line was drawn around the posterior cell. Then for each region, the area, mean fluorescence intensity (MFI) and total fluorescence intensity (TFI) were calculated. We used an anti-CENP-A antibody as a normalizing control. CENP-A intensity was also measured using the same

method: the MFI of CENP-A staining in the AB and P_1 cells, were calculated and then we made an average of them (CENP-A). In addition, we also subtracted the background coming from nonspecific binding of a variety of primary and secondary antibodies. In each case, three different areas outside the embryos were taken as backgrounds (Figure 2.3B). Then the average MFI or TFI of backgrounds was subtracted from the MFI or TFI of the P granule and CENP-A staining. After background subtractions, we made a ratio of PGL-1 staining over CENP-A. The value of the anterior ratio (rA) is the ratio of MFI or TFI of PGL-1-A over the average of MFI or TFI of CENP-A, while the value of the posterior ratio (rP) is the ratio of PGL-1-P intensity over CENP-A staining of the embryo.

CENP-A

PGL-1
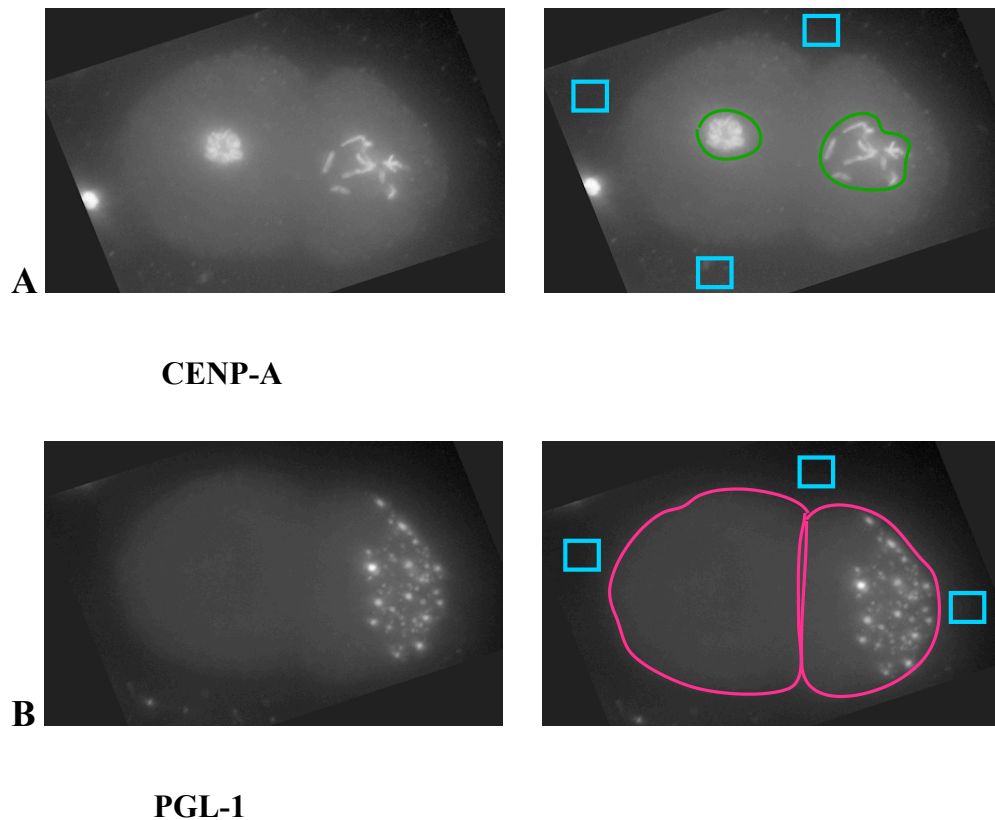
Figure 2.3: Fluorescence intensity measurements and background subtraction. (A) Embryos were stained with a histone marker (CENP-A) and the fluorescence intensity of histones at the anterior and posterior cells was measured (shown by green circles). (B) P granule marker (OIC1D4) was also used. P granule fluorescence intensity was measured in the anterior and posterior cells (shown by red circles). In each case, three different areas outside the embryos (shown by blue squares) were taken as backgrounds.

2.3. Sterility Assays:

10 adult hermaphrodites were picked on plates and were shifted from 15°C to 26°C for 5 hr. After 5 hr, the mothers were removed and their F1 progeny were remained on the same plates until they become adults. Worms that contained material in their uterus were transferred to individual plates for 5 hr, to lay progeny. F1 mothers were removed after 5 hr and the F2 hermaphrodites were scored for sterility. We used a dissecting microscope to examine sterility or fertility. Worms that lacked embryos in their uterus, or that failed to lay eggs were scored as sterile, whereas worms that contained embryos in their uterus or lay progeny were scored as fertile.

3. Results

3.1. The number of P granules is decreased in *nhl-2* mutant embryos

The overlapping localization of NHL-2 with the constitutive P granule component PGL-1 during early embryonic stages makes it possible that NHL-2 functions in regulating P granule stability. Thus in order to test this possibility, we first investigated the effect(s) of *nhl-2* loss of function on the number of P granules, using immunofluorescence and imaging techniques. In order to observe P granules, we used a well-characterized antibody against one of the core components of P granules, PGL-1. We also used an anti-PAR-6 antibody as a permeabilization control. Using confocal microscopy, we acquired images from wild type and *nhl-2* mutant embryos with the same acquisition parameters. P granules are only segregated to the posterior pole of the embryo and therefore we applied a threshold to eliminate the background until no puncta were observed in the anterior. We then counted the number of P granules in each embryo using a double-blind approach. Quantifying the number of P granules in immunostained fixed specimen revealed a two-fold reduction (P value= 5.37711E-06) in the number of P granules in *nhl-2* embryos (n=27) compared to wild type (n=18) (Figure 3.1.A). These results suggest that NHL-2 is important to regulate the number of P granules in early embryos.

To determine whether or not loss of *nhl-2* function (P granule reduction in number) depends on the embryonic cell stage, we classified the embryos based on their developmental stage into three groups of one-cell, two-cell and four-cell stage embryos and then examined the number of P granules in each group. Using the same methods, our P granule quantification revealed that P granules are reduced in number in the absence of *nhl-2* during the three early stages of embryogenesis in *C. elegans*. This result demonstrates that P granules of *nhl-2* mutants are decreased in number at all developmental stages (Figure3.1.B).

Interestingly, during P granule quantification assays, we noticed that some *nhl-2* mutants have almost no P granules (Figure 3.2). Our further analysis demonstrates that there are two different populations of *nhl-2* embryos in terms of the number of P

granules. P granules number of the first population of *nhl-2* embryos (51%) is similar to that of wild type embryos, while there is a dramatic reduction in P granules number in the second population of *nhl-2* embryos (49%) (Figure 3.2). This suggests that NHL-2 is a regulator of P granule stability and is likely to function redundantly with other components of P granule stability and assembly pathway.

A

B
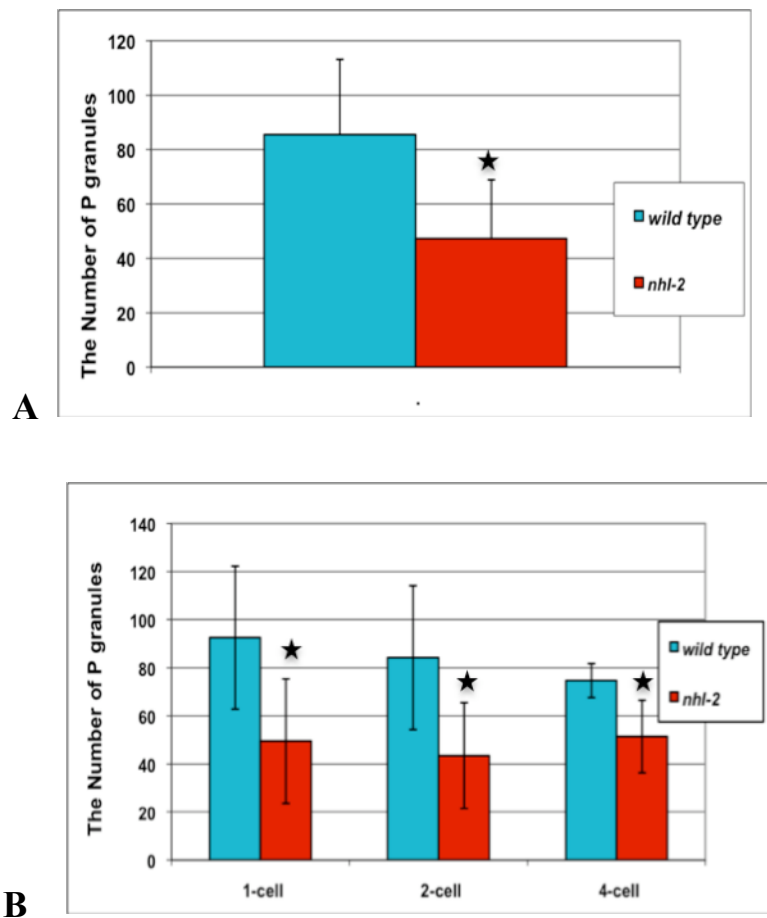
Figure 3.1: NHL-2 is important to regulate the P granule stability in early embryos. (A) P granules are reduced in number in *nhl-2* embryos (P value = 5.37711E-06) (B) Embryos were classified into three groups: one-cell, two-cell and four-cell stage embryos. Quantification analysis revealed a P granule reduction in *nhl-2* embryos in all groups. Error bars represent standard deviation. Stars denote statistical significance compared to control (p< 0.05, student t-test).

Figure 3.2: Different populations of *nhl-2* mutants. Analysis of 2-cell stage embryos showed that two populations of *nhl-2* mutant embryos exist. The first population shows a wild type phenotype in terms of the number of P granules while there is a dramatic reduction in the number of P granules in the second population. Note that two *nhl-2* mutants had less than 10 P granules per embryo.
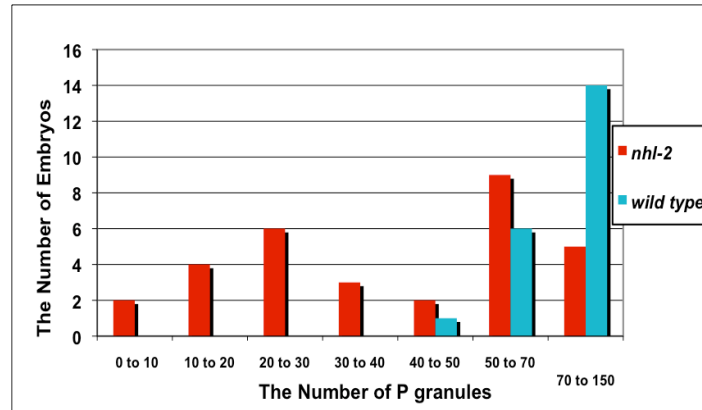
3.2. PGL-1 Fluorescence Intensity is significantly reduced in *nhl-2* mutant embryos

We were interested in whether the P granule reduction observed in *nhl-2* mutants is associated with a decrease in levels of one of the components of P granules, PGL-1. Thus we set out to test NHL-2 function in P granule stability more directly by examining the fluorescence intensity of the stained P granules. Embryos were stained with an antibody against PGL-1 and therefore the observed P granules are PGL-1 puncta. However this time, we used as control, an antibody against CENP-A, which is a histone H3-like protein. Both AB and P_1 cells contain almost the same amount of CENP-A, making CENP-A a proper control to normalize our values. We utilized a Delta vision microscope to acquire images from 2-cell stage wild type, *pgl-1* and *nhl-2* embryos using identical acquisition parameters. We measured the mean fluorescence intensity (MFI) of PGL-1 at anterior (PGL-1-A) and at posterior (PGL-1-P) as well as CENP-A MFI. In order to answer our question, we developed an image analysis assay: we made a ratio of PGL-1 staining over CENP-A. Briefly, the value of the anterior ratio (rA) is the ratio of MFI of PGL-1-A

over MFI of CENP-A, while the value of the posterior ratio (rP) is the ratio of PGL-1-P intensity over CENP-A staining of the embryo.

To assess whether our image analysis approach is a valid method to answer our question, we used wild type (as a positive control) and *pgl-1* (as a negative control) embryos. P granules are only segregated to the posterior cell and therefore the anterior cell should have less average PGL-1 intensity than the posterior cell. We expect however not to observe such a difference in our *pgl-1* mutants. Our image analysis revealed that the anterior average intensity of PGL-1 (rA) staining is significantly smaller than the posterior average intensity of PGL-1 staining (rP) in wild type embryos (P value= 0.002). On the contrary, in *pgl-1* mutants, the anterior PGL-1 intensity is almost the same as the posterior PGL-1 intensity (P value= 0.9). In addition, the posterior PGL-1 intensity of *pgl-1* mutants is significantly decreased compared to wild type (P value= 4.8194E-05), which is consistent with the fact that *pgl-1* mutants lack PGL-1 and therefore have no PGL-1 staining. This data suggests that our analysis assay for measuring PGL-1 intensity is robust (Figure 3.3.B).

We therefore used this approach to assay whether NHL-2 loss of function affects PGL-1 intensity levels. As shown in figure 3.3.B, we found that removal of *nhl-2* leads to a significant reduction in the posterior PGL-1 intensity as compared with wild type embryos (P value= 0.006). This result suggests that NHL-2 removal causes a reduction in the level of PGL-1 intensity (Figure 3.3.B). Our MFI analysis correlates with our previous results concerning the quantification of the number of P granules and shows a decrease in PGL-1 intensity levels in the absence of NHL-2.

Surprisingly, we observed some PGL-1 intensity in the anterior cells of all our embryos. Because P granules segregation is restricted to the posterior cell in wild type backgrounds, we defined the observed anterior PGL-1 intensity as an internal background of our P granule staining. We then postulated that the intensity of PGL-1 internal background is equal throughout the cytoplasm of the embryos and therefore in order to

measure the intensity of PGL-1 found in P granule foci (PGL-1 MFI) at posterior, we subtracted the rP from rA. Our data showed that the MFI of PGL-1 staining at posterior of *nhl-2* mutants is significantly diminished compared to wild type (P value= 0.03) (Figure 3.4.A). We then classified our embryos, based on the values obtained for our negative control, *pgl-1*. Interestingly, our data revealed that *nhl-2* mutants are distributed in different groups, as we observed variations in the MFI of P granules at the posterior pole from one embryo to another (figure 3.4.B). This finding shows a distribution in *nhl-2* mutants in terms of their PGL-1 intensity levels.
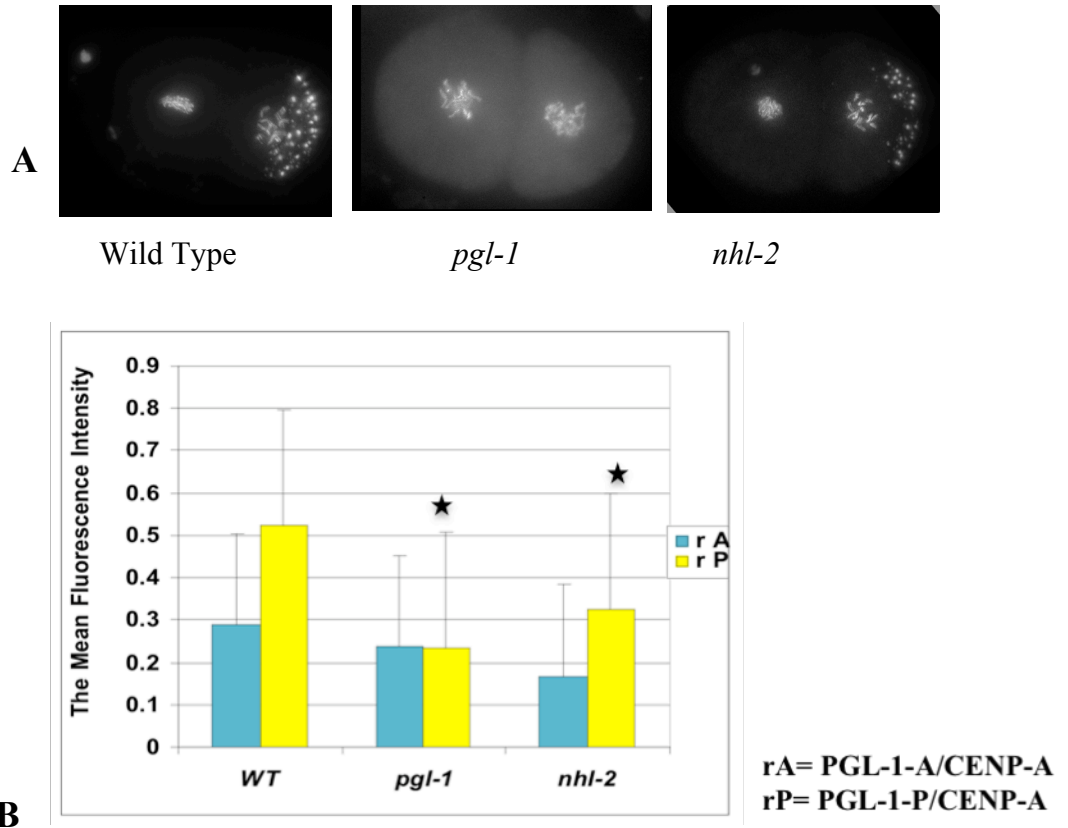
Figure 3.3: *nhl-2* mutants have less PGL-1 intensity. (A) Delta-vision images of fixed 2-cell stage wild type (n=30), *pgl-1* (n=30) and *nhl-2* (n=30) embryos, double-stained with anti-CENP-A and anti-PGL-1 antibodies. CENP-A mean intensity and PGL-1 mean intensity have been measured. In addition, in each case three different areas outside the embryos were taken as backgrounds. Then the average Mean Fluorescence Intensity (MFI) of backgrounds was subtracted from the MFI of the P granule and CENP-A staining. (B) Mean Fluorescence Intensity (MFI) measurements revealed that posterior MFI of *nhl-2* mutants was significantly reduced comparing to the posterior MFI of wild type embryos (P value= 0.006). The anterior PGL-1 MFI reduction observed in *nhl-2* mutants is not significant. Note that in *pgl-1* mutants, where there is no PGL-1 staining, the posterior MFI equals the anterior MFI. Stars denote statistical significance compared to rP value of the control (p< 0.05, student t-test).
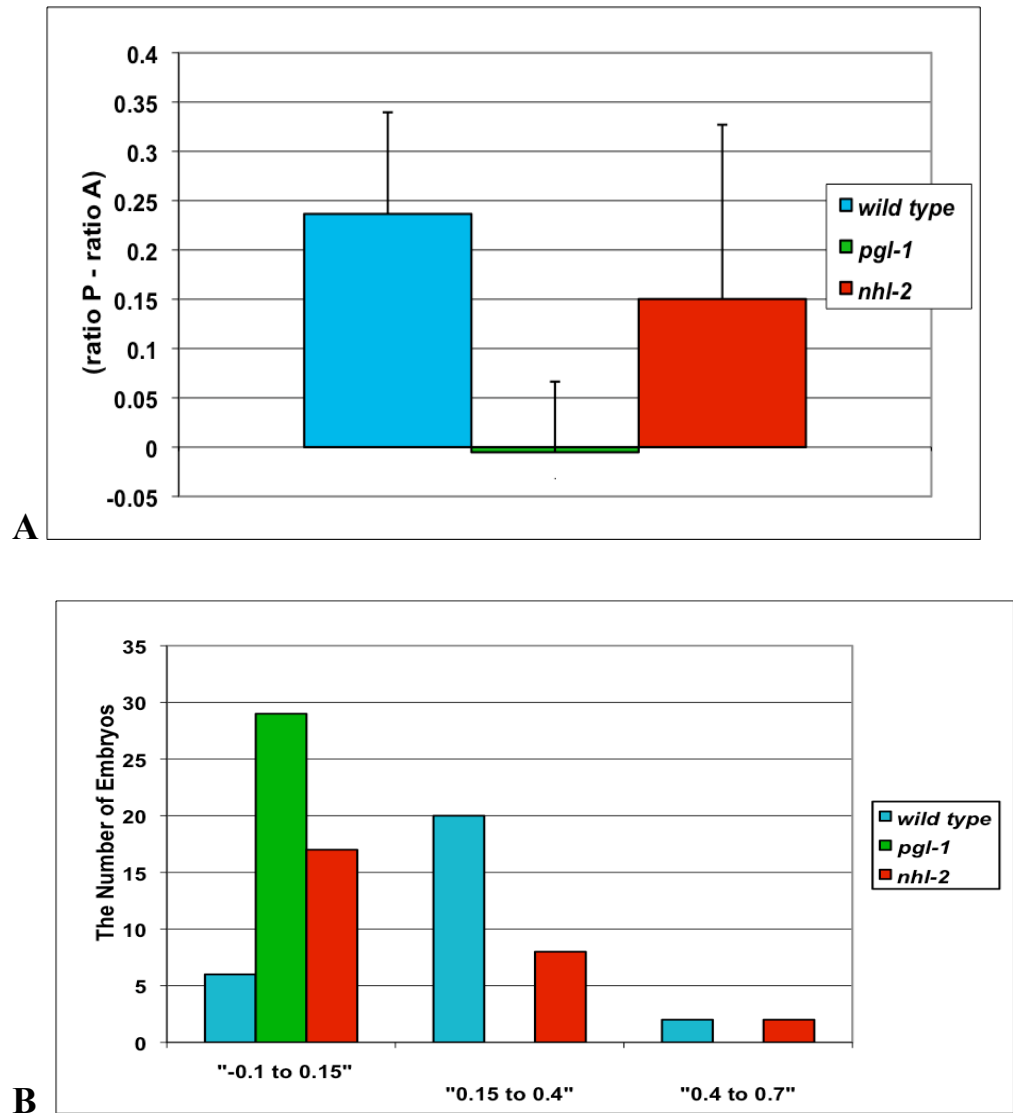
Figure 3.4: **Different populations of *nhl-2* mutants.** (A) We subtracted the posterior PGL-1 MFI (rP) from the anterior PGL-1 MFI (rA) in order to have the fluorescence intensity of P granules at the posterior cells and we observed that the intensity of P granule is significantly decreased after NHL-2 depletion. (B) NHLL-2 loss of function has different effects on P granule stability in embryos. P granule MFI (rP-rA) of *pgl-1* mutants ranges from -0.1 to 0.15. Based on the values obtained for *pgl-1* mutants we classified our wild type and *nhl-2* mutant embryos and we observed different populations of 2-cell stage *nhl-2* mutants, in terms of the intensity of P granules in the posterior.

3.3. The underlying mechanisms of P granule reduction

Our results support the idea that NHL-2 is involved in P granule biogenesis or stability during several early stages of *C. elegans* embryonic development and that its function may be partially redundant with other regulators of P granule stability.

To understand the mechanisms of P granule reduction (in both number and intensity) caused by NHL-2 loss of function, we postulated two hypotheses: (1) *fusion* or (2) *recruitment*. **Fusion:** One possibility for how NHL-2 regulates P granule stability is that NHL-2 prevents the accumulation or fusion of P granules so that *nhl-2* embryos have less but larger P granules. **Recruitment:** Alternatively, NHL-2 may be actively contributing to the recruitment of PGL-1 to P granules in *C. elegans* and therefore *nhl-2* depletion will affect proper P granule formation (Figure 3.5). To test these hypotheses, we measured and compared PGL-1 Total Fluorescence Intensity (TFI) using same methods as described before (Materials and Methods).
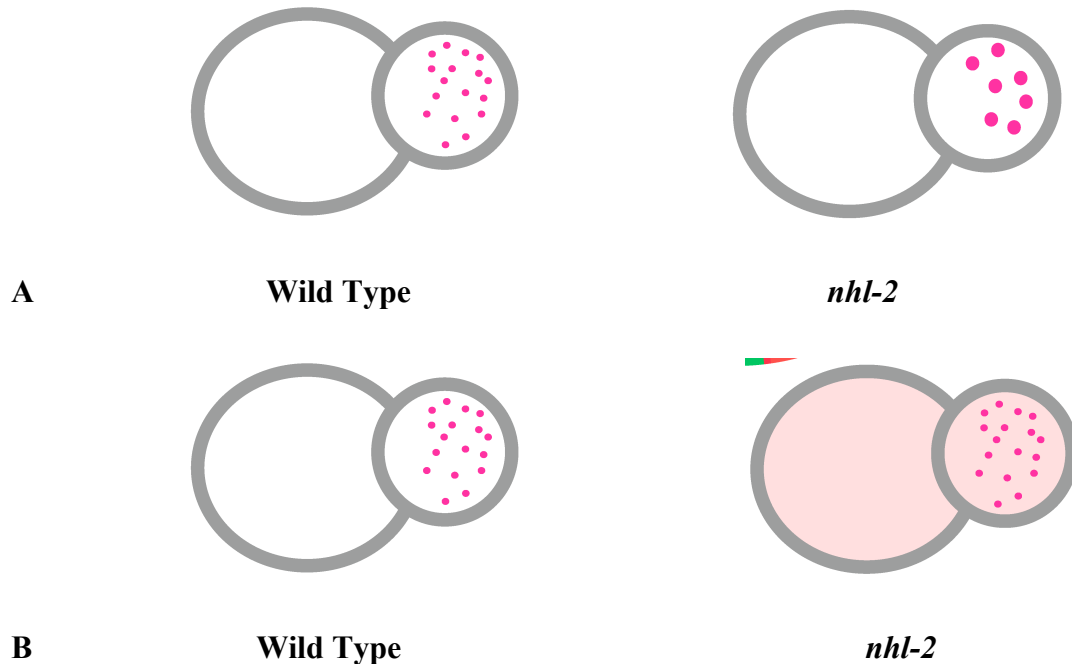
Figure 3.5: Two models for P granule reduction in *nhl-2* mutants. Schematic 2-cell embryos with P granules in pink. (A) Fusion hypothesis: The amount of P granule components and therefore P granules does not change in *nhl-2* mutants but P granules might tend to fuse together and form big clusters in the absence of NHL-2. If this is the case although the *nhl-2* loss of function leads to P granule reduction in number, the posterior P granule total fluorescence intensity (TFI) of wild type must be the same as that of the *nhl-2* embryos. (B) Recruitment hypothesis: another possibility is that the NHL-2 alters the proper PGL-1 assembly onto P granules in the posterior and therefore causing in an increase in the level of diffused PGL-1 (shown as a light pink background) in the anterior and posterior cytoplasm.

3.3.1. NHL-2 loss of function phenotype is not due to P granule fusion

If P granules fuse to form larger clusters in *nhl-2* mutants, then the amount of P granules material and thus the posterior TFI in *nhl-2* mutants should be the same as the one in wild type embryos. In order to assess if this is the case, we measured and compared the posterior PGL-1/ CENP-A TFIs in different backgrounds. Our TFI measurements showed that the posterior TFI in *nhl-2* mutants is reduced significantly (P value= 0.004) compared to the posterior TFI in wild type embryos (Figure 3.6). Interestingly the posterior TFI of *nhl-2* embryos is comparable to that of *pgl-1*. This result suggests that although in the

absence of NHL-2 P granules decrease in number, they do not accumulate to form larger granules. Therefore we concluded that NHL-2 functions in P granule stability not via fusing P granules.
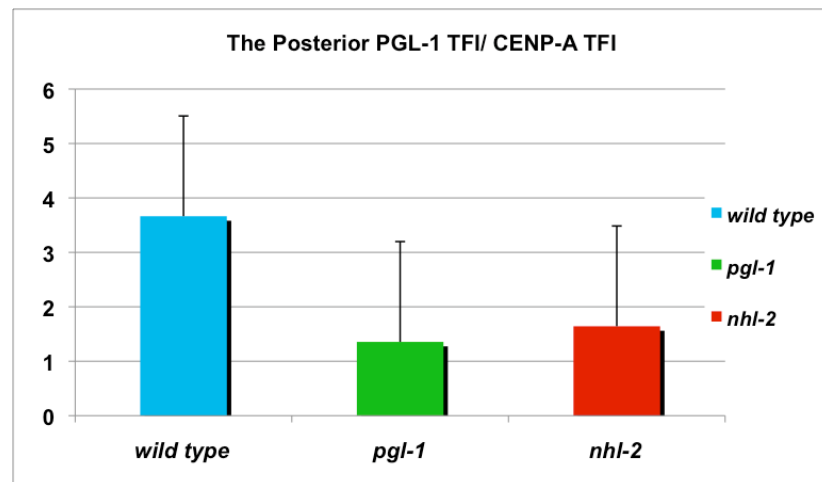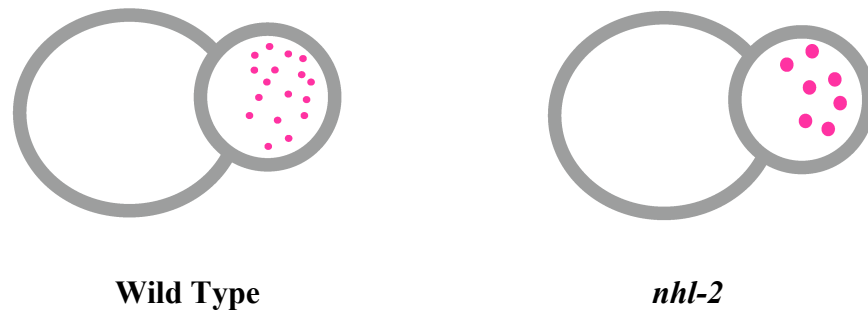
Wild Type   *nhl-2*

Figure 3.6: P granules do not fuse in the absence of NHL-2. The posterior TFI (Total Fluorescence Intensity) of PGL-1 has been measured and compared in wild type (n=30), *pgl-1* (n=30) and *nhl-2* (n=30) embryos. The posterior TFI in wild type is significantly higher than the posterior TFI of *nhl-2* mutants, suggesting that there is less amount of PGL-1 in the posterior pole of the embryo in the absence of NHL-2. This result indicates that the decrease in P granules and PGL-1 fluorescence intensity in *nhl-2* mutants is not due to P granules fusion.

3.3.2. *nhl-2* loss of function causes a general decrease in PGL-1 intensity levels at 2-cell stage embryos

The fact that the observed P granule reduction (number and intensity) in *nhl-2* mutants is not due to P granules fusion to one another, prompted us to test our second hypothesis, recruitment. If NHL-2 functions in PGL-1 assembly into P granules, there must be some P granule-free PGL-1 protein distributed throughout the cytoplasm instead of localizing to P granules in the posterior. If this is the case, we expect to observe an increase in the anterior TFI levels of *nhl-2* mutants and therefore a general increase in the PGL-1 TFI in the *nhl-2* mutants comparing to wild type. Thus in order to test whether disrupting the function of *nhl-2* affects PGL-1 recruitment, using the same approach, we measured the anterior TFI, the posterior TFI and the total TFI (both anterior and posterior) of PGL-1 staining in our wild type, *pgl-1* and *nhl-2* embryos. In each case we normalized our data, making a ratio of PGL-1 intensity over the total CENP-A intensity. In addition, we had to consider one important fact; the size of the embryos influences their TFI: the anterior AB cell is bigger than the posterior P1 cell and therefore although P granules are only partitioned to the posterior cell, the anterior TFI is always higher than the posterior TFI. Thus in order to solve this problem we assumed that the anterior cell had the same area as the posterior cell and according to its original fluorescence intensity, we then find out its new PGL-1 intensity. The comparison of the anterior TFI and the posterior TFI within each strain revealed that there is no increase in the PGL-1 fluorescence intensity in the anterior of the *nhl-2* mutants. In addition, the total TFI of *nhl-2* mutant embryos is significantly less than the total TFI of wild type embryos (P value=0.0002). This result indicates that NHL-2 does not function in PGL-1 recruitment to P granules at 2-cell stage (Figure 3.7.A).

Furthermore, after area subtraction, our total TFI measurement showed that there is a significant decrease in the intensity of P granule foci at the posterior of *nhl-2* mutants (Figure 3.7.B), confirming that there is a general decrease in the PGL-1 levels in the absence of NHL-2.
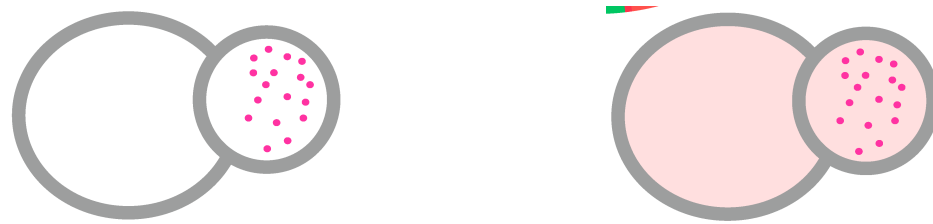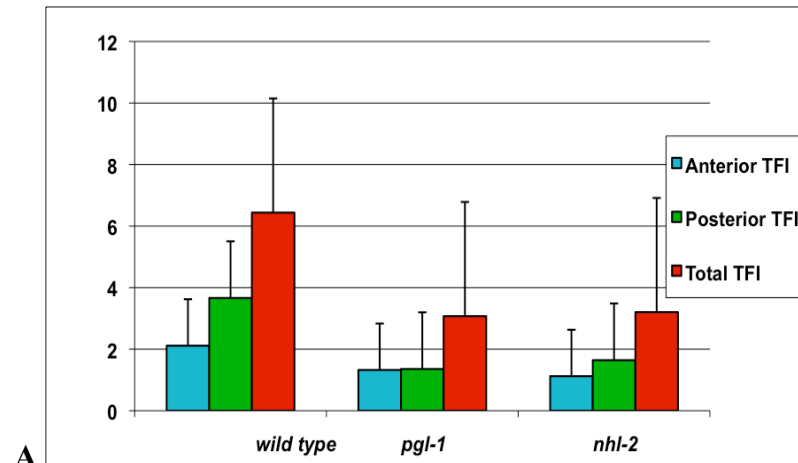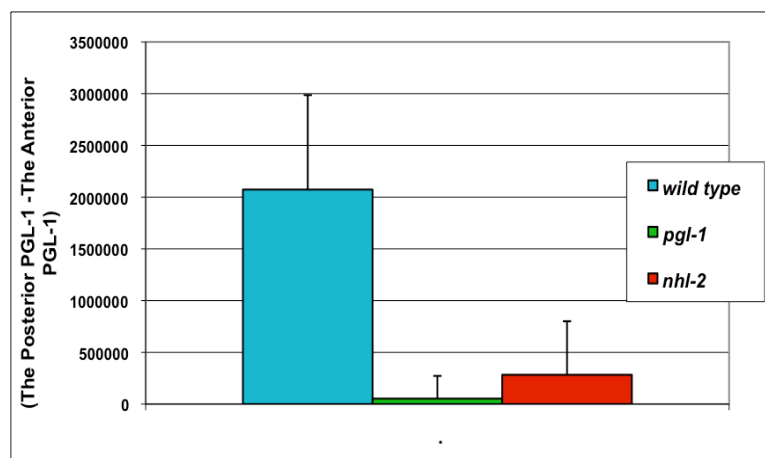
Figure 3.7: A general reduction of PGL-1 intensity levels in *nhl-2* mutants. (A) The Total Fluorescence Intensity (TFI) of PGL-1 and CENP-1 has been measured in wild type (n=30), *pgl-1* (n=30) and *nhl-2* (n=30) embryos. In each group, we assumed that the anterior cell and the posterior cell have the same area and we then compared their intensities regarding to their new size. (B) P granule intensity is significantly decreased in the absence of NHL-2.

3.4. NHL-2 is required for fertility at elevated temperatures

Depletion of any of the three constitutive P granule components PGL-1, DEPS-1 and GLH-1 causes germ cell underproliferation and a 100% temperature-sensitive sterility at F2 (Kawasaki et al, 1998, 2004). NHL-2 localized to P granules, we therefore asked whether loss of *nhl-2* activity causes sterility defects similar to *pgl-1*, *glh-1* and *deps-1* mutants. Thus in order to elucidate the possible regulatory role of NHL-2 in patterning the germ-line, we performed sterility assays in wild type (negative control for sterility) and *pgl-1* (positive control for sterility) and *nhl-2* mutant backgrounds. In addition, in order to investigate whether NHL-2 loss of function, enhances the sterility phenotype observed in *pgl-1* mutants, we generated *nhl-2; pgl-1* double mutants. Mothers (P0's) were shifted from 15°C to 26°C as adults for 5 hrs and their fertility/sterility at F1 and F2 has been examined: animals do not produce progeny or any viable progeny, were scored as sterile.

When grown at elevated temperature of 26°C, 100% of *pgl-1* and *nhl-2; pgl-1* mutants at F2 generation, develop into sterile adults. Our analysis showed that *nhl-2* loss of function affects fertility at F2 generation. However, one interesting feature of our sterility analysis of *nhl-2* mutants is the variability observed in germline defects between experiments. Our analysis revealed that there are two populations of *nhl-2* mutants for sterility phenotype at F2. The first population of *nhl-2* animals developed into sterile worms at F2, while the second group showed a wild type phenotype (Figure 3.8). To further investigate the possible genetic interaction between *nhl-2* and other CeBrats, we generated a quadruple mutant strain: *nhl-2* and *nhl-3; nhl-2, ncl-1, nhl-1*. Our analysis revealed that compared to *nhl-2* single mutants, the quadruple strain displayed enhanced sterility at F2 (100%). This finding suggests that NHL-2 and other CeBrats function redundantly in regulating germline proliferation.

To investigate the underlying mechanisms of the observed sterility, using the Axio-Imager microscope we examined the gonads of animals raised at the restrictive temperature of 26°C. Our analysis showed that *pgl-1* and the first population of *nhl-2* animals raised at

26°C, are sterile and contain under-proliferated germline, while wild type animals and the second group of *nhl-2* mutants are fertile and contain a well-proliferated germline with healthy-looking embryos (Figure 3.9). Altogether, our data shows that NHL-2 affects fertility at 26°C and might function redundantly with other regulators of P granule assembly pathway.
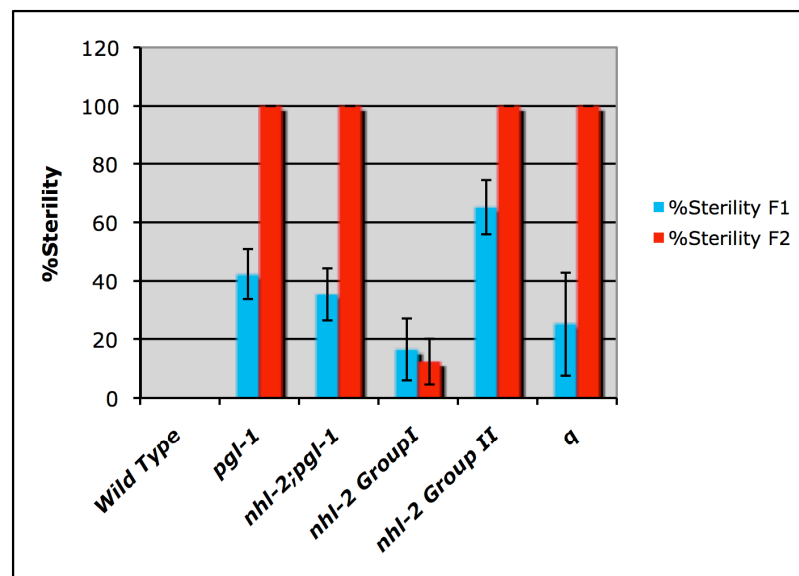
Figure 3.8: Removing of NHL-2 and other CeBrats affects fertility at 26°C. *nhl-3; nhl-2, ncl-1, nhl-1* quadruple mutants (q) display temperature-sensitive sterility at F2, a phenotype that also observed at F2 generation of *nhl-2* and *nhl-2;pgl-1* mutants. Interestingly however, *nhl-2* single mutants show variability in the sterility defect.

Wild Type

pgl-1

nhl-2

q
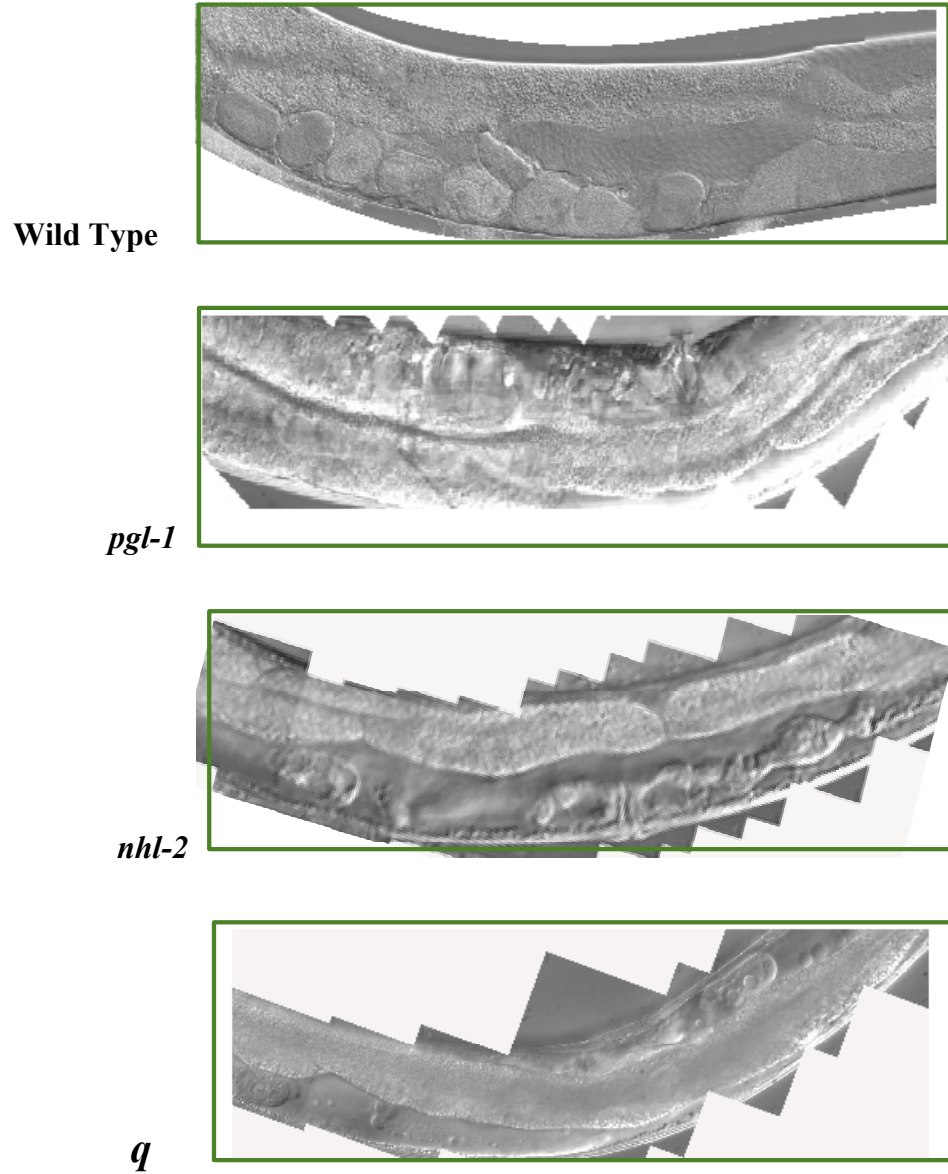
Figure 3.9: Normarski images of wild type and mutant hermaphrodites grown a 26°C. *nhl-2* mutants of group I   and *nhl-3; nhl-2, ncl-1, nhl-1* quadruple mutants (*q*) similar to *pgl-1* mutants lack gametes in their uterus when compared with wild type animals. This defect is similar to those observed in *pgl-1* mutants.

4. Discussion

4.1. NHL-2 loss of function and P granules Defects

Our studies showed that loss of NHL-2, a P granule-associated protein causes a reduction in P granules number. In addition, consistent with our P granules number quantification approach, our P granule intensity analysis showed that PGL-1 intensity levels also decrease significantly in the absence of NHL-2. Interestingly we also observed a reduction in PGL-1 intensity levels in the anterior cytoplasm of 2-cell stage *nhl-2* mutants, suggesting that *nhl-2* loss of function results in a general PGL-1 decrease. Altogether, our results suggest that NHL-2 is important for P granule biogenesis or stability. In addition, we also showed that, *nhl-2* loss of function does not lead to P granules aggregation. This data suggests that NHL-2 functions in P granule biogenesis perhaps through its role in the assembly of PGL-1 to P granules. However, the precise function of NHL-2 in regulating P granule stability remains to be elucidated.

We only investigated the PGL-1 intensity levels at 2-cell stage embryos, where P granules are already partitioned to the posterior cell, but it is likely that NHL-2 loss of function affects P granule stability before or during P granule segregation to the posterior pole. Thus two explanations seem likely:

(1) One explanation is that the observed general decrease in the levels of PGL-1 in *nhl-2* mutants is due to the role of NHL-2 in regulating P granule dissolution/condensation. In other words, NHL-2 might be involved in increasing the posterior P granule condensation that occurs after symmetry breaking. To assess this possibility, we plan to develop a 3D particle tracking method (Brangwynne et al, 2009) in order to analyze the localization dynamics and fluorescent levels of GFP-fused PGL-1, taking advantage of our *nhl-2*: PGL1-GFP and PGL-1:GFP strains embryos using time-lapse fluorescence microscopy.

(2) P granules are passed on to the embryo with the cytoplasm inherited from oocytes. If NHL-2 loss of function causes a reduction in PGL-1 levels during oogenesis, the

embryos will also have less PGL-1 and therefore less P granules. To understand whether the P granule reduction defect comes from oocytes, we will perform immunostaining and imaging assays (described in the materials and methods) to quantify the number of P granules in oocytes and also to measure the PGL-1 fluorescence intensity in the germline and oocytes of wild type, *pgl-1* and *nhl-2* embryos.

Another explanation is that, NHL-2's function in P granule biogenesis, takes place in oocytes causing a decrease in PGL-1 levels. During embryogenesis, this PGL-1 reduction in turn can increase the dissolution rate throughout the cytoplasm in a way that P granules are not formed robustly in the posterior of the embryo.

4.2. NHL-2 might function redundantly with other components of P granule assembly pathway

One interesting aspect of the NHL-2 loss of function is the variability of the *nhl-2* mutant phenotype. For instance, there are two populations of *nhl-2* mutants in terms of the number of P granules. Such distributions are also observed in the PGL-1 fluorescence intensity levels of *nhl-2* mutants. *nhl-2* is a null loss of function mutation and therefore the variabilities observed might be due to functional redundancy of NHL-2 with other members of CeBrtas or with key components of P granule assembly pathway.

In order to investigate whether NHL-2 interacts physically with any of these proteins, we will use western blot and co-immunoprecipitation techniques. To this end Marianne Desrosiers in our lab has generated antibodies against NHL-2 and we plan to characterize the proteins that interact directly or indirectly with NHL-2 by mass-spectrometry. We will also try to identify the possible genetic interaction between *nhl-2* and other genes involved in P granule stability. Our candidates:

CeBrats: To assess whether other Brats also play a role in P granule biogenesis, we will use immunofluorescence staining using anti-PGL-1 antibodies and quantify the number of P granules and the intensity of PGL-1 protein in *nhl-1;nhl-3;ncl-1;nhl-2* and *nhl-*

1;ncl-1;nhl-3 embryos. The observed phenotypes will be compared with those observed in *nhl-2 embryos*. The observation of mislocalized P granules or greater reduction in the number of P granules or staining intensities of PGL-1 compared to *nhl-2* embryos would suggest that other CeBrats function redundantly with *nhl-2*. On a contrary, no enhancement of the *nhl-2* phenotype after removal of *nhl-1*, *nhl-3* and *ncl-1* would indicate that only NHL-2 contributes to the P granule biogenesis in *C. elegans*. Moreover, we are planning to examine the fertility of triple mutants at elevated temperature to test whether other Brats recapitulate the sterility phenotype.

P granule Components: So far we looked at PGL-1, which is one of the most downstream components of the P granule assembly pathway. One possibility that is consistent with the available data is that GLH-1 or DEPS-1 can interact with NHL-2 to control P granule biogenesis and stability. In order to better define the molecular role of NHL-2 in P granule assembly pathway and to further investigate the molecular mechanisms by which, P granules are reduced in the absence of NHL-2, the following experiments will be done. *nhl-2* mutants and *deps-1*and *glh-1* RNAi embryos will be fixed and stained against PGL-1, GLH-1 or DEPS-1 antibodies using the same methods explained above. Then the localization and intensity of each protein will be quantified and compared with the wild type. One possibility is that NHL-2 localizes to P granules dependently of GLH-1 and/or DEPS-1. To test this, NHL-2 localization will be examined in *glh-1* RNAi, *deps-1* RNAi and RNAi treated embryos against both *glh-1* and *deps-1*. For instance, NHL-2 mislocalization in any of these backgrounds would indicate that GLH-1 or/and DEPS-1 is required for the proper localization of NHL-2 to P granules, suggesting that NHL-2 is downstream of other components in P granule assembly pathway. If that is the case we will also quantify the number of P granules and measure PGL-1 intensity to test whether NHL-2 localization affects its role in P granule stability. On the other hand, similar experiments will be done to test the localization of GLH-1 and DEPS-1 in *nhl-2* mutants. No change in GLH-1 and DEPS-1 intensity levels or localization will indicate that NHL-2 functions downstream of GLH-1 and DEPS-1 in the P granule assembly pathway.

To better investigate the role of NHL-2 in *C. elegans* development and to assess its functional redundancy with other P granule components, we intend to quantify the number of P granules and measure PGL-1, GLH-1 or DEPS-1 intensity levels in wild type embryos, *nhl-2* mutants or the generated RNAi embryos. Any decrease in the number of P granules or protein intensity would suggest that GLH-1 or/and DEPS-1 collaborate with NHL-2 in order to regulate the P granule stability. Altogether, these experiments will allow us to position NHL-2 in the P granule assembly pathway.

PAR-1: PAR-1 is one of the first molecular markers of Anterior-Posterior polarity, which localizes to the posterior pole of the *C. elegans* embryo. Several lines of evidence indicate that *par-1* plays roles in determining germline protein and P granule localization (Boyd et al., 1996; Morton et al., 2002). Cheeks et al. showed that PAR-1, through its kinase activity, stabilizes the asymmetric localization of those P granules that reach the posterior of the embryo. Interestingly, a homolog of PAR-1 in *Drosophila* localizes to the posterior pole of the embryo and phosphorylates and stabilizes the germline protein Oskar (Shulman et al., 2000). It has been shown that mutations that disrupt the expression of PAR-1 in *Drosophila*, affect the proper localization of pole-plasm components (equivalent of P granules in *C. elegans*) and results in the reduction of the number of pole cells (Tomancak et al., 2000). PAR-1's function in stabilizing P granules might be a conserved mechanism for regulating the localization of germline determinants (Cheeks et al. 2004, Riechmann et al, 2002). PAR-1 function is essential for germline specification, differentiation, or maintenance. In addition, NHL-2 is also involved in P granule stability raising the possibility that PAR-l might associate with NHL-2 to regulate P granule stability. In order to test this possibility we will apply immunofluorescence approaches using *par-1* embryos and compare the number and localization of P granules and intensity of PGL-1 in these embryos with that of the wild type and *nhl-2* embryos. Furthermore, in order to investigate the possible genetic interaction between PAR-1 and NHL-2, we plan to test P granule localization and number in *par-1;nhl-2* embryos. For instance, if we see no difference in P granule number or PGL-1 mean intensity in *par-1;nhl-2* compared to *nhl-2*

mutants, we can conclude that PAR-1 and NHL-2 are both involved in P granule stability, but through different mechanisms. On the contrary, a dramatic increase in *nhl-2* phenotype in double mutants would suggest that PAR-1 functions with or in parallel to NHL-2 to regulate P granule stability during early embryogenesis. Additionally we can use protein phosphorylation assays in order to uncover PAR-1 substrates in P granule stability pathways and also to examine if NHL-2 is one of them.

CAR-1: CAR-1 is a conserved RNA-binding protein that associates with CGH-1 within P granules of *C. elegans*. It has been shown that the brood size of *car-1* (RNAi) animals is reduced due to the increased physiological apoptosis (Boag et al., 2005). We showed that *nhl-2* loss of function affects proper germline proliferation. In addition, NHL-2 interacts with CGH-1 (Hammell et al., 2009) and therefore it might be possible that NHL-2 and CAR-1 associate with each other through CGH-1 in order to regulate germline proliferation.

4.3. How could RNA regulation function in P granule stability?

P granules are RNA-rich organelles that specifically accumulate in the germ cells in *C. elegans*. In addition, P granules contain several mRNA binding proteins, many of which are also present in P bodies. Although P granules are essential for germ cell identity and germline development, the mechanisms they use to control developmental regulation are still unknown. One possibility is that, due to their perinuclear positioning, P granules might function as centers for mRNA storage depending on the developmental stage: P granules capture mRNAs as they pass through nuclear pores in order to repress them. Interestingly, several lines of evidence link P granules and their components to RNA-related processes. For instance, it has been shown that RNA-induced silencing complex (RISC) proteins involved in microRNA processing, associate with homologs of Vasa in flies and mice (Kotaja et al., 2006, Megosh et al., 2006). It has been also shown that Brat and Mei-P26 (a member of the Brat family in *Drosophila*) bind to Argonaute-1, a member of the RISC

complex (Neumuller et al., 2008). Furthermore, it has been reported that NHL-2 physically interacts with the core miRISC components (*alg-1* and *ain-1*) and the P-body protein CGH-1 and thereby functions in the posttranscriptional repression in *C. elegans* larvae (Hammell et al., 2009). All these evidences led us to the hypothesis that RNA regulation might be involved in regulating P granule stability in *C. elegans*.

In order to assess this possibility, we are interested in whether NHL-2 is involved in RNA regulation during *C. elegans* embryogenesis. It is already known that NHL-2 enhances the translational repression in larvae in *C. elegans* (Hammell et al., 2009), suggesting that NHL-2 might play the same role in *C. elegans* embryos. If NHL-2 is involved in repression of maternal mRNAs and repressing them to P granules in *C. elegans* embryos, it could explain why P granule reduction occurs in *nhl-2* mutants: NHL-2 loss of functions affects mRNA repression, leading to a decrease in the number of P granules. To examine this hypothesis we intend to investigate the translational level of some maternal mRNAs, known to be silenced during early embryogenesis. For instance, *nos-2* is one maternal mRNA that is translationally repressed in oocytes and early embryos. This repression depends on the 3' UTR of *nos-2* (D'agostino et al., 2006). Therefore we are planning to make use of a GFP:H2B:*nos-2* 3'UTR transgene construct generated by D'agostino et al, in order to detect *nos-2* repression in wild type and *nhl-2* embryos. To assess that we can measure the GFP: H2B fluorescence intensity in oocytes and early embryos of both wild type and *nhl-2*. An increase in the relative GFP fluorescence intensity in *nhl-2* compared to wild type would indicate that NHL-2 is required for the translational repression of *nos-2* during early embryogenesis.

Another possibility is that NHL-2 granules interact with other RNA granules in order to regulate P granule stability. One class of RNA granules contains markers of stress granules such as TIA-1 (*C. elegans* ortholog of T-cell restricted intracellular antigen) and PAB-1 (Poly (A) binding protein). It has recently been shown that P granules also contain TIA-1 and PAB-1 (Gallo et al. 2008). Interestingly, the TIA-1 and PAB-1 granules have the same distribution pattern as observed for NHL-2 granules (Gallo et al., 2008; Hyenne et

al., 2008), suggesting that NHL-2 granules might interact with P granules and other RNA granules to stimulate the mRNA exchange between these granules in order to function in posttranscriptional gene regulation. In order to assess this hypothesis we plan to test the TIA-1 and PAB-1 localization in wild type and *nhl-2* embryos. We will also examine the distribution pattern and number of NHL-2 granules and P granules in *tia-1*(RNAi) and *pab-1*(RNAi) embryos. To further investigate the interaction between NHL-2 and TIA-1 and/or PAB-1 granules, we will use time-lapse microscopy. This will help us to examine the dynamics of NHL-2:GFP, TIA-1:GFP, PAB-1:GFP and PGL-1:GFP in live embryos of wild type, *nhl-2*, *pab-1*(RNAi) and *tia-1*(RNAi).

References

Amiri, A., Keiper, B.D., Kawasaki, I., Fan, Y., Kohara, Y., Rhoads, R.E., and Strome, S. (2001). An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in C. elegans. Development *128*, 3899-3912.

Anderson, P., and Kedersha, N. (2006). RNA granules. J Cell Biol *172*, 803-808.

Arama, E., Dickman, D., Kimchie, Z., Shearn, A., and Lev, Z. (2000). Mutations in the beta-propeller domain of the Drosophila brain tumor (brat) protein induce neoplasm in the larval brain. Oncogene *19*, 3706-3716.

Audhya, A., Hyndman, F., McLeod, I.X., Maddox, A.S., Yates, J.R., 3rd, Desai, A., and Oegema, K. (2005). A complex containing the Sm protein CAR-1 and the RNA helicase CGH-1 is required for embryonic cytokinesis in Caenorhabditis elegans. J Cell Biol *171*, 267-279.

Barbee, S.A., Lublin, A.L., and Evans, T.C. (2002). A novel function for the Sm proteins in germ granule localization during C. elegans embryogenesis. Curr Biol *12*, 1502-1506.

Bilder, D. (2004). Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes Dev *18*, 1909-1925.

Boag, P.R., Atalay, A., Robida, S., Reinke, V., and Blackwell, T.K. (2008). Protection of specific maternal messenger RNAs by the P body protein CGH-1 (Dhh1/RCK) during Caenorhabditis elegans oogenesis. J Cell Biol *182*, 543-557.

Boag, P.R., Nakamura, A., and Blackwell, T.K. (2005). A conserved RNA-protein complex component involved in physiological germline apoptosis regulation in C. elegans. Development *132*, 4975-4986.

Boyd, L., Guo, S., Levitan, D., Stinchcomb, D.T., and Kemphues, K.J. (1996). PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in C. elegans embryos. Development *122*, 3075-3084.

Brangwynne, C.P., Eckmann, C.R., Courson, D.S., Rybarska, A., Hoege, C., Gharakhani, J., Julicher, F., and Hyman, A.A. (2009). Germline P granules are liquid droplets that localize by controlled dissolution/condensation. Science *324*, 1729-1732.

Brengues, M., Teixeira, D., and Parker, R. (2005). Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. Science *310*, 486-489.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Cheeks, R.J., Canman, J.C., Gabriel, W.N., Meyer, N., Strome, S., and Goldstein, B. (2004). C. elegans PAR proteins function by mobilizing and stabilizing asymmetrically localized protein complexes. Curr Biol *14*, 851-862.

Cinalli, R.M., Rangan, P., and Lehmann, R. (2008). Germ cells are forever. Cell *132*, 559-562.

Cohen, E.E. (2006). Role of epidermal growth factor receptor pathway-targeted therapy in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol *24*, 2659-2665.

Cougot, N., Babajko, S., and Seraphin, B. (2004). Cytoplasmic foci are sites of mRNA decay in human cells. J Cell Biol *165*, 31-40.

Dernburg, A.F., McDonald, K., Moulder, G., Barstead, R., Dresser, M., and Villeneuve, A.M. (1998). Meiotic recombination in C. elegans initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. Cell *94*, 387-398.

Donson, A.M., Banerjee, A., Gamboni-Robertson, F., Fleitz, J.M., and Foreman, N.K. (2000). Protein kinase C zeta isoform is critical for proliferation in human glioblastoma cell lines. J Neurooncol *47*, 109-115.

Draper, B.W., Mello, C.C., Bowerman, B., Hardin, J., and Priess, J.R. (1996). MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. Cell *87*, 205-216.

Eder, A.M., Sui, X., Rosen, D.G., Nolden, L.K., Cheng, K.W., Lahad, J.P., Kango-Singh, M., Lu, K.H., Warneke, C.L., Atkinson, E.N., Bedrosian, I., Keyomarsi, K., Kuo, W.L., Gray, J.W., Yin, J.C., Liu, J., Halder, G., and Mills, G.B (2005). Atypical PKCiota contributes to poor prognosis through loss of apical-basal polarity and cyclin E overexpression in ovarian cancer. Proc Natl Acad Sci U S A *35*, 12519-24.

Francis, R., Barton, M.K., Kimble, J., and Schedl, T. (1995). gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans. Genetics *139*, 579-606.

Frank, D.J., and Roth, M.B. (1998). ncl-1 is required for the regulation of cell size and ribosomal RNA synthesis in Caenorhabditis elegans. J Cell Biol *140*, 1321-1329.

Frey, S., Richter, R.P., and Gorlich, D. (2006). FG-rich repeats of nuclear pore proteins form a three-dimensional meshwork with hydrogel-like properties. Science *314*, 815-817.

Gallo, C.M., Munro, E., Rasoloson, D., Merritt, C., and Seydoux, G. (2008). Processing bodies and germ granules are distinct RNA granules that interact in C. elegans embryos. Dev Biol *323*, 76-87.

Guedes, S., and Priess, J.R. (1997). The C. elegans MEX-1 protein is present in germline blastomeres and is a P granule component. Development *124*, 731-739.

Hammell, C.M., Lubin, I., Boag, P.R., Blackwell, T.K., and Ambros, V. (2009). nhl-2 Modulates microRNA activity in Caenorhabditis elegans. Cell *136*, 926-938.

Hay, B., Jan, L.Y., and Jan, Y.N. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell *55*, 577-587.

Hay, B., Jan, L.Y., and Jan, Y.N. (1990). Localization of vasa, a component of Drosophila polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. Development *109*, 425-433.

Henderson, M.A., Cronland, E., Dunkelbarger, S., Contreras, V., Strome, S., and Keiper, B.D. (2009). A germline-specific isoform of eIF4E (IFE-1) is required for efficient translation of stored mRNAs and maturation of both oocytes and sperm. J Cell Sci *122*, 1529-1539.

Horvitz, H.R., and Herskowitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell *68*, 237-255.

Hird, S.N., Paulsen, J.E., and Strome, S. (1996). Segregation of germ granules in living Caenorhabditis elegans embryos: cell-type-specific mechanisms for cytoplasmic localisation. Development *122*, 1303-1312.

Hird, S.N., and White, J.G. (1993). Cortical and cytoplasmic flow polarity in early embryonic cells of Caenorhabditis elegans. J Cell Biol *121*, 1343-1355.

Hirsh, D., Oppenheim, D., and Klass, M. (1976). Development of the reproductive system of Caenorhabditis elegans. Dev Biol *49*, 200-219.

Hyenne, V., Desrosiers, M., and Labbe, J.C. (2008). C. elegans Brat homologs regulate PAR protein-dependent polarity and asymmetric cell division. Dev Biol *321*, 368-378.

Kawasaki, I., Amiri, A., Fan, Y., Meyer, N., Dunkelbarger, S., Motohashi, T., Karashima, T., Bossinger, O., and Strome, S. (2004). The PGL family proteins associate with germ granules and function redundantly in Caenorhabditis elegans germline development. Genetics *167*, 645-661.

Kawasaki, I., Shim, Y.H., Kirchner, J., Kaminker, J., Wood, W.B., and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans. Cell *94*, 635-645.

Kedersha, N., and Anderson, P. (2002). Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc Trans *30*, 963-969.

Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fritzler, M.J., Scheuner, D., Kaufman, R.J., Golan, D.E., and Anderson, P. (2005). Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol *169*, 871-884.

Kedersha, N.L., Gupta, M., Li, W., Miller, I., and Anderson, P. (1999). RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. J Cell Biol *147*, 1431-1442.

Kemphues, K.J., Priess, J.R., Morton, D.G., and Cheng, N.S. (1988). Identification of genes required for cytoplasmic localization in early C. elegans embryos. Cell *52*, 311-320.

Kimble, J., and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. Dev Biol *70*, 396-417.

Lall, S., Piano, F., and Davis, R.E. (2005). Caenorhabditis elegans decapping proteins: localization and functional analysis of Dcp1, Dcp2, and DcpS during embryogenesis. Mol Biol Cell *16*, 5880-5890.

Lasko, P.F., and Ashburner, M. (1988). The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. Nature *335*, 611-617.

Leacock, S.W., and Reinke, V. (2008). MEG-1 and MEG-2 are embryo-specific P-granule components required for germline development in Caenorhabditis elegans. Genetics *178*, 295-306.

Lee, M., and Vasioukhin, V. (2008). Cell polarity and cancer--cell and tissue polarity as a non-canonical tumor suppressor. J Cell Sci *121*, 1141-1150.

Lehmann, R. (1992). Germ-plasm formation and germ-cell determination in Drosophila. Curr Opin Genet Dev *2*, 543-549.

Maddox, P.S., Hyndman, F., Monen, J., Oegema, K., and Desai, A. (2007). Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. J Cell Biol *176*, 757-763.

Mahowald, A.P. (1968). Polar granules of Drosophila. II. Ultrastructural changes during early embryogenesis. J Exp Zool *167*, 237-261.

Mello, C.C., Schubert, C., Draper, B., Zhang, W., Lobel, R., and Priess, J.R. (1996). The PIE-1 protein and germline specification in C. elegans embryos. Nature *382*, 710-712.

Morton, D.G., Shakes, D.C., Nugent, S., Dichoso, D., Wang, W., Golden, A., and Kemphues, K.J. (2002). The Caenorhabditis elegans par-5 gene encodes a 14-3-3 protein required for cellular asymmetry in the early embryo. Dev Biol *241*, 47-58.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

Navarro, R.E., Shim, E.Y., Kohara, Y., Singson, A., and Blackwell, T.K. (2001). cgh-1, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in C. elegans. Development *128*, 3221-3232.

Neumuller, R.A., Betschinger, J., Fischer, A., Bushati, N., Poernbacher, I., Mechtler, K., Cohen, S.M., and Knoblich, J.A. (2008). Mei-P26 regulates microRNAs and cell growth in the Drosophila ovarian stem cell lineage. Nature *454*, 241-245.

Parker, R., and Sheth, U. (2007). P bodies and the control of mRNA translation and degradation. Mol Cell *25*, 635-646.

Pitt, J.N., Schisa, J.A., and Priess, J.R. (2000). P granules in the germ cells of Caenorhabditis elegans adults are associated with clusters of nuclear pores and contain RNA. Dev Biol *219*, 315-333.

Rajyaguru, P., and Parker, R. (2009). CGH-1 and the control of maternal mRNAs. Trends Cell Biol *19*, 24-28.

Regala, R.P., Weems, C., Jamieson, L., Khoor, A., Edell, E.S., Lohse, C.M., and Fields, A.P. (2005). Atypical protein kinase C iota is an oncogene in human non-small cell lung cancer. Cancer Res *65*, 8905-8911.

Riechmann, V., Gutierrez, G.J., Filardo, P., Nebreda, A.R., and Ephrussi, A. (2002). Par-1 regulates stability of the posterior determinant Oskar by phosphorylation. Nat Cell Biol *4*, 337-342.

Schisa, J.A., Pitt, J.N., and Priess, J.R. (2001). Analysis of RNA associated with P granules in germ cells of C. elegans adults. Development *128*, 1287-1298.

Schubert, C.M., Lin, R., de Vries, C.J., Plasterk, R.H., and Priess, J.R. (2000). MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos. Mol Cell *5*, 671-682.

Schwamborn, J.C., Berezikov, E., and Knoblich, J.A. (2009). The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell *136*, 913-925.

Sengoku, T., Nureki, O., Nakamura, A., Kobayashi, S., and Yokoyama, S. (2006). Structural basis for RNA unwinding by the DEAD-box protein Drosophila Vasa. Cell *125*, 287-300.

Seydoux, G., and Braun, R.E. (2006). Pathway to totipotency: lessons from germ cells. Cell *127*, 891-904.

Seydoux, G., and Fire, A. (1994). Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans. Development *120*, 2823-2834.

Seydoux, G., Mello, C.C., Pettitt, J., Wood, W.B., Priess, J.R., and Fire, A. (1996). Repression of gene expression in the embryonic germ lineage of C. elegans. Nature *382*, 713-716.

Sheth, U., and Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science *300*, 805-808.

Shimada, M., Yokosawa, H., and Kawahara, H. (2006). OMA-1 is a P granules-associated protein that is required for germline specification in Caenorhabditis elegans embryos. Genes Cells *11*, 383-396.

Shulman, J.M., Benton, R., and St Johnston, D. (2000). The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. Cell *101*, 377-388.

Slack, F.J., and Ruvkun, G. (1998). A novel repeat domain that is often associated with RING finger and B-box motifs. Trends Biochem Sci *23*, 474-475.

Sonoda, J., and Wharton, R.P. (2001). Drosophila Brain Tumor is a translational repressor. Genes Dev *15*, 762-773.

Spike, C., Meyer, N., Racen, E., Orsborn, A., Kirchner, J., Kuznicki, K., Yee, C., Bennett, K., and Strome, S. (2008a). Genetic analysis of the Caenorhabditis elegans GLH family of P-granule proteins. Genetics *178*, 1973-1987.

Spike, C.A., Bader, J., Reinke, V., and Strome, S. (2008b). DEPS-1 promotes P-granule assembly and RNA interference in C. elegans germ cells. Development *135*, 983-993.

Spike, C.A., and Strome, S. (2003). Germ plasm: protein degradation in the soma. Curr Biol *13*, R837-839.

Squirrell, J.M., Eggers, Z.T., Luedke, N., Saari, B., Grimson, A., Lyons, G.E., Anderson, P., and White, J.G. (2006). CAR-1, a protein that localizes with the mRNA decapping component DCAP-1, is required for cytokinesis and ER organization in Caenorhabditis elegans embryos. Mol Biol Cell *17*, 336-344.

Stallings-Mann, M., Jamieson, L., Regala, R.P., Weems, C., Murray, N.R., and Fields, A.P. (2006). A novel small-molecule inhibitor of protein kinase Ciota blocks transformed growth of non-small-cell lung cancer cells. Cancer Res *66*, 1767-1774.

Strome, S., and Lehmann, R. (2007). Germ versus soma decisions: lessons from flies and worms. Science *316*, 392-393.

Strome, S., and Wood, W.B. (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans. Proc Natl Acad Sci U S A *79*, 1558-1562.

Subramaniam, K., and Seydoux, G. (1999). nos-1 and nos-2, two genes related to Drosophila nanos, regulate primordial germ cell development and survival in Caenorhabditis elegans. Development *126*, 4861-4871.

Tabara, H., Hill, R.J., Mello, C.C., Priess, J.R., and Kohara, Y. (1999). pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans. Development *126*, 1-11.

Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K.C., Kemphues, K.J., and Ephrussi, A. (2000). A Drosophila melanogaster homologue of Caenorhabditis elegans par-1 acts at an early step in embryonic-axis formation. Nat Cell Biol *2*, 458-460.

Updike, D.L., and Strome, S. (2009a). A Genome-wide RNAi Screen for Genes That Affect the Stability, Distribution, and Function of P Granules in Caenorhabditis elegans. Genetics.

Updike, D.L., and Strome, S. (2009b). P Granule Assembly and Function in C. elegans Germ Cells. J Androl.

Wodarz, A. (2005). Molecular control of cell polarity and asymmetric cell division in Drosophila neuroblasts. Curr Opin Cell Biol *17*, 475-481.

Wodarz, A., and Nathke, I. (2007). Cell polarity in development and cancer. Nat Cell Biol *9*, 1016-1024.

Wolf, N., Priess, J., and Hirsh, D. (1983). Segregation of germline granules in early embryos of Caenorhabditis elegans: an electron microscopic analysis. J Embryol Exp Morphol *73*, 297-306.

Zorio, D.A., Cheng, N.N., Blumenthal, T., and Spieth, J. (1994). Operons as a common form of chromosomal organization in C. elegans. Nature *372*, 270-272.

Mahowald, A.P., 1968. Polar granules of *Drosophila* II. Ultrastructural changes during early embryogenesis. *J. Exp. Zool.* **167**, pp. 237–262.